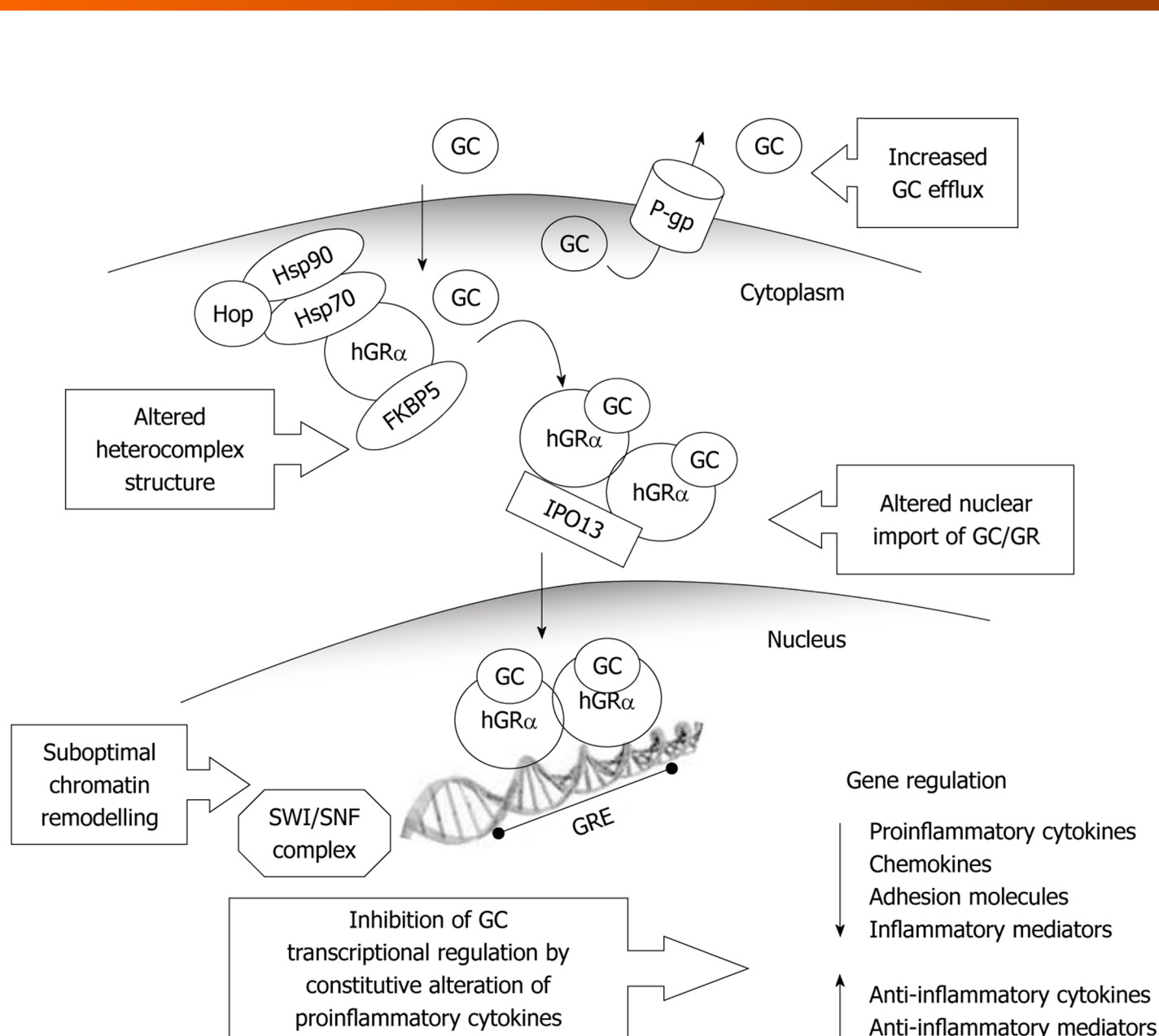


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Contents

Weekly Volume 17 Number 9 March 7, 2011

EDITORIAL

- 1091 Diagnosis of spontaneous bacterial peritonitis: An update on leucocyte esterase reagent strips
Koulaouzidis A
- 1095 Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease
De Iudicibus S, Franca R, Martelossi S, Ventura A, Decorti G

TOPIC HIGHLIGHT

- 1109 Digestive oncologist in the gastroenterology training curriculum
Mulder CJJ, Peeters M, Cats A, Dahele A, Terhaar sive Droste J

REVIEW

- 1116 Recent results of laparoscopic surgery in inflammatory bowel disease
Kessler H, Mudter J, Hohenberger W

ORIGINAL ARTICLE

- 1126 CT diagnosis of recurrence after pancreatic cancer: Is there a pattern?
Heye T, Zausig N, Klauss M, Singer R, Werner J, Richter GM, Kauczor HU, Grenacher L
- 1135 Octreotide ameliorates gastric lesions in chronically mild stressed rats
Nassar NN, Schaalán MF, Zaki HF, Abdallah DM
- 1143 Effects of prostaglandin F_{2α} on small intestinal interstitial cells of Cajal
Park CG, Kim YD, Kim MY, Koh JW, Jun JY, Yeum CH, So I, Choi S
- 1152 Hepatoma cell line HepG2.2.15 demonstrates distinct biological features compared with parental HepG2
Zhao R, Wang TZ, Kong D, Zhang L, Meng HX, Jiang Y, Wu YQ, Yu ZX, Jin XM

BRIEF ARTICLE

- 1160 Gastroesophageal reflux disease management according to contemporary international guidelines: A translational study
Pace F, Riegler G, de Leone A, Dominici P, Grossi E, the EMERGE Study Group
- 1167 Interleukin-24 is correlated with differentiation and lymph node numbers in rectal cancer
Choi Y, Roh MS, Hong YS, Lee HS, Hur WJ
- 1174 Long-term efficacy of infliximab maintenance therapy for perianal Crohn's disease
Uchino M, Ikeuchi H, Bando T, Matsuoka H, Takesue Y, Takahashi Y, Matsumoto T, Tomita N

- 1180** Prognostic factors of T4 gastric cancer patients undergoing potentially curative resection
Fukuda N, Sugiyama Y, Wada J
- 1185** Diagnostic value of antigenemia assay for cytomegalovirus gastrointestinal disease in immunocompromised patients
Nagata N, Kobayakawa M, Shimbo T, Hoshimoto K, Yada T, Gotoda T, Akiyama J, Oka S, Uemura N
- 1192** Strong expression of CD133 is associated with increased cholangiocarcinoma progression
Leelawat K, Thongtawee T, Narong S, Subwongcharoen S, Treepongkaruna S
- 1199** Prevalence and risk factors of asymptomatic peptic ulcer disease in Taiwan
Wang FW, Tu MS, Mar GY, Chuang HY, Yu HC, Cheng LC, Hsu PI
- 1204** Coffee drinking and pancreatic cancer risk: A meta-analysis of cohort studies
Dong J, Zou J, Yu XF
- 1211** p53 codon 72 polymorphism and liver cancer susceptibility: A meta-analysis of epidemiologic studies
Chen X, Liu F, Li B, Wei YG, Yan LN, Wen TF
- 1219** Metastasis-associated protein 1 induces VEGF-C and facilitates lymphangiogenesis in colorectal cancer
Du B, Yang ZY, Zhong XY, Fang M, Yan YR, Qi GL, Pan YL, Zhou XL
- 1227** *TP53* Arg72Pro polymorphism is associated with esophageal cancer risk: A meta-analysis
Jiang DK, Yao L, Wang WZ, Peng B, Ren WH, Yang XM, Yu L

CASE REPORT

- 1234** *Helicobacter pylori*-negative Russell body gastritis: Case report
Del Gobbo A, Elli L, Braidotti P, Di Nuovo F, Bosari S, Romagnoli S

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APPENDIX I Meetings
I-VI Instructions to authors

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Diagnosis of spontaneous bacterial peritonitis: An update on leucocyte esterase reagent strips

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Abstract

Ascites remain the commonest complication of decompensated cirrhosis. Spontaneous bacterial peritonitis (SBP) is defined as the infection of ascitic fluid (AF) in the absence of a contiguous source of infection and/or an intra-abdominal inflammatory focus. An AF polymorphonuclear (PMN) leucocyte count $\geq 250/\text{mm}^3$ -irrespective of the AF culture result- is universally accepted nowadays as the best surrogate marker for diagnosing SBP. Frequently the results of the manual or automated PMN count do not reach the hands of the responsible medical personnel in a timely manner. However, this is a crucial step in SBP management. Since 2000, 26 studies (most of them published as full papers) have checked the validity of using leucocyte esterase reagent strips (LERS) in SBP diagnosis. LERS appear to have low sensitivity for SBP, some LERS types more than others. On the other hand, though, LERS have consistently given a high negative predictive value ($> 95\%$ in the majority of the studies) and this supports the use of LERS as a preliminary screening tool for SBP diagnosis. Finally, an AF-tailored dipstick has been developed. Within the proper setting, it is set to become the mainstream process for handling AF samples.

INTRODUCTION

Ascites remains the commonest of the three major complications of advanced or decompensated cirrhosis (along with hepatic encephalopathy and variceal haemorrhage). Cirrhotics with ascites have, over a one-year period, 10% probability of developing the first episode of spontaneous bacterial peritonitis (SBP)^[1]. Conn first introduced the term SBP, publishing his clinical findings just one year after Kerr *et al* described (in 1963) 11 cases of seemingly unexplained infection of the ascitic fluid (AF)^[2].

SBP is defined as the infection of AF in the absence of a contiguous source of infection and/or an intra-abdominal (and potentially surgically treated) inflammatory focus. Depending on the patient population examined (outpatients or hospitalised), the prevalence of SBP varies from 3.5% and 30%^[3]. Around 50% of SBP episodes are present at the time of hospital admission, whilst the remainder are acquired during the hospitalisation period^[2]. The mortality of untreated SBP remains high ($> 80\%$), and a satisfactory patient course and clinical outcome is based on an aggressive approach aiming to rapid diagnosis and prompt initiation of antibiotic therapy.

DIAGNOSIS OF SBP

The clinical manifestations of SBP can be subtle and in-

sidious, and its diagnosis requires a high index of clinical suspicion. Abdominal paracentesis is considered necessary for all patients with ascites on hospital admission, in-patient cirrhotics with ascites who develop clinical signs of sepsis, hepatic encephalopathy, (sudden or unexplained) renal impairment and/or all cirrhotics who develop GI bleeding^[4]. Unfortunately, a clinical diagnosis of infected AF without a paracentesis is not adequate^[1].

An AF polymorphonuclear (PMN) leukocyte count $\geq 250/\text{mm}^3$, irrespective of the AF culture result, is universally accepted nowadays as the best surrogate marker for diagnosing SBP^[5]. The presence of positive AF cultures is confirmatory, but by no means a necessary prerequisite for instigation of antibiotic therapy. In fact, it is considered a “fatal” mistake to wait 48 h for culture results before initiating therapy, where it is indicated.

Frequently the results of the manual PMN count do not reach the hands of the responsible medical personnel in a timely manner^[6]. Such situations include busy night or weekend shifts, small hospitals with off-site laboratory facilities, or units with limited case-load and liver disease expertise. We have recently showed that the mean delay from paracentesis to a validated PMN result out-of-hours was more than 4 h^[7]. Furthermore, manual AF PMN counting is laborious and costly. The use of automated cell counters has now been backed-up by sufficient published evidence to become the common practice^[5,8].

However, even automated cell counts suffer generally from similar constraints to those described above for manual techniques. Therefore, any alternative test that may provide or, more importantly, exclude a diagnosis of SBP at the bedside and reduce the “tap-to-first shot” time is considered welcome. The leukocyte esterase reagent strips (LERS), commonly used in every day practice for the rapid diagnosis of urinary tract infections (UTIs), were certainly featuring as a promising candidate.

LERS IN SBP

LERS had already been successfully evaluated in the diagnosis of infection in other sterile body fluids i.e. synovial, pleural, cerebrospinal fluid and peritoneal dialysate^[9-11]. The LERS test is based on the esterase activity of the leucocytes. A pyrrole, esterified with an amino-acid is used as the substrate; hydrolysis of the ester (mediated by the esterase) releases the pyrrole which in turn reacts with a diazonium salt yielding a violet or purple azo dye in the relevant pad of the strip^[11]. LERS are not specific for PMNs and the interpretation of the colorimetric reaction is inherently subjective, therefore the method is considered qualitative or semi-quantitative at best. Butani *et al*^[12] were the first to present their results on the use of LERS in SBP diagnosis as an abstract in DDW 2000.

Since then, 26 publications followed (23 as full, peer-reviewed papers and 3 as either an abstract or a letter to Editor; of them, 22 are in English, 2 in French, 1 in Chinese & 1 in Korean), with the first full paper that of Vanbiervliet *et al*^[13] validating the Multistix[®]8SG.

Their results were very encouraging. Thus, various

LERS were eventually validated in what were mostly single or two-centre studies (Table 1), with one notable exception in the French multicentre study (Nousbaum *et al*^[28], 70 centres) published initially as an abstract and later as a full paper in 2007. It is important to note here that the grading is different for each dipstick, and therefore the cut-off leucocyte count should be used instead, in order to draw meaningful conclusions.

The French multicentre study pointed out the weakness of Multistix[®]8SG and, to a certain extent, of the concept of using dipstick in SBP diagnosis overall. Furthermore, 2 systematic reviews^[9,10] have been published in 2008, both pointing out that the heterogeneity in the number of patients included in each study, the AF samples tested and SBP episodes observed, as well as in all measures of LERS performance, did not allow pooling of the results via meta-analysis. Overall, the Aution[®] and Combur[®] dipsticks have performed better^[38] (in regards to the negative predictive value) than the Multistix[®]. The spectrophotometric analyser Clinitek[®] 50, compatible with the Multistix[®] dipstick, was used in only 6 studies.

The rather intense research on the field has brought up important details on the limitations of LERS. First, the results seem to be influenced by the number of PMNs in the AF, LERS performing less well if the PMN count $< 1000/\mu\text{L}$ ^[39]. Second, all LERS validated in the SBP studies were initially designed for use in the diagnosis of UTIs; in infected urine though, both the number of leucocytes and the protein content are quite different, the first being significantly higher than in most SBP^[39], while the latter does not exceed the 1 g/L level^[35]. The above 2 factors are considered significant for the observed low sensitivity of some LERS. I need to mention again here that, aside the fact there is significant inter-study variability in terms of the LERS brands used, as well as to the cut-off level examined, LERS are not specific for PMNs and the interchangeable use of PMNs and leucocytes (seen in the majority of the studies) is confusing to the reader. Finally, LERS are not suitable for the few cases of chylous ascites or peritoneal tuberculosis.

On the other hand, LERS have consistently given a high negative predictive value (NPV) of above 95% in the majority of the studies and, as in SBP, a false positive result (which might eventually lead to the ‘adverse’ administration of a single dose of an overall well-tolerated antibiotic^[28]) is considered ethically and medically acceptable advocating the use of LERS as a preliminary screening tool for SBP diagnosis. In addition, Castelote *et al*^[33] only recently showed that LERS, despite their qualitative nature, could be well used in the clinical management of SBP. The low cost of the strips can only be considered a significant advantage.

Only one study has checked the combine use of the LERS with the relevant pad for nitrites. There was no additional advantage by combining the two results. Finally, despite clear evidence to support its use^[5], no study has validated the combination results of LER pad with that of the pH^[3].

CONCLUSION

In conclusion, there is reasonable amount of evidence

Table 1 Studies, patients included, ascitic fluid samples tested, inpatients/outpatients, type of leukocyte esterase reagent strips used, leukocyte esterase reagent strips cut-off grade of the study with Sens, Spec, PPV and NPV

Study	Patients	Samples	In/Out	M/F	SBP	LEERS	LEERS cut-off	Sens (%)	Spec (%)	PPV (%)	NPV (%)
Vanbiervliet <i>et al</i> ^[13]	72	78	72/0	44/28	9	Multistix8®SG	70 leuc/μL-G2	100	100	100	100
Castelote <i>et al</i> ^[14]	128	228	128/0	91/37	52	Aution® sticks	75 leuc/μL-G2	96	89	74	99
Thévenot <i>et al</i> ^[15]	31	100	23/8	13/18	9	Multistix8®SG	125 leuc/μL-G3	89	100	100	99
						Combur2LN®	75 leuc/μL-G2	89	100	100	99
Butani <i>et al</i> ^[16]	75	136	n/s	n/s	12	Multistix10®SG	70 leuc/μL-G2	83	99	91	98
Sapey <i>et al</i> ^[11]	34 (s-group)	55 (s-group)	n/s	51/15	13	Multistix10®SG	25 leuc/μL-G1	83/100	96/100	83/100	96/100
	76	184				Nepheur-test®	25 leuc/μL-G1	86/100	92.5/100	75/100	99/100
Sapey <i>et al</i> ^[17]	51	245	9/42		17	Multistix10®SG	25 leuc/μL-G1	64.7	99.6	91.7	97.4
						Nepheur-test®	25 leuc/μL-G1	88.2	99.6	93.8	99.1
Kim <i>et al</i> ^[18]	257	257	257/0	187/70	79	UriSCAN®	75 leuc/μL-G2	100	99	98	100
Kim <i>et al</i> ^[19]	53	75	53/0	36/17	18	Multistix10®SG	75 leuc/μL-G2	50	100	100	87
						UriSCAN®	75 leuc/μL-G2	100	100	100	100
Sarwar <i>et al</i> ^[20]	214	214	214/0	116/98	38	Combur10®	75 leuc/μL-G2	95	92	72	99
Wisniewski <i>et al</i> ^[21]	47	90	47/0	27/20	6	Multistix8®SG	15 leuc/μL-G1	83	83	42	97
Braga <i>et al</i> ^[22]	42	100	35/7	10/32	9	Combur® UX	75 leuc/μL-G2	100	98.9	92.3	100
Rerknimitr <i>et al</i> ^[23]	127	200	106/21	75/52	42	Combur10M®	25 leuc/μL-G1	88	81	55	96
Campillo <i>et al</i> ^[24]	116	443	n/s	76/40	33	Multistix8®SG	70 leuc/μL-G2	45.7	98	75	93.3
						Combur2LN®	75 leuc/μL-G2	63	99.2	91	92.9
Li <i>et al</i> ^[25]	84	84	84/0	47/37	25	Multistix10®SG	15 leuc/μL-G1	92.8	84.7	71.8	96.1
Ribeiro <i>et al</i> ^[26]	106	200	80/26	82/24	11	Multistix10®SG	15 leuc/μL-G1	86	96	60	99
Gaya <i>et al</i> ^[27]	105	173	71/34	71/34	17	Multistix10®SG	15 leuc/μL-G1	100	91	50	100
Nousbaum <i>et al</i> ^[28]	1041	2123	686/355	748/293	117	Multistix8®SG	70 leuc/μL-G2	45.3	99.2	77.9	96.9
Torun <i>et al</i> ^[29]	63	63	63/0	38/25	15	Aution® sticks	75 leuc/μL-G2	93	100	100	98
Nobre <i>et al</i> ^[30]	55	109	55/0	33/22	9	H-T Combina®	75 leuc/μL-G2	78	88	37	98
de Araujo <i>et al</i> ^[31]	71	155	43/28	57/24	17	Multistix10®SG	15 leuc/μL-G1	80	98.5	90.9	96.2
		159				Choiceline 10®	75 leuc/μL-G2	76.9	97.7	87	95.6
Balogopal <i>et al</i> ^[32]	175	n/f	n/f	146/29	n/f	Magistix10®	125 leuc/μL-n/f	92	100	n/f	n/f
Castellote <i>et al</i> ^[33]	51	n/s	51	n/s	53	Aution® sticks	75 leuc/μL-G2	89	86	62	97
Rerknimitr <i>et al</i> ^[34]	143	250	n/s	91/52	30	Multistix10®SG	?25 leuc/μL-G1	80	94.5	66.7	97.2
						Aution® sticks	?250 leuc/μL-G3	90	93.2	64.3	98.6
						Combur10®	?75 leuc/μL-G2	90	93.2	64.3	98.6
[letter]Gülberg <i>et al</i> ^[35]	n/s	194	n/s	n/s	16	Multistix10®SG	n/s	31	n/s	n/s	n/s
						Combur®	n/s	44	n/s	n/s	n/s
[letter]Farmer <i>et al</i> ^[36]	256	311	n/s	161/95	59	Multistix8®SG	70 leuc/μL-G2	96	96.5	90.7	99.4
[abstract] Delaunay-Tardy <i>et al</i> ^[37]	n/f	n/f	n/f	n/f	n/f	Multistix8®SG	n/f	60	n/f	n/f	n/f

Sens: Sensitivity; Spec: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; LEERS: Leukocyte esterase reagent strips; SBP: Spontaneous bacterial peritonitis; n/s : Not stated; n/f : Not found; G : Grade (as per LEERS); In/out: Inpatients/outpatients; M/F : Male/female; s-group: subgroup

to support the use of LEERS in the work-up of patients suspected of having SBP. The PMN count (be it manual or automated) is not to be abolished from SBP diagnostic algorithm. Remote hospitals, less affluent health systems and busy junior clinicians should realise the benefit of LEERS and incorporate them in their AF handling routine. A “new kid on the block” has just appeared^[40] in the race against SBP; if further validation studies worldwide are supportive, it is set to become the mainstream process for handling AF samples^[41].

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Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease

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Abstract

Natural and synthetic glucocorticoids (GCs) are widely employed in a number of inflammatory, autoimmune and neoplastic diseases, and, despite the introduction of novel therapies, remain the first-line treatment for inducing remission in moderate to severe active Crohn's disease and ulcerative colitis. Despite their extensive therapeutic use and the proven effectiveness, considerable clinical evidence of wide inter-individual differences in GC efficacy among patients has been reported, in particular when these agents are used in inflammatory diseases. In recent years, a detailed knowledge of the GC mechanism of action and of the genetic variants affecting GC activity at the molecular level has arisen from several studies. GCs interact with their cytoplasmic receptor, and are able to repress inflammatory gene expression through several distinct mechanisms. The glucocorticoid receptor (GR) is therefore crucial for the effects of these agents: mutations in the GR gene (NR3C1, nuclear receptor subfamily 3, group C, member 1) are the primary cause of a rare, inherited form of GC resistance; in addition, several polymorphisms of this gene have been described and associated with GC response and toxicity.

However, the GR is not self-standing in the cell and the receptor-mediated functions are the result of a complex interplay of GR and many other cellular partners. The latter comprise several chaperonins of the large cooperative hetero-oligomeric complex that binds the hormone-free GR in the cytosol, and several factors involved in the transcriptional machinery and chromatin remodeling, that are critical for the hormonal control of target genes transcription in the nucleus. Furthermore, variants in the principal effectors of GCs (e.g. cytokines and their regulators) have also to be taken into account for a comprehensive evaluation of the variability in GC response. Polymorphisms in genes involved in the transport and/or metabolism of these hormones have also been suggested as other possible candidates of interest that could play a role in the observed inter-individual differences in efficacy and toxicity. The best-characterized example is the drug efflux pump P-glycoprotein, a membrane transporter that extrudes GCs from cells, thereby lowering their intracellular concentration. This protein is encoded by the ABCB1/MDR1 gene; this gene presents different known polymorphic sites that can influence its expression and function. This editorial reviews the current knowledge on this topic and underlines the role of genetics in predicting GC clinical response. The ambitious goal of pharmacogenomic studies is to adapt therapies to a patient's specific genetic background, thus improving on efficacy and safety rates.

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Key words: Glucocorticoids; Inflammatory bowel disease; Pharmacogenomics

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INTRODUCTION

Glucocorticoids (GCs) are a well-accepted therapy for inflammatory, autoimmune and proliferative diseases^[1-3]. Despite the large clinical use, the benefits of these agents are often narrowed by inter-individual variability. Indeed, some patients show a poor or absent response, and in these subjects, GCs have to be employed in high doses. The clinical use of these compounds is associated with a number of serious complications, including osteoporosis, metabolic disease and increased risk of cardiovascular disease^[4-6]; therefore, patients, and in particular those who respond poorly to these agents, are at high risk of side effects. In addition, besides being a problem for patients and a challenge to clinicians, inadequate GC therapy also represents a socio-economic matter because of the consequent considerable impact on health care costs^[7,8].

GCs are effective inhibitors of cytokine secretion and T-cell activation, and are consequently largely employed in different inflammatory conditions, including inflammatory bowel disease (IBD). In these diseases, GC resistance or dependence is particularly frequent. Clinical reports in pediatric IBD patients have shown that up to 90% of subjects have a rapid improvement of symptoms when prednisone is given^[9]. However, after 1 year, only 55% of early steroid-treated patients are still in remission and can be considered steroid-responsive. Around 38% of patients are not able to discontinue the therapy and experience an increase in disease activity when the dose is reduced (steroid-dependent); 7% of subjects are resistant and do not respond to GC therapy^[9,10]. Among the adult IBD population, a prospective analysis has described the 1-year outcome in patients with Crohn's disease (CD) treated with a first oral prednisolone course (40-60 mg/d) and tapering to a maintenance dose of 10-15 mg/d^[11]. In this study, prolonged steroid response was obtained in 44% of patients, 36% of subjects were steroid dependent and 20% steroid resistant, and a high frequency of surgery was reported within 1 mo after steroid treatment. Similar results have been obtained in a retrospective American study: immediate outcomes for CD and ulcerative colitis (UC) respectively, were complete remission in 58% and 54%, partial remission in 26% and 30%, resistance in 16% of patients^[12]. Outcome at 1 year showed a prolonged response in 32% of CD patients, GC dependence in 28%, and surgical intervention in 38%. In UC patients, 1-year outcomes were prolonged response in 49%, dependence in 22%, and surgery in 29% of subjects.

In 1999, a pivotal study by Hearing *et al.*^[13] demonstrated a poor *in vitro* response to GCs of circulating lymphocytes in UC patients who exhibited a reduced or absent clinical response to these agents. *In vitro* lymphocyte resistance to GCs has been demonstrated to correlate with clinical outcome in other inflammatory diseases such as asthma^[14], systemic lupus erythematosus^[15], rheumatoid arthritis^[16] and renal allograft rejection^[17]. A wide variation in lymphocyte steroid sensitivity is evident even in healthy individuals^[18]. This suggests that the observed variability is a stable intrinsic property of an individual that becomes important when the subject has to be treated with GCs for

an inflammatory disease^[19], and is therefore likely to have a genetic basis.

Over the past years, significant advances have been made in understanding the molecular mechanisms by which GCs act and the basis of such inter-patient variability. This editorial describes the mechanisms of GC anti-inflammatory action and discusses the molecular and genetic basis for GC resistance in chronic inflammatory diseases, especially IBD.

MOLECULAR MECHANISM OF GC ACTION

Due to their high lipophilicity, exogenous GCs are widely bioavailable; these agents, as well as the endogenous compound cortisol, are transported in the blood predominantly bound to the corticosteroid-binding globulin and, to a lesser extent, to albumin^[20]. Free GCs are able to diffuse passively across plasma membranes and interact specifically with a cytosolic receptor (GR), expressed in virtually all tissues. The GC receptor is a member of the nuclear receptor (NR) superfamily, which includes receptors for steroid hormones (e.g. corticosteroids, androgens, estrogens and progesterone), as well as other hydrophobic molecules (such as bile acids, vitamins A and D, retinoic acid and thyroid hormones). All these molecules induce their actions by the same molecular mechanism: at the basis of this mechanism stands the physical interaction between the lipophilic ligand and its own cytosolic/nuclear receptor that, in turn, activates a multistep signal transduction pathway to end up in specific genomic transcriptional effects^[21]. Receptors belonging to the NR superfamily are highly homologous to each other and share structural features with a common modular domain organization: they present a transactivation domain at the N-terminal part (NTD), a central zinc finger DNA-binding domain (DBD) and a ligand-specific binding domain (LBD) at the C terminus.

The ligand-free GR is not self-standing in the cell, but exists in heteromeric complex with molecular chaperones and co-chaperones. In the functionally mature form of the heterocomplex, the free GR is associated with an Hsp90 dimer, with p23 and with any of the Hsp90 co-chaperones, except Hsp70/Hsp90 organizing protein. Such associations are essential for keeping the receptor in the correct folding for a hormone-responsive state^[22]. Upon ligand binding, receptors undergo conformational changes and expose the DBD, which is otherwise hidden in the ligand-free conformation. Cytosolic receptors also provide nuclear localization signals (NLSs) that are comprised of a closely spaced arrangement of 5-8 basic amino acids^[23], which co-localize with DBD and, once exposed, interact with nuclear transport factors (importins).

Nuclear heterocomplex translocation is necessary for transactivating target genes. Translocation is a tightly regulated process that occurs through nuclear pore complexes in a ligand- and energy-dependent manner, and is mediated by specific nuclear transport factors that belong to the evolutionarily conserved importin β family of nuclear transporters^[23]. Among these, importin-13 (IPO13) has

Table 1 Factors involved in glucocorticoid resistance

GR	Reduced GR mRNA expression ^[42] Reduced GC binding affinity ^[43] Polymorphisms in the <i>NR3C1</i> gene ^[45]
GR heterocomplex	Altered expression of the chaperones and co-chaperones that make up the heterocomplex ^[74-78] Polymorphisms in the <i>Hsp90</i> gene ^[79] Genetic variations in the <i>STIP1</i> gene coding for Hop ^[79]
Nuclear transport factors	Polymorphisms in the Importin 13 gene ^[86]
Transcription machinery	Lower expression of SMARCB1 in resistant leukaemia cells ^[97]
Pro-inflammatory mediators	Polymorphisms in the <i>NALP1</i> gene ^[72] TNF- α promoter SNP correlated with steroid response in several disease ^[149-154] IL-10 polymorphisms related with a positive prednisone response in children with ALL ^[159]
P-gp	Hyperexpression of P-gp in lymphocytes and epithelial cells from GC-resistant IBD patients ^[167]

GR: Glucocorticoid receptor; GC: Glucocorticoid; Hsp: Heat-shock protein; Hop: Hsp70/Hsp90 organization protein; P-gp: P-glycoprotein; NR3C1: Nuclear receptor subfamily 3, group C, member 1; NALP1: NACHT leucine-rich-repeat protein 1; SNP: Single nucleotide polymorphism; TNF- α : Tumor necrosis factor α ; IL: Interleukin; ALL: Acute lymphoblastic leukemia; IBD: Inflammatory bowel disease.

been functionally characterized as a primary regulator of the translocation of the GC-bound GR across the nuclear membrane^[23].

The zinc finger motifs of the DBD allow the interaction of the activated receptor with specific DNA sequences, termed GC-responsive elements (GRE, 5'-GGTA-CAnnnTGTTCT-3' where n refers to any nucleotide^[24]), located within regulatory regions of GC-responsive genes. GR homodimerizes on GRE and recruits transcriptional co-activators, as well as basal transcription machinery, to the transcription start site. These co-activators, that include CREB (cAMP response element-binding) binding protein (CBP), steroid receptor co-activator-1 (SRC-1), GR-interacting protein (GRP-1), p300 and switching/sucrose non fermenting chromatin remodeling complex (SWI/SNF)^[25], induce histone acetylation, thus allowing the transactivation of GC-responsive genes. Although some GC anti-inflammatory effects are achieved through induction of anti-inflammatory genes, such as interleukin (IL)-10, annexin 1 and the inhibitor of nuclear factor (NF)- κ B^[26,27], transactivation enhances mainly the expression of genes involved in metabolic processes^[28,29], and is therefore, responsible for the majority of unwanted side effects^[30,31].

Although positive GREs mediate transcriptional up-regulation in response to GCs, negative GREs (5'-ATY-ACnnTnTGATCn-3'^[32]) downregulate the transcription of responsive genes. Indeed, the presence of GR on GRE might competitively prevent the binding of activator protein (AP)-1 and NF- κ B on the same promoter regions or might transactivate their inhibitor proteins. Furthermore, GRE-independent mechanisms of transrepression also exist: the GR physically interacts with AP-1^[33], NF- κ B^[34] and signal transducers and activators of transcription^[35]. Transrepression is believed to be responsible for the majority of the beneficial anti-inflammatory effects of GCs^[30,36-38].

The molecular mechanisms of GC action are further complicated by the realization that these hormones can also induce rapid non-genomic effects within the cytoplasm; for example, they induce the release of Src kinase from the GR heterocomplex, which results in lipocortin activation and inhibition of arachidonic acid release^[39], and altered cytoplasmic ion content^[40].

MOLECULAR MECHANISM OF GC RESISTANCE IN IBD

The phenomenon of GC resistance in chronic inflammatory diseases is, as stated above, quite common, however the precise molecular mechanism is still unclear. First of all, it should be separated from the rare familial condition of primary generalized GC resistance, for which the name of Chrousos syndrome has been recently proposed^[41]. This is a rare, sporadic or familial syndrome caused by mutations in the nuclear receptor subfamily 3, group C, member 1 (NR3C1, Nuclear Receptor Nomenclature Committee, 1999) gene. The disease is characterized by target tissue insensitivity to GCs due to reduction or lack of functional GC receptors and by compensatory elevation in adrenocorticotrophic hormone (ACTH). This results in an increased secretion of cortisol, albeit in the absence of signs of Cushing syndrome, as well as of other adrenal hormones with mineralocorticoid and androgenic activities, which is responsible for the main symptoms (hypertension and signs of hyperandrogenism). As stated above, this syndrome is, however, extremely rare, and no cases in IBD patients have been described in the literature.

In consideration of the complexity of GC mechanisms of action (Figure 1), the most common forms of resistance observed in chronic inflammatory conditions, and in IBD in particular, may occur at several levels, and some candidate areas have been identified (Tables 1 and 2): (1) the GR receptor heterocomplex and proteins involved in nuclear translocation and transcription; (2) the pro-inflammatory mediators in the downstream signaling pathway of the GC-GR complex; and (3) P-glycoprotein (P-gp) and other proteins involved in the extrusion and metabolism of GCs.

The GR heterocomplex and proteins involved in nuclear translocation and transcription

The GR is an interesting candidate for GC resistance in IBD, and a significant lower expression of GR mRNA has been reported in the intestinal mucosa in patients with steroid-resistant UC^[42]; in addition, GC binding affinity in mononuclear cells is reduced in patients with GC resistant diseases^[43].

Table 2 Factors involved in glucocorticoid resistance in inflammatory bowel disease

GR	Significantly lower expression of GR mRNA in the intestinal mucosa in patients with GC-resistant IBD ^[42] In colonic biopsies of UC patients, significantly more GR β positive cells in the resistant group than in the GC sensitive control group ^[60]
Pro-inflammatory mediators	Significantly higher frequency of <i>BclI</i> polymorphism in GC-responsive IBD patients ^[61] TNF- α G-308A polymorphism significantly associated with steroid resistance and requirement for surgery ^[152] Association between this polymorphism and steroid dependency in a large population of CD patients ^[155]
P-gp	Higher probability of non-response to GCs in pediatric IBD patients carriers of the NALP1 variant genotype ^[72] Hyperexpression of P-gp in circulating lymphocytes and epithelial cells from patients with GC resistant IBD ^[167] Association between GC refractory CD and P-gp intronic polymorphisms ^[173] C3435T polymorphism in pediatric IBD Italian patients treated with GC not associated with response to therapy ^[152]

GR: Glucocorticoid receptor; GC: Glucocorticoid; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; TNF- α : Tumor necrosis factor α ; NALP1: NACHT leucine-rich-repeat protein 1; P-gp: P-glycoprotein; CD: Crohn's disease.

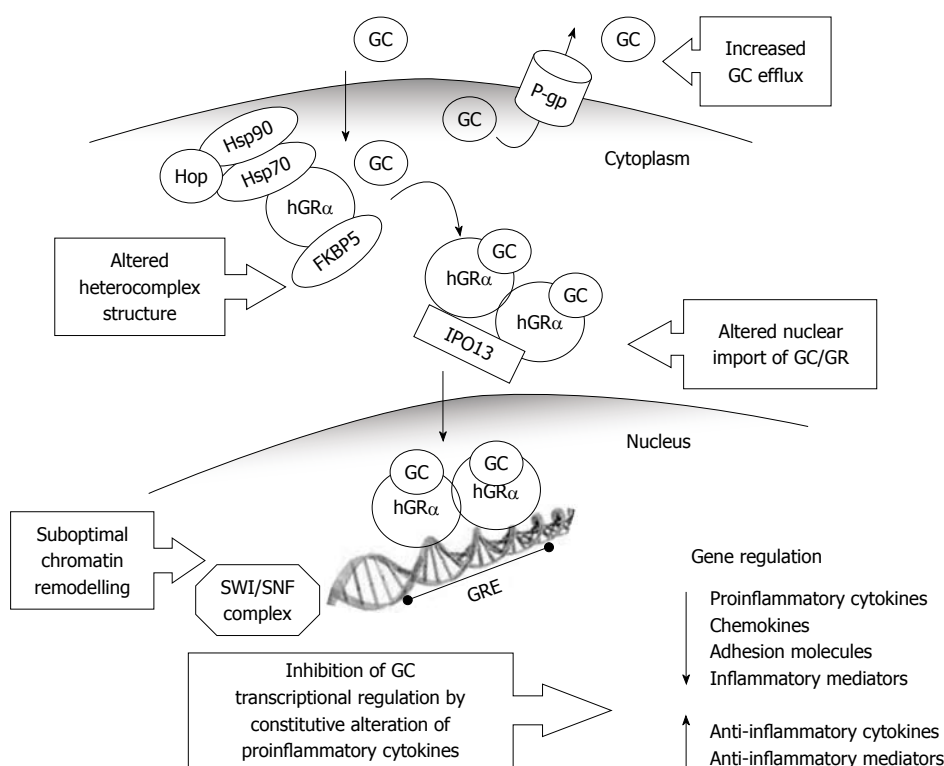


Figure 1 Mechanism of glucocorticoid resistance in inflammatory diseases. GR: Glucocorticoid receptor; GC: Glucocorticoid; FKBP5: FK506-binding protein 5; GRE: Glucocorticoid responsive elements; hGR α : Human glucocorticoid receptor α ; Hsp: Heat-shock protein; Hop: Hsp70/Hsp90 organization protein; IPO13: Importin 13; P-gp: P-glycoprotein; SWI/SNF complex: Switching/sucrose non-fermenting chromatin remodeling complex.

GR: The human GR gene NR3C1 is located on chromosome 5q31.3 and includes nine exons^[44]. Polymorphisms, that is, variations in the DNA sequence with a frequency of more than 1% in the healthy population, of the human GR gene may impair the formation of the GC-GR complex and subsequently alter transactivation and/or transrepression processes.

A large number of polymorphisms in this gene have been described: according to the dbSNP build 130 database of National Center for Biotechnology Information (NCBI), 1152 entries are known at the moment, but only a few are functionally relevant. The *TthIII* (rs10052957), ER22/23EK (rs6189/rs6190), N363S (rs6195), *BclI* (rs41423247) and GR-9 β (rs6198) polymorphisms have been the most studied and have been associated with differences in metabolic parameters and body composition,

and with autoimmune and cardiovascular disease. These genetic variants have been also related with changes in GC sensitivity or altered cortisol level^[45] and may therefore account for the variability in the response to GC therapy.

Three polymorphisms are associated with a reduced sensitivity to endogenous and exogenous GCs.

TthIII is a restriction fragment length polymorphism (RFLP) caused by a C>T change in the GR gene promoter region; it is located in a large intron of approximately 27 kb, 3807 bp upstream of the GR start site^[22]. The polymorphism has been associated with elevated diurnal cortisol levels^[46] and with a reduced cortisol response to 1 mg dexamethasone (DEX), as well as lower insulin and cholesterol levels^[47].

The ER22/23EK polymorphisms are located in the N-terminal transactivation domain of the GR and involve

two nucleotide changes in codon 22 and 23 of exon 2 (GAG AGG to GAA AAG), which are changing the amino acid sequence from glutamic acid-arginine (E-R) to glutamic acid-lysine (E-K). Since the polymorphism is located in the transactivation domain, the amino acid change might affect the tertiary structure of the receptor, which influences the transactivational and/or transrepressional activity on target genes^[48]. A relative GC resistance, with a reduction of GR transcriptional activity in transfected COS-1 cells and in peripheral blood mononuclear leukocytes of homozygous carriers, has been described^[49]. *In vivo*, an association with higher post-DEX cortisol levels as well as less cortisol suppression after a 1 mg DEX suppression test in ER22/23EK carriers, has also been shown. In addition, the polymorphism is associated with a better metabolic and cardiovascular health profile and an increased survival^[50,51].

The GR-9 β polymorphism is located in the 3'-untranslated region (UTR) of exon 9 β , where an ATTTA sequence is changed into GTTTA. This polymorphism was first characterized by Derijk *et al.*^[52] and functional studies have revealed a stabilizing effect on the mRNA of the GR β isoform, which leads to enhanced expression of the inactive GR β protein. GR β is one of several GR protein isoforms and is generated through an alternative splicing pathway that links further downstream sequences of exon 9, termed exon 9 β , to the end of exon 8. In contrast to the functionally active and most abundant isoform GR α ^[53], GR β is unable to bind ligand, is transcriptionally inactive, and exerts a dominant negative effect on transactivation by interfering with the binding of GR α to the DNA^[54-58]. Honda *et al.*^[59] have reported GR β -specific mRNA expression in lymphocytes of 83% of patients with steroid-resistant UC, compared to only 9% in responsive subjects and 10% in healthy controls and chronic active CD patients. This observation has recently been confirmed by Fujishima *et al.*^[60] who have found, in colonic biopsies of UC patients, significantly more GR β -positive cells in the resistant group than in the GC sensitive and control groups. However, in IBD, GR β is expressed 100-1000 times less than GR α , and this challenges its role in the genesis of steroid resistance in this disease.

Only a few studies, so far, have evaluated the role of the *TibIII*, ER22/23EK and GR-9 β polymorphisms in the response to exogenous GCs in IBD or in other diseases. In 119 pediatric patients with IBD, no association has been found between the ER22/23EK polymorphism and GC response^[61]. In addition, the polymorphism did not appear to confer protection against the occurrence of respiratory distress syndrome in 62 preterm infants born to mothers treated with a complete course of betamethasone^[62], or to play a role in the toxicity induced by GCs in 36 children with acute lymphoblastic leukemia (ALL)^[63]. The three polymorphisms have been also studied in haplotype: in 646 patients with multiple sclerosis treated with GCs, the haplotype consisting in *TibIII*, ER22/23EK, and 9 β -G was associated with GC resistance, with a more rapid disease progression. However this seemed to result from the presence of ER22/23EK, and not from the other two polymorphisms^[64].

Two single nucleotide polymorphisms (SNPs) in the *NR3C1* gene, the N363S and *BcI* polymorphisms, are associated with an increased sensitivity to GCs.

The N363S polymorphism is located in exon 2 and consists of an AAT>AGT nucleotide change at position 1220, which results in an asparagine to serine change in codon 363. A significantly higher transactivating capacity of the mutant has been described *in vitro* in human peripheral blood mononuclear cells^[65] and is associated with an increased sensitivity to GCs *in vivo*^[66]. Few studies have investigated the role of this polymorphism in the response to exogenous GCs. Szabó *et al.*^[67] have investigated whether variants of the *NR3C1* gene may contribute to steroid-induced ocular hypertension. In 102 patients who underwent photorefractive keratectomy and received topical steroids as part of postoperative therapy, a significant correlation was found between N363S heterozygosity and ocular hypertension. Furthermore, in 48 patients with Duchenne muscular dystrophy treated with prednisolone or deflazacort, the N363S carriers showed a trend towards a later age at loss of ambulation in comparison with non-carrier patients^[68]. Only one study so far has evaluated the role of this polymorphism in GC response in IBD: but no relation was observed between the presence of this SNP and response to GCs in a population of 119 pediatric patients^[61].

As extensively reported in the literature, the most clinically relevant polymorphism of the *NR3C1* gene is the *BcI* SNP. Initially described as a polymorphic restriction site inside intron 2, the nucleotide alteration was subsequently identified as a C>G substitution, 646 nucleotides downstream from exon 2^[69]. This polymorphism is also associated with hypersensitivity to GCs in both heterozygous and homozygous carriers of the G allele. An association with unfavorable metabolic characteristics, such as increased body mass index and insulin resistance has been also described^[70,71].

The *BcI* SNP has been studied in 119 pediatric patients with IBD (64 with CD, and 55 with UC). Patients were divided into two groups based on their response to GC treatment: GC dependence (45 patients) was defined by an initial response to prednisone with relapse on dose reduction, not allowing steroid discontinuation, and GC-responsiveness (67 patients), equivalent to therapeutic success, was defined as GC withdrawal without the need for steroids for at least 1 year. A significantly higher frequency of *BcI* mutated genotype was observed in the GC-responsive patients than in the GC-dependent group, which confirms an increased sensitivity to GCs in subjects with the *BcI* mutated genotype^[61]. These results have been subsequently confirmed in a larger cohort of young patients with IBD^[72].

GR heterocomplex: The integrity of the mature GR heterocomplex is required for optimal ligand binding and subsequent activation of the transcriptional response, and abnormalities in the chaperones and co-chaperones that make up the heterocomplex may contribute to altered GC responsiveness^[73].

Although no study has considered the role of these abnormalities in IBD, altered levels of Hsp90 have been

found in peripheral blood mononuclear cells from individuals with steroid-resistant forms of asthma^[74], multiple sclerosis^[75], and idiopathic nephrotic syndrome^[76]. Kojika *et al.*^[77] have shown that alteration in Hsp90 and Hsp70 is associated with decreased sensitivity to GCs in human leukemia cells. However, no relationship has been found between Hsp90 mRNA and resistance to GCs in spare bone marrow or peripheral blood samples taken from ALL patients at diagnosis^[78]. SNPs in the Hsp90 genes (HSPCA encoding hsp90-1 α , HSPCB encoding hsp90-1 β) have been recently described; however, in an adult asthmatic population, no correlation was found between SNPs in the HSPCA (3'-UTR+307, rs3736807, rs4906178, rs3809386, promoter -32) or HSPCB (rs504697 and rs3757286) genes and response to treatment^[79].

Generally, steroid receptors display functional instability when deprived of chaperones. FKBP51, coded by the FKBP5 gene, is a negative regulator of GC action and reduces GR binding affinity^[80]. However, when GCs binds to the GR, the receptor complex is activated and FKBP51 is replaced by FKBP52 (coded by FKBP4 gene), a positive regulator of GR signaling^[81,82]. The relative levels of FKBP51 and FKBP52 have been shown to be important determinants of GC cellular sensitivity in various systems. One recent study has investigated the role of FKBP5 genetic variants in the response to GCs in asthmatic patients^[79], however the studied polymorphisms (rs3800373, rs9394309, rs938525, rs9470080, rs9368878 and rs3798346) were not correlated with response. In this study, genetic variations in the *STIP1* gene, which codes for the co-chaperone Hsp70/Hsp90 organizing protein, on the contrary, seemed to have a role in identifying asthmatic subjects who were more responsive to GC therapy^[79].

Nuclear transport factors: Importins represent important players in GC pharmacology, as they mediate nuclear translocation through nuclear pore complexes. Importin 13 (IPO13) has been functionally characterized as a primary regulator of GC-bound GR across the nuclear membrane^[23]. IPO13 was first discovered as a GC-inducible gene that is important in lung development^[83], therefore, its functional characterization has been performed in airway epithelial cells and lung-derived transformed cell lines^[84]. Inhibition of lung epithelial cell IPO13 production reduces the nuclear translocation of GR from the cytoplasm, and subsequent GC-mediated silencing of inflammatory cytokine production^[84], which suggests that the normal anti-inflammatory response induced by GCs is dependent on normal IPO13 expression levels or activity. Therefore, irregular GC-GR signaling, ascribable to IPO13 variation, might affect the therapeutic responsiveness to GCs. IPO13 is expressed in other tissues as well, such as fetal brain, heart, kidneys and intestine^[83,85], therefore, it is feasible to hypothesize that dysregulation of IPO13-mediated processes might account for the variable response to GCs also in other diseases, such as IBD.

Importins are interesting candidates for pharmacogenomic studies, although little is known about their genetic variants. Recently, the association of 10 IPO13 polymorphisms with responsiveness to inhaled GCs, measured by

change in methacoline dose required for a 20% drop in FEV1, has been investigated in children with mild to moderate asthma^[86]. Unexpectedly, the genetic effects conferred by IPO13 variants were clinically significant in subjects who were randomized to placebo and nedocromil treatment (control group), rather than those treated with the inhaled GC budesonide, either in single SNP or in haplotype analysis. No other study on IPO13 genetic variants is available to date, therefore, these results require confirmation and further investigations are needed to shed light on the putative role of IPO13 mutants in GC-resistant or GC-hyper-responsive cases in other chronic inflammatory diseases.

Transcription machinery: In eukaryotic cells, gene transcription is inhibited by condensed chromatin structure, which prevents the interaction between gene-specific transcription factors and their DNA recognition sequences, thus blocking the access of the transcriptional machinery to DNA^[87]. Dynamic chromatin remodeling is therefore a fundamental mechanism in mediating genomic effects, and a large family of protein complexes that promote chromatin restructuring in an ATP-dependent manner, by disrupting histone-DNA contacts, has been described^[88-90]. Among these, the highly conserved mammalian SWI/SNF chromatin remodeling complex is the one recruited during GC-dependent gene activation^[91-93]. The complex is composed of several subunits^[94,95], including a catalytic ATPase subunit (either SMARCA4/BRG1 or SMARCA2/BRM) and Brahma-related gene 1 (BRG1)-associated proteins^[96], such as the SWI/SNF-related, matrix-associated, actin-dependent regulators of chromatin (SMARCC1, SMARCC2, SMARCD1-3, SMARCE1, SMARCB1 and ACTL6-A-B^[94,95]).

Alterations in any component of the SWI/SNF complex might be responsible for GC resistance, if access of GC-bound GR to DNA is compromised because of an impaired or suboptimal chromatin remodeling and nucleosome disruption. Using a genome-wide approach in patients with newly diagnosed childhood B-lineage ALL, differential expression of a relatively small number of genes has been shown to be associated with drug resistance and treatment outcome^[97]. Lower expression of SMARCB1 is related to resistant leukemia cells and, vice versa, higher levels are associated with GC-sensitive ALL^[97]. Decreased expression of other core subunits of the SWI/SNF complex, such as SMARCA4 and ARID1A, is also associated with GC resistance in primary ALL cells^[98].

To date, only a few studies have addressed the functional effects of SMARC polymorphisms. By using the human CEPH (Centre d'Etude du Polymorphisme Humain) cell lines from 90 individuals and sequencing the SMARCB1 promoter region, a regulatory SNP (-228G>T) has been discovered: the TT genotype has been functionally associated with higher SMARCB1 expression at both mRNA and protein levels^[99], and a positive association has been found between the SMARCB1 mRNA level and prednisolone sensitivity, as evaluated by MTT assay. In addition, knockdown of SMARCB1 in human ALL cell lines has confirmed that reduced SMARCB1 expression induces GC resistance^[99].

Pro-inflammatory mediators in the downstream signaling pathway of the GC-GR complex

Inflammation can be seen as a physiological homeostatic process elicited by the body in response to injurious agents, to protect tissues and to help in recovery. However, inflammation itself can potentially lead to tissue damage, if the organism does not properly control the process. Endogenous GCs are involved in the regulation of many physiological functions and assist the innate and adaptive immunity^[100-102] by balancing pro- and anti-inflammatory mediators, thus preventing overwhelming inflammation^[103].

According to gene expression analysis of human peripheral blood mononuclear cells from healthy donors, approximately 20% of genes, particularly those involved in the immune response, are regulated either positively or negatively in response to DEX treatment^[104]. GCs downregulate the expression of pro-inflammatory cytokines [such as IL-1 α , IL-1 β , IL-8, interferon (IFN)- α and IFN- β ^[104,105]], chemokines^[106,107], adhesion molecules, inflammatory enzymes and receptors. At the same time, GCs upregulate the expression of other cytokines that suppress the production of inflammatory mediators (such as transforming growth factor- β 3 and IL-10), thus boosting the anti-inflammatory effects.

Pro-inflammatory cytokines are involved in the pathogenesis of several chronic inflammatory diseases such as rheumatoid arthritis, osteoarthritis, asthma and IBD^[108,109]. Their excessive expression is generally efficiently counteracted by GCs^[110]. Individuals with steroid-non-responsive forms often show higher levels of local and/or systemic pro-inflammatory cytokines^[111-113].

Research of the cytokine profile in relation to GC resistance has been mainly performed in steroid-resistant asthma, and only a few studies have involved patients with IBD. Several *in vitro* data suggest that cytokines modify GC effects by interference with the GR signaling^[114]. IL-1 α has been reported to inhibit DEX-induced GR translocation in mouse fibroblasts^[115] and to downregulate the GR in a rat hepatoma cell line^[116], whereas IL-1 β inhibits GR function in colonic epithelial cells^[117]. Tumor necrosis factor (TNF)- α decreases corticosteroid sensitivity in monocytes by downregulation of GR^[118], and, in patients with UC, mucosal levels of this cytokine, as well as of IL-6 and IL-8 are higher in steroid-resistant patients^[119]. In a recent study, a large panel of cytokines was studied and their expression was correlated with steroid response. In particular, high IL-10 expression significantly enhanced steroid action, while IL-2 appeared to have the greatest antagonistic effect on the antiproliferative activity of steroids^[19]. This cytokine has been also related to increased GR β expression^[120], decreased translocation of the GR-GC complex to the nucleus^[121], and increased AP-1 levels, with abnormal interaction with the GR^[122]. IL-2 and IL-4 reduce GR affinity and T-cell response to GCs *in vitro*^[43], by a mechanism that involves p38 mitogen activated protein kinase (MAPK) activation^[123]. GCs inhibit the MAPK signaling pathway, through the induction of MAPK phosphatase 1 (MKP1), and this could result in inhibition of expression of various inflammatory genes^[124]. In

conclusion, a complex circular interplay between GCs and cytokines takes place, with GCs downregulating pro-inflammatory cytokines and cytokines limiting GC action.

Basal cytokine expression levels are fine-tuned by genetic profile. Polymorphisms in the cytokine regulatory regions determine a "lower/higher cytokine producers" phenotype that might in part be responsible of inter-individual variations, in terms of severity of inflammation and therapy responses. Higher cytokine producers might become GC-resistant or they might be less responsive at standard doses, therefore requiring dose adjustment to overcome the inflammation; however, only a few studies have focused on the effect of these genetic polymorphisms on GC response.

IL-1 β : Among the major inflammatory cytokines, IL-1 has a pivotal role. IL-1 is constituted by two distinct polypeptides, IL-1 α and IL-1 β . IL-1 β can promote the production of matrix metalloproteinases and the synthesis of prostaglandins^[110,125], as well as the production of other inflammatory mediators such as IL-6, IL-8 and TNF- α , thus amplifying the inflammation cascade^[126]. IL-1 β is therefore a key player in prompting and maintaining inflammation^[119,127] and polymorphisms in the *IL-1 β* gene might be of primary relevance in the modulation of GC response.

A cluster of genes encoding for IL-1 α , IL-1 β and for the natural receptor antagonist IL-1Ra has been mapped on chromosome 2q13^[128,129]. Polymorphisms in this gene cluster have been associated with a large variety of human diseases^[130] and with altered levels of IL-1^[131-134]. So far, the dbSNP build 130 database of NCBI reports 158 submissions for human IL-1, among which, several SNPs are described^[131,135]. Two SNPs in the IL-1 β promoter region (C-511T and T-31C) exhibit almost complete linkage disequilibrium. The C-511T SNP (rs16944) results in the loss of AP-2 binding site, while the T-31C (rs1143627) results in the loss of the first T in TATA box. The latter SNP appears to cause a paradoxical increase in IL-1 β in the presence of steroids^[136], which could be relevant to the occurrence of GC resistance. Carriers of the haplotype composed of IL-1 β -31C allele and -511T allele showed higher plasmatic concentrations of the cytokine, compared to subjects with IL-1 β wild-type genotype in Caucasians^[132,137], but decreased mRNA and protein levels in Japanese subjects^[138]. A recent report from this laboratory has investigated the role of the C-511T polymorphism in the response to GCs in 154 young IBD patients, but no relation with GC resistance (14 patients) or dependence (54 patients) was found^[72].

IL-1 β is released as an inactive precursor (pro IL-1 β , p35) by monocytes and macrophages in response to inflammatory stimuli, and is cleaved to the active mature form (p17) by caspase-1^[139,140]. Caspase-1 is part of multiprotein cytoplasmic complexes, called NACHT leucine-rich-repeat protein 1 (NALP1) and NALP3 inflammasomes. Jin *et al.*^[141,142] have recently shown that variants of NALP1 are associated with several autoimmune diseases, and have suggested that mutations in NALP1 gene may result in deregulated secretion of IL-1 β . The rs12150220

non-synonymous polymorphism, previously reported to confer susceptibility to autoimmune and autoinflammatory diseases^[143] results in the Leu > His amino acid variation in position 155, between the N-terminal pyrin and NACHT domains of the human NALP1 protein. This region has been highly conserved through primate evolution, which suggests a critical role in protein function^[142]. Data from our laboratory have shown that pediatric IBD patients, carriers of the NALP1 homozygote rs12150220 variant genotype, exhibit a higher probability of non-response to GC therapy^[72].

TNF- α : TNF- α is a potent pro-inflammatory cytokine released by cells of the immune system upon stimulation, and is almost not detectable in resting conditions^[144]. The gene encoding TNF- α is located in the class III region of the major histocompatibility complex within the human leukocyte antigen (HLA) on chromosome 6p21.3^[145]. This part of the genome is one of the most polymorphic in humans and contains many genes that encode proteins involved in inflammatory and immune responses^[146].

The G-308A (rs1800629) is one of the best documented polymorphisms of the TNF- α gene^[147]. This SNP lies in a binding site for the transcription factor AP-1 and the A allele has been shown to have higher transcriptional activity than the G allele, increasing TNF- α production *in vitro*^[148]. The A allele carriers show an enhanced susceptibility to several autoimmune and inflammatory disorders, such as systemic lupus erythematosus, celiac disease^[149], Alzheimer disease^[150,151], IBD^[152], asthma^[153] and rheumatoid arthritis^[154].

The polymorphism has been correlated also with steroid response in several diseases. In a cohort of 386 pediatric IBD patients, the mutated allele was significantly associated with steroid resistance and requirement for surgery in the subset of 200 CD patients^[152]. In addition, previous evidence in a large population of CD patients has shown that this polymorphism is more frequent in steroid-dependent subjects; possibly as a consequence of a more intense TNF- α -driven inflammatory reaction at the mucosal level^[155]. The AA genotype has been associated with reduced response to steroids also in other diseases, such as idiopathic nephrotic syndrome^[156], rejection episodes in HLA-DR mismatched transplant patients^[157] and solid and lymphoid malignancies^[158,159].

IL-10: IL-10, known as human cytokine synthesis inhibitory factor, is a cytokine that is produced primarily by monocytes and to a lesser extent by lymphocytes. IL-10 has pleiotropic effects in immunoregulation and inflammation^[160-162]. It inhibits the production of inflammatory mediators, and can be considered as a natural immunosuppressant of TNF^[163]. GCs upregulate the expression of IL-10^[104], and conversely, IL-10 acts synergistically with GCs, improving the ability of DEX to reduce IL-6 secretion in whole-blood cell cultures. In addition, the cytokine increases the concentration of DEX-binding sites in these cells, with no effect on the binding affinity^[118]. The human IL-10 gene is located on chromosome 1q31-q32. Previous studies have demonstrated that an A>G polymorphism at

nucleotide position -1082 within the IL-10 gene promoter region (rs1800896) influences the cytokine plasma levels, which are significantly higher in patients homozygous for the G allele^[159]. No data exist on the role of IL-10 polymorphisms in GC response in IBD, however, the mutated genotype is related to a positive prednisone response in childhood ALL^[159]. In accordance, Marino and colleagues^[63] have found a better response to GC remission induction therapy in Italian pediatric ALL patients with the mutated allele. In patients affected by rheumatoid arthritis, the high IL-10 producer genotype (-1082GG) is also associated with a favorable outcome, specifically to prednisone therapy^[164].

The multidrug resistance gene and other proteins involved in the pharmacokinetics of GCs

Cellular extruding pumps and metabolizing enzymes are the two key pathways involved in the elimination of many drugs. P-gp is one of the major transporters that extrude xenobiotics and drugs out of the cells. The cytochrome P450 (CYP) enzyme superfamily, that catalyzes phase I reactions, is active in the metabolism of many exogenous and endogenous compounds. P-gp and CYP3A are expressed in the intestinal epithelial cells, in lymphocytes and in the liver and have been reported to be involved in the transport and metabolism of GCs.

P-gp: P-gp is a 170-kDa transport membrane glycoprotein, which is responsible for resistance to a number of structurally and functionally unrelated drugs in clinical use. Human P-gp is a phosphorylated and glycosylated protein that consists of 1280 amino acids and two homologous and symmetric sequences, each containing six transmembrane domains and an ATP-binding motif. ATP hydrolysis provides the energy for active drug transport against a steep concentration gradient^[165]. The protein plays an important role in the absorption, distribution, and elimination of drugs that are its substrates, among which GCs, and P-gp inhibitors reduce cortisol efflux from human intestinal epithelial cells and T cells^[166]. The protein is highly expressed in circulating lymphocytes and epithelial cells from CD and UC patients with GC-resistant IBD^[167], however a relationship between GC administration and P-gp expression has been described in monocytes from IBD patients^[168]. It remains therefore to be clarified if the increased expression of P-gp reflects a primary phenomenon in IBD or a secondary one, influenced by GC therapy.

P-gp is encoded by the ABCB1 (MDR1) gene, located on human chromosome 7q21.12^[169], and several studies have demonstrated that genetic polymorphisms in this gene lead to functional alterations and phenotypic variations in P-gp expression^[170,171]. The first systematic screening for ABCB1 polymorphisms was performed in 2000^[170]: a synonymous SNP in exon 26 (C3435T) was the first variation to be associated with altered protein expression. P-gp expression in the duodenum of individuals with the homozygous variant T allele was decreased when compared with individuals with the C allele (wild-type). Other studies have shown that SNPs at exons 12 (C1236T), 21 (G2677T/A) and 1b (T-129C) may also be associated with altered

transport function or expression^[172]. A weak association between GC refractory CD and MDR1 polymorphisms in introns 13 and 16 has been initially described^[173], however, subsequent studies have not confirmed this observation. In 2007, Cucchiara and colleagues studied the C3435T MDR1 polymorphism in 200 pediatric Italian patients with CD and 186 patients with UC treated with GCs, and demonstrated that this SNP was not associated with response to medical therapy^[152]. In confirmation of these results, a large study performed on an adult Italian IBD population did not find any association between the polymorphisms of the MDR1 gene (C3435T and G26677T/A) and clinical response to GC therapy^[174]. Further studies are therefore needed to clarify if MDR1 polymorphisms play a role in steroid resistance in IBD.

CYP3A: CYP3A is the primary CYP subfamily in humans and is responsible for the phase I metabolism of > 50% of drugs currently in use^[175], including GCs^[176]. Many drug substrates of CYP3A4 are also substrates of P-gp, and the overlap between CYP3A4 and P-gp is also emphasized by the genomic proximity of these genes (7q22.1 and 7q21.1 for CYP3A4 and MDR1, respectively)^[172]. The CYP3A activity of the adult human liver is the sum activity of all subfamily members, including CYP3A5, which is highly polymorphic in Caucasians^[177], and genetic variants may play an important role in inter-individual differences in the pharmacokinetics of drugs metabolized by CYP3A5.

The most frequent and functionally important SNP in the *CYP3A5* gene consists of an A6986G transition within intron 3 (*CYP3A5*3*)^[175]: this mutation creates an alternative splice site in the pre-mRNA and results in production of an aberrant mRNA (SV1-mRNA) that contains 131 bp of intron 3 sequence (exon 3B) inserted between exons 3 and 4. The exon 3B insertion turns out in a frameshift and predicted truncation of the translated protein at amino acid 102. Additional intronic or exonic mutations (*CYP3A5*5*, **6*, and **7*) may alter splicing and result in premature stop codons or exon deletion^[178,179].

No report is present in the literature about the role of CYP3A5 polymorphisms in GC resistance in IBD, but plasma prednisolone concentrations, measured by HPLC in 95 renal transplant recipients treated with repeated doses of triple therapy immunosuppression, consisting of prednisolone, tacrolimus and mycophenolate mofetil, have been recently studied^[180]. The AUC₀₋₂₄ of prednisolone in recipients having both MDR1 3435CC and *CYP3A5*3*/**3* genotypes tends to be higher than the MDR1 3435TT plus *CYP3A5*3*/**3* genotype. The *CYP3A5*3* polymorphism has also been included in a study to identify pharmacogenomic predictors of outcome in 70 pediatric heart transplant patients followed for at least 1 year post-transplantation, but in this population, no correlation of the *CYP3A5*3* SNP with steroid response after transplantation was observed^[181].

CONCLUSION

GCs are potent anti-inflammatory drugs and, despite con-

siderable adverse effects, still remain the first-line therapy for inducing a rapid remission in moderate to severe active CD and UC. In addition, high inter-individual variability is observed when these agents are administered to patients with IBD and other chronic inflammatory diseases. A main goal in this area of medicine is therefore to improve the efficacy and safety of these agents and, when possible, to reduce steroid exposure and to employ a non-steroid option. This is particularly important in patients that do not respond, who will suffer considerable steroid-dependent morbidity without any clinical gain.

Pharmacogenomics is a relatively novel branch of investigation and represents an innovative frontier of medicine. Its ambitious goal is to identify genetic determinants that will help physicians in diagnostic decisions, and supply reliable genetic tools to adjust treatment *a priori*. A knowledge of genetic profiles with an impact on drug response would improve cure rates, avoid inadequate regimens or time wasting, and reduce overall health-care costs.

Pharmacogenetic research in IBD has witnessed only modest success; in particular, because treatment response in this disease is influenced by many factors, such as disease duration, behavior and severity, and at present, despite intensive investigation, none of the potential pharmacogenetic markers is strong enough to be used in clinical practice. Reported genetic associations have not yet shown consistent or robust results, and for most of the considered SNPs, results are controversial. More studies with larger sample size and well-characterized patient cohorts, uniformly treated and systematically evaluated, are therefore needed. These studies should support findings with greater statistical confidence that should be hopefully translated into clinical practice in the near future.

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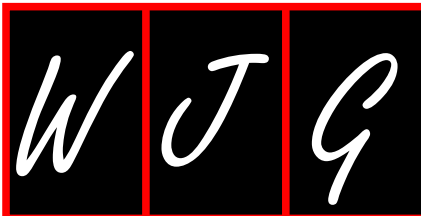
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Digestive oncologist in the gastroenterology training curriculum

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Abstract

Until the late 1980s, gastroenterology (GE) was considered a subspecialty of Internal Medicine. Today, GE also incorporates Hepatology. However, Digestive Oncology training is poorly defined in the Hepatogastroenterology (HGE)-curriculum. Therefore, a Digestive Oncology curriculum should be developed and this document might be a starting point for such a curriculum. HGE-specialists are increasingly resisting the paradigm in which they play only a diagnostic and technical role in the management of digestive tumors. We suggest minimum endpoints in the standard HGE-curriculum for oncology, and recommend a focus year in the Netherlands for Digestive Oncology in the HGE-curriculum. To produce well-trained digestive oncologists, an advanced Digestive Oncology training program with specific qualifications in Digestive Oncology (2 years) has been developed. The schedule in Belgium includes a period of at least 6 mo to be spent in a medical oncology department. The goal of

these programs remains the production of well-trained digestive oncologists. HGE specialists are part of the multidisciplinary oncological teams, and some have been administering chemotherapy in their countries for years. In this article, we provide a road map for the organization of a proper training in Digestive Oncology. We hope that the World Gastroenterology Organisation and other (inter)national societies will support the necessary certifications for this specific training in the HGE-curriculum.

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Key words: Gastroenterology; Training; Digestive oncologist; Curriculum; Chemotherapy; Immunotherapy; Oncology; Targeted therapy

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INTRODUCTION

Until the late 1980s, gastroenterology (GE) was considered a subspecialty of Internal Medicine. However, since then, GE has become more complex, in both endoscopy and drug therapy, and the speciality now also incorporates Hepatology. We now train Hepatogastroenterologists (HGE-specialists). It is a challenge to develop a training program that will produce HGE-specialists who are competent in all aspects of HGE by the end of their program^[1,2].

In 2002, the Dutch Board for Hepatogastroenterol-

ogy extended GE training to 4 years, with a Common Internal Medicine Trunk of 2 years^[3]. In the final year of training, a fellow subspecializes, if possible/desirable, in advanced endoscopy, neuromotility, hepatology, or (digestive) oncology. Training in hepatology has been well defined^[4,5]. Digestive Oncology training is currently poorly defined^[1]. The European Board for Gastroenterology and Hepatology has defined subspecialties in advanced endoscopy, hepatology, clinical nutrition, and, indeed, Digestive Oncology. However, a proper curriculum is lacking (<http://www.eubog.org>)^[6]. The World Gastroenterology Organisation (WGO) formulated a document outlining standards for HGE training^[5]. However, again Digestive Oncology was poorly defined. Therefore, a Digestive Oncology curriculum needs to be developed and subjected to regular revision. This document serves as a starting point for such a curriculum.

DIGESTIVE ONCOLOGY

Today, there are remarkable opportunities for those seeking a career in Digestive Oncology^[1,7]. The revolution in molecular biology, advances in interventional endoscopy and anti-cancer therapies, as well as the management of subsequent treatment-related side effects, have permanently changed the way we care for our patients with digestive tumors. HGE-specialists are increasingly resisting the paradigm in which they play only a diagnostic or technical role in the care of digestive tumors. They now perceive the empty space they need to fill with their knowledge. HGE-specialists are pre-eminently competent to organize supportive care around the Digestive Oncology patient. They recognize the caveats in the poor nutritional condition of the patient and are able to immediately take supporting measures that are necessary for completion of the patient's treatment. This also holds true for situations in which patients experience toxicity induced by different anti-cancer treatments. A new generation seeks to assume this more central role in the multidisciplinary care of our patients. With proper training, we see no reason why future generations of gastroenterologists should be barred from the delivery of anti-cancer therapies, because some might term it "chemotherapy".

Minimally invasive laparoscopic approaches have been developed for almost all digestive tumors, and image-guided intervention is providing an innovative therapeutic option for early cancers^[8]. Modern evidence-based "outcomes research" provides an objective tool for assessing clinical results. Patient-completed questionnaires and standardized assessment of individual preferences have helped us to understand survivorship issues among Digestive Oncology patients, including the long-term effects of treatment on quality of life^[9,10].

The HGE-specialist has the immense advantage that he/she has can both recognizing malnutrition or obstructing symptoms threatening the patient's condition in an early state, can subsequently visualize this endoscopically, and can then take the necessary measures to resolve

such devastating situations. Obviously, full insight into the possible treatment plan for this vulnerable patient group is warranted.

Surgical excellence remains critically important in the field of Digestive Oncology. Despite the development of modern anti-cancer drug therapy, (intra-operative) radiotherapy, and the combined use of these treatment modalities, the need for optimal surgery has not been eliminated. Rather, the pre-operative use of such therapies has challenged surgeons to develop the necessary skills to operate on highly pre-treated tumors, whilst simultaneously reducing the burden of surgical morbidity. Similarly induction chemo(radio)therapy for oesophageal and gastric cancers has also expanded the role of surgery, by allowing the resection of some cancers previously considered inoperable^[11,12]. Future leaders in Digestive Oncology must be skilled gastroenterologists and surgeons capable of advanced endoscopy, laparoscopic techniques, and image-guided therapy. They must also be scholars, well versed in the nuances of modern diagnostic and staging procedures, fully appreciative of the benefits and limitations of anti-cancer therapy (chemotherapy, immunological, and targeted therapy) and radiation therapy (including chemoradiation).

Comprehensive training also requires exposure to research, either in the basic sciences, translational research, and/or clinical trials. This will prepare future HGE-specialists to explore hypotheses about digestive tumors in an effort to improve patient care.

SOCIETIES FOR DIGESTIVE ONCOLOGY

The European Society of Digestive Oncology (ESDO) was founded in 2008 (<http://www.esdo.com>). Currently, more and more academic centres are offering fellowships in Digestive Oncology. According to the ESDO membership details, essential requirements of the digestive oncologist include: expertise in multidisciplinary care, the ability to perform and understand the limitations of complex tumor treatments, a clear understanding of the biology and science of digestive tumors, and the ability to collaborate in (translational) research. We suggest that active membership of ESDO should be encouraged for HGE-specialists who devote > 50% of their total professional activity to the field of digestive tumors, who have presented at least one oncology paper in a national meeting, are board certified by the European Board of Gastroenterology and Hepatology (<http://www.eubog.org>) or the respective equivalent in their country of origin, and have additional oncology experience following formal HGE training. The goal of advanced training in Digestive Oncology is to enhance knowledge and skills beyond the expertise obtained during a normal HGE residency program. The duration of advanced fellowship training might be 24 mo, with a minimum of 12 mo of clinical exposure during the formal 6 years of HGE training (focus year) and 12 mo of formal Digestive Oncology. If applicants have completed research in Diges-

tive Oncology, for example a PhD under the auspices of governing bodies such as ESDO (or equivalent in their country), they might apply to have the 6 mo research requirement waived during their 24 mo of advanced fellowship training in Digestive Oncology: this should be discussed.

PROGRAMS IN DEVELOPMENT

All training programs are required to provide a structured educational experience at an advanced level to ensure that trainees acquire the knowledge and skills necessary to gain expertise beyond that acquired in the standard HGE residency^[1]. Access to patient care and multidisciplinary team discussions with medical oncologists, radiation oncologists, surgeons, pathologists, and radiologists who have expertise in digestive tumors are an additional requirement for the trainees. Programs must provide structured clinical opportunities for trainees to develop advanced skills in interventional endoscopy. Each fellow on the advanced Digestive Oncology program should be involved in at least 100 major interventional procedures for the treatment of digestive tumors. Additionally, a research component should be discussed with structured supervision for at least 6 mo. If basic science laboratory training is offered, the necessary facilities must be available under the supervision of a trainer who has demonstrated at least a national reputation in research, as evidenced by publications in peer-reviewed journals, and membership of digestive oncological societies. As an example of one of the Dutch HGE-approved fellowships for a focus year in formal HGE training, the following section describes the suggested curriculum for Digestive Oncology.

DIGESTIVE ONCOLOGY IN THE DUTCH TRAINING OF HGE-SPECIALISTS (FOCUS YEAR)

In the Netherlands, during the last year of HGE training, it is possible to focus on Digestive Oncology^[3]. In this year, a minimum of six sessions per week must be spent on oncology-related activities in the context of the focus area. The content of the training within the professional body and the terms for the fellowship have been designed under the auspices of the National Board for Hepato-Gastroenterology.

END OF TRAINING COMPETENCIES

Knowledge

Knowledge of the duties/remit as described by the end of training competencies for general HGE-specialists, with particular emphasis on anti-cancer drug treatment and radiotherapy treatment possibilities; knowledge of primary and secondary prevention of digestive tumors; knowledge of hereditary cancer and polyposis syndromes

affecting the digestive tract; knowledge of the rarer digestive tumors, such as anal carcinomas, hepatocellular carcinomas, GI-lymphomas (MALT-lymphomas and Enteropathy Associated T-cell lymphomas), GIST tumors, neuro-endocrine tumors, cystic pancreas tumors; knowledge of advanced endoscopic techniques for diagnosis, staging, and treatment of pre-malignant disorders of the gastrointestinal tract, such as chromo-endoscopy, Endoscopic Ultrasonography (EUS), ablative techniques, and endoscopic mucosal resection; knowledge of palliative care for malignant digestive disorders and early recognition patients in need of nutritional support; and knowledge of side-effects of different anti-cancer treatment modalities.

Skills

Skill in procedures such as those described by the end of training general HGE-specialists; optional (overlap with focus year of advanced endoscopy); endoscopic treatment of malignant stenoses of the oesophagus, stomach, duodenum, and colon; recognition and identification of premalignant lesions; endoscopic treatment of anastomotic leakages after surgery; percutaneous endoscopic gastrostomy (PEG) placement; endoscopic ultrasound (EUS) and EUS-guided fine needle aspiration (FNA); endoscopic ablative treatment, such as Photo-Dynamic Therapy, electrocoagulation, argon plasma coagulation (APC), and intraluminal radiotherapy; and endoscopic mucosal resections in the oesophagus, stomach, duodenum, and colorectum.

Learning environment

A minimum of two HGE-specialists with an interest in oncology, one of whom is recognised as a mentor by the National Society in the focus area; weekly multidisciplinary oncology meetings; and practice within the department in EUS, including diagnostic biopsy, endoscopic ablative treatment methods, intraluminal radiotherapy, endoscopic treatment of malignant stenoses in the oesophagus, stomach, duodenum, and colorectum, and endoscopic mucosal resections. The department should be active in the development of newly targeted endoscopic diagnostic and therapeutic modalities for pre-malignant abnormalities. Within the hospital the personnel should include: registered medical oncologists (internist-oncologist), minimum two; registered surgical oncologists (surgeon-oncologist), minimum two; radiation oncologists, minimum two; and a clinical geneticist, possibly seconded or on a consultative basis.

Training

(Endoscopic) activities, based on the end of training targets; medical oncology, minimum 40 sessions; radiation oncology, minimum 10 sessions; attendance at oncological/surgical interventions (oesophageal resection, gastric resection, pylorus-preserving pancreatoduodenectomy (PPPD), liver resection, colon resection, total mesorectal excision (TME)-resection, only once); a minimum of 10

sessions of pathology; and a minimum of 10 sessions of clinical genetics.

Participation in discussions and structured consultations

At least weekly multidisciplinary oncology meetings with minimum participants to include an internist-oncologist, an oncological surgeon, a radiotherapist, a radiation oncologist, a pathologist, and a nuclear medicine specialist. At least one meeting per quarter should include a clinical geneticist.

Scientific activities

Conference attendance: minimum of one attendance at an international clinical oncology conference (ASCO, ESMO, ESDO) or an international interdisciplinary Digestive Oncology conference. The fellow should take an active part in a research project in the area of oncology, preferably in the context of a research degree. The fellow should write at least one scientific publication on an oncology-related subject and present at least one abstract concerning an oncology-related subject at a national or international conference.

Quality control

GI training and quality inspection at a regular interval of at least 5 years, organized and supervised by the national society for HGE.

Educational supervision

Trainers are required to provide appraisal during training and assessment that contributes to the evidence of competence of the fellow, who is recommended to provide a portfolio of assessed cases. Trainers must provide adequate on-site supervision for trainees at all times, as defined in the curriculum. Satisfactory assessments from trainers and completed log books that demonstrate that the fellow meets the criteria of competence are required for a fellow to be assessed as competent in Digestive Oncology (focus year).

Advanced Digestive Oncology training in Belgium (2 years)

The HGE-specialist of the future, or at least some of them, desire continuous care for their digestive tumor patients. To treat them, however, HGE-specialists should acquire skills. The necessary skills are currently beyond those taught during the HGE training in most countries. Since the beginning of 2010, there has been official recognition of HGE-specialists with a specific qualification in Digestive Oncology in Belgium^[13]. To obtain this qualification, the candidate has to do one oriented year (focus year) during the main training years for HGE and one additional year. Six months of this training period needs to be spent in a medical oncology department. The curriculum will be focused on: (1) the pathophysiology of the different types of gastrointestinal tumors; (2) diagnostic management; (3) development of a multidisciplinary treatment plan; (4) the correct administration of systemic treatments, including chemo- and immunothera-

py, biological and genetic treatments; (5) the management of side effects; (6) the management of tumor-related and iatrogenic complications; (7) cancer registration; (8) multidisciplinary approaches; (9) the evaluation and conception of clinical trials; and (10) active participation in palliation. To maintain their recognition, more than 50% of the specialist's working time has to focus on oncology.

Standards for safe administration of treatment

Chemotherapy ordering, preparation and administration; patient education and informed consent; staff education and training; assessing how patients respond to treatment; monitoring patient-related toxicity.

DIGESTIVE TUMORS

Risk factors in HGE-cancers; indicators for endoscopy in diagnosis; indicators for endoscopy in staging; indicators for nutritional support; combined modality therapy; role of palliative chemotherapy; chemoprevention; (family) screening; and genetic testing. The Curriculum of the European Society for Medical Oncology (ESMO) and the American Society of Clinical Oncology (ASCO) described in the Global Core Curriculum for training in Medical Oncology provides the main framework for the training for Digestive Oncology^[14].

FUTURE EUROPEAN BOARD PROGRAM DESCRIPTION

The goal of the future European HGE-Board fellowship program remains to produce, for the local countries, a framework of well-trained digestive oncologists who will be leaders in academic Digestive Oncology and be qualified to promote improvements in national care to reduce the incidence, morbidity, and mortality of digestive cancer, and improve quality of life^[6]. Disease management teams include specific cancer sites (oesophageal, gastric, colorectal cancer (CRC), pancreatic, liver, hepatobiliary, anal, neuroendocrine, and gastrointestinal stroma cell tumors) and comprise surgeons, (digestive) oncologists, radiation oncologists, palliative care physicians, dieticians, pathologists, radiologists, nuclear physicists, clinical geneticists, and laboratory researchers. In case of GI-lymphomas, such management teams should also include hematologists. Working in these disease management teams, fellows have an opportunity to participate in the development of multidisciplinary management plans for patients, as well as the design and pursuit research opportunities, including clinical trials. The role of these teams includes, for example, the enhancement and promotion of CRC-screening, early tumor detection, primary and secondary prevention, and management of pre-malignant or malignant HGE diseases, as well as the translation of research into the clinical setting.

CLINICAL EXPERIENCE

The future program of the European Board's curriculum

should be designed to provide the fellow with a practical knowledge of the most up-to-date diagnostic and therapeutic strategies for digestive tumors^[15]. The aim is to develop familiarity with, and stimulate interest in, clinical and laboratory studies designed to advance knowledge in the field. The responsibility for clinical care is shared by the faculty, fellows, residents, medical students, and nurse practitioners. Fellows are directly involved in the management of patients with digestive tumors. The volume and nature of the clinical experience is such that the fellow has an opportunity to participate in the management of patients with all types and stages of digestive tumors.

TRAINING CRITERIA

During the general HGE training, fellows should acquire better theoretical knowledge of the etiology, pathogenesis, natural history, clinical manifestation, work-up, and treatment of digestive tumors^[15]. Such knowledge should include nutrition, if necessary pre-operative, during treatment, and for palliative care. Knowledge of logistics, health care economics, and medical ethics seems mandatory^[16]. The fellow must have observed, and been responsible for, adequate numbers of patients with digestive tumors, both as inpatients and outpatients. Such patients include those with complications encountered during work-up, surgery, chemotherapy and radiotherapy. They should also include those in need of enteral and parenteral nutritional support, such as nasogastric feeding, PEG-/percutaneous endoscopic jejunostomy (PEJ)-feeding. Close cooperation with a hospital dietician should be part of this.

Clinical experience must be gained in paid positions, obtained by open and transparent competition. Teaching must be an integral part of this Digestive Oncology program. Supervision of clinical work should be defined locally.

DIDACTIC EDUCATION

The goal of the advanced 24-mo educational program of the European Board of HGE is to provide a broad view of all aspects of digestive tumours, and a familiarity with diagnostic and therapeutic approaches. Rotations in medical oncology for at least 4-8 mo are supplemented by lectures on systemic therapy. In an oncology setting, staff work in the presence of patients with cancer every day. The emotional impact of this work must be recognized, and fellows should be coached on this, not only to appreciate the impact of the cancers on the patients, but also on the impact of oncology care on non-licensed support staff^[17].

ONCOLOGY IN THE STANDARD HGE-CURRICULUM

The description of the end of training competency, learning environment, and the training are as indicated in

the Gastroenterology Consensus Document in the Netherlands dated 14 December 2004^[1,3].

With relation to oncology, we can quote from the current terms the following passages: (1) General end of training competency: medical aspects: prevention; knowledge of preventative medicine with emphasis on pre-malignant disorders of the digestive tract; and (2) specific syndromes: (a) the specialist is experienced in the area of malignant disorders of the digestive tract; and (b) during the training in HGE there should be sufficient attention and space for the following basic courses: (i) Clinical genetics; and (ii) Clinical epidemiology.

An important part of the workload of HGE-specialists involves oncology. Areas in which the HGE-specialist must have expertise and skills include: (1) prevention, screening and surveillance; (2) diagnosis and staging; (3) endoscopic therapy, both curative and palliative; (4) supportive treatment, for example in relation to nutrition; and (5) diagnosis and treatment of both short-term and long-term complications of oncological therapies, for example radiation damage and drug-induced treatment induced mucositis.

CRITERIA FOR THE END OF THE TRAINING COMPETENCY IS SPECIFIED AS FOLLOWS

Knowledge

Basic knowledge of epidemiology and pathogenesis of the frequently occurring digestive tumors; knowledge of symptomatology, diagnosis and staging of frequently occurring digestive tumors; basic knowledge of rare digestive tumors; basic knowledge of molecular tumor biology; knowledge of familial tumor syndromes, such as familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC), and MYH-associated polyposis (MAP), including surveillance advice and knowledge of indications for referral to clinical genetic centres; knowledge of surveillance (pro's and cons) of premalignant disorders, such as Barrett's oesophagus, intestinal metaplasia and atrophy in the stomach, adenomatous polyps, and ulcerative colitis; knowledge of policy with relation to the follow-up after anti-cancer treatment; knowledge of the screening of the general population for pre-malignant and malignant disorders of the digestive tract; knowledge of the current range of abdominal imaging techniques with relation to oncological diagnosis, and their indications; familiarity with microscopic pre-malignant and malignant disorders of the digestive tract; knowledge of endoscopic treatment possibilities with curative and palliative intent; basic knowledge of other treatment modalities, such as surgery, radiotherapy, and chemotherapy; basic knowledge with relation to the development of neo-adjuvant and adjuvant treatments of the different digestive tumors; knowledge of nutrition as an integral part of treatment and of the different ways that feeding can be administered; and knowledge of di-

gestive problems in patients with non-gastroenterological tumors, for example radiation enteritis and proctitis, and the metastasis and ingrowth of other tumors into the digestive tract (breast cancer, melanoma).

Skills

Conventional endoscopic diagnosis of tumors of the digestive tract; global assessment of current abdominal imaging techniques in the context of oncological diagnosis; endoscopic resection of colorectal and gastric polyps; treatment of mild to moderately severe radiation proctitis with the use of APC; PEG placement; drainage of malignant ascites; delivery of bad news and the counselling of patients and their family members.

Learning environment

Each HGE training clinic must have the necessary facilities to integrate oncological aspects of digestive medicine into general training. The HGE-specialist should participate in multidisciplinary oncological discussions concerning abdominal tumors.

Training

There is no reason to introduce a separate oncological training period into the general HGE training program. This is only required for those individuals choosing to specialize in Digestive Oncology (focus year). Through local, regional and national teaching courses, much of the knowledge that is not accumulated in daily practice can be acquired to fulfil the end of training competency.

Review

To develop anonymous national assessment tests. Test frequency of once every 2 years.

Monitoring

Through HGE trainer.

Quality control

HGE training and quality inspection.

HGE-TRAINING AROUND THE WORLD

Differences in HGE training between the USA/Europe, India/China and, for example Sub Saharan Africa, are magnified by the obvious resource gaps between Third and First World countries. Redesigning training programs more efficiently would be another goal based on a common trunk system for HGE^[18]. India and China, with a common trunk of Internal Medicine for 2-3 years, have incorporated private hospitals into their programs. Well-equipped private hospitals should be used during Digestive Oncology focus years in the East and West. In Eastern Europe, Ministers of Health recently suggested that training for HGE should be reduced to 4 years in total^[19]. Arguments about specialist training are getting louder as governments get poorer because the current credit crisis. However, a properly organized scheme for Digestive On-

cology is mandatory to give patients the best chance of benefiting from national resources.

CONCLUSION

In this article, we present the critical elements of multidisciplinary fellowship training for the digestive oncologist, and suggest minimal competencies for the standard HGE-curriculum. Digestive Oncology is substantially under-represented in the undergraduate and postgraduate curricula of present day HGE training programs. Cancer prevention and screening, in particular, have suffered from poor exposure in the past^[20,21].

At the present time, the HGE specialist organizes the work-up, endoscopic-intervention of early digestive tumors, nutritional support and palliation of HGE-oncological patients. Interestingly, HGE-tumors are diagnosed by endoscopists often at the tip of their scope. Chemotherapy (neo-adjuvant) should be included based on competence^[11,12,22]. HGE-specialists have an essential role in the treatment of complications of digestive cancer and cancer therapy, from the placement of stents to the relief of obstructions and feeding tubes/PEG's to help anorexia and malnutrition, to the management of radiotherapy-induced bowel disorders. Malnutrition increases complications and reduces tolerance to systemic treatment^[23]. The HGE-specialist has a critical role in primary and secondary prevention of digestive tumors. However, just preventing a polyp from becoming a tumor, just making a diagnosis, just staging a tumor by EUS, just making referrals, just treating early complications of the oncologist, radiotherapist or surgeons, just taking care of palliation are unlikely to be "only" roles of HGE-specialists nowadays. It is this idea of expansion of the scope of HGE-units that will necessitate advanced Digestive Oncology training for a minority of HGE-specialists. What about immunotherapy in cancer, such as is performed for IBD? What about chemotherapy, a red line for HGE-specialists? Subspecialization within medical oncology has occurred to provide the required expertise needed to provide optimal patient care. The field of Digestive Oncology is also rapidly evolving. It requires specialized knowledge and skills to appropriately and safely administer various anti-cancer drug agents, with subsequent management of their toxicities. Some HGE-specialists trained as sub-specialists of Internal Medicine already administer chemotherapy in their countries. Maintaining the continuity of patient care is certainly a worthwhile goal.

In our opinion, HGE-specialists with advanced oncology training should be part of the gastrointestinal oncology team and be able to administer anti-cancer therapy in the years to come. In this article we provide a road map to organize this training. The scope and personalization of the HGE-curriculum is our major challenge, and one that will change HGE once again in the coming years. The proper positioning of Digestive Oncology is an important part of this. We hope that the WGO, American Gastroenterology Association, the European Board of

Hepatogastroenterology, and other (inter)national societies will organize the necessary certification for this HGE-specialization.

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Recent results of laparoscopic surgery in inflammatory bowel disease

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Abstract

Inflammatory bowel diseases are an ideal indication for the laparoscopic surgical approach as they are basically benign diseases not requiring lymphadenectomy and extended mesenteric excision; well-established surgical procedures are available for the conventional approach. Inflammatory alterations and fragility of the bowel and mesentery, however, may demand a high level of laparoscopic experience. A broad spectrum of operations from the rather easy enterostomy formation for anal Crohn's disease (CD) to restorative proctocolectomies for ulcerative colitis (UC) may be managed laparoscopically. The current evidence base for the use of laparoscopic techniques in the surgical therapy of inflammatory bowel diseases is presented. CD limited to the terminal ileum has become a common indication for laparoscopic surgical therapy. In severe anal CD, laparoscopic stoma formation is a standard procedure with low morbidity and short operative time. Studies comparing conventional and laparoscopic bowel resections, have found shorter times to first postoperative bowel movements and shorter hospital stays as well as lower complication rates in favour of the laparoscopic approach. Even complicated cases with previous surgery, abscess formation and enteric fistulas may be op-

erated on laparoscopically with a low morbidity. In UC, restorative proctocolectomy is the standard procedure in elective surgery. The demanding laparoscopic approach is increasingly used, however, mainly in major centers; its feasibility has been proven in various studies. An increased body mass index and acute inflammation of the bowel may be relative contraindications. Short and long-term outcomes like quality of life seem to be equivalent for open and laparoscopic surgery. Multiple studies have proven that the laparoscopic approach to CD and UC is a safe and successful alternative for selected patients. The appropriate selection criteria are still under investigation. Technical considerations are playing an important role for the complexity of both diseases.

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Key words: Crohn's disease; Ulcerative colitis; Laparoscopic; Colorectal; Surgery

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INTRODUCTION

Laparoscopic techniques have rapidly gained acceptance since their first introduction into surgery for cholecystolithiasis. Other pathologies of the gastrointestinal tract have become indications within a short period of time. First attempts in minimally invasive approaches to colorectal surgery date back to the early 1990s. Potential benefits are evident: smaller incision size, improved

cosmesis, less postoperative pain, earlier return of bowel movements and tolerance to diet. These factors may be translated into a faster recovery of the patient in general with reduced floor costs, and earlier return of the patient to normal activity^[1].

In inflammatory bowel diseases, Crohn's disease (CD) and ulcerative colitis (UC) have to be distinguished clearly regarding the introduction of laparoscopy as the operative procedures are varied. In CD, there is a wide range of potential procedures whereas in UC, restorative proctocolectomy is the standard operation in elective situations. Early reports about the introduction of laparoscopy to CD demonstrated the feasibility of laparoscopic surgery for the creation of stomas and for limited segmental bowel involvement. Rapidly, more complex procedures like ileocelectomies or subtotal colectomies were attempted successfully. First results of laparoscopic restorative proctocolectomy and ileal pouch-anal anastomosis (IPAA) for selected patients with UC or indeterminate colitis were not encouraging. Only around the year 2000, with newly-developed instruments, refined technique, and in specialized treatment centers, this comprehensive procedure was reappraised with improved results. Also, subtotal colectomies for acute inflammatory bowel diseases may be managed laparoscopically.

Complicated cases of CD may still be a special challenge, even for surgeons with excellent experience in operations for IBD and intensive laparoscopic training. Rates of conversion from laparoscopic to conventional surgery are comparatively high^[2,3]. There are high rates of unexpected findings like proximal strictures, stenoses, abscesses or phlegmons^[4]. In this article, an overview of the current status of the special surgical approach to CD and UC is provided.

CD

In CD, in general, surgery is normally reserved for patients who develop complications of the disease such as strictures and fistulas or who are unresponsive to or develop complications from aggressive medical therapy. The laparoscopic approach should be an ideal indication since it is a benign disease and the concerns related to laparoscopic cancer surgery do not apply. Additionally, it may provide an improved cosmetic result, which is an important factor in this mostly young patient population^[5]. Thickened bowel loops, thickened and friable mesentery, inflammatory phlegmons and masses, enteric fistulas, abscesses, and multiple adhesions from previous conventional surgeries have deterred surgeons from considering a laparoscopic approach. However, as most patients are aware of the fact that there is a high risk of further surgeries becoming necessary at some later point in their lives, they are motivated to prefer a type of surgery that offers them minimal scarring and faster recovery^[6,7]. Even cases with complications may be attempted and completed laparoscopically, depending on the individual situation and the surgeon's expertise.

Early postoperative results

The decision to perform a laparoscopic procedure in an individual case as well as the conversion rates during surgery are influenced by the expertise of the surgeon which also determines the immediate postoperative outcome. After a number of years of application of the new technique, several studies about short and long-term results have been published.

The purpose of a study at the University of Chicago^[8] was to compare short-term outcomes of laparoscopic colectomy (LC) *vs* open colectomy (OC) in patients with Crohn's colitis. Data on all patients undergoing colectomy for primary or recurrent CD confined to the colon during 6 years were collected. Patient and disease-specific characteristics and perioperative and short-term postoperative outcomes were prospectively collected and analyzed. A total of 125 patients underwent colectomy during the study period, 55 (44%) LC. There were six conversions (10.9%). Median operative time was shorter in the LC group ($P = 0.032$). Earlier return of bowel function was noted in the LC group (3 d *vs* 4 d, OC). Length of postoperative stay was shorter in the LC group ($P = 0.001$). There was one death in the OC group. Postoperative complications occurred in 8 (14.5%) LC patients *vs* 16 (22.9%) OC patients. Disease recurrence rate was 16%, 10.9% LC and 20% OC, respectively.

It was stated that LC was a safe and effective technique in the hands of experienced surgeons. Benefits of LC in CD included reduced operative blood loss, quicker return of bowel function, and shorter hospital length of stay. Very similar results were found in studies published by Soop *et al*^[9], Tanaka *et al*^[10] and Kroesen *et al*^[11] who investigated the results of laparoscopic proctocolectomies with an incisionless technique.

For a long time it has been known that there is a higher leak rate in bowel resections for CD than for other benign conditions. In order to investigate the safety of laparoscopic (Lap) colorectal surgery as reflected by the anastomotic bowel leak (ABL) rate compared with that seen in open surgery, the Cleveland Clinic Foundation has recently evaluated its data^[12]. Between 2000 and 2007, 1516 consecutive patients undergoing laparoscopic surgery with bowel anastomosis were covariate-adjusted to 3258 patients undergoing open surgery by pathology and site of anastomosis using the institutional review board-approved laparoscopic, diverticular, Crohn's, and colorectal cancer databases. Of these patients, 643 patients in each group were equally matched by pathology, site of anastomosis, date of surgery, age, gender, and body mass index (BMI). The clinical ABL rate was compared between the two groups by the location of bowel anastomosis and year of surgery. A total of 4774 patients (1516 laparoscopic, 3258 open; mean age, 55.8 ± 17.4 years; BMI, 27.8 ± 6.2 kg/m²) underwent colorectal resection with bowel anastomosis (cancer 45.3%, Crohn's 29.6%, diverticulitis 12.3%, other 12.8%). There were no differences in the overall clinical ABL rates between laparoscopic (2.6%) and open procedures (2.1%, $P = 0.5$), between laparo-

scopic right *vs* open right ($P = 0.6$), between laparoscopic left *vs* open left ($P = 0.8$), and between patients operated on during different time periods ($P = 0.4$). For the case-matched 643 patients, there were no differences in clinical ABL rates between laparoscopic *vs* open groups based on site of anastomosis, pathology, and year of surgery. A laparoscopic colorectal approach was not associated with a higher risk of clinical ABL.

The largest unicentric study published until now comes from the Mount Sinai Hospital in New York City^[13]. The authors reviewed their experience with 335 laparoscopic resections for CD over the past 15 years in a retrospective analysis of a prospective database. The mean age of the patients was 39 years, 54% of the patients were women. In most cases, the indication for surgery was intestinal obstruction (73%) or abdominal pain (16%). The most common operation was primary ileocolic resection, performed for 178 cases (49%). Secondary ileocolic resections were performed for 20% and small bowel resections for 11% of the cases. Of the 117 patients with enteric fistulas, 45% had multiple fistulas. There were 80 enteroenteric, 51 ileosigmoid, 33 enteroabdominal wall, and 22 ileovesical fistulas. Multiple resections were performed for 33 patients (9%). Eight conversions occurred (2%), primarily because of large inflammatory masses involving the intestinal mesentery. The mean length of hospital stay was 5 d, and the mean operative time was 177 min (range, 62–400 min). There were no mortalities. The complications were primarily bowel obstruction, anastomotic leak, and postoperative bleeding, resulting in a postoperative complication rate of 13%.

Long-term results

The question has been raised for a long time if in laparoscopic resection for CD occult segments of disease may be missed at surgery and if the long-term result may be impaired this way. There had also been concerns if less of an immune response may be induced by laparoscopic methods compared with conventional surgery.

Long-term results of laparoscopically assisted *vs* open ileocolic resection for CD were evaluated in a randomized trial by Eshuis *et al.*^[14]. Sixty patients who underwent ileocolic resection between 1999 and 2003 were followed prospectively. Primary outcomes were reoperation, re-admission and repeat resection rates for recurrent CD. Secondary outcomes were quality of life (QOL), body image and cosmesis. Median follow-up was 6.7 years (interquartile range, 5.7–7.9 years). Sixteen of 29 and 16 of 26 patients remained relapse free after ileocolic resection in the laparoscopic and open groups, respectively. Resection of recurrent CD was necessary in two of 29 *vs* three of 26 patients. Overall reoperation rates for recurrent CD, incisional hernia and adhesion-related problems were two of 29 *vs* six of 26. QOL was similar, whereas body image and cosmesis scores were significantly higher after laparoscopy ($P = 0.029$ and $P < 0.001$, respectively). It was concluded that laparoscopic assisted ileocolic resection resulted in better body image and cosmesis, whereas open surgery was more likely

to produce incisional hernia and obstruction.

Long-term results of a prospective randomized study previously conducted at the Cleveland Clinic Foundation comparing laparoscopic (LC) and open ileocelectomy (OC) for ileocolic CD were published by Stocchi *et al.*^[15]. The purpose was to analyze long-term recurrence rates and complications. Follow-up data were available on 56 of 60 patients. Demographic data, recurrence rates, need for additional surgery related to primary procedure, and medication uses were recorded. Mean follow-up for 56 patients (27 LC *vs* 29 OC) was 10.5 years and comparable between LC and OC (10.0 *vs* 11.0, respectively, $P = 0.64$). One patient died 8 years after OC of causes unrelated to CD. One patient underwent incisional hernia repair after LC (4%) *vs* 4 patients (14%) after OC ($P = 0.61$). Two patients in the LC group underwent adhesiolysis *vs* none after OC ($P = 0.23$). Incidences of anorectal disease, anorectal surgery, endoscopic or radiologic recurrence, and medication use were also similar between LC and OC. OC patients requiring operation during follow-up were significantly more likely than LC to require multiple operations ($P = 0.006$). As a conclusion, long-term data confirmed that LC is at least comparable to OC in the treatment of ileocolic CD.

Complex CD

Goyer *et al.*^[16] analyzed in a prospective study the feasibility of laparoscopic ileocolonic resection for complex CD, i.e. recurrence or complication from abscess and/or fistula, and compared postoperative outcomes in patients with and without complex CD. During 10 years, 124 laparoscopic ileocolonic resections were attempted for CD: 54 patients with complex CD (group I) and 70 patients without complex CD (group II). Indications for surgery in group I included fistula (43%), abscess (30%), and recurrent disease after ileocolonic resection (27%). Complex CD was significantly associated with increased mean operative time (214 min *vs* 191 min, $P < 0.05$), increased conversion rate to open procedure (37% *vs* 14%, $P < 0.01$), and increased use of temporary stoma (39% *vs* 9%, $P < 0.001$). No patients died. Overall postoperative morbidity was similar between both groups (17% *vs* 17%), including major surgical postoperative complications (7% *vs* 6%, $P = \text{NS}$). Mean hospital stay was not statistically different between both groups (8 d *vs* 7 d, $P = \text{NS}$). This large comparative study suggested that laparoscopic ileocolonic resection for complex CD was feasible and safe with good postoperative outcomes.

Recurrent disease

Laparoscopic surgery is increasingly performed for primary, especially ileocolic CD, but its application in patients with recurrent disease is less well described. The aim of a study of the Mayo Clinic^[17] was to assess the safety, feasibility and potential short-term benefits of a laparoscopic approach. Patients undergoing laparoscopic surgery for recurrent ileocolic disease were identified using a prospectively maintained database. Potential risk factors for con-

version to open surgery and overall patient outcomes were assessed with univariate analysis. Forty patients were identified, of which 30 (75%) were completed laparoscopically and 10 (25%) were converted to open surgery. The groups did not differ with respect to clinicopathological features. Converted patients were significantly more likely to require adhesiolysis (100% *vs* 67%, $P = 0.04$). The groups did not differ with respect to incidence of postoperative complications or frequency of readmission within 30 d. There was no mortality. Conversion increased the length of stay in the hospital. Similar results were found by other authors^[18-20].

Hand-assisted surgery

Hand-assisted laparoscopic surgery (HALS) has gained clinical acceptance as a practical alternative to purely laparoscopically assisted surgery (LAP) for the surgical treatment of complex colorectal diseases like in IBD. Its role in challenging operations for CD (subtotal or total colectomy) has yet to be established. A recent study of Nakajima *et al*^[21] aimed to evaluate the feasibility, safety, and potential benefit of HALS subtotal and total colectomy for Crohn's colitis. Thirty-eight consecutive patients who underwent subtotal or total colectomy as their initial abdominal surgery for Crohn's extensive colitis (involvement of 3 or more colonic segments) were evaluated. The patients were divided into three groups (open, LAP, and HALS), and their background and postoperative data were retrospectively analyzed. The reviews included 14 open, 6 LAP, and 18 HALS cases. The groups were comparable in terms of age at surgery, gender, BMI, extent and type of disease, indications, and procedures. The median operative time was significantly longer for LAP (330 min; range, 154-540 min) than for HALS (251 min; range, 165-340 min) or open surgery (200 min; range, 172-315 min). The blood loss was significantly less with LAP (170 mL; range, 115-257 mL) and HALS (225 mL; range, 35-890 mL) than with open surgery (438 mL; range, 280-780 mL). No difference was seen in postoperative complications, and no mortality occurred in the series. The authors concluded that HALS subtotal and total colectomies were feasible and safe. The HALS procedure seemed potentially beneficial for patients with extensive Crohn's colitis by reducing the operative time for laparoscopic surgery while retaining its less invasive nature.

Laparoscopic resection and transcolonic specimen retrieval

Recently, in numerous surgical meetings, "natural-orifice transluminal endoscopic surgery" and "single-incision laparoscopic surgery" have moved into the focus of interest. Either no or maximally one abdominal port incision are necessary for this. It has been demonstrated that ileocolic resection for CD is feasible entirely laparoscopically. However, normally, an incision is needed for specimen extraction (minilaparotomy). Eshuis *et al*^[22] recently reported an early observational study assessing the feasibility of endoscopic transcolonic specimen removal avoiding any type of minilaparotomy. Endoscopic specimen removal was attempted in a consecutive series of ten

patients scheduled for laparoscopic ileocolic resection. Primary outcomes were feasibility, operating time, reoperation rate, pain scores, morphine requirement and hospital stay. To assess applicability, outcomes were compared with previous data from patients who had laparoscopic assisted operations. Transcolonic removal was successful in eight of ten patients; it was considered not feasible in two patients because the inflammatory mass was too large (7-8 cm). Median operating time was 208 min and median postoperative hospital stay was 5 d. After surgery, two patients developed an intra-abdominal abscess, drained laparoscopically or percutaneously, and one patient had another site-specific infection. The operation took longer than conventional laparoscopy, with no benefits perceived by patients in terms of cosmesis or body image. The authors concluded that transcolonic removal of the specimen in ileocolic CD was feasible in the absence of a large inflammatory mass but infection might be a problem. It could not be stated that the technique offered any benefits compared with conventional laparoscopic surgery.

Influence of body weight

In obese patients, laparoscopic techniques may be impaired by difficulties in creating a sufficient pneumoperitoneum and by worse visualisation of the regular anatomy caused by masses of fatty tissue. At the Cleveland Clinic Florida^[23] a retrospective study of prospectively collected data was designed to evaluate the results of laparoscopic colorectal resections in normal weight patients compared with overweight and obese patients with IBD. All consecutive patients with IBD who underwent laparoscopy in 8 years were reviewed. BMI, age, gender, comorbidities, ASA classification, and surgical- and disease-related variables, including 60-d postoperative complications, were reviewed. A total of 213 patients were analyzed. Group I comprised 127 normal-weight patients (BMI, 18.5-24.9 kg/m²), and group II included 67 overweight patients (BMI, 25-29.9 kg/m²) and 19 obese patients (BMI ≥ 30 kg/m²). Procedures performed included ileocolic resection in 56% of patients in each group. Total colectomy with or without proctectomy was undertaken in 39.4% in group I and 40.7% in group II. The conversion rate was 18% for group I and 22.09% for group II ($P > 0.005$; not significant). The most common reason for conversion was failure to progress due to adhesions or phlegmon. There were no differences in major postoperative complication rates (wound infection, abscess, anastomotic leakage, or small-bowel obstruction) or mean hospital stay (6.7, 6.8, respectively), and there was no mortality. These results demonstrated that the benefits of laparoscopic bowel resection should not be denied to overweight or obese patients based strictly on their BMI.

Nationwide study

The fact that the laparoscopic approach to CD has demonstrated benefits in several small series was in incentive for Lesperance *et al*^[24] to examine its use and outcomes on a national level in the United States. A variety of patient-

and system-related factors were identified to influence the utilization of laparoscopy in CD. All admissions with a diagnosis of CD requiring bowel resection were selected from the 2000-2004 Nationwide Inpatient Sample. Regression analyses were used to compare outcome measures and identify independent predictors of undergoing laparoscopy. Of 396 911 patients admitted for CD, 49 609 (12%) required surgical treatment. They were predominantly Caucasian (64%), female (54%), and with ileocolic disease (72%). Laparoscopic resection was performed in 2826 cases (6%) and was associated with lower complications (8% *vs* 16%), shorter length of stay (6 d *vs* 9 d), lower charges (\$27 575 *vs* \$38 713), and mortality (0.2% *vs* 0.9%, all $P < 0.01$). Open surgery was used more often for fistulas (8% *vs* 1%) and when ostomies were required (12% *vs* 7%). Independent predictors of laparoscopic resection were age < 35 [odds ratio (OR) = 2.4], female gender (OR = 1.4), admission to a teaching hospital (OR = 1.2), ileocecal location (OR = 1.5), and lower disease stage (OR = 1.1, all $P < 0.05$). Ethnic category, insurance status, and type of admission (elective *vs* non-elective) were not associated with operative method ($P > 0.05$). In conclusion, laparoscopic resection was associated with excellent short-term outcomes compared to open surgery.

Meta-analyses

Several meta-analyses about the impact of laparoscopic surgery in CD have been published^[25-29]. Tan's most recent study was designed to determine the safety and feasibility of laparoscopic surgery in CD. A search of published studies in English between January 1990 and February 2006 was performed by using the MEDLINE and PubMed databases and the Cochrane Central Register of Controlled Trials. The studies were reviewed by two independent assessors. The rate of conversion from laparoscopic to open surgery was 11.2%. Laparoscopic procedures took longer to perform compared with open procedures, with a weighted mean difference of 25.54 min ($P = 0.03$). Patients who underwent laparoscopic surgery had a more rapid recovery of bowel function, with a weighted mean difference of 0.75 d ($P = 0.02$) and were able to tolerate oral intake earlier, with a weighted mean difference of 1.43 d ($P = 0.0008$). The duration of hospitalization was shorter, with a weighted mean difference of 1.82 d ($P = 0.02$). Morbidity was lower for laparoscopic procedures compared with open procedures (OR = 0.57, 95% CI: 0.37-0.87, $P = 0.01$). The rate of disease recurrence was similar for both laparoscopic and open surgery.

In all meta-analyses published to date, laparoscopic surgery for CD took longer to perform, but significant short-term benefits to the patient were observed. The morbidity was lower and the rate of disease recurrence was similar. Therefore, laparoscopic surgery for CD proved to be safely feasible.

UC

Under elective conditions, today, restorative procto-

colectomy with ileal J-pouch formation is the standard of surgery. In the early 1990s, initial experiences with a laparoscopic management were reported. However, these first results did not seem very promising, the laparoscopic technique in these comprehensive operations appeared too difficult to apply, too time-consuming, and comorbidity was too high^[30-33]. In the meantime, multiple technical innovations which have been introduced and increased surgical experience have advanced the field of complex surgery. In recent studies, more favourable results have been stated. Earlier return of bowel movements and shorter hospital stays have been observed in patients undergoing laparoscopy^[34-37]. In other studies, even more benefits like reduced pain, decreased morbidity and hospital stay, improved nutrition, preservation of immune response and decreased short and long-term complications have been observed. Functional outcomes and quality-of-life measurements have been comparable^[38-40]. Long operative times and learning curves, however, are still delaying a universal application of laparoscopy in the surgical management of UC^[41]. Boller *et al.*^[42] suggested breaking down comprehensive operations like the IPAA procedure in a stepwise fashion to simplify their complexity and allow the young surgeon to effectively reproduce this operation. The approach becomes viable by outlining the single steps in a systematic manner.

Early postoperative results

Total proctocolectomy with Brooke ileostomy has remained the classical traditional operation, also as a reserve for cases where an ileal pouch is not feasible or advisable. However, hardly any studies describe outcomes after the minimally invasive approach. Holubar *et al.*^[43] analyzed the safety and feasibility of these procedures by examining short-term (30-d) outcomes. Using a prospective database at the Mayo Clinic, a cohort of patients who underwent laparoscopic total proctocolectomy with Brooke ileostomy during 8 years was identified. Forty-four patients were included. Colitis duration was 66 mo (24-240 mo), and 40% had prior surgery. The indication for surgery was refractory colitis (82%) and neoplasia (18%). Factors influencing choice of total proctocolectomy with permanent ileostomy were advanced age in 18 (41%), lifestyle in 13 (30%), medical comorbidities in 11 (25%), fecal incontinence in 10 (23%), oncologic reasons in 3 (6.8%), and obesity in 3 (6.8%). Twenty-three (52%) operations were hand-assisted laparoscopic surgeries, 13 (30%) were laparoscopic-assisted, and 8 (18%) were "laparoscopic-incisionless" with transanal specimen extraction. Two laparoscopic-assisted cases (4.6%) were converted. Operative time was 329 (272-402) min, and length of stay 5 (4-6) d. Major post-operative complications occurred in 4 (9%); there were no perioperative mortalities.

At the same institution, a study was designed to compare short-term outcomes after laparoscopic IPAA with those of open IPAA in patients with both sclerosing cholangitis and UC^[44]. Sixteen patients with sclerosing cholangitis and UC undergoing laparoscopic IPAA were

matched with 16 open ileal pouch control subjects by sex, American Society of Anesthesiologists' score, age, and BMI. Operative mortality was zero. Operative time was longer in the laparoscopic group. Thirty-day complications were not significantly different between groups, but length of stay was significantly shorter in the laparoscopic group. Average return of gastrointestinal function was 2.5 d in the laparoscopic group and 4.8 d in the open group ($P = 0.001$). Time to soft diet was 3 d in the laparoscopic group and 6 d in the open group ($P < 0.001$). All patients were alive and all pouches were intact at last follow-up.

Satisfying mid-term outcomes have been reported in a series by Berdah *et al*^[45]. His prospective study aimed to analyze the functional outcome after a two-stage laparoscopic total proctocolectomy with IPAA. Over 9 years, 68 consecutive two-stage laparoscopic total proctocolectomies with IPAA were performed (UC: $n = 61$; familial adenomatous polyposis: $n = 7$). A covering ileostomy was used in all patients. Forty patients whose covering ileostomy had been closed for a minimum of 2 years were included in this series. Conversion to laparotomy was necessary in 4 of 40 patients (10%). Thirteen postoperative complications occurred in 13 of 40 patients (30%). At a median follow-up of 38 mo (range, 26-90 mo), the median number of bowel movements was 4 per 24 h (range, 2-10); 15 patients (38%) had no nighttime bowel movements. None of the patients had fecal incontinence or urgency. Thirty-four of the 40 patients (85%) experienced no soiling. Seven patients (18%) took regular antidiarrheal medication. All patients were able to resume all activities practiced prior to illness onset, and 36 of 40 (90%) were satisfied with their overall QOL (very good or good).

Long-term results

In contrast to short term results, long-term outcomes after laparoscopic IPAA have not been evaluated thoroughly. In a study published by Fichera *et al*^[46], short- and long-term results were compared prospectively. During 5 years, 73 laparoscopic and 106 open IPAA patients were enrolled. There were no differences in demographics, treatment, indication, duration of surgery, and diversion between groups. Laparoscopic patients had faster return of flatus ($P = 0.008$), faster resumption of a liquid diet ($P < 0.001$), and less blood loss ($P = 0.026$). While complications were similar, the incidence of incisional hernias was lower in the laparoscopic group ($P = 0.011$). Mean follow-up was 24.8 mo. The average number of bowel movements was 6.8 ± 2.8 per day for laparoscopy and 6.3 ± 1.7 for open ($P = 0.058$). Overall, 68.4% of patients were fully continent at 1 year, up to 83.7% long term without differences between groups. Other indicators of defecatory function and QOL remained similar over time.

Self-reported sexual function, body image and QOL after laparoscopic and open IPAA were compared and analyzed in a study by Larson *et al*^[47]. At the Mayo Clinic, between 1978 and 2004, 100 laparoscopic and 189 open operations were performed in patients who were identi-

fied from a previously published cohort. Patients were surveyed 1 year after operation to evaluate sexual function, body image, and QOL. A total of 125 of 289 patients (43%) returned completed surveys. There were no significant differences in terms of demographics, complications, or long-term functional outcomes between those who completed the surveys and those who did not. There were no clinical differences in results between laparoscopic and open patients using the three survey instruments. Orgasmic function scores were lower in men who underwent laparoscopic IPAA ($P < 0.05$) compared with open IPAA. Overall, sexual function scores were equal to or better than normal values for men but were lower in women. Finally, overall body image and QOL scores were above the means published for the United States.

Postoperative adhesions are an expected outcome for the majority of open abdominal operations. Adhesions are responsible for more than 75% of small bowel obstruction cases. Another study from the Mayo Clinic^[48] was initiated to evaluate adhesions to the anterior abdominal wall and adnexal organs after laparoscopic IPAA. Patients who underwent laparoscopic IPAA for UC had laparoscopic evaluation of adhesions at loop ileostomy closure for assessment of adhesions to the anterior abdominal wall and for adhesions to the adnexae in the case of women. Adhesions to the adnexae were quantified using the American Fertility Society adhesion score. Data were maintained prospectively. In this study, 34 patients (21 women) ranging in age from 19 to 78 years (median, 36 years) underwent laparoscopic IPAA. Twenty-three patients (68%) had no adhesions to the anterior abdominal wall, and the remaining 11 patients had few adhesions (filmy, avascular). No patients had dense adhesions to the abdominal wall. Of the 21 women, 15 (71%) had no adnexal adhesions, 5 had filmy adhesions enclosing less than one-third one adnexa, and one had filmy adhesions enclosing one-third to two-thirds of one adnexa. No patient had adhesions affecting both adnexae. It was concluded that laparoscopic IPAA results in few adhesions to the anterior abdominal wall or to gynecologic organs. These adhesions were significantly fewer than previously reported for open operations with or without the use of a glycerol hyaluronate/carboxymethylcellulose bioresorbable adhesion barrier.

Hand-assisted surgery

The role of hand-assistance in laparoscopic restorative proctocolectomies has not yet been defined. In a few comparative and randomized studies, hand-assisted laparoscopic restorative proctocolectomy (HALS-RP) maintained the advantages of a minimally invasive approach with some potential benefits. The aim of a study by Tsuruta *et al*^[49] from Keio University in Tokyo was to evaluate the effectiveness of HALS-RP compared with a conventional laparoscopic restorative proctocolectomy (LAP-RP) in patients with UC. A retrospective study was conducted using a prospectively maintained database to compare a consecutive series of 30 patients who underwent HALS-

RP during 3 years with 40 patients who underwent LAP-RP during 10 years. Both groups were well matched. The median operative time was significantly shorter for HALS-RP [356 min (range, 176-590 min)] than for LAP-RP [505 min (range, 360-785 min), $P < 0.001$]. The median length of incision was significantly longer for HALS-RP [8 cm (range, 7.5-8 cm)] than for LAP-RP [5.5 cm (range, 5-8 cm)]. The estimated blood loss and the length of hospital stay were similar between the two groups. The incidence of postoperative complications including anastomotic leakage did not differ between the both groups ($P = 0.437$).

Hand-assistance in emergency subtotal colectomies for cases of severe UC was analyzed by Watanabe *et al.*^[50]. The medical records of 60 patients who underwent emergency subtotal colectomy with hand-assisted laparoscopic technique (30 cases) or open technique (30 cases) were reviewed. One patient in the laparoscopic group required conversion to open surgery. The median operative time was significantly longer in the laparoscopic group (242 min *vs* 191 min, $P < 0.001$). The rate of early postoperative complications in the laparoscopic group was significantly less than that in the open group (37% *vs* 63%, $P = 0.041$). In the open group, four patients required relaparotomy because of peritoneal abscess or strangulation ileus, whereas no patient required relaparotomy in the laparoscopic group ($P = 0.040$). HALS was found to be an acceptable alternative to conventional open surgery.

Fulminant colitis and emergency surgery

Several institutions have published their data about their experience with minimally-invasive procedures applied in emergency cases. At the Mayo Clinic, safety, feasibility, and short-term outcomes of three-stage minimally invasive surgery for fulminant UC were evaluated^[51]. All patients with UC who underwent minimally invasive surgery for both subtotal colectomy and subsequent IPAA from 2000 to 2007 were identified. Fifty patients underwent minimally invasive subtotal colectomy for fulminant UC; 50% were male, with a median age of 34 years. All patients had refractory colitis: 96% were taking steroids, 76% were recently hospitalized, 59% had ≥ 5 kg weight loss, 57% had anemia that required transfusions, 30% were on biologic-based therapy, and 96% had ≥ 1 severe Truelove and Witts' criteria. Of these 50 procedures, 72% were performed by using laparoscopic-assisted and 28% with hand-assisted techniques. The conversion rate was 6%. Subsequently, minimally invasive completion proctectomy with IPAA was performed in 42 patients with a 2.3% conversion rate. Median length of stay after each procedure was 4 d. There was one anastomotic leak and no mortality.

At Washington University in St. Louis, short-term outcomes of laparoscopic *vs* open total abdominal colectomy and end ileostomy for severe UC were investigated^[52]. The impact of the initial surgical approach on subsequent operations for three-stage restorative proctocolectomy was evaluated. Thirty-seven patients underwent laparoscopic, 41 open total abdominal colectomy at the initial stage of

a three-stage restorative proctocolectomy. Each stage was analyzed independently by using two-tailed *t*-tests and analysis of covariance. The laparoscopic total abdominal colectomy patients underwent subsequent restorative proctectomy 49 d sooner ($P = 0.0044$) and ileostomy closure 17 d sooner ($P = 0.00003$) than the open total abdominal colectomy patients. Laparoscopic abdominal colectomy for severe UC in selected patients was safe and associated with short-term benefits that may lead to faster recovery and progression to completion of restorative proctocolectomy.

In a very similar retrospective review of 90 patients at the Mount Sinai Hospital in New York City, laparoscopic subtotal colectomy was safely feasible and conferred the benefits of improved cosmesis, reduced intraoperative blood loss and shorter hospital stay^[53].

Comparing patients' outcome after laparoscopic IPAA with and without previous emergency subtotal colectomy, McAllister *et al.*^[54] found the pouch procedure not only safely feasible in the virgin abdomen but also in patients with previous OC.

Several studies suggested that infliximab may increase postoperative complication rates for patients who later require a restorative proctocolectomy with IPAA. This question was investigated in a study by Coquet-Reinier *et al.*^[55] aimed to assess the postoperative course of patients after laparoscopic IPAA, comparing those who had and those who had not received infliximab before surgery. No significant difference was found between patients treated with and those treated without infliximab for mean operative time (353 min *vs* 355 min), complication rate (23% *vs* 38%), and mean hospital stay (22 d *vs* 25 d). No adverse impact from previous infliximab therapy on the laparoscopic IPAA postoperative course was detected.

Meta-analysis and cochrane review

Wu *et al.*^[56,57] published a meta-analysis comprising sixteen controlled trials. There was only one prospective randomized study among the studies selected. Outcome effects of laparoscopic and open surgery were pooled. A fixed effect model or random effect model was respectively used depending on the heterogeneity test of trials. Postoperative fasting time and postoperative hospital stay were shorter in laparoscopic surgery for UC [-1.37 (-2.15, -0.58), -3.22 (-4.20, -2.24), respectively, $P < 0.05$]. The overall complication rate was higher in open surgery, compared with laparoscopic surgery (54.8% *vs* 39.3%, $P = 0.004$). However, duration of laparoscopic surgery for UC was extended compared with open surgery (weighted mean difference 69.29 min, $P = 0.04$). As to recovery of bowel function, as indicated by peritoneal abscess, anastomotic leakage, postoperative bowel obstruction, wound infection, blood loss, and mortality, laparoscopic surgery did not show any superiority over open surgery. Re-operation rate was almost even (5.2% *vs* 7.3%). The whole conversion to open surgery was 4.2%.

The presumed benefits of the laparoscopic approach were analyzed systematically in a Cochrane review by

Table 1 Overview of most important study results dealing with laparoscopic and open surgical management of inflammatory bowel diseases

Author	Yr	Patients	Findings
Crohn's disease			
Umanskiy <i>et al</i> ^[8]	2010	55 lap colect 70 open colect	Early postop. results favourable for laparoscopy
El-Gazzaz <i>et al</i> ^[12]	2010	643 matched cases open and laparosc	Anastomotic leak rates even for open and lap surgery
Nguyen <i>et al</i> ^[13]	2009	335 laparoscopic	Postop compl. rate 13% (leaks, obstruction, bleeding)
Eshuis <i>et al</i> ^[14]	2010	29 laparoscopic 26 open	Better cosmesis for laparoscopy, more hernias and obstructions in open surgery
Stocchi <i>et al</i> ^[15]	2008	Follow-up, randomized study 27 lap, 29 open	Open colectomy patients significantly more frequently requiring multiple reoperations, otherwise results similar
Goyer <i>et al</i> ^[16]	2009	54, complex CD 70, uncomplicated	In complex disease, significantly longer OR time, conversion rate and stoma frequency
Holubar <i>et al</i> ^[17]	2010	30 lap completed 10 converted	Significantly more adhesions and length of stay in conversion, complication rates even
Nakajima <i>et al</i> ^[21]	2010	14 open 6 lap 18 hand-assisted	Significantly longer OR time in laparoscopic cases, significantly less blood loss in lap. and hand-assisted cases
Canedo <i>et al</i> ^[23]	2010	127 BMI < 25 kg/m ² 67 BMI > 25 kg/m ² , < 30 kg/m ² 19 BMI > 30 kg/m ²	More conversions in obesity, no differences in postop. complications and hospital stays
Lesperance <i>et al</i> ^[24]	2009	Nationwide study 49.609 surg. cases 2.826 lap cases	Less complications, shorter hospital stays, lower charges and mortality in laparoscopy, applied mainly in younger female patients with ileocecal disease at lower stage
Tan <i>et al</i> ^[25]	2007	Metaanalysis 14 studies 881 patients	Conversion rate 11.2%, lap. surgery with significantly longer OR time, more rapid recovery, shorter hospital stay and lower morbidity, similar recurrence rates
Ulcerative colitis			
Holubar <i>et al</i> ^[43]	2009	Total proctocolect 23 hand-assisted 13 lap-assisted 8 lap-"incisionless"	Median OR time 329 min, hospital stay 5 d, major complications in 9%, no mortality
Berdah <i>et al</i> ^[45]	2009	68 RPC + pouch	30% complication rate, all patients resuming preop. grade of activity, 90% satisfaction "good/very good"
Fichera <i>et al</i> ^[46]	2009	RPC + pouch 73 lap, 106 open	In laparoscopy, faster resumption of bowel function, less blood loss and lower rate of hernias in follow-up
Larson <i>et al</i> ^[47]	2008	RPC + pouch 100 lap, 189 open	Long-term data, worse sexual functional results in lap. cases, better body image and quality of life
Indar <i>et al</i> ^[48]	2008	34, RPC + pouch	Adhesion evaluation at ileostomy closure : 68% no and 32% few adhesions, lower than in open surgery
Tsuruta <i>et al</i> ^[49]	2009	30, HALS-RPC 40, Lap-RPC	OR time significantly longer for lap-RPC, incision length significantly longer for HALS-RPC
Watanabe <i>et al</i> ^[50]	2009	Emergency colect 30 HALS, 30 open	For HALS, OR time significantly longer, postop. complication rate significantly lower
Holubar <i>et al</i> ^[51]	2009	Fulminant colitis, 36 lap-assist. colect	Conversion rate 6%, median lengths of stay 4 d in both 14 HALS colect. groups, subsequent completion proctectomy in 42 pat
Chung <i>et al</i> ^[52]	2009	Severe UC 37 lap., 41 open	Faster recovery and progression to completion of RPC in lap. colectomies patients
Wu <i>et al</i> ^[56,57]	2008	Metaanalysis	Overall complication rate higher in open surgery duration of lap surgery significantly extended
Ahmed Ali <i>et al</i> ^[58]	2010	16 controlled trials	OR time significantly longer in lap. surgery, no differences in postop. course or recovery between lap. and open procedures
Ahmed Ali <i>et al</i> ^[58]	2009	Cochrane review 11 studies, 607 pat 253 laparoscopic	

CD: Crohn's disease; UC: Ulcerative colitis; OR: Operation room; BMI: Body mass index; HALS: Hand-assisted laparoscopic surgery; RPC: Restorative proctocolectomy.

Ahmed Ali *et al*^[58]. The aim was to compare the beneficial and harmful effects of laparoscopic *vs* open IPAA for patients with UC and FAP. The authors searched The Cochrane IBD/FBD Group Specialized Trial Register (April 2007), The Cochrane Library (Issue 1, 2007), MEDLINE (1990 to April 2007), EMBASE (1990 to April 2007), ISI Web of Knowledge (1990 to April 2007) and the

web casts of the American Society of Colon and Rectal Surgeons (up to 2006) for all trials comparing open *vs* laparoscopic IPAA. All trials in patients with UC or FAP comparing any kind of laparoscopic IPAA *vs* open IPAA were included. No language limitations were applied. Two authors independently performed selection of trials and data extraction. The methodological quality of all included

trials was evaluated to assess bias risk. Analysis of randomized and non-randomized controlled trials was performed separately. Analyses were based on the intention-to-treat principle. Sensitivity and subgroup analyses were performed if appropriate. Eleven trials were identified which included 607 patients, of whom 253 (41%) were in the laparoscopic IPAA group. Only one of the included trials was a randomized controlled trial. There were no significant differences in mortality or complications between the two groups. Reoperation and readmission rates were not significantly different. Operative time was significantly longer in the laparoscopic group both in the randomized and meta-analysis of non-randomized controlled trials (weighted mean difference 91 min). There were no significant differences between the two groups regarding postoperative recovery parameters. Total incision length was significantly shorter in the laparoscopic group, while two trials evaluating cosmesis found significantly higher cosmesis scores in the laparoscopic group. Short-term advantages of the laparoscopic approach seemed to be limited and their clinical significance arguable.

Table 1 summarizes the most important study results dealing with laparoscopic and open surgical management of inflammatory bowel diseases.

CONCLUSION

Laparoscopic surgery for CD has been established in numerous major centers. A wide range of procedures may be performed laparoscopically from stoma formation to extended colon and small bowel resections. The minimally-invasive approach shows short-term advantages in complex cases with previous conventional operations, recurrences, enteric fistula and abscess formations. Only limited indications or contraindications may be seen in cases like ileus where no sufficient visualization is obtained during laparoscopy and in acute and fulminant cases demanding a quick and safe surgical solution of the emergency situation. The size of the specimen which needs to be removed may restrict any type of minimally-invasive approach in extended disease.

In UC, laparoscopic restorative proctocolectomy with IPAA is carried out in major surgical centers for elective surgery, but also in cancer and emergency cases. Short and long-term results are comparable to open surgery; in some studies shorter hospital stays and earlier postoperative recovery have been observed. Long operative times and the long learning curve are still factors restricting a broad use of the minimally-invasive approach. A considerable variety of individual laparoscopic techniques is still being observed among different institutions. An important task for future studies will be the analysis of which case selection has the best benefit from a laparoscopic approach in inflammatory bowel diseases.

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CT diagnosis of recurrence after pancreatic cancer: Is there a pattern?

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Abstract

AIM: To investigate predilection sites of recurrence of pancreatic cancer by computed tomography (CT) in follow-up after surgery.

METHODS: Seventy seven patients with recurrence after pancreatic cancer surgery were retrospectively identified. The operative technique, R-status, T-stage and development of tumor markers were evaluated. Two radiologists analyzed CT scans with consensus readings. Location of local recurrence, lymph node recurrence and organ metastases were noted. Surgery and progression of findings on follow-up CT were con-

sidered as reference standard.

RESULTS: The mean follow-up interval was 3.9 ± 1.8 mo, with a mean relapse-free interval of 12.9 ± 10.4 mo. The predominant site of recurrence was local (65%), followed by lymph node (17%), liver metastasis (11%) and peritoneal carcinosis (7%). Local recurrence emerged at the superior mesenteric artery ($n = 28$), the hepatic artery ($n = 8$), in an area defined by the surrounding vessels: celiac trunk, portal vein, inferior vena cava ($n = 22$), and in a space limited by the mesenteric artery, portal vein and inferior vena cava ($n = 17$). Lymph node recurrence occurred in the mesenteric root and left lateral to the aorta. Recurrence was confirmed by surgery ($n = 22$) and follow-up CT ($n = 55$). Tumor markers [carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA)] increased in accordance with signs of recurrence in most cases (86% CA19-9; 79.2% CEA).

CONCLUSION: Specific changes of local and lymph node recurrence can be found in the course of the cardinal peripancreatic vessels. The superior mesenteric artery is the leading structure for recurrence.

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Key words: Pancreatic cancer; Recurrence; Computed tomography; Follow-up; Tumor marker

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INTRODUCTION

Pancreatic cancer is a disease with a poor survival rate after curative surgical therapy^[1]. Local recurrence after resection with curative intent is frequently observed within 2 years for the majority of patients^[2]. The median survival in resectable pancreatic cancer is reported to range from 11 mo for surgery alone to 20 mo for surgery in combination with adjuvant chemotherapy^[3-6]. These data are reflected by a 5-year survival rate of 10%-25%^[7,8]. The mean disease-free period in imaging studies is 267 ± 158 d with negative surgical margins but 72 ± 47 d with positive margins^[9].

So far there have not been many imaging studies in the literature focusing on detection of pancreatic cancer recurrence. One reason may be that recurrence of pancreatic cancer was not treated, but in recent years radiochemotherapy and, in rare cases, surgery for local recurrence has been advocated^[10]. A major problem in patients with pancreatic cancer is that extensive postoperative changes with scar tissue formation as well as lymph node enlargement are present after surgical therapy that may be mistaken for disease recurrence^[11]. This study was conducted to investigate whether recurrence of pancreatic cancer shows a specific pattern of regrowth on regular follow-up computed tomography (CT) examinations after surgery. The goal was to identify predilection sites of recurrence on CT in the follow-up of pancreatic cancer surgery.

MATERIALS AND METHODS

This retrospective study was approved by the local ethics committee.

Patients

A total of 641 patients, who underwent surgery for a primary malignant tumor of the pancreas in a local surgical department from January 2002 to March 2007, were identified for this study. Of these, 245 had at least one follow-up CT in our department. In all patients with CT follow-up postoperative changes such as enlarged lymph nodes, and soft tissue formation in the resection area and along the cardinal mesenteric vessels were initially present. We excluded 168 patients because further consecutive follow-up CT imaging was not available. In 77 patients (47 male, 30 female; mean age: 67.8 years; range, 41-86 years) with a baseline CT examination 3-6 mo postoperatively, follow-up imaging revealed progression of soft tissue surrounding the peripancreatic vessels, progression of lymph nodes, or appearance of liver metastasis as an indication of tumor recurrence. All these patients also showed clinical signs of tumor recurrence with rising tumor marker levels and deterioration of their physical condition. Apart from 3 patients that

Table 1 Patient characteristics

Gender	47 male, 30 female
Age	mean 67.8 yr; range, 41-86 yr
T-stage ¹	4 T2, 70 T3, 1 T4
N-stage ¹	18 N0, 57 N1
R-stage ²	54 R0, 10 R1, 5 R2
Histology	<i>n</i>
Adenocarcinoma of the papilla	5
Ductal adenocarcinoma	65
Mucinous-papillary carcinoma	4
Adenocarcinoma of the bile duct	1
Neuroendocrine carcinoma	1
Acinar cell carcinoma	1
Localization	
Papilla	5
Head	59
Body	9
Tail	4
Resection	
Whipple	60
Left resection	11
Total pancreatectomy	6

¹In 2 cases TNM-stage was not obtainable from medical records; ²In 8 cases R-status not obtainable from medical records.

are alive to date, all other patients died in the course of further progression of tumor recurrence. Tumor and relevant surgical data including the operative technique, TNM stage, the R-status and the development of postoperative tumor markers were taken from the postoperative database of all pancreatic cancer patients (Table 1). If disease recurrence was detected on follow-up imaging, surgery was considered as standard of reference if performed. The surgical report as well as the histological workup was evaluated. If no surgery was performed the progression of findings on further follow-up imaging studies served as standard of reference.

Surgery for recurrence was performed in 22 cases confirming recurrence. In 55 cases no surgery was performed but further follow-up imaging by CT (*n* = 53) and by positron emission tomography (PET)-CT (*n* = 2) showed further progression of disease recurrence. In 9 patients a potential curative approach for surgery was pursued with 4 hemihepatectomies, 3 lymphadenectomies and 2 resections of the remnant pancreas. In 6 other patients a potential curative surgical approach was not feasible but intraoperative radiotherapy, and in one case a combination of radiochemotherapy with additional tumor reduction was performed. In all patients with a diagnosis of tumor recurrence, second line chemotherapy or radiation therapy was administered for disease control.

As tumor marker values during follow-up might have a great interindividual variability, the factor for the tumor marker increase over time was calculated from the value at the time of tumor recurrence divided by the initial postoperative (2-4 mo) value. In 48 cases tumor marker values for carcinoembryonic antigen (CEA) were measured during the initial postoperative period and the time of tumor recurrence; in 57 cases carbohydrate antigen 19-9 (CA19-9) levels were available.

Table 2 Types of tumor recurrence: isolated or combined occurrence with other manifestations of recurrence

	Isolated	In combination with				Total
		Local	Lymph node	Liver metastasis	Peritoneal carcinosis	
Local recurrence	43		11	5	5	64
Lymph node recurrence	6	11				17
Liver metastasis	5	5			1	11
Peritoneal carcinosis	1	5		1		7
	55					99 ¹

¹One manifestation with a combination of local recurrence, liver metastasis and peritoneal carcinosis not listed.

Image evaluation

All image data were evaluated by two radiologists (with 5 and 10 years experience of pancreas imaging) on diagnostic workstations (Centricity PACS, GE, USA) with consensus readings. Disease recurrence was classified into local recurrence, lymph node recurrence, liver metastasis and peritoneal carcinosis. For each case, tumor recurrence could either appear as a singular finding, such as local recurrence, or as a combination, e.g. local recurrence and liver metastasis concurrently on follow-up imaging. The criteria for local disease recurrence were defined as a soft tissue formation that increased in size over time in the resection area or along the cardinal visceral vessels around the pancreatic bed. An increase in lymph node size over multiple follow-ups was classified as lymph node recurrence. Disease recurrence was classified as liver metastasis if the appearance of new liver lesions with specific image characteristics, e.g. irregular margins, hypodensity, was observed. Peritoneal carcinosis was considered as another possible form of disease recurrence if typical, e.g. nodal peritoneal, changes were present. The exact location of each form of disease recurrence was noted.

CT protocol

All patients were given 1-1.5 L of water orally prior to the examination. The standard hydro-pancreas protocol consists of an unenhanced (5 mm slice thickness/4 mm increment), arterial and venous phase (3 mm slice thickness/2 mm increment, axial and coronal reconstructions) imaging after injecting 130 mL contrast agent (Ultravist 370, Bayer-Schering, Berlin, Germany) at a flow rate of 5 mL/s *via* a cubital vein. Scanning was performed on different CT scanner generations with 4, 16, 64 slices (Volume Zoom, Sensation, Definition; Siemens, Forchheim, Germany).

Data analysis

All data are presented as absolute numbers and percentages. Statistical analysis was performed using the paired Student *t*-test and one-way analysis of variance test for tumor marker values with commercially available software (SPSS Statistics 17.0, SPSS Inc., Chicago, USA).

RESULTS

Patients

The mean follow-up time between CT examinations was

3.9 ± 1.8 mo (range, 2-12 mo). The mean relapse-free time interval was 12.9 ± 10.4 mo (range, 3-76 mo).

Tumor recurrence

In 55 cases a solitary presentation of tumor relapse was present and in 22 cases combinations of different types of tumor recurrence (*n* = 45) resulting in a total of 100 types of recurrence were present (Table 2). The predominant type of tumor recurrence was local recurrence in 65 cases (65%), with isolated manifestations in 43 cases and in combination with other types of tumor recurrence in 22 cases (Table 2). Lymph node recurrence (17%) was found in 6 cases as an isolated finding but in 11 cases in combination. Liver metastasis (11%) was commonly seen in combination with local recurrence and with isolated tumor presentation in 5 cases. Peritoneal carcinosis (7%) was, except for one case with an isolated manifestation, mainly associated with other forms of tumor recurrence. In cases where the initial resection status was R1, the most common tumor recurrence type was local recurrence in 8 cases, lymph node recurrence in one case, and in another case peritoneal carcinosis. The R2 status presented as local recurrence in all 5 cases.

Localization of tumor recurrence

Local recurrence was found most often along the superior mesenteric artery (SMA, *n* = 28), either as a localized tumor mass or as a diffuse cuff-like tissue formation (Figure 1). The second most common site for tumor recurrence (*n* = 22) was an area defined by the celiac trunk (cT) as the medial border, the portal vein (PV) as the anterior border and the inferior vena cava (IVC) as the dorsal border (Figure 2). This is followed by an area (*n* = 17) which is just caudal to the aforementioned space where the medial boundary is the SMA while the other limiting structures, the PV and IVC, remain the same. In 10 cases, tumor regrowth or a secondary tumor occurred at the resection margin of the residual pancreatic parenchyma. In 8 cases the tumor presented as a cuff-like tissue formation along the common or proper hepatic artery (HA). One case was identified with tumor recurrence in the very distal mesenteric root.

Lymph node recurrence was found in 12 cases in the mesenteric root mainly in close proximity to the superior mesenteric artery. In 5 cases, lymph node recurrence was found to the left of the aorta and in one case anterior to

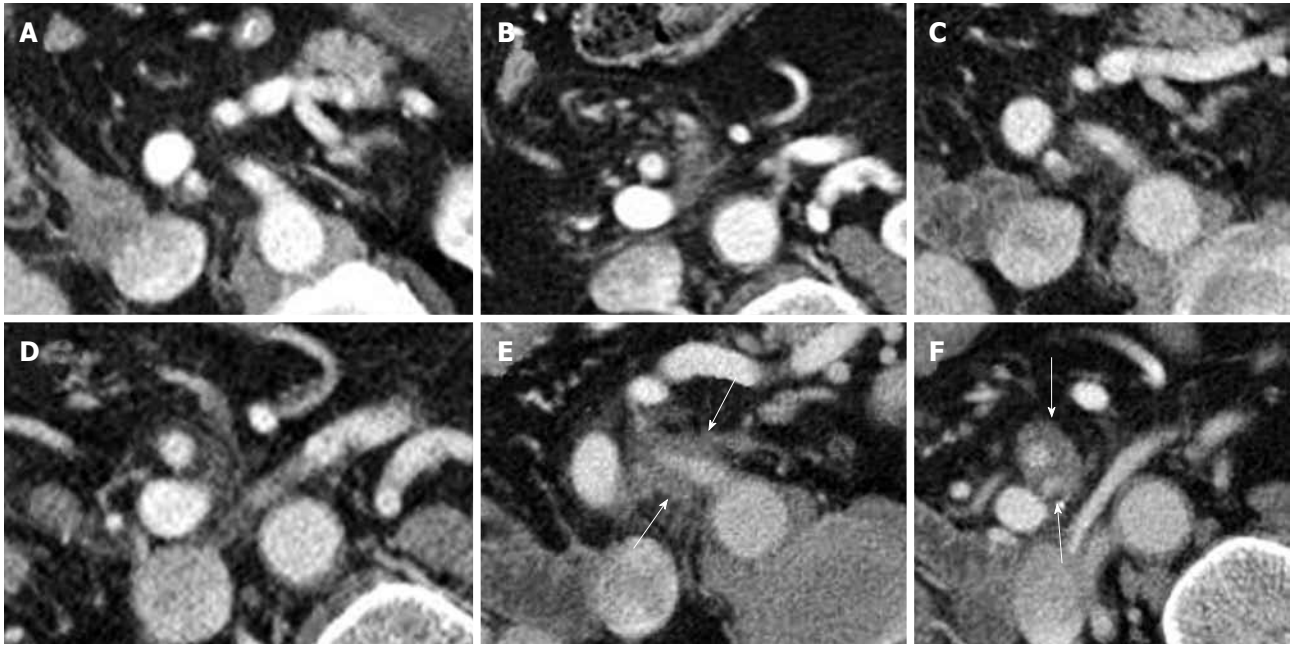


Figure 1 Computed tomography follow-up after Whipple's procedure for pancreatic cancer of the head in a 69-year-old patient (T3, N1). A, B: Follow-up imaging after 3 mo; C, D: 11 mo; E, F: 22 mo. Recurrence at the origin of the superior mesenteric artery (E) and more distally in the mesenteric root (F: white arrows).

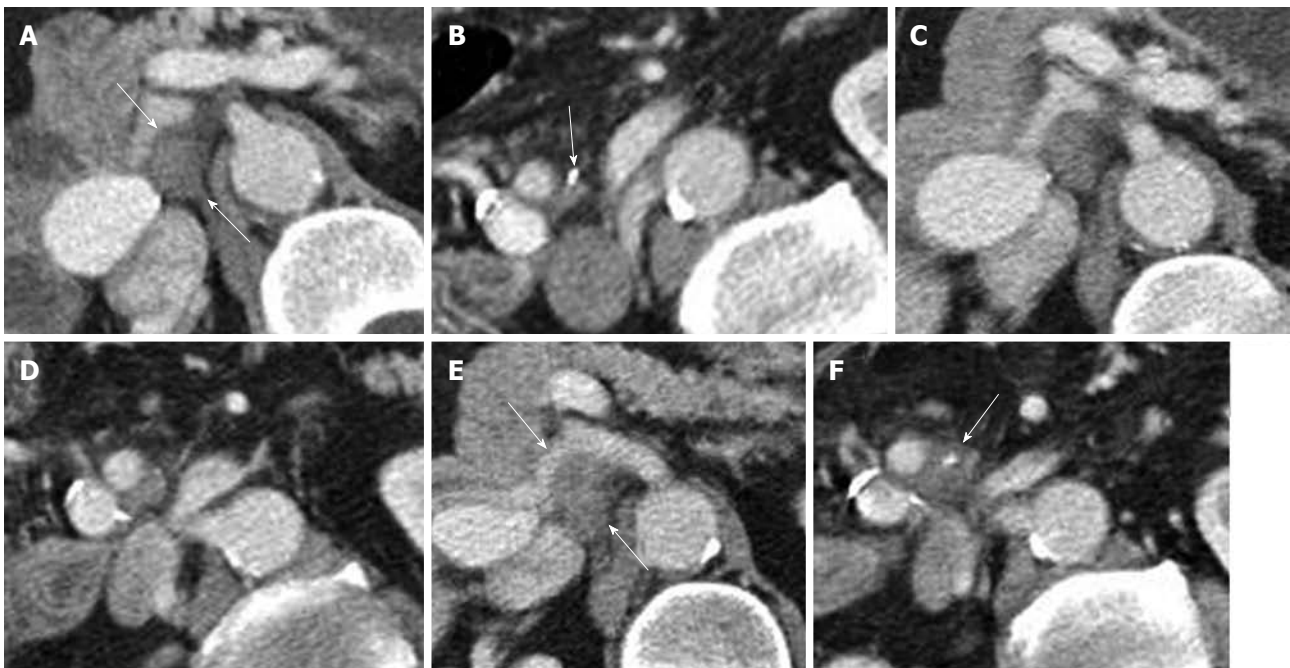


Figure 2 Sixty-eight-year-old patient after surgery for ductal adenocarcinoma of the pancreatic head (T3, N1). A, B: Follow-up imaging after 6 mo; C, D: 8 mo; E, F: 12 mo. Initial soft tissue formation (A, B: white arrows) with a carbohydrate antigen 19-9 (CA19-9) level of 9 U/mL increased (E, F: white arrows) with concurrent increase in CA 19-9 to 56 U/mL.

the aorta (Figure 3).

Tumor marker development

The mean initial postoperative tumor marker level for CEA was 3.12 ± 2.7 $\mu\text{g/L}$ and for CA19-9 was 66.98 ± 501.2 U/mL. At the time of tumor recurrence, the mean value for CEA was 12.3 ± 18.1 $\mu\text{g/L}$ and for CA19-9 was 587.65 ± 4475.4 U/mL. CEA tumor marker values at the time of tumor recurrence were significantly different

from the mean initial values ($P = 0.001$). The mean values of CA19-9 showed no significant differences in this comparison ($P = 0.067$). The mean factor for the tumor marker increase was 5.6 ± 1.6 for CEA and 51.5 ± 309.6 for CA19-9. There was no significant difference ($P > 0.05$) for mean tumor marker values of CEA and CA19-9 stratified by type of recurrence (Table 3), except for the level of CA19-9 in the case of an isolated appearance of liver metastasis compared with mean values in local recurrence

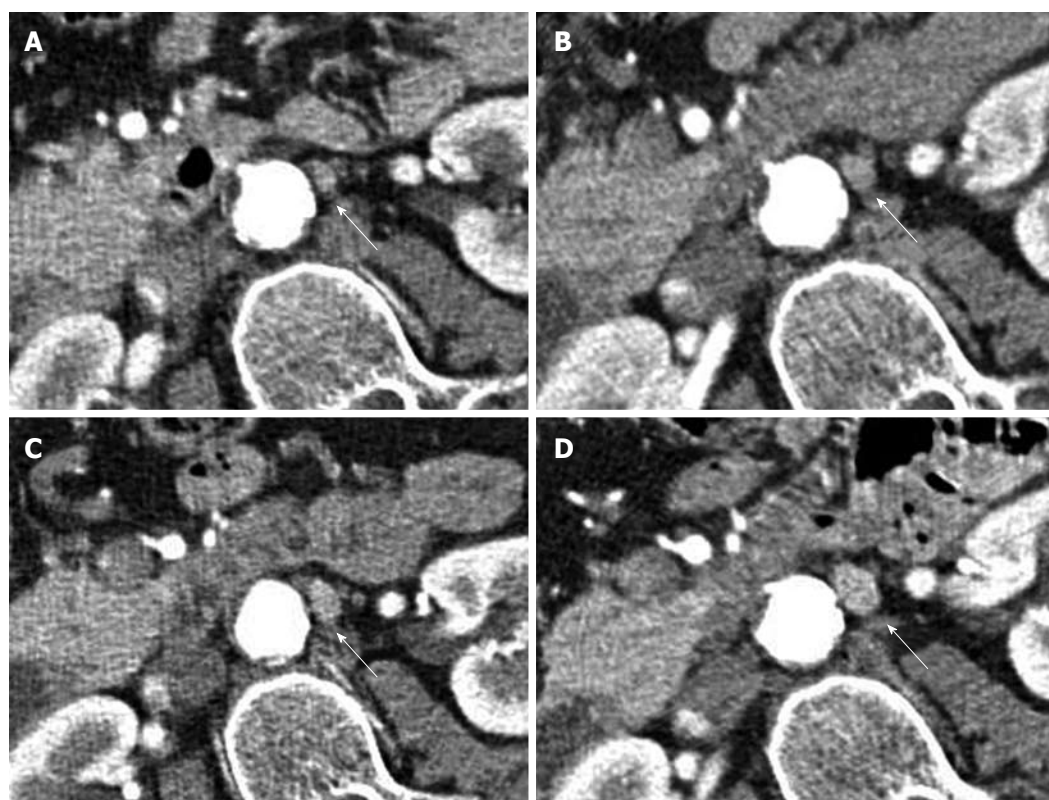


Figure 3 Sixty-year-old patient after resection of the pancreatic tail for a neuroendocrine carcinoma. Follow-up imaging after A : 4 mo; B: 7 mo; C: 9 mo; D: 12 mo. A normal size lymph node (white arrows) of 9 mm initially at the left of the aorta (A) increased to 16 mm (D) indicating lymph node recurrence.

Table 3 Mean tumor marker values at the time of recurrence stratified by type of recurrence

Type of recurrence	Tumor marker			
	n	CA 19-9 (U/mL)	n	CEA (μg/L)
Local	32	781.67 ± 2090.28	26	9.95 ± 15.57
Lymph node	5	244.02 ± 413.03	4	3.13 ± 2.35
Liver metastasis	5	6674.80 ± 14338.02	5	18.1 ± 18.26
Local & lymph node	7	399 ± 738.15	6	4.28 ± 4.03
Local & liver metastasis	5	1157.0 ± 1802.0	5	21.88 ± 26.76
Local & peritoneal carcinosis	3	351.0 ± 257.66	2	46.0 ± 39.6

No statistically significant differences in tumor markers between types of recurrence (one-way analysis of variance) except comparison of mean values of carbohydrate antigen 19-9 (CA19-9) for local recurrence and liver metastasis ($P = 0.025$, Student *t*-test). CEA: Carcinoembryonic antigen.

($P = 0.025$). The mean factor for the increase in CEA and CA19-9 values did not show any significant differences stratified by type of recurrence (Figure 4B and D). In a direct comparison of local recurrence with liver metastasis, there was a significant difference for both CEA and CA19-9 increase factors ($P < 0.05$). In 8 of 57 (14.0%) cases CA19-9 did not increase, and in 10 of 48 (20.8%) CEA values did not increase with detection of recurrence on CT scans.

DISCUSSION

This study indicated that a specific pattern of disease

recurrence in pancreatic cancer exists, especially of local recurrence, and that regular follow-up CT examinations are able to identify this pattern and detect recurrence in accordance with an increase in tumor markers (Figure 5).

To improve the prognosis of pancreatic cancer it is necessary to focus on the situation when tumor recurrence takes place. Second-line chemotherapy or radiotherapy in case of disease relapse seek to control tumor progression and improve the survival rate^[12]. In rare cases a potentially curative surgical approach for liver metastasis or local tumor recurrence was shown to be successful^[13-22].

It is essential to identify tumor recurrence early in order to offer patients further disease controlling measures or potentially curative options. Early intervention with chemotherapy in case of tumor recurrence has a higher chance of improving survival. The case for follow-up imaging is to identify tumor recurrence as early as possible in order to intervene appropriately. A major problem is that extensive postoperative changes, such as scar tissue formation, and enlarged and increased lymph nodes in the resection area are difficult to distinguish from real tumor relapse^[23].

Ruf *et al*^[24] compared ¹⁸F-fluorodeoxyglucose (FDG)-PET with CT/magnetic resonance imaging (MRI) in 25 patients with suspected disease recurrence and reported a 96% detection rate for FDG-PET in comparison to 39% with CT/MRI. Mortelé *et al*^[25] reported a diagnostic accuracy of 93.5% for CT in detecting recurrent pancreatic cancer, but pointed out that no predilection site for tumor recurrence was found in their series of 32 patients.

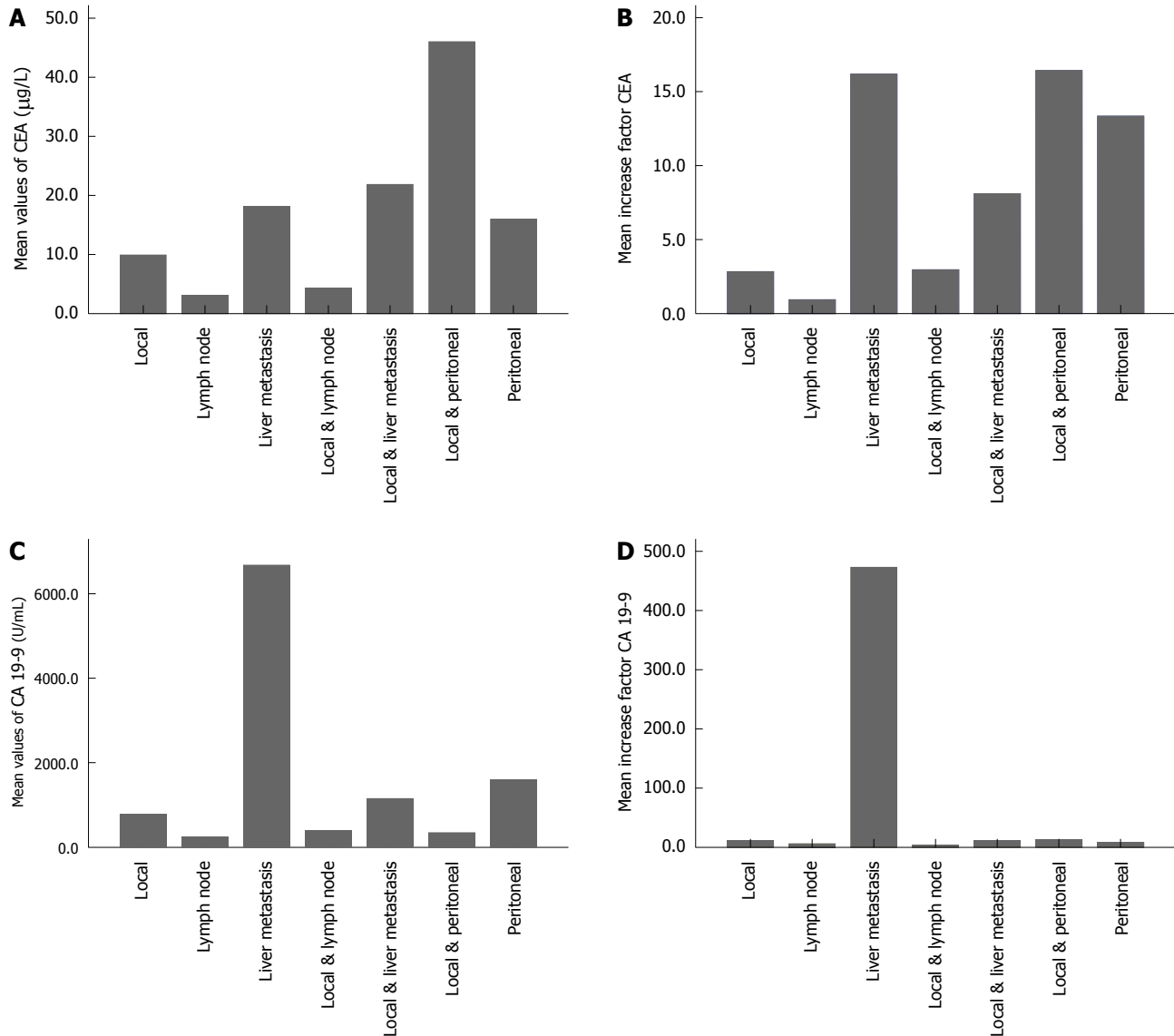


Figure 4 Bar graphs of mean values and mean increase factors for carcinoembryonic antigen and carbohydrate antigen 19-9 stratified by type of recurrence. A: Mean values of carcinoembryonic antigen (CEA) ($\mu\text{g/L}$) at the time of recurrence stratified by type of recurrence; B: Mean increase factor of CEA ($\mu\text{g/L}$) (values at the time of recurrence divided by initial values) stratified by type of recurrence; C: Mean values of carbohydrate antigen 19-9 (CA 19-9) (U/mL) at the time of recurrence stratified by type of recurrence; D: Mean increase factor of CA 19-9 (U/mL) (values at the time of recurrence divided by initial values) stratified by type of recurrence.

Coombs *et al*^[26] investigated 19 patients after pancreato-duodenectomy and detected tumor recurrence by follow-up CT in 12 patients. Larger studies on the diagnostic accuracy of follow-up imaging after surgical treatment for pancreatic cancer are lacking.

CT is a morphology-based imaging method and does not offer functional or metabolic information such as FDG-PET or PET-CT. Regular postoperative PET-CT imaging may be the optimal method to identify early tumor relapse but this modality is costly and not widely available. CT imaging does offer high resolution and artifact-free imaging which is widely available, safe and fast. In order to render CT the method of choice for follow-up of patients after surgery for pancreatic cancer there is a need to gather morphological information from follow-up studies to determine the pattern of disease recurrence.

Local pancreatic tumor re-growth spreads along the

cardinal visceral vessels, mainly the SMA and the HA, but can also present as a mass in specific spaces that are defined by the surrounding vasculature (SMA, HA, PV, IVC, cT). This behavior is consistent with the fact that pancreatic cancer is known to propagate along neurovascular structures on a histological level^[27]. Makino *et al*^[28] found a pattern of invasion into pancreatic and extrapancreatic nerve plexuses depending on the ventral or dorsal location of the tumor in the pancreatic head. Interestingly, in cases of invasion into the extrapancreatic nerve plexus of the SMA and HA, invasion to the adventitia of these vessels was a frequent finding. The distribution of local recurrence in the current study, which is primarily defined by the cardinal visceral vessels, may support speculations that tumor recurrence follows the same patterns of spread as the primary tumor. Noto *et al*^[29] investigated the SMA in 6 patients with *en bloc* resection of the pancreatic head

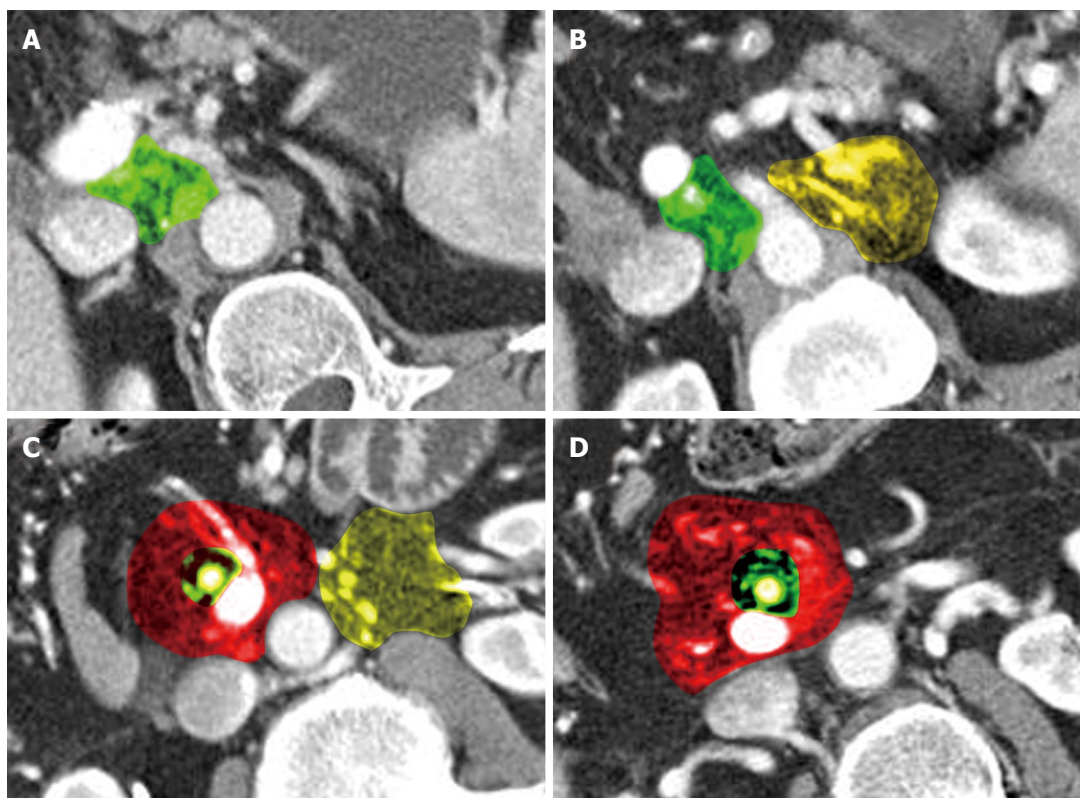


Figure 5 Schematic summary of localizations of local and lymph node recurrence patterns on contrast enhanced computed tomography at different levels of the upper abdomen. A: Offspring celiac trunk; B: Offspring superior mesenteric artery; C: Middle part of mesenteric root; D: Distal mesenteric root. Light green: Local recurrence in the area limited by cT (celiac trunk), (portal vein) PV, (inferior vena cava) IVC (top left); light green: Local recurrence in the area limited by superior mesenteric artery (SMA), PV, IVC (top right); yellow: Lymph node recurrence to the left of the aorta above/below the level of the renal vein; dark green: Local recurrence along the SMA; red: Lymph node recurrence in the mesenteric root close to the SMA.

for cancer, and found that the SMA was directly invaded in 3 cases and in 4 cases there was invasion to the perivascular nerve plexus. Furthermore invasion extended upwards along the SMA for the celiac nerve plexus. This corresponds with our results of the SMA being the most frequent site for tumor recurrence but also the space defined by the celiac trunk as the second most frequent site of recurrence. Additionally, Noto *et al*^[29], Kayahara *et al*^[30] found nodal metastasis around the SMA in all cases. Again this correlates with our findings that lymph node recurrence was mainly seen to the left of the aorta just superior or inferior to the left renal vein, as well as in the mesenteric root within a certain boundary of the SMA. Thus, for primary cancer of the pancreatic head, the SMA seems to serve as the main leading structure for disease propagation. As a hypothesis one could transfer these facts from initial tumor spread to tumor recurrence. This concept is even more convincing looking at recent discussions that the high rate of local tumor recurrence in pancreatic cancer is due to the fact that neurovascular tumor invasion may be left behind during the initial surgery. In other words, most pancreatic cancer resections are R1 (= microscopic residual tumor) resections, as recent studies with standardized pathological examinations have shown^[31,32]. According to recent articles there is no international consensus on the resection margins, and pathological reporting of pancreatic cancer specimens has resulted in a false low rate of R1 sta-

tus^[32]. Following Verbeke^[32], the critical resection margins for microscopic tumor residuals, the posterior margin but especially the medial margin, are very close to the SMA. In the case of R1 status, the remaining tumor cells would re-grow along these vascular structures and coincide with the locations of macroscopic tumor relapse on follow-up imaging. The results of our study seem to support this thesis since all sites of recurrence are mainly defined by vascular structures and locations of extrapancreatic nerve plexuses. These results are supported by the work of Ishigami *et al*^[33] showing malignant perivascular soft tissue along the HA and SMA. However, it must be mentioned that most of the included patients were initially operated on before standardized pathological reporting was introduced at the local pathology institute, explaining the low rate of R1 resections^[31].

In our study, 9 patients underwent surgical resection of the recurrent tumor with potential curative intent. Three of these patients, with initial surgery performed in 2000, 2003 and 2004 and second-line surgery in 2004 and 2007, are alive to date and are seen for regular follow-up. In all patients with the diagnosis of recurrence, second-line chemotherapy was recommended. This strongly emphasizes that there are multiple therapy options for disease recurrence at hand. In the future it will be necessary to develop new multi-modal approaches to therapy for the scenario of disease recurrence in order to improve

survival even in cases with advanced disease presentations.

Tumor marker (CEA, CA19-9) measurement showed that the increase in levels were concordant with the detection of recurrence on CT in most cases. The mean values for CEA and CA19-9 increased from local and lymph node recurrence to liver metastasis and combinations of recurrence, indicating an association between tumor burden and tumor markers (Figure 4A-D). This gives an estimation of the type of recurrence but does underline the need for imaging at this point if an increase in tumor marker levels is detected. However, this deduction from the present data is limited since local recurrence is over-represented compared to other types of recurrence, and the variability of the tumor marker values is high.

Another limitation of this study is a possible selection bias as it was not possible for practical reasons to include all patients after surgery, e.g. follow-up at their local hospitals, *etc.* Therefore it cannot be concluded that the remaining 168 patients with initial CT imaging did not develop tumor recurrence at some point. Also pathologic proof was not obtained in all cases owing to the retrospective nature of the study and also for ethical and practical reasons. The deductions of this work reflect mainly experience with ductal adenocarcinoma of the pancreatic head after Whipple's procedure. However this is the natural distribution of pancreatic tumors.

CT is a valuable tool that allows identification of tumor recurrence in accordance with an increase in tumor marker level. If tumor marker levels do not increase, recurrence can only be detected by imaging studies. We suggest a protocol for postoperative follow-up for pancreatic cancer that includes regular CT imaging and tumor marker measurement as a cost-effective and secure way to monitor these patients. A baseline CT examination 3 mo post-operatively is a prerequisite for follow-up imaging. This baseline examination is the template for comparisons with follow-up studies to distinguish postoperative changes from local tumor growth. PET-CT should be performed in addition if clinical suspicion of tumor recurrence is high, tumor markers increase and CT findings are inconclusive or negative for disease recurrence.

In conclusion, specific changes in local and lymph node recurrence of pancreatic cancer after surgery are present along the path of the peripancreatic cardinal vessels. These findings may also help differentiate non-specific postoperative changes from actual recurrence. Local tumor regrowth spreads along the cardinal visceral vessels but can also present as a mass-like recurrence at specific spaces that are defined by the surrounding vasculature. This is consistent with the known behavior of pancreatic cancer propagating along neurovascular structures. The SMA plays a major role as a leading structure for tumor regrowth. CT follow-up examinations in combination with tumor marker measurement are crucial diagnostic tools to identify local recurrence and lymph node metastasis at an early stage, allowing as many patients as possible to have second, potentially curative, surgical therapy or second-line radio/chemotherapy.

COMMENTS

Background

Pancreatic cancer has a poor 5-year survival rate of 10%-25%. Local recurrence is observed within 2 years after surgery for the majority of patients. Detection of recurrence of pancreatic cancer by imaging is challenging since extensive postoperative changes are present in the resection area after pancreatic surgery. It is crucial to identify signs of recurrence early in order to provide second line chemotherapy or even reoperation.

Research frontiers

Detection of recurrence patterns by CT imaging of pancreatic cancer after surgery to facilitate differentiation of postoperative changes from true recurrence. Development of a follow-up imaging protocol for pancreatic cancer after surgery.

Innovations and breakthroughs

This study describes a recurrence pattern of pancreatic cancer on CT imaging that has not been identified so far. Detection of recurrence by imaging was concordant with significant rise in tumor markers. The pattern demonstrates that pancreatic cancer reoccurs mainly along the cardinal neurovascular structures such as the superior mesenteric artery and hepatic artery. This is the same propagation pattern as the primary pancreatic cancer exhibits during tumor extension. The high rate of tumor recurrence is thought to arise because of residual tumor at the medial resection margin which is very close to the superior mesenteric artery. Previous articles have shown that many tumor resections were R1 resections as there was no international consensus on the histopathological workup. As a consequence a standardized histopathological workup for pancreatic cancer was recently introduced. This study confirms the relationship between residual microscopic tumor after surgery and its presentation as macroscopic recurrence on CT imaging.

Applications

The results of this study, demonstrating a distinct pattern of tumor recurrence, can be applied to optimize the radiological follow-up of pancreatic cancer after surgery. A baseline CT imaging study 3 mo after surgery is recommended for further follow-up imaging. It remains to be determined if early detection of recurrence leads to new therapy options and ultimately an improved survival rate.

Peer review

This well written and well structured paper reports the results of a retrospective single-center study on the role of CT imaging after surgical resection of pancreatic cancer. Adjuvant treatment strategies as well as novel methods for the follow-up of patients with this high risk disease are currently topics of a high clinical and scientific interest.

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Octreotide ameliorates gastric lesions in chronically mild stressed rats

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Abstract

AIM: To evaluate the effect of chronic mild stress (CMS) on the emergence of gastric ulcers and possible modulation by octreotide, a synthetic somatostatin analogue.

METHODS: Adult male Wistar rats were subjected to nine different unpredictable random stress procedures for 21 d, a multifactorial interactional animal model for CMS. Octreotide was administered daily for 21 d at two dose levels (50 and 90 µg/kg) before exposure to stress procedure. Macro- and microscopical assessments were made, in addition to quantification of plasma corticosterone and gastric mucosal inflammatory, oxidative stress, and apoptotic biomarkers.

RESULTS: Exposure to CMS elevated plasma corticosterone (28.3 ± 0.6 µg/dL, $P = 0.002$), an event that was accompanied by gastric lesions (6.4 ± 0.16 mm, $P = 0.01$) and confirmed histopathologically. Moreover, the insult elevated gastric mucosal lipid peroxides (13 ± 0.5 nmol/g tissue, $P = 0.001$), tumor necrosis factor- α ($3008.6 \pm$

78.18 pg/g tissue, $P < 0.001$), prostaglandin E2 (117.1 ± 4.31 pg/g tissue, $P = 0.002$), and caspase-3 activity (2.4 ± 0.14 OD/mg protein, $P = 0.002$). Conversely, CMS mitigated interleukin-10 (627.9 ± 12.82 pg/g tissue, $P = 0.001$). Furthermore, in animals exposed to CMS, octreotide restored plasma corticosterone (61% and 71% from CMS, $P = 0.002$) at both dose levels. These beneficial effects were associated with a remarkable suppression of gastric lesions (38% and 9% from CMS, $P = 0.01$) and reversal of derangements in gastric mucosa.

CONCLUSION: The current investigation provides evidence that exposure to CMS induces gastric ulceration, which was alleviated by administration of octreotide possibly possessing antioxidant, anti-inflammatory, and anti-apoptotic actions.

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Key words: Gastric ulcer; Chronic mild stress; Octreotide; Inflammation; Oxidative stress; Apoptosis; Histopathology

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INTRODUCTION

Severe life stressors frequently antedate the onset of functional gastrointestinal disorders. The stomach, in particular, is extremely sensitive to various stress stimuli and peptic ulcer has often been described as a stress disease^[1].

Stress *per se*, alters the mechanisms of neurohormonal regulation, which results in lesions earliest found in the stomach, as a consequence of a general adaptation syndrome^[2]. Chronic mild stress (CMS) is a paradigm where animals are exposed to a combination of mild unpredictable stressors^[3]. Moreover, in the CMS model, several reports implicate important roles of reactive oxygen species (ROS)^[4-6], and enhanced production of inflammatory mediators, which are also prime events in acute stress models^[7,8]. Markedly, stress induces gastric ulceration *via* different factors, *viz.* stimulation of brain gut axis, reduction of mucosal blood flow, and leukocyte infiltration^[7,9,10]. The latter contributes to free radical and pro-inflammatory cytokines formation, which further recruit more inflammatory cells, thus augmenting ROS production and maximizing mucosal damage^[7,8,11-13]. Imbalances in the production of interleukin (IL)-10 and tumor necrosis factor (TNF)- α play crucial roles in gastric ulceration^[7,14]. Furthermore, TNF- α activates extrinsic apoptotic pathway *via* caspase-3 induction, ultimately resulting in gastric injury^[15]. On the contrary, inhibition of TNF- α *via* the cytoprotective prostaglandin (PG) highlights its anti-inflammatory properties in the gastric mucosa^[13].

Somatostatin is secreted from D-cells in the stomach where it suppresses acid secretion directly from parietal cells and indirectly by inhibiting the release of histamine and gastrin^[16]. Furthermore, somatostatin inhibits pepsin secretion and reduces gastroduodenal mucosal blood flow, which are important entities in the pathophysiology of peptic ulcer bleeding^[17]. Conversely, gastric ulcers are linked to decreased levels of this hormone^[18,19]. The effectiveness of synthetic somatostatin analogues as gastro-protective agents is advocated by inhibition of leukocyte adhesion^[20] and antioxidant^[21] properties, beside their anti-secretory potential^[22].

The possibility that animals exposed to chronic stressors may develop gastric lesions has not been extensively investigated. Hence, the present study aimed to assess the potential modulatory effect of CMS on the stomach integrity assessed macro- and microscopically. In addition, the gastric mucosal redox status, as well as the inflammatory process that might accompany exposure to CMS were determined herein. Moreover, the study evaluated the effect of octreotide, a synthetic cyclic octapeptide somatostatin analogue, on CMS-induced gastric mucosal alterations.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (175 \pm 15 g; National Research Center Laboratory, Cairo, Egypt) were kept in a controlled environment, at a constant temperature (23 \pm 2°C), humidity (60% \pm 10%), and light/dark (12/12 h) cycle, lights on at 5:00 am. Rats were singly housed and acclimatized for 1 wk before any experimental procedures and were allowed standard rat chow and tap water *ad libitum*. Experimental protocols were approved by the Research Ethical Committee of Faculty of Pharmacy Cairo University (Cairo, Egypt).

Induction of CMS, treatment, and experimental groups

Rats were randomly assigned to 6 groups (n = 10-12; each). Animals in group I received an ip injection of saline and served as control group, while groups II and III rats received octreotide (Novartis Pharmaceuticals, Basle, Switzerland) at two dose levels (50 and 90 μ g/kg, ip)^[23]. Group IV rats were exposed to CMS, as detailed below, while animals of groups V and VI were subjected to CMS during daily treatment with octreotide at the indicated doses. The somatostatin analogue was dissolved in saline and its administration started from the first day 2 h before exposure to stressors between 9:00 am and 12:00 pm. CMS was induced by exposure of animals to unpredictable repetitive random stress procedures for 21 d following the protocol described by Bekris *et al.*^[24]. Briefly, the stressors comprised high-speed agitation for 10 min; deprivation of either food and/or water for 24 h; either 45°C heat stimulus for 5 min or 4°C cold exposure for 1 h; immobilization for 2 h; interrupted noise for 3 h; continuous illumination for 24 h; and tilted cage for 12 h. On the other hand, unstressed control animals were housed undisturbed under constant conditions, without contact with the stressed animals.

Plasma corticosterone level measurement

At the beginning of the following dark cycle, blood was collected in chilled EDTA-tubes. Plasma corticosterone levels were determined using a commercially available radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA).

Measurement of ulcer index

Rats were euthanized and their stomachs were rapidly removed and opened along the greater curvature to assess the extent of gastric damage in a double blind fashion. The length of each lesion along its greatest diameter was measured and the sum of lengths was expressed as ulcer index (mm)^[8].

Biochemical determinations

Gastric mucosa was scraped and homogenized either in ice-cold saline for assessment of lipid peroxides, total antioxidant capacity (TAC), TNF- α , IL-10, and capase-3 or in 0.1 mol/L phosphate (pH 7.4) buffer, containing 1 mmol/L EDTA and 0.1 μ mol/L indomethacin for PGE₂ measurement and frozen at -70°C until assayed.

Lipid peroxides and TAC

The thiobarbituric acid reaction of Mihara and Uchiyama^[25] was adopted for estimation of lipid peroxides level, using malondialdehyde (MDA) as a standard. The method for the assessment of TAC of gastric mucosa was based on that of Koracevic *et al.*^[26].

TNF- α , IL-10, PGE₂, and caspase-3 activity estimations

Gastric mucosal TNF- α , IL-10, and PGE₂ were measured by ELISA kits purchased from Invitrogen (California, USA), Bender MedSystems (Vienna, Austria), and Cayman

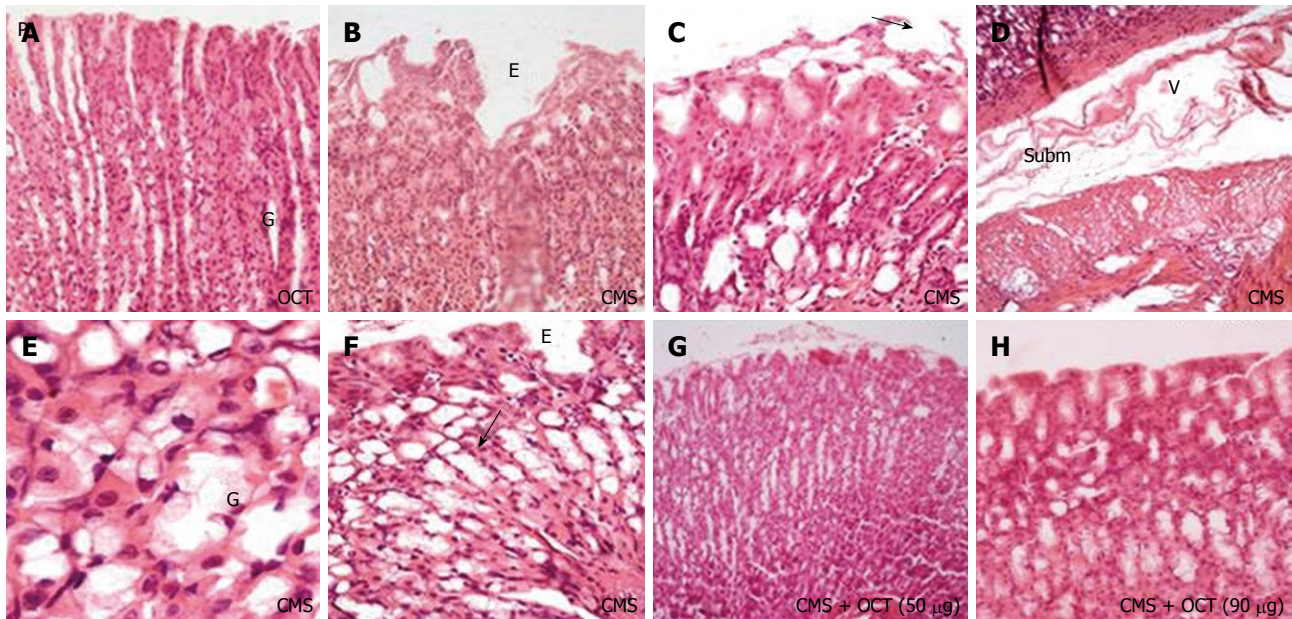


Figure 1 Representative photomicrographs of stomach fundus mucosa obtained at the beginning of the following dark cycle after last exposure to mild stressors with or in absence of octreotide (50 and 90 µg/kg, ip) treatment. A: Octreotide (OCT) administered to normal rats showing normal gastric architecture with gastric pits (P) and gastric glands (G); B: Chronic mild stress (CMS) sections showing mucosal erosion designated by letter E; C: CMS showing hydropic degeneration with erosion and epithelial desquamation (arrow); D: CMS with submucosal edema (V) as represented by wide separation of connective tissue between mucosa and muscularis mucosa infiltrated macrophages and eosinophils (E); E: CMS showing goblet cell (G) metaplasia indicative of mucous secretion and increase in activity of mucous secretion due to fasting, irritation and stress; F: CMS showing surface erosions (E) and marked goblet cell metaplasia with inflammatory cell infiltration (arrow) in the deep mucosa; G and H: Improvement of epithelial lining as indication for surface regeneration with OCT at both dose levels in CMS rats (HE stain, × 40 and 100).

Table 1 Effect of octreotide on ulcer index and plasma corticosterone level in chronic mild stressed rats

Groups	Parameters	
	Ulcer index (mm)	Plasma corticosterone (µg/dL)
Control	0 ± 0	15.4 ± 1.0
OCT (50 µg/kg)	0 ± 0	17.3 ± 0.7
OCT (90 µg/kg)	0 ± 0	16.0 ± 1.2
CMS	6.4 ± 0.16 ^a	28.4 ± 0.6 ^a
CMS + OCT (50 µg/kg)	2.4 ± 0.06 ^{a,b}	17.2 ± 0.7 ^b
CMS + OCT (90 µg/kg)	0.6 ± 0.01 ^{a,b}	20.4 ± 1.1 ^b

Rats were subjected to mild stressors for 21 d; octreotide was given 2 h before the insults or to normal animals. Data are mean of 6-8 rats ± SE. ^{a,b}*P* < 0.05 compared to control and chronic mild stress (CMS) groups respectively. For comparisons among treatment groups, one-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test was used. OCT: Octreotide.

Chemical (MI, USA), respectively. In addition, the activity of gastric mucosal caspase-3 was measured by using a colorimetric assay kit (Biosource International, California, USA). Briefly, the levels of the chromophore p-nitroanilide (pNA) released by caspase-3 activity in the tissue lysates were quantified spectrophotometrically at 405 nm. All the procedures of the used kits were performed following the manufacturer's instruction manual.

Stomach histopathological examination

The stomach was removed from representative animals (*n* = 4) in each group and immediately fixed in 10% phosphate buffered formaldehyde. Subsequently sections were

embedded in paraffin, and 5 µm sections were prepared. The sections were stained with haematoxylin and eosin (HE) and examined microscopically.

Statistical analysis

Data were expressed as mean of 6-8 experiments ± SE. The comparisons of data were carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test. All analysis utilized SPSS 16.0 statistical package for Windows (SPSS Inc., Chicago, IL, USA). A probability level of less than 0.05 was accepted as statistically significant.

RESULTS

Exposure of animals to CMS elevated plasma corticosterone (28 ± 0.6 µg/dL, *P* = 0.002, Table 1), an effect that was associated with erosion formation in the glandular region of the stomach, manifested by gross inspection (6.4 ± 0.16 mm, *P* = 0.01), as well as microscopically (Table 1 and Figure 1). Treatment with 50 and 90 µg/kg octreotide, on the contrary, restored plasma corticosterone level (61% and 71%, *P* = 0.002, respectively) and ameliorated gastric lesion formation (38%, *P* = 0.01, 50 µg/kg), which was more evident at the higher dose (9%, *P* = 0.01) in rats subjected to CMS.

Daily administration of octreotide at the indicated dose levels (50 and 90 µg/kg) for 3 wk to rats did not show any statistical difference from non-treated control animals except for a decrease in PGE₂ (64.9 ± 2.2 and 62.95 ± 3.57 pg/g tissue, *P* = 0.002, respectively) and in-

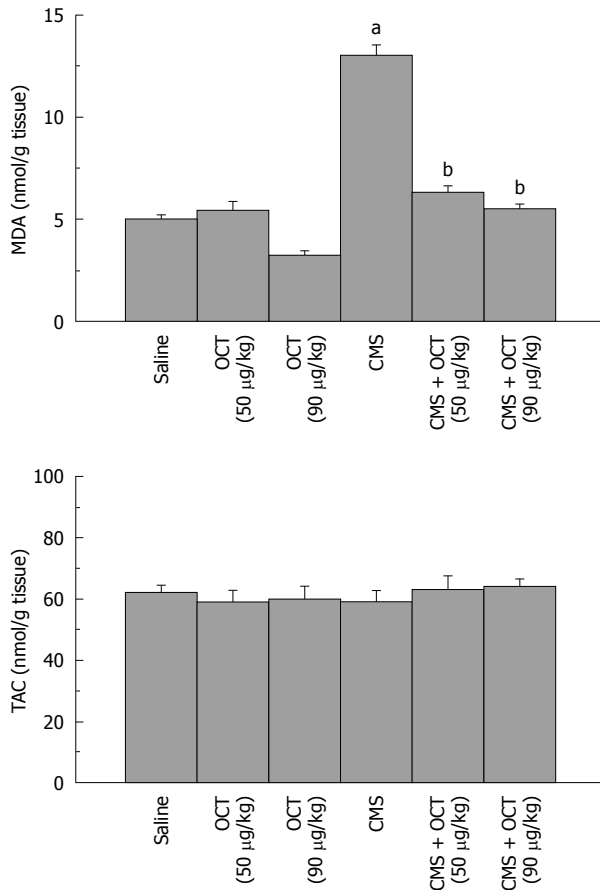


Figure 2 Effect of octreotide (50 and 90 µg/kg, ip) given 2 h prior to chronic mild stress exposure on mucosal malondialdehyde content (upper panel) and total antioxidant capacity (lower panel) in normal rats and those subjected to chronic mild stress. Values are mean \pm SE, $n = 6-8$ each. ^{a,b} $P < 0.05$ vs control and chronic mild stress (CMS), respectively, using one-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test. OCT: Octreotide; MDA: Malondialdehyde content; TAC: Total antioxidant capacity.

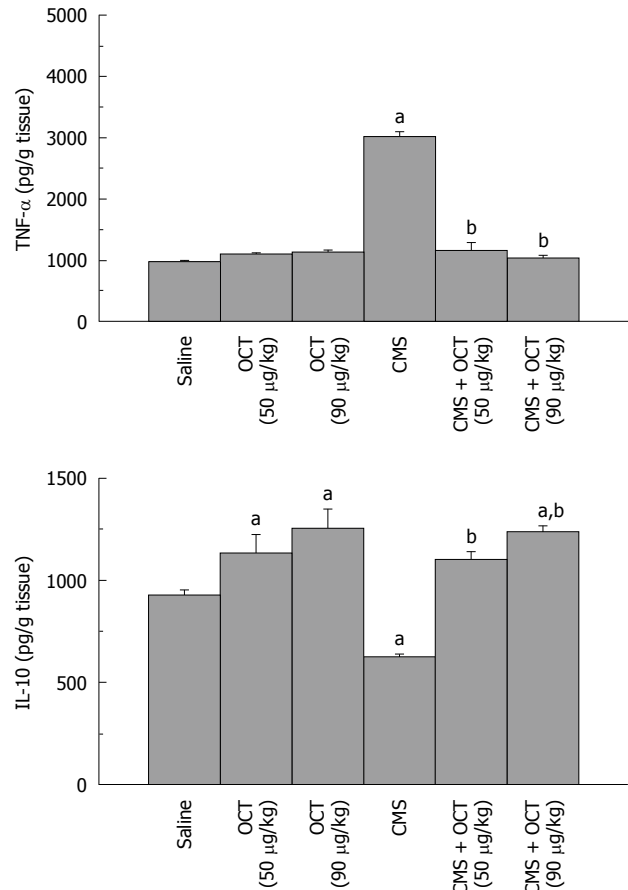


Figure 3 Effect of octreotide (50 and 90 µg/kg, ip) on gastric mucosal tumor necrosis factor- α (upper panel) and interleukin-10 (lower panel) content in normal rats and those subjected to chronic mild stress, where octreotide was administered 2 h before chronic mild stress exposure. Values are mean \pm SE, $n = 6-8$ each. ^{a,b} $P < 0.05$ vs control and chronic mild stress (CMS), respectively, using one-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test. OCT: Octreotide; TNF: Tumor necrosis factor; IL: Interleukin.

creased IL-10 (1128.5 ± 94.1 and 1250.53 ± 95.21 pg/g tissue, $P = 0.001$, respectively) levels (Figures 2-5).

CMS elevated gastric mucosal MDA (13 ± 0.5 nmol/g tissue, $P = 0.001$, Figure 2), TNF- α (3008.6 ± 78.18 pg/g tissue, $P < 0.001$, Figure 3), PGE₂ (117.1 ± 4.31 pg/g tissue, $P = 0.002$, Figure 4), and caspase-3 (2.4 ± 0.14 OD/mg protein, $P = 0.002$, Figure 5) as compared to control values. Furthermore, reduction in the level of IL-10 (627.9 ± 12.82 pg/g tissue, $P = 0.001$, Figure 3) in the gastric mucosa was detected, while TAC ($P = 0.099$) was not altered significantly (Figure 2). Octreotide at both dose levels (50 and 90 µg/kg) suppressed gastric mucosal MDA (58% and 50%, $P = 0.001$, respectively, Figure 2), TNF- α (39% and 34%, $P < 0.001$, respectively, Figure 3), PGE₂ (73% and 67%, $P = 0.002$, respectively, Figure 4) and caspase-3 activity (67% and 79%, $P = 0.002$, respectively, Figure 5), as compared to animals subjected to CMS. Furthermore, octreotide evoked an increment in gastric mucosal IL-10 (175% and 197%, $P = 0.001$, respectively, Figure 3), as compared to stressed rats; however TAC ($P = 0.099$, Figure 2) was unaltered.

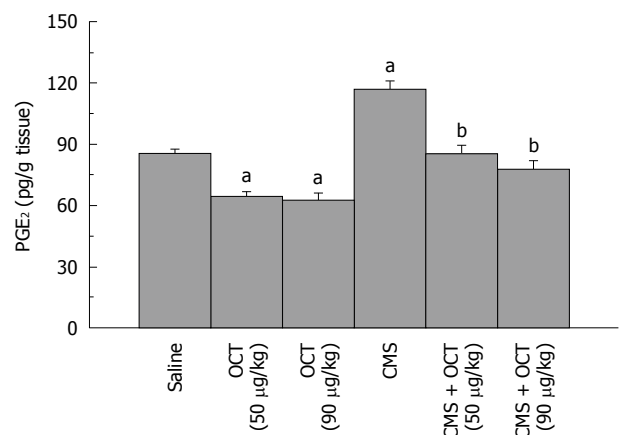


Figure 4 Effect of octreotide (50 and 90 µg/kg, ip) on gastric mucosal PGE₂ content in normal rats and those subjected to chronic mild stress (mean of 6-8 animals \pm SE). Octreotide was administered 2 h before chronic mild stress (CMS) exposure. ^{a,b} $P < 0.05$ vs control and CMS, respectively, using one-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test. OCT: Octreotide.

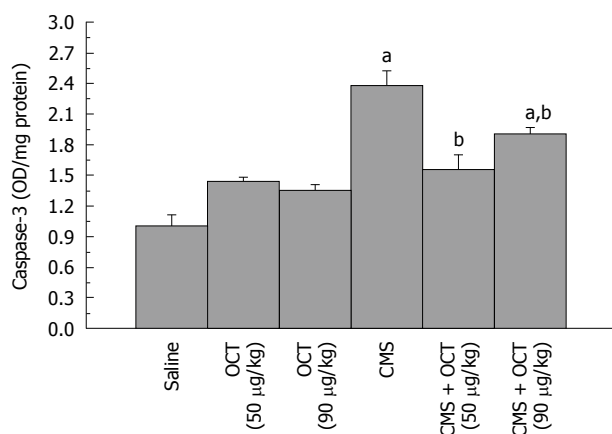


Figure 5 Effect of octreotide (50 and 90 µg/kg, ip) on gastric mucosal caspase-3 activity in normal rats and those subjected to chronic mild stress (mean of 6-8 animals \pm SE). Octreotide was administered 2 h before chronic mild stress (CMS) exposure. ^{a,b} $P < 0.05$ vs control and CMS, respectively, using one-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test. OCT: Octreotide.

DISCUSSION

Previous studies have implicated the influence of acute stress exposure in gastric induced ulcerations^[7,8,10]; however the effect of chronic exposure to mild environmental stressors on gastric mucosal integrity have not been fully delineated. The current investigation extends previous findings, using other stress procedures^[6,27,28], that CMS exposure induces gastric lesions as evidenced by macro- and microscopical besides mechanistic pathways. The most important findings of the current study demonstrate that CMS for 21 d (1) increased plasma corticosterone; (2) induces gastric mucosal erosions; (3) deranges mucosal oxidant status, as well as pro- and anti-inflammatory cytokines; (4) activates caspase-3 mediated apoptosis; and (5) surprisingly enhances PGE₂ production in the gastric mucosa. On the other hand, concomitant administration of octreotide reduced ulcer formation and efficiently reinstated most of the changes associated with CMS.

Stress, on one hand, causes the activation of the brain gut axis and stimulates the stomach both sympathetically and parasympathetically^[29]. The former produces arteriolar vasoconstriction, thus reducing blood flow to the stomach, while the latter enhances gastric motility and muscular contraction leading to vascular compression with consequent mucosal ischemia^[30]. Interestingly, in the present study we demonstrate hydropic degeneration with erosion and epithelial desquamation suggestive of ischemic outcome. Consequently, following the ischemic event, superoxide anion (O₂⁻) leakage from mitochondrial electron transport chain is triggered, which further augments hydroxyl radical (OH[•]) production, with subsequent oxidative damage of macromolecules^[31].

Stress ulcers, on the other hand, are linked to leukocyte infiltration, which further exacerbates free radicals production and TNF- α generation^[7,8,10]. This inflammatory cytokine further recruits more neutrophils resulting in a feed forward damaging cycle^[7]. Both effects were evidenced in

the current study by an increase in lipid peroxides, as well as TNF- α . The increment in lipid peroxides corroborates similar findings in other organs when exposed to CMS^[4,5] and several reports utilizing acute stress models^[8,31], as well as another chronic restraint stress ulcer model^[6]. Notably, lipid peroxides formation is an indicative marker of a vicious ROS cycle; however, there was no change in TAC levels from baseline. A plausible explanation for the latter is the adaptation of the gastric mucosa to CMS-induced ROS, where the presence of either decreased^[6,8,31] or increased^[31,32] levels of endogenous antioxidants, with a net unchanged concentration, was previously reported in ulcer models including acute stress. Meanwhile, we observed a decline in the level of the anti-inflammatory cytokine IL-10, which accounts for the overwhelming antagonistic effect of TNF- α as reported by Brossart *et al.*^[33], which may additionally aggravate gastric lesion formation. Recently, genetic IL-10 polymorphism has been found to predispose individuals to peptic ulcer^[34], thus lending further support to our findings.

Evidence exists that the synthetic somatostatin analogue, octreotide, possesses antiulcerogenic activity in other gastric lesions models^[20,21]. Notably, this protective effect may be attributed to maintaining of mucosal blood flow, an essential gastroprotective factor thus preserving tissue integrity. The gastroprotective effect was associated with reduction in MDA levels, which is in line with the reported findings utilizing octreotide in another model of gastric injury by Sener *et al.*^[21]. In addition, the study of Scheiman *et al.*^[20] provided evidence that octreotide affords gastroprotection by inhibition of neutrophil infiltration, which lends further support to the reduction in inflammatory cells recruitment in the gastric mucosa, more evident with the higher dose of octreotide in this study.

Octreotide also reinstated cytokine levels in the gastric mucosa of animals subjected to a combination of various stressors. Somatostatin analogues were shown to have significant anti-inflammatory effects *in vivo* associated with suppression of inflammatory cytokines as TNF- α ^[35]. Meanwhile, a recent *in vitro* report of ter Veld *et al.*^[36] displayed that octreotide increased IL-10 dose-dependently, an effect that supports the current finding in animals treated with the somatostatin analogue.

Apoptosis is largely implicated in the pathogenesis of gastric ulcers^[37]. Increased TNF- α , as well as free radicals activate caspase-3, one of the effector caspases involved in apoptotic cell death^[38]. Caspases, in turn, elicit neutrophil activation through increased expression of chemoattractants^[10] thus a vicious cycle exists, which further aggravates gastric damage. Therefore, the enhanced caspase-3 activity by CMS is consistent with the noted increase in TNF- α content of the gastric mucosa and the disturbance in oxidants/antioxidants mucosal homeostasis. Moreover, Esplugues *et al.*^[39] depicted that stress itself inhibits gastric acid secretion through a central nervous reflex mechanism; however, the present study documents that atrophy and degeneration of gastric glands as shown in photomicrographs of gastric mucosa of rats subjected to CMS may also be a cause. Additionally, this event, as

well as sloughing of the gastric epithelial layer, may thus pin down the contribution of cell death in this study. Although somatostatin has been reported as an inducer of apoptosis^[40], evidence supports its ability to upregulate Bcl-2, a major inhibitor of apoptosis^[41].

Despite the fact that some studies supported an ulcerogenic action of the endogenous glucocorticoids^[42], other reports^[42,43] showed that these steroids are released as an adaptive response to stress rather than being a significant ulcerogenic component of the brain-gut axis. The present study supports this notion, where CMS was shown to increase corticosterone level. In agreement with the adaptive response to stress, the current investigation revealed that rats subjected to CMS surprisingly elevated PGE₂, an action that is similar to cold-restraint stress^[44]. Exposure to mild stressors is known to cause preconditioning, which contributes to gastroprotective effects against more severe stressors in several gastric ulcer models including exposure to stressful conditions^[45]. Under stress conditions, the enhanced resistance to subsequent challenges by other irritants is attributed, in part, to increased endothelial growth factor (EGF) expression and release, as well as gastric mucosal cell proliferation^[46]. Moreover, phospholipase A₂ is activated by mild stress, which releases arachidonic acid to be metabolized to PGs by both COX-1 and -2 activation^[28]. Hence, PGs formed by preconditioning stress may attenuate stress induced gastric injury^[47]. Such an action could thus account for the erosions seen in the histopathological study rather than deep ulcerations that invade the muscularis layer. PGE₂ is among the factors that regulate gastric blood flow^[47] and enhance mucus, as well as bicarbonate synthesis^[48], which are important gastric defensive factors. The current study shows dilated gastric pits, which reflect increased mucus production in the gastric mucosa of rats exposed to CMS. Such an effect may be a consequence of elevated PGE₂ levels, hence confirming the adaptation theory. Since an imbalance between protective and aggressive factors in the stomach accounts for peptic ulcer formation, the present diverse conditions may favor ulcer formation rather than gastroprotection. On the other hand, treatment with octreotide restored PGE₂ in the gastric mucosa, an effect that highlights its efficacy in intercepting preconditioning of the gastric mucosa to mild stressful procedures.

This study reveals that exposure to chronic mild stressors, such as those present in the environment, may increase susceptibility of the gastric mucosa to aggressive factors, resulting ultimately in gastric lesions. Hence, rats subjected to CMS could serve as a chronic model for stress-induced peptic ulceration that can be used for the evaluation of compounds possessing antiulcer activity. Gastroprotective mechanisms of octreotide are probably due to its antioxidant capacity with concomitant anti-inflammatory and anti-apoptotic effects.

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COMMENTS

Background

Stress has been linked to the etiopathogenesis of various diseases, ranging from psychiatric disorders to several ailments of the gastrointestinal tract. Evidence exists that exposure to acute, as well as chronic, stressful conditions is linked to gastric injury. However, animal models for chronic induction of gastropathy are limited. Izgüt-Uysal *et al* showed that a chronic restrained model for 21 d induced gastric lesions. Moreover, Bhattacharya *et al* introduced another chronic mild stress (CMS) procedure using a rat footshock model as a modification of that adopted by Conti *et al* by adding the element of unpredictability. This model, as well as other stress procedures have been correlated to gastric injury. The current study aimed to evaluate the effect of random exposure to nine different unpredictable stress procedures for 21 d, a multifactorial interactional animal model for CMS, on the emergence of hemorrhagic gastric ulcers and the possible modulatory effects of octreotide, a synthetic somatostatin analogue.

Research frontiers

Gastric ulcer formation is attributed to an imbalance between aggressive and protective factors overweighing the effect of the former. Several hormones regulate gastric mucosal functions among which is somatostatin, a hormone secreted from D-cells in the stomach. Somatostatin suppresses acid secretion directly from parietal cells and indirectly by inhibiting the release of histamine and gastrin. It is used clinically in peptic ulcer bleeding due to its inhibitory effects on pepsin secretion and gastroduodenal mucosal blood flow. Conversely, gastric ulcers are linked to decreased levels of this hormone. However, somatostatin has a short half life, thus synthetic analogues are used efficiently. The effectiveness of synthetic somatostatin analogues as gastroprotective agents is advocated by inhibition of leukocyte adhesion and antioxidant properties, beside their antisecretory potential. Thus the current study utilized octreotide, a synthetic cyclic octapeptide somatostatin analogue, on a chronic model of mild stress exposures for its well documented gastroprotective effect.

Innovations and breakthroughs

A vast majority of acute models for stress-induced gastric ulcerations exists; however, workable models for chronicity and excessive exposure to stressors are limited. To further understand mechanistic pathways and identify new targets for ulcer treatments, the current study aimed to evaluate the effect of random exposure to nine different unpredictable stress procedures for 21 d, a multifactorial interactional animal model for CMS, on the emergence of hemorrhagic gastric ulcers and the possible modulatory effects of a somatostatin analogue, octreotide, utilizing two dose levels.

Applications

The introduction of a CMS gastric lesion model can give better insights for understanding the mechanisms involved in exposure to stressful stimuli in the environment on daily basis that produces gastric injury, as well to facilitate management of such hassle events.

Peer review

The authors presented data of some pathophysiological and morphologic alterations of gastric mucosa associated with experimental chronic stress in rats. They also tried to use a somatostatin analogue, octreotide, to protect the animals from stress-caused ulceration in gastric mucosa. The results appear to be very interesting. They may be helpful for fully understanding chronic stress and gastric lesions. It may indicate a possible therapeutic usage of similar chemicals in prevention of stress-associated gastric ulcer in high-risk individuals.

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Effects of prostaglandin F_{2α} on small intestinal interstitial cells of Cajal

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Abstract

AIM: To explore the role of prostaglandin F_{2α} (PGF_{2α}) on pacemaker activity in interstitial cells of Cajal (ICC) from mouse small intestine.

METHODS: In this study, effects of PGF_{2α} in the cultured ICC cells were investigated with patch clamp technology combined with Ca²⁺ image analysis.

RESULTS: Externally applied PGF_{2α} (10 μmol/L) produced membrane depolarization in current-clamp mode and increased tonic inward pacemaker currents in voltage-clamp mode. The application of flufenamic acid (a non-selective cation channel inhibitor) or niflumic acid (a

Cl⁻ channel inhibitor) abolished the generation of pacemaker currents but only flufenamic acid inhibited the PGF_{2α}-induced tonic inward currents. In addition, the tonic inward currents induced by PGF_{2α} were not inhibited by intracellular application of 5'-[thio]diphosphate trilitium salt. Pretreatment with Ca²⁺ free solution, U-73122, an active phospholipase C inhibitor, and thapsigargin, a Ca²⁺-ATPase inhibitor in endoplasmic reticulum, abolished the generation of pacemaker currents and suppressed the PGF_{2α}-induced tonic inward currents. However, chelerythrine or calphostin C, protein kinase C inhibitors, did not block the PGF_{2α}-induced effects on pacemaker currents. When recording intracellular Ca²⁺ ([Ca²⁺]_i) concentration using fluo-3/AM, PGF_{2α} broadly increased the spontaneous [Ca²⁺]_i oscillations.

CONCLUSION: These results suggest that PGF_{2α} can modulate pacemaker activity of ICC by acting non-selective action channels through phospholipase C-dependent pathway *via* [Ca²⁺]_i regulation

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Key words: Prostaglandin F_{2α}; Interstitial cells of Cajal; Tonic inward currents; Intestinal motility

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INTRODUCTION

Prostaglandins (PGs) of the E, F, and I series are widely

distributed in all body tissues, including the gastrointestinal (GI) tract and have been shown to affect water and electrolyte transport, mucous secretion and blood flow^[1-3]. Also, there is much evidence that PGs may be involved in the control of the contractions of intestinal smooth muscle. Inhibition of endogenous PG synthesis by indomethacin appears to enhance intestinal motility by inducing a fed-like pattern^[4-6]. These reports suggest that endogenous PGs may play an important role in the modulation of intestinal motility.

In general, PGE₂ is known to contract longitudinal muscle and to relax circular muscle, whereas PGF_{2α} induces contractions of both muscular layers^[6-9]. PGF_{2α} causes contractions of both colonic muscle layers in the guinea pig and in humans and rats^[10,11]. Endogenous PGF_{2α} is suggested to modify contractile activity of antral smooth muscle and intestinal muscle^[12,13]. Therefore, exogenous PGF_{2α} and PGF_{2α} synthesized in the intestinal smooth muscle cells may affect motor activities.

The interstitial cells of Cajal (ICC) have functions of pacemaker cells and neuromediator cells in the tunica muscularis of the GI tract^[14]. The ICC generate the rhythmic oscillations in membrane potential known as slow waves and this generation of slow waves is due to spontaneous inward currents called pacemaker currents^[15,16]. Although the exact mechanisms regarding these events are still unclear, many reports have suggested that the activation of non-selective cation channels, Cl⁻ channels, and spontaneous intracellular Ca²⁺ ([Ca²⁺]_i) activities in ICC are involved in the production of pacemaker activity^[15-17]. Also, it is well known that many endogenous agents such as neurotransmitters, hormones and paracrine substances modulate GI tract motility by influencing ICC.

There are many reports that PGF_{2α} has a role in GI motility by acting on smooth muscles but no studies have been performed to determine the effects of PGF_{2α} on electrical events in ICC. Therefore, the purpose of our study was to investigate the signal transduction effects of PGF_{2α} on pacemaker activity in cultured ICC.

MATERIALS AND METHODS

Ethics

All experiments were carried out according to the guiding principles for the care and use of animals approved by the ethics committee of Chosun University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the number of animals used and their suffering.

Solutions and drugs

The cells were bathed in a solution containing: 5 mmol/L KCl, 135 mmol/L NaCl, 2 mmol/L CaCl₂, 10 mmol/L glucose, 1.2 mmol/L MgCl₂, and 10 mmol/L HEPES, adjusted to pH 7.2 with Tris. The pipette solution contained 140 mmol/L KCl, 5 mmol/L MgCl₂, 2.7 mmol/L K₂ATP, 0.1 mmol/L Na₂GTP, 2.5 mmol/L creatine phosphate disodium, 5 mmol/L HEPES, 0.1 mmol/L EGTA, adjusted to pH 7.2 with Tris.

Drugs used were: PGF_{2α}, guanosine 5'-[thio]diphosphate trilithium salt (GDP-β-S), U-73122, Calphostin C, Chelethrine, and Thapsigargin. All drugs were purchased from the Sigma Chemical Co (St. Louis, MO, USA). Flufenamic acid and niflumic acid were purchased from Calbiochem (San Diego, CA, USA).

All drugs were dissolved in DW or DMSO to prepare stock solutions (10 or 100 mmol/L), and were either added to the bath solution or applied to the whole-cell preparations by superfusion. The final concentration of DMSO was less than 0.05%.

Preparation of cells and tissues

Balb/C mice (3- to 7-d-old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa removed by sharp dissection. Small strips of intestinal muscle were equilibrated in Ca²⁺-free Hank's solution for 30 min and the cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 1.3 mg/mL; bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 2 mg/mL; trypsin inhibitor (Sigma), 2 mg/mL; and ATP, 0.27 mg/mL. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/mL, Falcon/BD) in 35 mm culture dishes. The cells were then cultured at 37°C in a 95% O₂-5% CO₂ incubator in smooth muscle growth medium (SMGM, Clonetics Co., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/mL, Sigma). Interstitial cells of Cajal (ICC) were identified immunologically with a monoclonal antibody for Kit protein (ACK2) labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA).

Patch clamp experiments

The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC. Currents or potentials were amplified by use of an Axopatch 1-D (Axon Instruments, Foster, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor and a pen recorder (Gould 2200, Gould, Valley view, OH, USA).

Results were analyzed using pClamp and Sigma plot (version 9.0) software. All experiments were performed at 30°C.

Measurement of the [Ca²⁺]_i concentration

Changes in the [Ca²⁺]_i concentration were monitored by using fluo-3/AM, which was initially dissolved in dimethyl

sulfoxide and stored at -20°C. The cultured ICC on coverslips (25 mm) were rinsed twice with a bath solution (5 mmol/L KCl, 135 mmol/L NaCl, 2 mmol/L CaCl₂, 10 mmol/L glucose, 1.2 mmol/L MgCl₂ and 10 mmol/L HEPES, adjusted to pH 7.4 with Tris). The coverslips were then incubated in the bath solution containing 5 μmol/L fluo-3 with 5% CO₂ at 37°C for 5 min, rinsed two more times with the bath solution, mounted on a perfusion chamber, and scanned every 0.4 s with a Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera (× 400). Fluorescence was determined with an excitation wavelength of 488 nm, and emitted light was observed at 515 nm. During scanning of the Ca²⁺ imaging, the temperature of the perfusion chamber containing the cultured ICC was kept at 30°C. The variations of [Ca²⁺]_i fluorescence emission intensity were expressed as F1/F0 where F0 is the intensity of the first imaging.

Statistical analysis

Data are expressed as the mean ± SE. Differences in the data were evaluated by Student's *t*-test. *P* < 0.05 was taken as a statistically significant difference. The *n* values reported in the text refer to the number of cells used in the patch-clamp experiments.

RESULTS

Effect of PGF_{2α} on pacemaker potentials and currents in cultured ICC

ICC, identified by Kit immunofluorescence, had a distinctive morphology that was easily recognized in cultures. We thus performed the electrophysiological recording from cultured ICC under current (*I* = 0) and voltage clamp mode. Under current clamp mode, ICC showed spontaneous pacemaker potentials. The resting membrane potential was -53 ± 4 mV and amplitude was 27 ± 2 mV. In the presence of PGF_{2α} (10 μmol/L), membrane potentials were depolarized to -29 ± 3.4 mV and the amplitude of pacemaker potentials was decreased to 3.9 ± 1.6 mV (*n* = 5, Figure 1A, bar graph not shown). These results are in agreement with previous studies showing that ICC have spontaneous pacemaker activity and we also found PGF_{2α} to have action on this electrical activity of ICC. Under a voltage clamp at a holding potential of -70 mV, the ICC generated spontaneous inward currents. Treatment with various concentrations of PGF_{2α} in cultured ICC produced tonic inward currents and decreased the frequency and the amplitude of pacemaker currents in a dose-dependent manner (Figure 1B-D). As shown in Figure 1E-G, the values of frequency, amplitude and resting currents with regard to pacemaker currents under control conditions were significantly different from those obtained in the presence of PGF_{2α}.

Effects of non-selective cation channel blocker or Cl⁻ channel blocker on PGF_{2α}-induced responses in cultured ICC

In order to characterize the tonic inward currents in-

duced by PGF_{2α}, we used flufenamic acid, a non-selective cation channel blocker, or niflumic acid, a Cl⁻ channel blocker. Figure 2A shows that treatment with flufenamic acid (10 μmol/L) abolished the generation of pacemaker currents and blocked the PGF_{2α}-induced tonic currents in ICC. The summarized bar graph (Figure 2B) indicates that the resting currents produced by PGF_{2α} were -21 ± 9 pA in the presence of flufenamic acid and that this value was significantly different when compared with control values obtained in the absence of flufenamic acid (*n* = 4). In the presence of niflumic acid (10 μmol/L), the pacemaker currents were abolished. Under this condition, PGF_{2α} still produced the tonic inward currents (Figure 2C). In the presence of niflumic acid, the resting currents produced by PGF_{2α} were -98 ± 12 pA; this value was not significantly different when compared with control values obtained in the absence of niflumic acid (*n* = 5, Figure 2D).

No involvement of G proteins in the PGF_{2α}-induced tonic inward currents in cultured ICC

The effects of GDP-β-S, a nonhydrolysable guanosine 5'-diphosphate analogue which permanently inactivates GTP binding proteins, were examined to determine whether the G-protein is involved in the effects of PGF_{2α} in ICC. When GDP-β-S (1 mmol/L) was in the pipette, PGF_{2α} (10 μmol/L) still showed the tonic inward currents (Figure 3A). In the presence of GDP-β-S in the pipette, the resting currents in the control were -23 ± 9 pA. The resting currents during treatment with PGF_{2α} in the presence of GDP-β-S were -159.9 ± 36 pA (*n* = 4, Figure 3B), which were not significantly different from those obtained by treatment with PGF_{2α} in the absence of GDP-β-S.

External Ca²⁺-free solution and Ca²⁺-ATPase inhibitor of endoplasmic reticulum suppress PGF_{2α} effects in cultured ICC

To investigate the role of external Ca²⁺ or internal Ca²⁺, PGF_{2α} was tested under external Ca²⁺-free conditions and in the presence of thapsigargin, a Ca²⁺-ATPase inhibitor of endoplasmic reticulum. The application of external Ca²⁺-free solution completely inhibited the pacemaker currents in voltage clamp mode at a holding potential of -70 mV and in this condition, PGF_{2α} (10 μmol/L)-induced effects on pacemaker currents were blocked (*n* = 5, Figure 4A). The value of resting currents with PGF_{2α} (10 μmol/L) in Ca²⁺-free solution was significantly different when compared with control values obtained in normal solution (Figure 4B). In addition, the treatment with thapsigargin (5 μmol/L) inhibited the pacemaker currents in ICC and blocked the PGF_{2α}-induced tonic inward currents (Figure 4C). In the presence of thapsigargin, the value of resting currents during treatment with PGF_{2α} was significantly different from those obtained by treatment with PGF_{2α} in the absence of thapsigargin (*n* = 6, Figure 4D).

Effects of phospholipase C inhibitor on the PGF_{2α}-induced tonic inward currents in cultured ICC

To investigate whether the PGF_{2α}-induced effects on

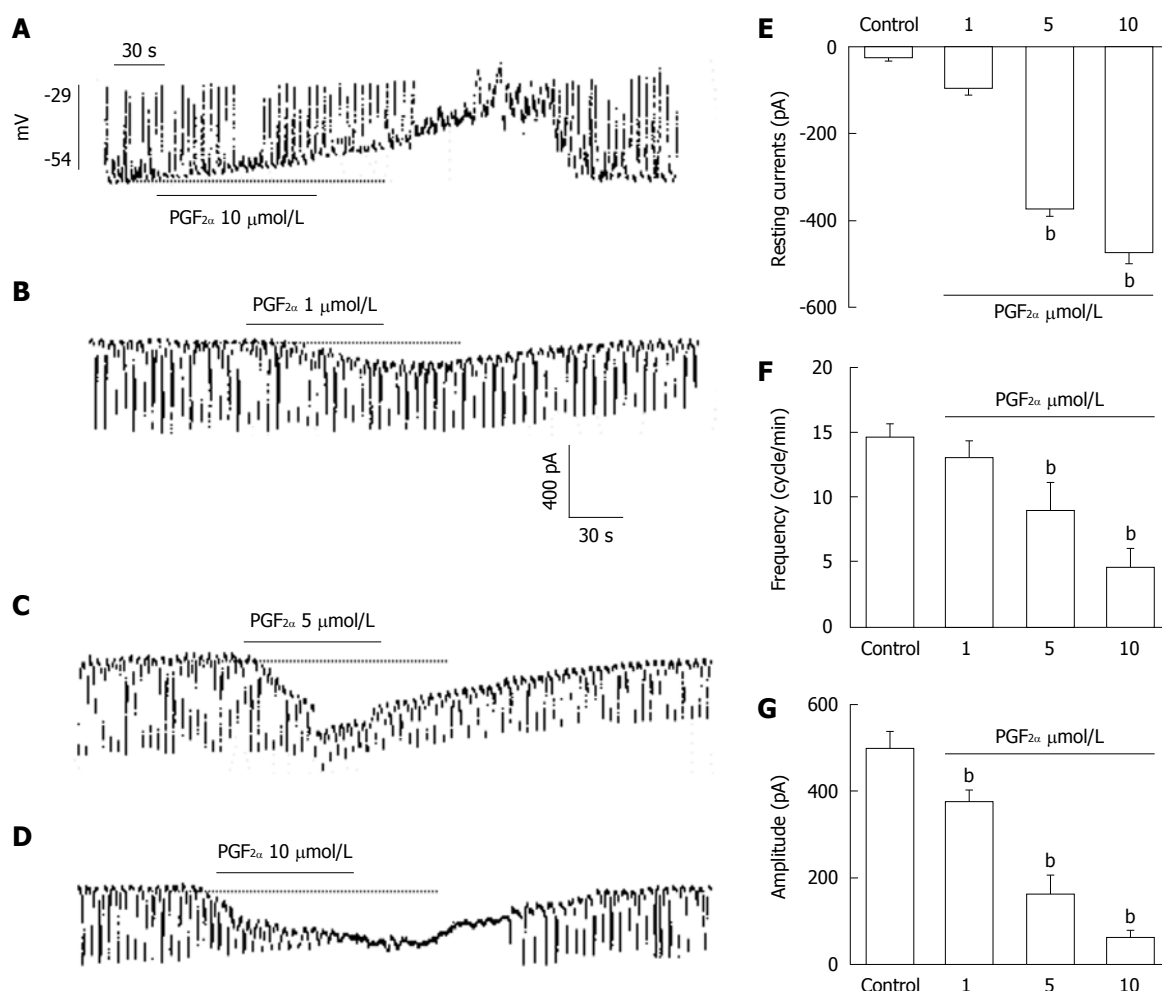


Figure 1 The effects of Prostaglandin F_{2α} on pacemaker potentials and pacemaker currents recorded in cultured interstitial cells of Cajal from mouse small intestine. A: Pacemaker potentials of interstitial cells of Cajal (ICC) exposed to Prostaglandin F_{2α} (PGF_{2α}) (10 μmol/L) in the current-clamping mode (*I* = 0). Vertical solid line scales denote amplitude of pacemaker potential and horizontal solid line scales denote duration of recording (s) pacemaker potentials; B-D: Pacemaker currents of ICC recorded at a holding potential of -70 mV exposed to various concentrations of PGF_{2α} (1, 5, and 10 μmol/L). The dotted lines indicate zero current levels. Vertical solid line scales denote amplitude of pacemaker current and horizontal solid line scales denote duration of recording (s) pacemaker currents. The responses to PGF_{2α} are summarized in E-G. The bars represent mean ± SE. ^b*P* < 0.01 vs the untreated control.

pacemaker currents are mediated by the activation of phospholipase C (PLC), we treated the ICC with U-73122, a PLC inhibitor. U-73122 (5 μmol/L) abolished the generation of pacemaker currents and blocked the PGF_{2α}-induced tonic inward currents (Figure 5A). In the presence of U-73122, the tonic inward currents produced by PGF_{2α} (10 μmol/L) were -21 ± 11 pA. The value of resting currents induced by PGF_{2α} was significantly different from those obtained by treatment with PGF_{2α} in the absence of U-73122 (*n* = 5, Figure 5B). These results suggest that the PGF_{2α}-induced tonic inward currents may be a PLC-dependent mechanism.

Effects of protein kinase C inhibitor on PGF_{2α}-induced responses in cultured ICC

We tested the effects of chelerythrine or calphostin C, inhibitors of protein kinase C (PKC), to investigate whether PGF_{2α}-induced responses to pacemaker currents are mediated by the activation of PKC. Chelerythrine (1 μmol/L) or calphostin C (10 μmol/L) did not have an effect on

tonic inward currents induced by PGF_{2α} (10 μmol/L) (Figure 6A and C) and the value also was not significantly different when compared with the tonic inward currents induced by PGF_{2α} obtained in the absence of chelerythrine or calphostin C (*n* = 5, Figure 6B and D).

Increasing of [Ca²⁺]_i intensity by PGF_{2α}

Because many reports have suggested that [Ca²⁺]_i oscillations in ICC could be considered to be the primary mechanism for the pacemaker activity in GI activity, we examined the effect of PGF_{2α} on [Ca²⁺]_i oscillations in ICC. In this study, we measured spontaneous [Ca²⁺]_i oscillations of ICC which are connected with cell clusters. Spontaneous [Ca²⁺]_i oscillations observed in ICC which were loaded with fluo3-AM and the time series data show the spontaneous regular [Ca²⁺]_i oscillations. In the presence of PGF_{2α} (10 μmol/L), the basal points of [Ca²⁺]_i oscillations were increased but the peak points of [Ca²⁺]_i oscillations were slightly decreased (Figure 7A). The data of the time series are summarized in Figure 7B. These results suggest

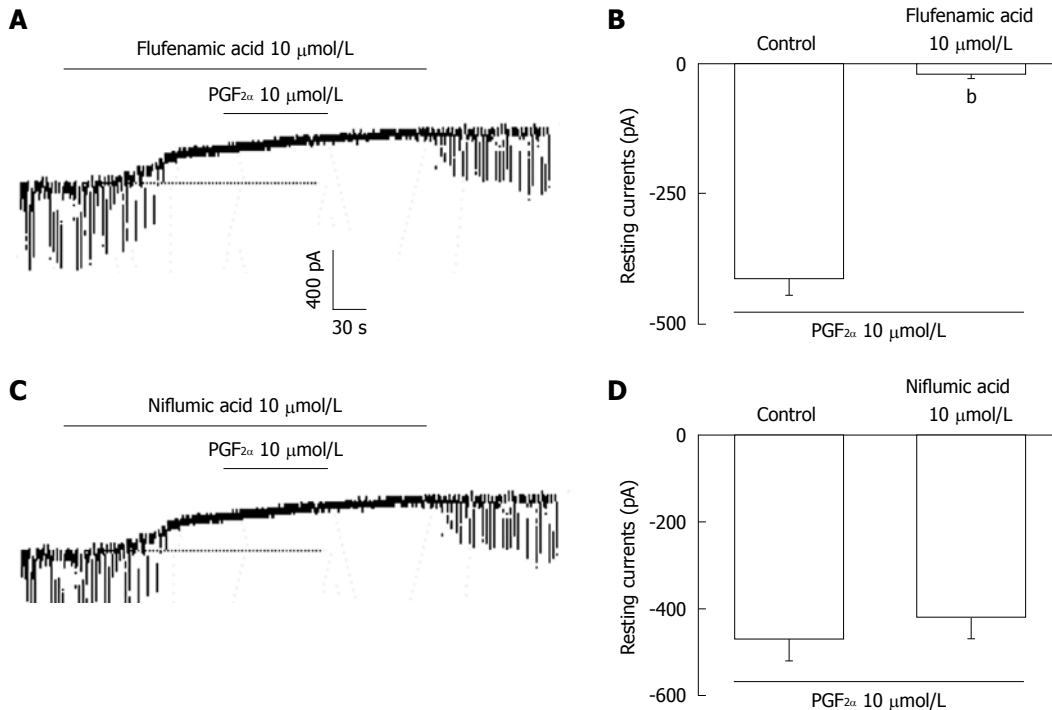


Figure 2 The effects of flufenamic acid or niflumic acid on prostaglandin $\text{F}_{2\alpha}$ -induced responses in pacemaker currents in cultured interstitial cells of Cajal from mouse small intestine. **A:** Application of flufenamic acid (10 $\mu\text{mol/L}$) abolished the generation of pacemaker currents. Under these conditions, the prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (10 $\mu\text{mol/L}$) did not produce tonic inward currents; **C:** Niflumic acid (10 $\mu\text{mol/L}$) also abolished the generation of pacemaker currents. However, niflumic acid did not block the $\text{PGF}_{2\alpha}$ (10 $\mu\text{mol/L}$)-induced tonic inward currents. The dotted lines indicate zero current levels. Responses to the $\text{PGF}_{2\alpha}$ in the presence of flufenamic acid or niflumic acid are summarized in **B** and **D**. Vertical solid line scales denote amplitude of pacemaker current and horizontal solid line scales denote duration of recording (s) pacemaker currents. The bars represent mean \pm SE. ^b $P < 0.01$ vs the untreated control.

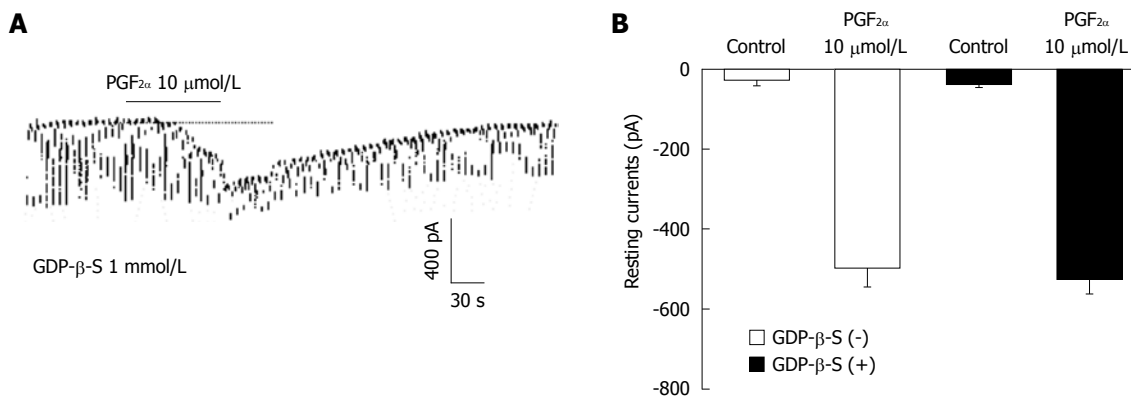


Figure 3 The effects of 5'-[thio]diphosphate trilithium salt on response to prostaglandin $\text{F}_{2\alpha}$ -induced pacemaker currents from interstitial cells of Cajal from mouse small intestine. **A:** Pacemaker currents from interstitial cells of Cajal exposed to prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (10 $\mu\text{mol/L}$) in the presence of 5'-[thio]diphosphate trilithium salt (GDP- β -S) (1 mmol/L) in the pipette. The tonic inward currents with suppressed amplitude and frequency induced by $\text{PGF}_{2\alpha}$ remained unchanged during internally applied GDP- β -S (1 mmol/L). The dotted lines indicate the zero current levels. The effects of $\text{PGF}_{2\alpha}$ in the presence of GDP- β -S are summarized in **B**. Vertical solid line scales denote amplitude of pacemaker current and horizontal solid line scales denote duration of recording (s) pacemaker currents. Bars represent mean \pm SE. The effects of GDP- β -S on $\text{PGF}_{2\alpha}$ -induced pacemaker currents were not significantly different from the $\text{PGF}_{2\alpha}$ -induced pacemaker currents in the absence of GDP- β -S.

that the action of $\text{PGF}_{2\alpha}$ on ICC may involve the regulation of spontaneous $[\text{Ca}^{2+}]_i$ oscillations.

DISCUSSION

Although the actions of $\text{PGF}_{2\alpha}$ have been demonstrated with regard to GI motility in tissue and smooth muscle cells, this is the first study in ICC in which an attempt has

been made to determine the effects of $\text{PGF}_{2\alpha}$ on electrical activity in the small intestine.

The results of the present study demonstrate that $\text{PGF}_{2\alpha}$ regulates intestinal motility by modulating the pacemaker currents of ICC and that this modulation is mediated *via* the action on non-selective cation channels and $[\text{Ca}^{2+}]_i$ mobilization in a PKC-independent manner.

Most regions of the GI tract generate spontaneous elec-

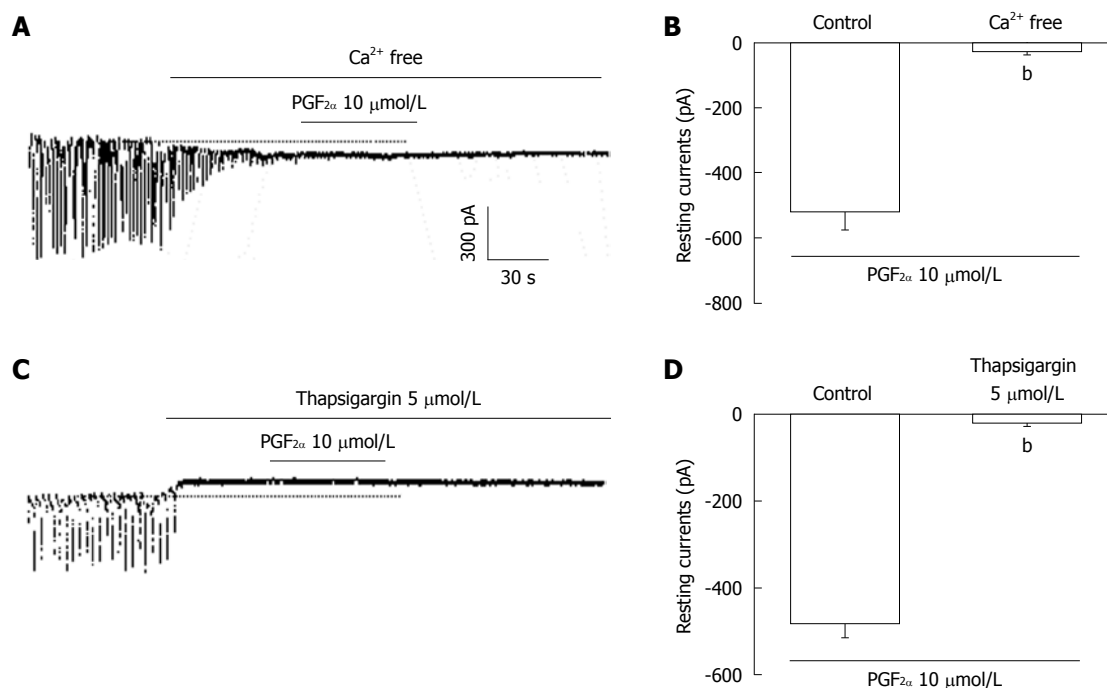


Figure 4 The effects of an external Ca^{2+} -free solution or thapsigargin on the prostaglandin $\text{F}_{2\alpha}$ -induced response in pacemaker currents in cultured interstitial cells of Cajal from mouse small intestine. A: External Ca^{2+} -free solution abolished the generation of pacemaker currents. Under these conditions, the prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (10 $\mu\text{mol/L}$)-induced tonic inward currents were blocked; C: Thapsigargin (5 $\mu\text{mol/L}$) abolished the generation of pacemaker currents. Thapsigargin also blocked the $\text{PGF}_{2\alpha}$ (10 $\mu\text{mol/L}$)-induced tonic inward currents. The dotted lines indicate the zero current levels. Responses to the $\text{PGF}_{2\alpha}$ in the external Ca^{2+} -free solution and in the presence of thapsigargin are summarized in B and D. Vertical solid line scales denote amplitude of pacemaker current and horizontal solid line scales denote duration of recording (s) pacemaker current. The bars represent mean \pm SE. ^b $P < 0.01$ vs the untreated control.

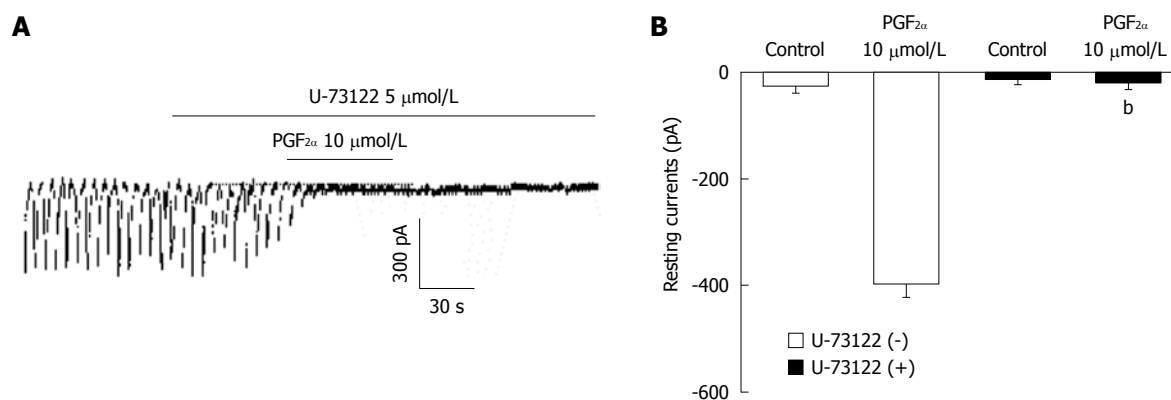


Figure 5 The effects of U-73122 on the prostaglandin $\text{F}_{2\alpha}$ -induced response in pacemaker currents of cultured interstitial cells of Cajal from mouse small intestine. A: U-73122 (5 $\mu\text{mol/L}$) abolished the generation of pacemaker currents. U-73122 also blocked the Prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (10 $\mu\text{mol/L}$)-induced tonic inward currents. The dotted lines indicate the zero current levels. Responses to the $\text{PGF}_{2\alpha}$ are summarized in B. Vertical solid line scales denote amplitude of pacemaker current and horizontal solid line scales denote duration of recording (s) pacemaker current. The bars represent mean \pm SE. The effect of U-73122 on $\text{PGF}_{2\alpha}$ -induced pacemaker currents was significantly different from the $\text{PGF}_{2\alpha}$ -induced pacemaker currents in the absence of U-73122 (^b $P < 0.01$).

trical and mechanical activity in the absence of stimulation. When electrical recordings are made from smooth muscle cells lying in the GI tract, a regular discharge of long lasting waves of depolarization, called slow waves, is detected. It has recently become apparent that slow waves are generated by a specialized population of smooth muscle cells, known as ICC^[18]. ICC generate spontaneous pacemaker inward currents that depolarize membrane, this spreads to smooth muscle *via* gap junctions resulting in depolarization of membrane in smooth muscle leading to contraction by

generating acting potentials through voltage-dependent Ca^{2+} channel activation^[18]. From previous studies, many reports suggested that $\text{PGF}_{2\alpha}$ usually showed contractile actions in *in vivo* and *in vitro* studies^[19,21]. These reports indicate the possibility that $\text{PGF}_{2\alpha}$ may have stimulatory functions on the electrical activity of ICC. In the present study we found that ICC produced spontaneous pacemaker inward currents under voltage clamp mode and that the application of $\text{PGF}_{2\alpha}$ evoked the tonic inward currents of pacemaker currents. This result offers the new suggestion that the

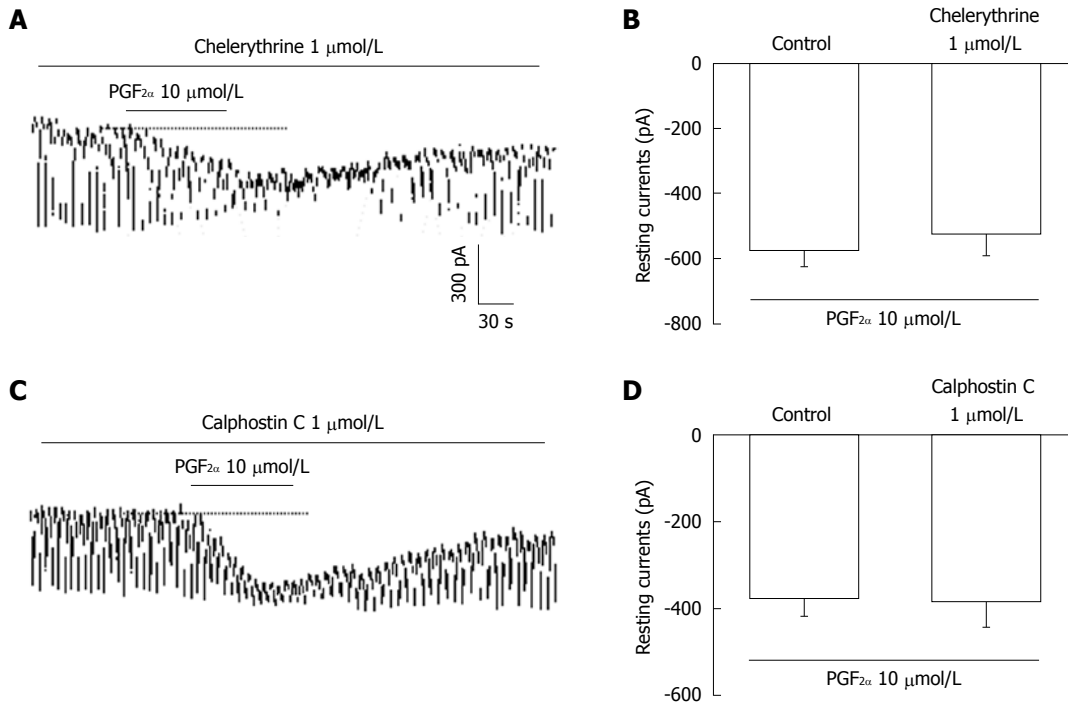


Figure 6 The effects of chelerythrine or calphostin C on the prostaglandin $\text{F}_{2\alpha}$ -induced response in pacemaker currents in cultured interstitial cells of Cajal from mouse small intestine. A and C: Pacemaker currents of interstitial cells of Cajal exposed to prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (10 $\mu\text{mol/L}$) in the presence of chelerythrine (1 $\mu\text{mol/L}$) or calphostin C (1 $\mu\text{mol/L}$). Under these conditions, the $\text{PGF}_{2\alpha}$ caused tonic inward currents. The dotted lines indicate the zero current levels. Responses to the $\text{PGF}_{2\alpha}$ in the presence of chelerythrine or calphostin C are summarized in B and D. Vertical solid line scales denote amplitude of pacemaker current and horizontal solid line scales denote duration of recording (s) pacemaker current. The bars represent mean \pm SE. No significant difference from the untreated control.

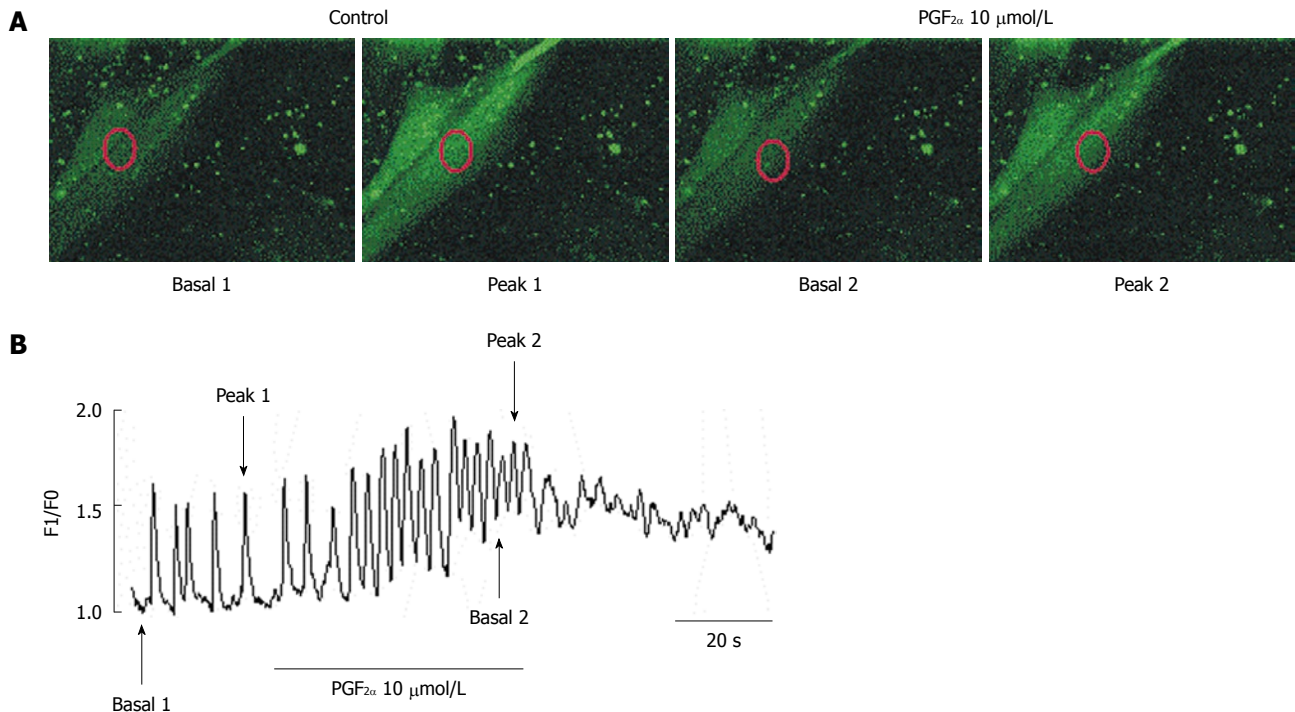


Figure 7 Effects of Prostaglandin $\text{F}_{2\alpha}$ on intracellular Ca^{2+} oscillations in cultured interstitial cells of Cajal from mouse small intestine. A: Sequential fluorescence intensity images of fluo-3-loaded cultured interstitial cells of Cajal under normal conditions. The representative frames indicate in (B) basal 1, 2, peak 1 and 2. The exposure time of each frame was 500 ms; B: Fluorescence intensity change plotted in (A) red marker in absence and presence of Prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (10 $\mu\text{mol/L}$). The horizontal solid line scales denote duration of fluorescence intensity change (s).

regulation of electrical activity in ICC may be involved in the contractile effects of $\text{PGF}_{2\alpha}$ in the GI tract.

Until now, the exact mechanism of pacemaker activity generation has not been fully understood in ICC.

Two suggestions exist: that pacemaker currents of ICC are mediated by the activation of voltage-independent non-selective cation channels and that inwardly rectifying Cl⁻ channels can be generated by the rhythmic inward currents^[15-17]. For checking of these suggestions, we used the blockers of non-selective cation and inwardly rectifying Cl⁻ channels and we could find that flufenamic acid, a non-selective cation blocker, abolished pacemaker current generation and that PGF_{2α}-induced tonic inward currents were blocked by flufenamic acid. However, although niflumic acid, a Cl⁻ channel blocker, also abolished pacemaker generation, niflumic acid did not block the PGF_{2α}-induced tonic inward currents. Furthermore, we have already found that substance P and bradykinin may modulate intestinal motility acting on ICC through the activation of non-selective cation channels^[22,23]. Therefore, these data strongly provide support for the suggestion that, and it is likely that, both Cl⁻ channels and non-selective cation channels are essentially needed for the generation of the spontaneous pacemaker currents in ICC. However, the agent-induced tonic inward currents in ICC may modulate the pacemaker currents by regulating non-selective cation channels in ICC.

The effects of PGs are mediated by specific receptors, classified into basic types (DP, EP, FP, IP, and TP) according to the PG ligand that each binds with the greatest affinity. They have different cell- and tissue-specific functions, as determined by selective coupling to G proteins and by the expression of splicing isoforms. In the case of PGF_{2α}, PGF_{2α} induces inositol (1,4,5) trisphosphate (IP₃) production and increases Ca²⁺ levels *via* FP receptors, which are coupled to G proteins for functional action in various tissues. For example, PGF_{2α} induces phosphoinositol (PI) turnover in isolated luteal cells and the effect of PGF_{2α} in inducing Ca²⁺ mobilization in mouse fibroblasts occurs in conjunction with formation of IP₃, and is pertussis toxin-insensitive^[24,25]. These findings suggest that FP receptors can interact with a member of the G protein family to activate phospholipase C (PLC), leading ultimately to an IP₃-mediated mobilization of Ca²⁺ from intracellular pools. However, there also appears to be a secondary phase of Ca²⁺ release by PGF_{2α}-treated mouse fibroblasts that is likely due to extracellular Ca²⁺ entry. In this present study, when GDP-β-S was present in the pipette, the tonic inward pacemaker currents induced by PGF_{2α} were still present. This means that the effects of PGF_{2α} on electrical activity in ICC may be not related to G proteins. Additionally, since many proposals have been made that PGF_{2α} may have biological activity through mobilization of [Ca²⁺]_i and PLC activation, we used thapsigargin, a potent endoplasmic reticulum Ca²⁺-ATPase inhibitor and U-73122, an active PLC inhibitor, and found that these inhibitors suppressed the PGF_{2α}-induced tonic inward currents. These results strongly support the proposition that the release of Ca²⁺ from internal storage and PLC activation by PGF_{2α} are essential to produce tonic inward currents. Also, these findings have correlations with previous suggestions. In addition, it is well known

that the generation of pacemaker currents is dependent upon [Ca²⁺]_i oscillation and that the periodic release of Ca²⁺ from endoplasmic reticulum is essential for generating pacemaker currents. We believe that our experiments using thapsigargin and U-73122 underscore this information.

In many tissues, it is well known that the binding of PGF_{2α} to its receptor results in not only the activation of PLC (and PLC is the initial step of the PI cascade that generates diacylglycerol (DAG), IP₃, and Ca²⁺ release) but also in the activation of a PKC-dependent pathway. The increase in [Ca²⁺]_i not only promotes translocation of some PKC isozymes to the plasma membrane, but, in concert with DAG, is essential in activating the conventional isoforms of PKC^[22]. However, in the present study, chelerythrine or calphostin C, specific and potent PKC inhibitors, did not block PGF_{2α}-induced effects, suggesting that PKC is not involved in the actions of PGF_{2α} in ICC.

The periodic pacemaker activity of ICC is dependent on [Ca²⁺]_i oscillations. The pacemaker mechanism is initiated by release of Ca²⁺ from the endoplasmic reticulum and is followed by reuptake of Ca²⁺ into the mitochondria^[26]. In our results, we found spontaneous [Ca²⁺]_i oscillations in ICC and this means that the spontaneous pacemaker activity of ICC is closely involved with [Ca²⁺]_i oscillations in this experiment; the treatment with PGF_{2α} in ICC increased the basal point of [Ca²⁺]_i oscillation and decreased the peak point. However, the [Ca²⁺]_i intensity was broadly increased as the action of tonic inward currents reversed. Our previous report suggested that PGE₂ inhibited [Ca²⁺]_i oscillations by ATP-sensitive K⁺ channel activation in cultured ICC^[27]. The observed actions of PGE₂ on [Ca²⁺]_i oscillation in ICC support the suggestion that [Ca²⁺]_i oscillations are important actions of pacemaker activity. Namely, PGE₂ and PGF_{2α} have opposing actions in ICC. However, both PGE₂ and PGF_{2α} have the same target for modulating the pacemaker activity of ICC; that is, the [Ca²⁺]_i in ICC. Therefore, these results suggest that the spontaneous oscillation of [Ca²⁺]_i is essential for pacemaker activity of ICC and that the [Ca²⁺]_i can be the main regulatory target for various endogenous agents or neurotransmitters in ICC.

In the present study, externally applied PGF_{2α} depolarized ICC membranes and formed spike potentials which would result in muscle contraction. As the ICC are electrically coupled with nearby smooth muscles through the gap junctions, the resulting contraction propagates around and along the gut in a coordinated manner and ultimately regulates GI motility.

In conclusion, this study describes the effects of PGF_{2α} on ICC in the mouse small intestine. PGF_{2α} depolarized the membrane with increased tonic inward currents, which were activated by non-selective cation channels *via* external Ca²⁺ influx, PLC, and internal Ca²⁺ mobilization, in a PKC-independent manner. Thus, PGF_{2α} may play a very important role in regulating the rhythm and contraction of small intestinal smooth muscles by acting on ICC.

COMMENTS

Background

Prostaglandins (PGs) of the E, F, and I series are widely distributed in all body tissues, including the gastrointestinal (GI) tract. Many reports have suggested that PGF_{2α} plays an important role in the modulation of intestinal motility.

Research frontiers

There are many reports that PGF_{2α} has a function in GI motility by acting on smooth muscles but no studies have been performed to determine the effects of PGF_{2α} on electrical events in interstitial cells of Cajal (ICC). Therefore, the purpose of our study was to investigate the signal transduction effects of PGF_{2α} on pacemaker activity in cultured ICC.

Innovations and breakthroughs

This study showed the actions of PGF_{2α} on ICC in the mouse small intestine. PGF_{2α} depolarized the membrane with increased tonic inward currents, which were activated by non-selective cation channels via external Ca²⁺ influx, phospholipase C, and internal Ca²⁺ mobilization, in a protein kinase C-independent manner.

Applications

The role of PGF_{2α} in ICC may be one theory for understanding the excitatory action of PGF_{2α} in GI motility.

Terminology

ICC have functions of pacemaker cells and neuromediator cells in the tunica muscularis of the GI tract. The ICC generate rhythmic oscillations in membrane potential known as slow waves and this generation of slow waves is due to spontaneous inward currents called pacemaker currents.

Peer review

It is an interesting paper dealing with a demanding subject. They found that PGF_{2α} can regulate intestinal motility through the modulation of ICC pacemaker activities.

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Hepatoma cell line HepG2.2.15 demonstrates distinct biological features compared with parental HepG2

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and the pathological analysis of tumor formation was performed. Two cytoskeletal proteins were detected by Western blotting.

RESULTS: Compared with HepG2 cells, HepG2.2.15 cells showed organelle degeneration and filopodia disappearance under electron microscope. HepG2.2.15 cells proliferated and migrated slowly *in vitro*, and hardly formed tumor and lung metastasis in nude mice. Flow cytometry showed that the majority of HepG2.2.15 cells were arrested in G1 phase, and apoptosis was minor in both cell lines. Furthermore, the levels of cytoskeletal proteins F-actin and Ezrin were decreased in HepG2.2.15 cells.

CONCLUSION: HepG2.2.15 cells demonstrated a lower proliferation and invasion ability than the HepG2 cells due to HBV transfection.

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Key words: HepG2.2.15; HepG2; Hepatitis B virus; Biological feature; Tumor

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Abstract

AIM: To investigate the biological features of hepatitis B virus (HBV)-transfected HepG2.2.15 cells.

METHODS: The cell ultrastructure, cell cycle and apoptosis, and the abilities of proliferation and invasion of HBV-transfected HepG2.2.15 and the parent HepG2 cells were examined by electron microscopy, flow cytometry, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and trans-well assay. Oncogenicity of the two cell lines was compared *via* subcutaneous injection and orthotopic injection or implantation in nude mice,

INTRODUCTION

Hepatocellular carcinoma (HCC) is the primary malignancy of the liver. It is the third leading cause of cancer

death in the world, and the second in China^[1,2]. It is generally accepted that hepatitis B virus (HBV) plays a major causative role in the development of HCC^[3,4]. To investigate the pathogenesis of HBV in HCC, several HBV expressing cell lines have been established by viral DNA transfection^[5,6]. Most of them are derived from HepG2 and HuH7^[7,8].

HepG2.2.15 cells are derived from the human hepatoblastoma cell line HepG2 and are characterized by having stable HBV expression and replication in the culture system^[9]. As a cell source that can produce HBV, HepG2.2.15 has been frequently used in studies of HBV infection.

In this study, to clarify the cellular and biological features associated with HBV transfection, we examined HBV-producing HepG2.2.15 and parental HepG2 cells in a variety of biological processes including cell proliferation, cell invasion, tumor development and metastasis. We also explored the underlying mechanism accounting for the different features between the two cell lines.

MATERIALS AND METHODS

Cell lines and culture

HepG2.2.15 and HepG2 cells were cultured in DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), in 5% CO₂ at 37°C. A final concentration of 380 mg/L G418 (Invitrogen) was added into the medium for the maintenance of HepG2.2.15 cells.

Enzyme-linked immunosorbent assay

To detect the expression and replication of HBV in HepG2.2.15 cells, hepatitis B surface antigen (HBsAg) and hepatitis B envelope antigen (HBeAg) levels in the medium at 24 h, 48 h and 72 h were determined semi-quantitatively using enzyme-linked immunosorbent assay (ELISA) kits (Sino-American Biotechnology Company, Shanghai, China) according to the manufacturer's instructions. All experiments were performed in triplicate.

Electron microscopy

HepG2.2.15 and HepG2 cells were collected and fixed in 2.5% glutaraldehyde (GA) overnight, dehydrated in a graded series of ethanol and embedded in Quetol-812. The ultrathin sections were cut and stained with lead citrate. The grids were examined under a JEM-1220 electron microscope.

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

HepG2.2.15 and HepG2 cells were seeded in triplicate into 96-well plates at 4×10^3 cells per well. Twenty microliters 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) was added to the medium and cultured for another 4 h. DMSO (150 μ L) was added and the absorbance of each well was read using a Bio-Rad model 550 microplate reader at a wavelength of 490 nm. It was tested for 5 d and the data were expressed as mean \pm SD.

Flow cytometry

Cell cycle assay was monitored using propidium iodide (PI) staining of the nuclei. The cells were fixed in 75% cold alcohol overnight, resuspended in 300 μ L PBS and stained with 500 μ L PI (250 μ g/mL) for 30 min in the dark. Annexin V/PI double staining was used for apoptosis assay. Annexin V and PI were added for incubation for 15 min at 4°C. The cells were analyzed by flow cytometry (BD BioSciences, San Jose, CA, USA).

Trans-well assay

The invasive abilities of HepG2.2.15 and HepG2 cells were determined using matrigel (BD) coated 24-well trans-well chambers (Corning Costar, NY, USA) as described previously^[10]. In brief, trans-well was coated with 10 μ L matrigel and dried in the air; and 5×10^4 cells in serum-free DMEM were seeded into the upper chamber, with the lower chamber supplemented with DMEM containing 10% FBS. The trans-well was incubated at 37°C in 5% CO₂. Incubation time was different due to different invasive abilities of the two cell lines. The cells that had penetrated through the pores were fixed, stained with hematoxylin and eosin (HE) and photographed under light microscope. The experiments were conducted in triplicate.

Western blotting analysis

Total protein extracts of cultured cells were performed routinely. Twelve-microgram samples were size fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with F-actin or Ezrin antibodies (Bioss, Beijing, China), and detected using Western blue (Promega, Madison, WI, USA). GAPDH (Calbiochem, Gibbstown, NJ, USA) was used as internal control.

Animals

Four-week-old female BALB/c nude mice were maintained in the laboratory for animal experiments under specific pathogen-free conditions. The experiments were conducted in accordance with the Guideline for Animal Experiments of the National Cancer Center of China.

Tumor formation assay via subcutaneous injection

HepG2.2.15 and HepG2 cells were harvested and resuspended to 2×10^7 /mL with PBS, and 300 μ L was injected subcutaneously into the left flank of each of eight nude mice. The tumor volume was calculated according to the formula: $V = \text{mean diameter}^3 \times \pi/6$ ^[11] and measured and recorded every 2 d.

Tumor development and metastasis assay via orthotopic implantation

Once the subcutaneous tumor reached 1 cm in diameter, it was removed and cut into 1 mm \times 1 mm \times 1 mm cubes freshly at 4°C. Another group of mice were anesthetized with an intra-peritoneal injection of pentobarbital at a dose of 60 mg/kg. Tumor cubes were implanted into the liver as described previously^[12]. The mice were

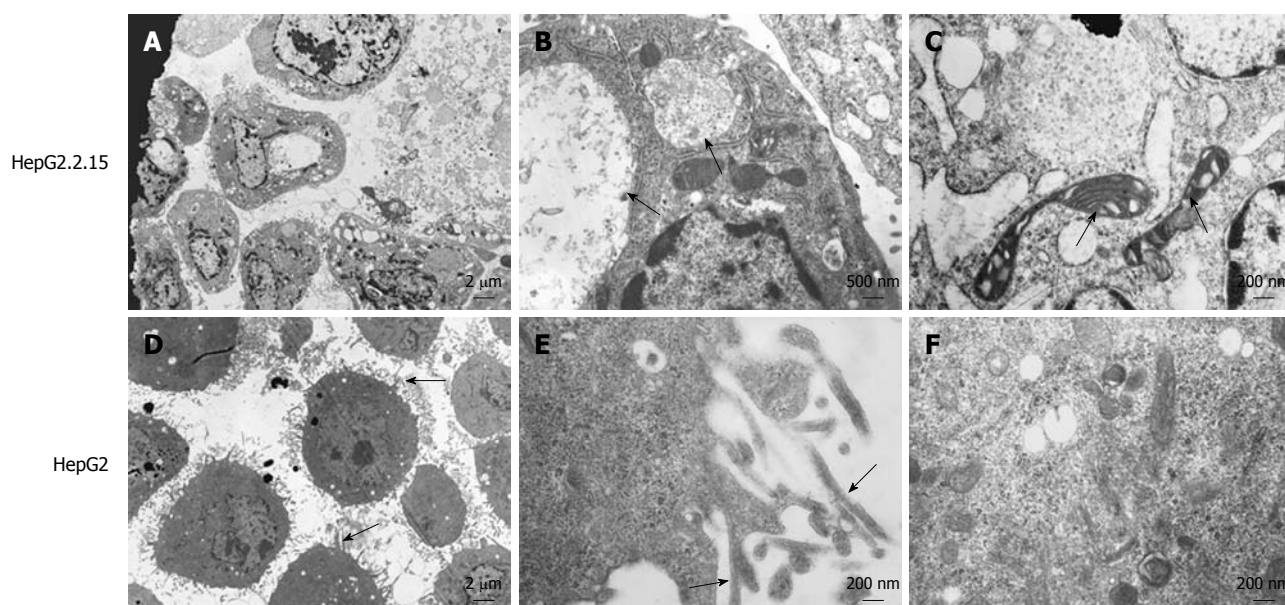


Figure 1 Ultrastructure of HepG2.2.15 and HepG2 cells. A: Filopodia disappearance in HepG2.2.15 cells (EM × 2500); B: Viral inclusion bodies in the cytoplasm of HepG2.2.15 cells. Arrows indicate the viral inclusion bodies (EM × 15000); C: Arrows indicate degenerated mitochondria (EM × 25000); D: Plentiful filopodia around HepG2 cells. Arrows indicate filopodia (EM × 2500); E: Microfilament appearance in filopodia in HepG2 cells in high power field. Arrows indicate microfilament (EM × 25000); F: Abundant organelles in the cytoplasm of HepG2 cells (EM × 25000).

killed 60 d after implantation to harvest the liver and lung. The volume of tumor was determined according to the method described by Janik *et al.*^[13]. The liver and lung were removed and fixed in 4% formalin for standard pathological studies.

Tumor development and metastasis assay via liver injection

HepG2.2.15 and HepG2 cells (1.5×10^6 cells/150 μ L) were prepared in PBS at 4°C for injection. The mice were anesthetized and the liver was exposed as mentioned above. The cells were injected into the left lobe of liver of ten nude mice. The mice were observed for 60 d. Liver and lung were sampled for standard pathological studies as described above.

Statistical analysis

Data were expressed as percentage, mean \pm SD. Comparisons between two groups were analyzed by the χ^2 and Student *t* test. Mann-Whitney *U*-test was employed for analysis of subcutaneous tumor growth. *P* < 0.05 was considered statistically significant.

RESULTS

Ultrastructure of HepG2.2.15 cells

Ultrastructural analysis demonstrated that HepG2.2.15 cells had obviously decreased filopodia (Figure 1A) compared with HepG2 cells (Figure 1D). Plentiful filopodia formed around HepG2 cells and higher amplification showed microfilaments in the filopodia (Figure 1E). Moreover, viral inclusion bodies existed in the cytoplasm of HepG2.2.15 cells (Figure 1B), and many organelles, such as mitochondria,

ribosome and endoplasmic reticulum, were found to be degenerated in HepG2.2.15 cells (Figure 1C). In contrast, HepG2 cells contained normal and abundant organelles including ribosome, glycogen, microfilament and microtubule (Figure 1F).

Lower proliferation ability of HepG2.2.15 cells

HBsAg and HBeAg were detected in the culture supernatant of HepG2.2.15 cells by ELISA. While the HBsAg level increased in a time-dependent manner, HBeAg level peaked at around 24 h and remained largely unchanged until 72 h (Figure 2A). As shown in Figure 2B, HepG2 cells had a significantly higher proliferation rate than HepG2.2.15 cells from Day 2 (*P* < 0.01), especially on Day 4 and Day 5 (*P* < 0.001).

Cell cycle G1/S arrest in HepG2.2.15 cells

To further investigate the reduced proliferation of HepG2.2.15, we tested cell cycle and apoptosis by flow cytometry. The results indicated that the percentage of the G1 phase of HepG2 was significantly lower than that of HepG2.2.15 (*P* < 0.01), but the HepG2 cells in S phase were increased significantly (*P* < 0.001) (Figure 2C), indicating cell cycle arrest at the G1/S phase in HepG2.2.15 cells. The apoptosis analysis showed no significant difference in apoptosis between HepG2.2.15 and HepG2 cells (Figure 2D).

Lower invasion ability of HepG2.2.15 cells in vitro

Trans-well analysis demonstrated that HepG2.2.15 and HepG2 cells were significantly different in invasion ability *in vitro*. HepG2 cells on the lower part of the membrane were detected as early as 2 h after incubation, with an in-

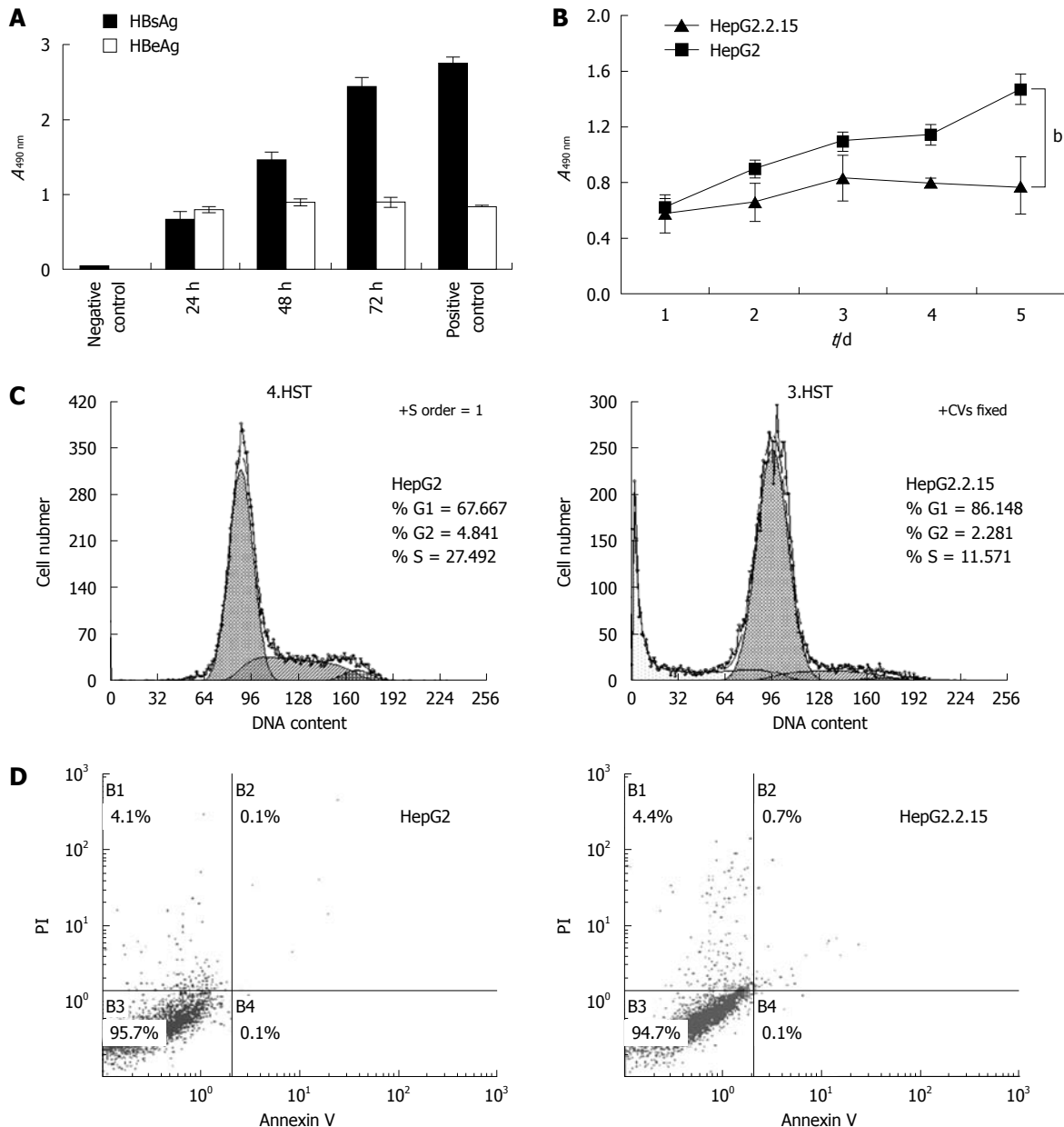


Figure 2 Cell proliferation and apoptosis flow cytometry. A: The levels of hepatitis B surface antigen (HBsAg) and hepatitis B envelope antigen (HBeAg) in HepG2.2.15 cell supernatant. The supernatant was collected every 24 h and tested by enzyme-linked immunosorbent assay; B: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of cell proliferation. The absorbencies of test wells were read every 24 h and the data represent the mean \pm SD ($^b P < 0.001$); C: Flow cytometry of cell cycle; D: Apoptosis percentages in B1, B2 and B4 areas. All experiments were repeated three times with similar results.

creasing number of cells invading through the membrane at 4 h, 6 h and 12 h (Figure 3A). In contrast, the invasion of HepG2.2.15 cells into the lower chamber was detected at 24 h, 36 h, 48 h and 60 h, respectively (Figure 3B). These results suggested that HepG2.2.15 cells had lower invasion ability and took longer time to go through the membrane.

Decreased expression levels of F-actin and Ezrin in HepG2.2.15 cells

To elucidate the mechanism accounting for the difference in cell invasion between HepG2.2.15 and HepG2 cells, we examined the expression of F-actin and Ezrin, which are both cytoskeleton proteins that play crucial roles in

maintaining cell shape and promoting cell invasion. Western blotting analysis showed a 0.7-fold decrease of F-actin level and a 3.8-fold decrease of Ezrin level in HepG2.2.15 cells compared with HepG2 cells (Figure 3C).

Lower tumorigenicity of HepG2.2.15 cells *in vivo*

To explore the biological features of HepG2.2.15 cells *in vivo*, we monitored subcutaneous tumor growth *in vivo*. Two days after injection of HepG2 cells, the nodules were visualized and the diameter reached 0.6 cm on Day 6, and 100% (8/8) mice formed tumors. In contrast, only 25% (2/8) mice injected with HepG2.2.15 cells formed tumors, significantly lower than that of HepG2 cells ($P < 0.01$), and tumor formation was slower than the mice injected

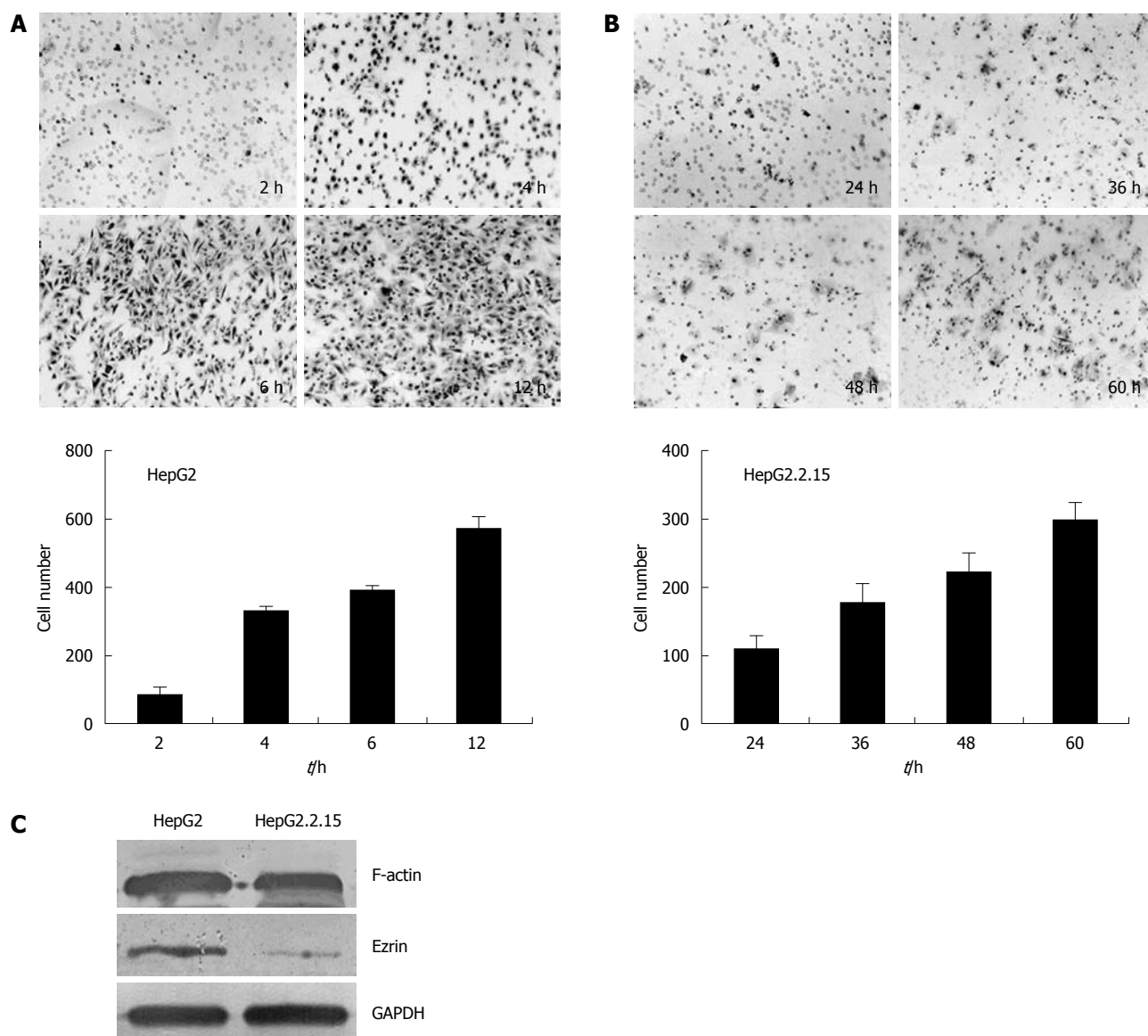


Figure 3 Invasion assays of HepG2 and HepG2.2.15 cells. The trans-well membranes were collected at different time points and the cells that went through the pores were stained with hematoxylin and eosin. The cell numbers were counted. A: HepG2 cells collected and stained at 2 h, 4 h, 6 h and 12 h after incubation; B: HepG2.2.15 cells collected and stained at 24 h, 36 h, 48 h and 60 h after incubation; C: Western blotting analysis of F-actin and Ezrin.

with HepG2 cells (Figure 4A). Notably, 100% (10/10) mice injected with HepG2 cells formed tumor in the liver 60 d after tumor cubes implantation, and the mean volume was $1.7 \pm 0.4 \text{ cm}^3$. Furthermore, all the mice (10/10) developed tumor in the liver after the injection of HepG2 cells and the mean volume of tumor was as big as $3.1 \pm 1.1 \text{ cm}^3$. Nevertheless, the incidence of tumor formation in HepG2.2.15 group (40%, 4/10) was significantly lower than the HepG2 group ($P < 0.05$). The mean volume was $2.3 \pm 0.3 \text{ cm}^3$ (Figure 4B). Only one case formed tumor (10%, 1/10) with a volume of 2.1 cm^3 (Figure 4B). The incidence of tumor formation in the liver was significantly higher in HepG2 implantation group ($P < 0.05$) and injection group ($P < 0.001$) when compared with HepG2.2.15 group. Taken together, these results indicated the low tumorigenicity of HepG2.2.15 cells *in vivo*, being consistent with their reduced cell proliferation and invasion *in vitro*.

Pathological analysis of tumor formation

Lung metastasis was observed under light microscope with a highest percentage of 50% (Figure 4E) in HepG2 group. The growth pattern (Figure 4C), invasion and changes in tumor and surrounding normal tissues were also analyzed in all the groups (Table 1). Most non-tumor livers showed obvious fatty changes in HepG2.2.15 groups (Figure 4D) and the invasion to surrounding organs occurred more frequently in HepG2 groups.

DISCUSSION

This study found that HepG2.2.15 cells had lower proliferation and invasion ability than the HepG2 cells. The majority of HepG2.2.15 cells were arrested at G1-S phase and the level of two important cytoskeletal proteins decreased.

Table 1 Pathological analysis *in vivo*

Tumor behavior	HepG2		HepG2.2.15	
	Cell injection (n = 10)	Tissue implantation (n = 10)	Cell injection (n = 10)	Tissue implantation (n = 10)
Formation (%)	100	100	10 ^d	40 ^a
Volume (cm ³)	3.1 ± 1.1	1.7 ± 0.4	2.1	2.3 ± 0.3
Growth	Expensive growth and central necrosis	Same	Same	Same
Surrounding tissue	Degeneration and necrosis	Same	Same	Same
Normal tissue	Spotty or piecemeal necrosis, scattered fat droplets and vacuolation, focal inflammatory infiltration	Spotty or piecemeal necrosis, diffuse fatty change 40% and focal inflammatory infiltration	Spotty or piecemeal necrosis, diffuse or scattered fatty change 50%, focal inflammatory infiltration and diffuse cytoplasmic swelling of liver cells	Spotty or piecemeal necrosis, diffuse fatty change 40%, focal inflammatory infiltration and mild cytoplasmic swelling of liver cells
Tumor invasion	Abdominal wall 100%; pancreas 37.5%; esophagus 12.5%	Abdominal wall 50%; pancreas 12.5%; diaphragm/ribs 10%	Abdominal wall 10%	Diaphragm/ribs 10%
Metastasis				
Intra-liver	75% (6/8)	30% (3/10)	0	0
Lung	50% (4/8)	0	10% (1/10)	10% (1/10)

^a*P* < 0.05 vs HepG2 tissue implantation group; ^d*P* < 0.001 vs HepG2 cell injected group, χ^2 test.

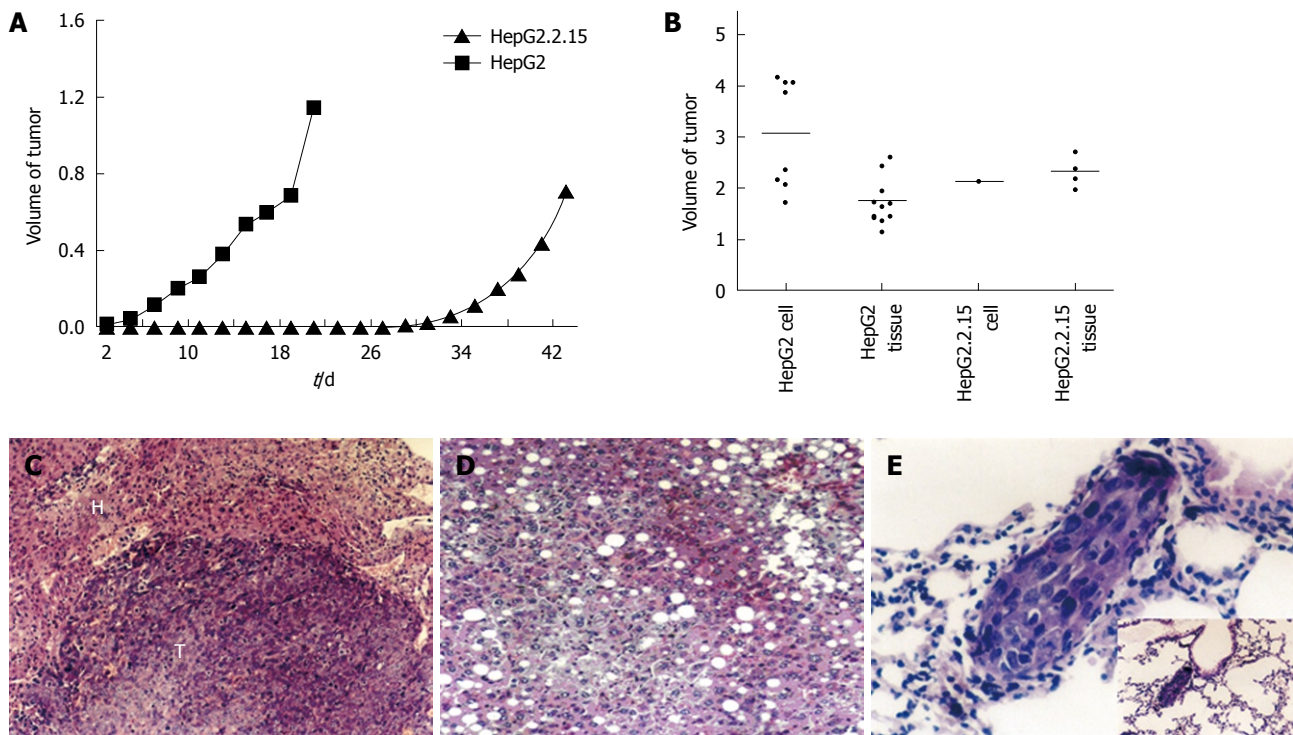


Figure 4 Tumor formation of HepG2 and HepG2.2.15 cells *in vivo*. A: The volume of subcutaneous tumors were measured and recorded every 2 d. The difference of tumor growth rate between HepG2 and HepG2.2.15 groups was significant (*P* < 0.01, Mann-Whitney *U* test); B: Tumor development in four groups *in vivo*; C: Expansive growth of tumor. The boundary of tumor and normal tissue is clear [hematoxylin and eosin (HE) stain, × 120]. T: Tumor; H: Liver tissue; D: Fatty changes in liver tissue of HepG2.2.15 groups (HE stain, × 120); E: Lung metastasis in HepG2 injected group (HE stain, × 460) and low power field.

HBV contains four open reading frames S, C, P, and X. Kanda *et al.*^[7] and Kim *et al.*^[14] showed that HBx transfection down-regulated cell viability and induced apoptosis. HBx suppressed tumor cell proliferation, induced apoptosis and caused cell cycle arrest at G1-S *in vitro*^[15]. These results are partly consistent with our findings in this study. It has been shown that HBV replication depends on the cell cycle and the decrease in S phase^[16], so HBV replica-

tion may affect cell cycle progression. This may partly explain the G1-S arrest in HepG2.2.15 cells. Proteome analysis of HepG2.2.15 and HepG2 cells displayed abundant differentially expressed proteins^[17]. In this study, we found that the expression level of Ezrin and F-actin was lower in HepG2.2.15 than in HepG2. F-actin is the major cytoskeletal element and Ezrin is a member of the ERM (ezrin-radixin-moesin) cytoskeleton-associated protein

family^[18]. Both of them have membrane-cytoskeleton linking functions^[19] and participate in cell migration, growth regulation^[20], filopodia formation^[21], and cancer metastasis^[22]. Therefore, reduced level of Ezrin and F-actin in HepG2.2.15 cells may contribute to the lower proliferation and invasion ability. Additionally, the lower expression of Ezrin was accompanied with reduced filopodia in HepG2.2.15 cells. The dysfunction of organelles and cytoskeleton in HepG2.2.15 cells may also contribute to the slower cell growth and invasion both *in vitro* and *in vivo*.

Notably, non-tumor liver tissues showed mild to severe hepatic fatty changes, necrosis and neutrophil infiltration in HepG2.2.15 groups. In the mice injected with HepG2.2.15 cells, the low rate of tumor formation was accompanied by severe degeneration and necrosis in liver tissues. Interestingly, similar results have been reported by other researchers. For example, overexpression of HBx caused negative accommodation of microsome triglyceride transfer protein and accumulation of intracellular triglyceride and cholesterol in hepatocyte^[23]. Liver tissue from the HBx transgenic mice showed mild to severe hepatic necrosis, fatty changes, mild to moderate chronic hepatitis and cytoplasmic vacuolation^[24]. So, the correlations between HBV, hepatic degeneration and inflammation are striking and need to be further investigated.

In summary, HepG2.2.15 cells demonstrated decreased proliferation and invasion ability compared with its parental HepG2 cells due to HBV transfection. HBV-induced cell cycle arrest and cytoskeletal alteration might be implicated in the mechanism. Our findings will help better understand the cellular and biological features of HepG2.2.15 cells associated with HBV, and select the most suitable cell lines for research. These findings also shed new light on the interaction between HBV and host cells.

ACKNOWLEDGMENTS

We are grateful to Professor Yu-Mei Wen for providing us the cell lines.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death in the world, and the second in China. It is generally accepted that hepatitis B virus (HBV) plays a major causative role in the development of HCC. Although considerable studies have been conducted, the precise mechanism remains unclear.

Research frontiers

HBV infection is considered a high risk for the development of HCC. To investigate the pathogenesis of HBV in HCC, several HBV expressing cell lines have been established by viral DNA transfection. HepG2.2.15 cell line is one of the most common models for HBV-associated disease, but the cellular and biological features associated with HBV in HepG2.2.15 cells is seldom considered. Little is known about the effects of HBV on the biological features of host cells.

Innovations and breakthroughs

In this study, the authors demonstrated that HepG2.2.15 cells had lower proliferation and invasion ability than their parental HepG2 cells. HBV-induced cell cycle arrest and cytoskeletal alteration might be implicated in the mechanism.

Applications

The findings in this study will help us better understand the cellular and biological

features of HepG2.2.15 cells associated with HBV and select the most suitable cell lines for research. They also shed new light on the interaction between HBV and host cells.

Terminology

HepG2.2.15 cells are derived from HepG2 cells transfected with a full-length HBV plasmid. It is characterized by having stable HBV expression and replication in the culture system.

Peer review

The study is well conducted and the methodology is sound.

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Gastroesophageal reflux disease management according to contemporary international guidelines: A translational study

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Abstract

AIM: To test the Genval recommendations and the usefulness of a short trial of proton pump inhibitor (PPI) in the initial management and maintenance treatment of gastroesophageal reflux disease (GERD) patients.

METHODS: Five hundred and seventy seven patients with heartburn were recruited. After completing a psychometric tool to assess quality of life (PGWBI) and a previously validated GERD symptom questionnaire (QUID), patients were grouped into those with esophagitis (EE, $n = 306$) or without mucosal damage (NERD, $n = 271$) according to endoscopy results. The study started with a 2-wk period of high dose omeprazole (omeprazole test); patients responding to this PPI test entered an acute phase (3 mo) of treatment with any PPI at the standard dose. Finally, those patients with a favorable response to the standard PPI dose were maintained on a half PPI dose for a further 3-mo period.

RESULTS: The test was positive in 519 (89.9%)

patients, with a greater response in EE patients (96.4%) compared with NERD patients (82.6%) ($P = 0.011$). Both the percentage of completely asymptomatic patients, at 3 and 6 mo, and the reduction in heartburn intensity were significantly higher in the EE compared with NERD patients ($P < 0.01$). Finally, the mean PGWBI score was significantly decreased before and increased after therapy in both subgroups when compared with the mean value in a reference Italian population.

CONCLUSION: Our study confirms the validity of the Genval guidelines in the management of GERD patients. In addition, we observed that the overall response to PPI therapy is lower in NERD compared to EE patients.

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Key words: Gastroesophageal reflux disease; Proton pump inhibitors; Nonerosive gastroesophageal reflux; Questionnaire; Quality of life

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INTRODUCTION

In 1999 an international panel of experts involved in the

management of gastroesophageal reflux disease (GERD) convened in Genval, Belgium, for a workshop which aimed to review the existing evidence concerning the definition, pathogenesis and natural history, and impact of the disease on the patient, and clinical manifestations as well as diagnostic and therapeutic strategies. The results of this workshop were published as the “The Genval Workshop Report”^[1], and had a substantial impact both on the management strategies in GERD and in the development of further guidelines, as for example the Marrakech Workshop^[2] and the Montreal Global Definition^[3].

The Genval recommendations constitute a comprehensive body of knowledge relevant to good management. In 2002 we designed a multicenter study on GERD patients with the primary aim of testing “in the field” the Genval recommendations and to prospectively evaluate: (1) the usefulness of a short trial of proton pump inhibitor (PPI) in the initial management of GERD; and (2) the usefulness of a step-down PPI in the maintenance treatment of GERD patients.

As secondary aims we wanted to assess the ability of PPI maintenance therapy to restore the quality of life of our patients and to assess the ability in differentiating between erosive and non erosive disease by a new GERD questionnaire, the QUESIONARIO Italiano Diagnostico (QUID), which has been created by application of artificial neural networks as reported elsewhere^[4]. The latter issue will be described in a separate paper.

MATERIALS AND METHODS

This study was a national multicenter collaborative investigation (the EMERGE project). The study was conducted on GERD patients with or without erosive esophagitis between June 2003 and June 2005. The study started with a 2-wk period of high dose omeprazole 20 mg *bid* (the so-called omeprazole test). Patients responding to this test entered an acute phase (3 mo) of treatment with any available PPI at a standard dose. Finally, those patients with a favorable response to the standard PPI dose were maintained on half the PPI dose for a further 3-mo period. Patients continued this regimen unless their symptoms relapsed: in this case, they could resume the previous dose. Patients requiring this change of therapy more than once were considered to have treatment failure and were excluded from the final analysis.

The study was conducted according to good clinical practices. All Ethics Committees of the participating centers granted authorization, and written informed consent was obtained from all patients.

Five hundred and seventy-seven adult outpatients (282 female, 295 male, mean age 43.5 ± 12.4 years), referred to the various centers in order to undergo upper GI endoscopy, were recruited by 60 gastroenterological Italian centers.

The demographic data and clinical characteristics of the whole patient sample are shown in Table 1, according to the endoscopic findings [erosive esophagitis (EE) vs nonerosive gastroesophageal reflux (NERD)].

Table 1 Proton pump inhibitor types and equivalent doses used for maintenance therapy *n* (%)

PPI types and doses	Acute phase	Maintenance phase
Esomeprazole 40 mg	149 (26.94)	10 (2.16)
Esomeprazole 20 mg	20 (3.62)	141 (30.45)
Omeprazole 20 mg	314 (56.78)	33 (7.13)
Omeprazole 10 mg	1 (0.18)	211 (45.57)
Lansoprazole 30 mg	13 (2.35)	4 (0.86)
Lansoprazole 15 mg	1 (0.18)	14 (3.02)
Pantoprazole 40 mg	39 (7.05)	1 (0.22)
Pantoprazole 20 mg	1 (0.18)	35 (7.56)
Rabeprazole 20 mg	15 (2.71)	1 (0.22)
Rabeprazole 10 mg		13 (2.81)

Inclusion criteria

Patients were included provided that, before endoscopic examination, the following criteria were fulfilled: (1) Age between 18 and 65 years; (2) Presence of heartburn symptoms for at least 15 d, at least once a day; (3) Referral by treating physician to a tertiary centre to undergo upper gastrointestinal (GI) endoscopy; (4) Ability to read and write Italian, > 5 years of schooling; and (5) Consent to voluntary participation with signature on the Informed Consent form before study screening.

Exclusion criteria

Patients who had one or more of these criteria at baseline were excluded from the study: (1) Treatment with PPI in the month preceding endoscopy; (2) Diagnosis of esophagitis established during the past 12 mo; (3) Pregnancy and/or lactation; and (4) Concomitant severe disease during the 4 wk preceding the study, requiring any pharmacological treatment.

Study design

Before undergoing endoscopy, patients were given a psychometric tool to assess quality of life (PGWBI-Psychological General Well Being Index) and the previously validated GERD symptom assessment questionnaire (QUID)^[4].

The PGWBI is a 22-item self-administered questionnaire designed to measure perception of general wellbeing through 6 domains: anxiety (5 items), depression (3 items), feeling of wellbeing (4 items), vitality (4 items), general health (3 items) and self-control (3 items). All the items were rated on a 6-point scale (0-5). The highest possible score (110) represented the best possible wellbeing^[5].

QUID is a 41-item questionnaire which investigates typical and atypical symptoms of GERD and relevant life habits for GERD. It was developed in a previous study^[4] and is partially derived from an Italian validated version of GERQ (Gastroesophageal Reflux Questionnaire by Mayo Clinic^[6]), in which the most relevant items were selected by means of artificial neural networks^[4].

Upper GI endoscopy was performed according to the standard protocol of each participating center, and biopsy samples for diagnosis of *Helicobacter pylori* infection were

not universally taken, nor was there any requirement to give eradication therapy.

Esophagitis was diagnosed and graded according to the Savary Miller classification as follows^[7]: Grade I -single or multiple erosions on a single fold; Grade II -multiple erosions on multiple folds; Grade III-multiple circumferential erosions; Grade IV-ulcer, stricture, and esophageal shortening.

Only omeprazole 20 mg *bid* was used for the initial PPI test, since the majority of studies existing in the literature at the time of study design were conducted with this drug^[8-14]. The choice of therapy following the PPI test, in accordance with Genval Guidelines^[2], was any available PPI at the standard dose. The same drug was subsequently used at half dose as maintenance treatment, again in accordance with Genval recommendations. In Table 1 the various PPIs used are shown with relative doses, according to the study phase. Only those patients who had a positive PPI test, defined as a reduction in heartburn severity greater than 50% at the end of the 15-d period of the test, continued the study^[8,9,14]. In the case of worsening of symptoms after any reduction of dose, the participating physicians were free to resume the previous therapy. The patient could resume the treatment schedule only once during the period of the trial. Additional therapeutic step-ups were considered failure of treatment and required withdrawal from the study. Patients taking concomitant medications for medical problems other than GERD could continue therapy, and this was recorded. Patients in the study were not allowed to take any other antisecretory or prokinetic drug. Over-the-counter (OTC) antacids were allowed, if needed because of insufficient symptom control and their use was recorded.

Daily diary

During the 15 d of the PPI test the patient had to report daily in a diary the characteristics of heartburn in terms of intensity and frequency: intensity was scored in a 5-point scale, ranging from 0 = absent to 4 = unbearable and intolerable, with 1 = mild, 2 = moderate, 3 = severe. Daily average frequency was expressed simply by giving the number of daily heartburn attack. This allowed a composite heartburn score to be constructed, by multiplying the daily average frequency by the intensity.

Heartburn intensity and frequency were also assessed at the end of the acute phase (3 mo after completion of the PPI test) and after 6 mo, at the end of the study. The entire bulk of symptoms, from regurgitation to extra-esophageal concomitant manifestations were assessed by means of the QUID questionnaire only, at baseline and at the end of study, at 6 mo.

RESULTS

The details of the study according to the various phases are shown in Figure 1.

Demographic and clinical features

Of the 577 patients recruited, 306 (53%) were diagnosed

Table 2 Study population demographics and features according to endoscopic results *n* (%)

	EE (306/577, 53%)	NERD (271/577, 47%)	<i>P</i>
Male	194 (63.4)	101 (37.3)	< 0.05
Female	112 (36.6)	170 (62.7)	< 0.05
Age (yr), mean (± SD)	43.6 (± 12.22)	43.42 (± 12.56)	NS
Height (cm), mean (± SD)	169.86 (± 8.63)	166.84 (± 8.14)	NS
Weight (kg), mean (± SD)	75.33 (± 15.40)	68.49 (± 12.41)	NS
Smoking	164 (48.1)	115 (39.2)	NS
Esophagitis severity (Savary-Miller classification)			
Grade 1	237 (77.5)	-	NS
Grade 2	52 (16.9)	-	NS
Grade 3	9 (2.9)	-	NS
Grade 4	8 (2.6)	-	NS
<i>H. pylori</i> presence	51 (14.9)	37 (12.6)	NS
Heartburn intensity			
1 = mild	28 (9.2)	33 (12.1)	NS
2 = moderate	181 (59.1)	166 (61.2)	NS
3 = severe	88 (28.7)	64 (23.6)	NS
4 = unbearable	9 (2.9)	8 (2.9)	NS
Regurgitation	248 (81)	227 (83.7)	NS
Chest pain	146 (47.7)	120 (44.3)	NS
Dysphagia	99 (32.3)	95 (35)	NS
Belching	198 (64.7)	181 (66.7)	NS
Chronic cough	102 (33)	85 (31.4)	NS
Hoarseness	89 (29)	102 (37.6)	NS

EE: Erosive esophagitis; NERD: Non erosive reflux disease; NS: Not significant.

with esophagitis while the remaining 271 (47%) showed no esophageal mucosal damage (Table 2). Two hundred and seventy-nine patients were smokers, 164 with EE and 115 with NERD, while 526 (289 with EE and 237 with NERD) were coffee drinkers. *H. pylori* status was investigated in a major subsample (454/577, 78.6%): *H. pylori* was found to be present in 88 patients (19.4%) and absent in 366 patients (80.6%). Eradication therapy was given in only 31 patients (16 with NERD and 15 with EE).

The severity of esophagitis, assessed by the Savary-Miller classification, was as follows: grade 1 = 237 (77.5%), grade 2 = 52 (16.9%), grade 3 = 9 (2.9%), and grade 4 = 8 (2.6%).

For various reasons, as shown in Table 2, 179 patients (31%) left the study early: of these, 87 had EE and 92 had NERD. Thus 398 patients (69%) completed the entire study period (Figure 1).

Symptoms, other than heartburn, which were evaluated by QUID questionnaire at baseline and at study completion, such as regurgitation, chest pain, dysphagia and belching, were present at baseline in percentages ranging from 83.1% for regurgitation to 33.3% for dysphagia (Table 2). Chest pain, chronic cough and hoarseness, were reported in 46.7% (296/634), 31.2% (202/634) and 33.1% (210/634) patients, respectively (Table 3).

PPI test

During the 2-wk PPI test phase, 24 patients dropped out (Figure 1) and, of 553 (95.8%) patients completing the

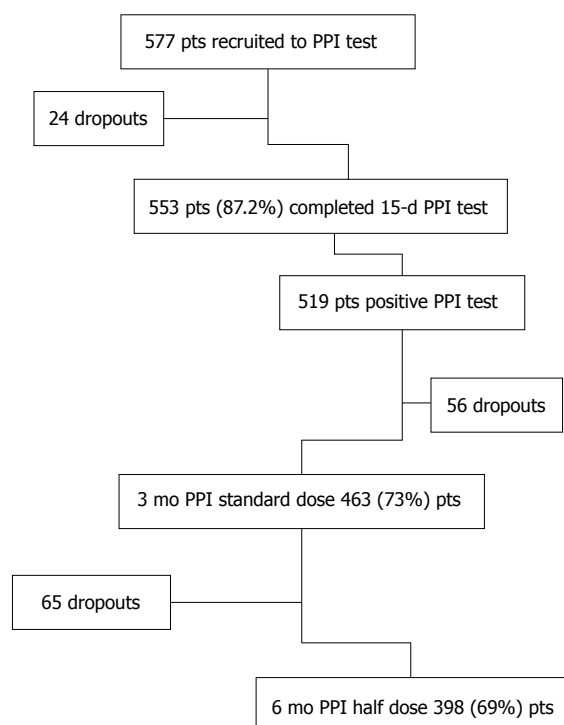


Figure 1 Study design (the percentages refer to recruited patients). PPI: Proton pump inhibitor. Pts: Patients.

Table 3 Dropout causes

Dropout cause	n (%)
Personal reasons	12 (6.7)
Therapy failure	1 (0.6)
Patient fails to follow-up visit	46 (25.7)
Prolonged time to visit	60 (33.5)
Adverse reaction	2 (1.1)
Other	58 (32.4)
Total	179 (100)

PPI test, 519 (89.9%) had a positive test, defined as a reduction in heartburn severity greater than 50% at the end of the 15-d period, and entered the acute study phase. The PPI test was positive in 224/271 (82.6%) of patients with NERD and in 295/306 (96.4%) of those with EE. The heartburn score at day 1 of the PPI test was 7.2 ± 16.09 (standard deviation) for the entire GERD population, while it was 5.94 ± 13.29 for EE patients and 8.8 ± 18.97 for NERD patients; the score was 1.37 ± 8.64 , 0.86 ± 6.50 and 1.99 ± 10.67 for the entire GERD population, EE patients and NERD patients, respectively, at day 16 (trend $P < 0.001$). The intensity of heartburn decreased over 16 d similarly in EE (mean delta variation 0.07 ± 0.1) and NERD (mean delta variation 0.08 ± 0.09) patients.

Acute and maintenance therapy

Five hundred and nineteen patients started the acute treatment period of 3 mo, with the standard PPI dose. After 3 mo, 463 (80.2%) patients were satisfactorily

treated and were admitted to the last 3-mo phase with half the PPI dose. Of these, 398 (69%) completed the full 6 mo of the study protocol (Figure 1). Corresponding figures for patients with EE after 3 mo and 6 mo were 261/577 (45.2%) and 219/577 (38%), respectively, and 202/577 (35%) and 179/577 (31%) respectively, for patients with NERD.

After 3 mo of acute therapy, 32/261 (12.3%) with EE and 40/202 (19.8%) NERD patients still complained of heartburn ($P = 0.02$), whereas after 6 mo, with half dose PPI therapy, the proportion was 27/219 (12.3%) and 45/179 (25.1%) for EE and NERD patients, respectively ($P < 0.001$). In other words, the percentage of completely asymptomatic patients, both at 3 and 6 mo, was significantly higher in the EE compared with NERD patients (Figure 2). The distribution of heartburn intensity among the 2 subgroups at 3 and 6 mo is shown in Figure 2.

Similarly the intensity of heartburn decreased with time differently in patients with EE and with NERD, the reduction of intensity being significantly greater in patients with EE (mean delta reduction 2.08 ± 0.77) compared with NERD patients (mean delta reduction 1.77 ± 0.83 , $P < 0.01$).

The other GERD symptoms, such as regurgitation, chest pain, dysphagia and belching, showed a consistent and significant decrease from baseline to the final visit (Figure 3), and the same happened with the 2 most frequently reported possible extraesophageal manifestations, chronic cough and hoarseness. None of these symptoms showed a different response in NERD as compared to EE patients.

PGWBI

Before therapy the mean PGWBI score of our GERD population was 72.4 ± 15.62 and it rose to 84.3 ± 14.27 after the 6 mo of therapy. When compared with the mean value of a reference Italian population^[15], which was 78 ± 17.89 , these values were significantly different before and after therapy, and both for EE and NERD patients. In other words, the quality of life was significantly worsened by GERD symptoms before therapy and it was fully restored, even above the reference values, by the therapy. If the effect size (ES) of this change is considered, i.e. the mean change found in a given variable divided by the standard deviation (where a positive value means improvement and a negative means worsening), we found a difference in ES of -0.31 between baseline GERD and the general population before therapy, and of 0.35 between study end and the general population, without significant differences between NERD and EE patients. Among the 6 domains of PGWBI for both NERD and EE patients, it appears that anxiety and general health showed the most important variation before and after therapy. Finally, in both NERD and EE subgroups, it was apparent that the decrease in anxiety was directly related to the improvement in symptoms, in particular heartburn and nausea (data not shown).

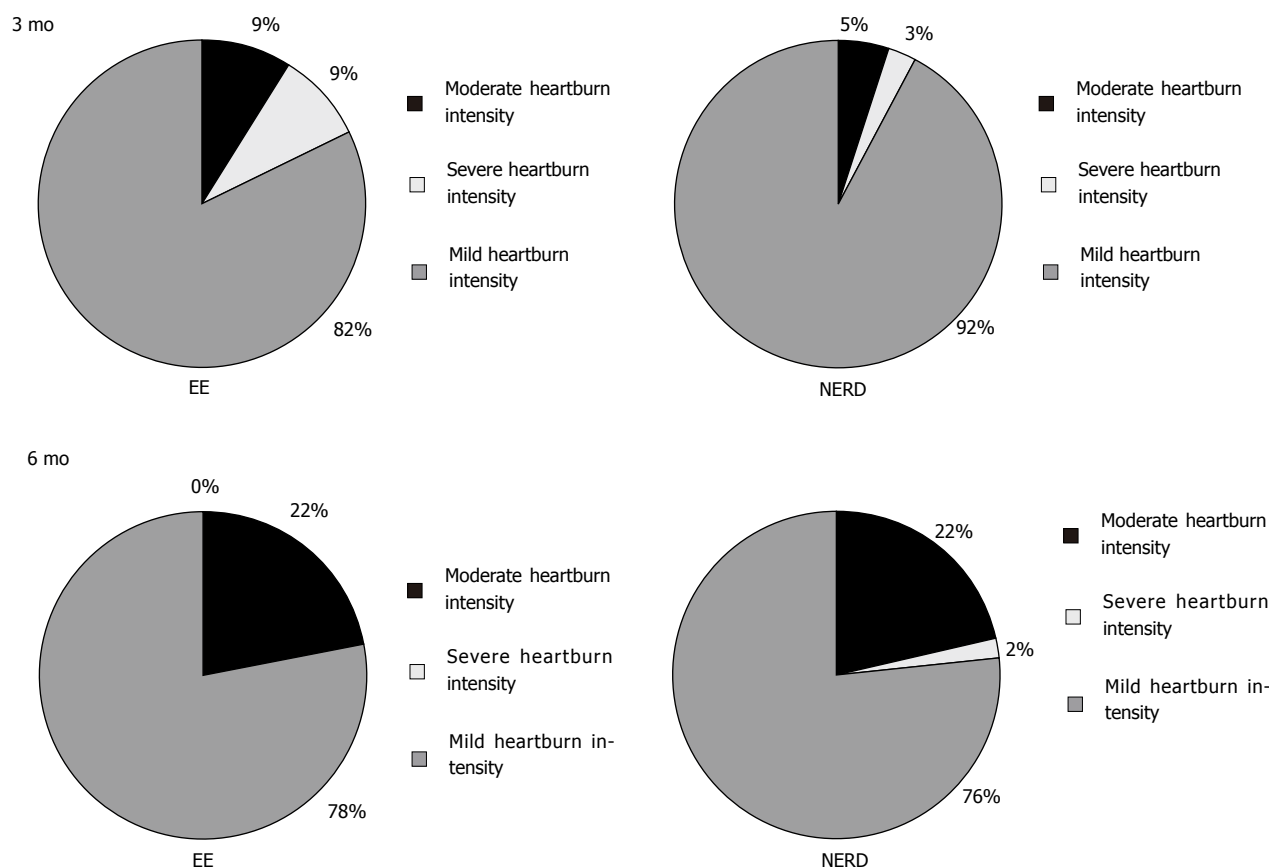


Figure 2 Distribution of heartburn intensity at 3 mo (top panels) and 6 mo (bottom panels) therapy in erosive esophagitis (left panels) and non erosive reflux disease patients (right panels). EE: Erosive esophagitis; NERD: Non erosive reflux disease.

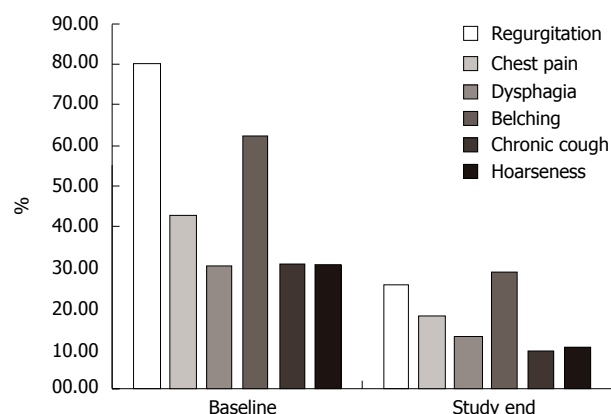


Figure 3 Additional symptoms assessed at baseline and at study end by QUESIONARIO ITALIANO DIAGNOSTICO questionnaire (data are presented for the 398 patients completing the study). Baseline vs study end, for all symptoms, $P < 0.001$.

DISCUSSION

Our study was specifically designed with the aim of assessing the validity of the Genval guidelines on GERD management in a population of patients referred for upper GI endoscopy in 60 Italian centers. In particular, we were interested in establishing, in patients with mild-to-moderate esophagitis or with NERD: (1) the symptomatic response to a brief, high dose PPI treat-

ment as initial management; (2) whether the step-down therapeutic approach is useful to optimally balance drug cost and symptom response. As far as the first aim was concerned, the omeprazole test was used for diagnostic purposes in NERD patients only, whereas we decided to use it as a therapeutic start-up also in the EE group in order to have comparable treatments in both groups. We observed a “positive” PPI test, as defined by a reduction in heartburn severity greater than 50% at the end of the 15-d period, in 96.4% of EE patients and in 82.6% of NERD patients. The latter patients were, according to the Genval guidelines, subjects with “clinically significant impairment in quality of life due to reflux-related symptoms”, and therefore could be diagnosed as GERD patients not only in accordance with Genval^[1], but also with Montreal^[3] guidelines, without any need to proceed with a PPI or other diagnostic test. However, it turned out that as many as 17.4% of our NERD patients did not respond to the PPI test and for the purpose of our study were not further treated with a PPI. We do not know how many of these “non responders” could in fact still be diagnosed as GERD patients by means of esophageal pH-metry or pH-impedance monitoring, since we did not apply such tests. On the other hand, by excluding those patients with presumably non acid-related problems, we selected in a very simple way those who could better respond to PPI treatment. The figure of about 80% of patients responding to PPI, and hence

categorizable as GERD patients, is in keeping with Genval statement 13, which states “When heartburn is a major or sole symptom, gastroesophageal reflux is the cause in at least 75% of individuals”. The Genval guidelines suggest, for non endoscoped patients, endoscopy-negative patients or Los Angeles grade A or B esophagitis patients, starting treatment with high dose PPI for 2-4 wk, which corresponds to our omeprazole test, and to check the symptomatic response thereafter^[1]. In studies conducted on NERD patients, the PPI test has been found to have a high sensitivity but a very low diagnostic specificity^[16], in particular when compared with objective measures of GERD, such as pH-metry, or with symptom questionnaires^[17]. Since our NERD population was recruited on the basis of clinical diagnostic criteria (e.g. the Genval criteria) and a positive response to the PPI test, the enrolled sample may not quite be representative of the NERD population at large. On the other hand, it is more homogeneous, because it includes only the acid-related segment of the NERD spectrum, and therefore by definition excludes patients with functional heartburn (cf the Rome criteria for functional heartburn)^[18]. As for the duration of the PPI test, we believe that our study proves that in GERD patients with typical symptoms, the suggestion to empirically treat with 2-4 wk of high dose PPIs^[1] could be temporally limited to 1 or 2 wk, since the reduction of heartburn was already near to the maximum after the first week of high dose omeprazole administration (Figure 2).

The Genval guidelines^[1] have been subsequently updated by the Marrakech recommendations^[2], which however do not differ much with regard to the indications and doses of PPI therapy, and the so called PPI test, in the management of GERD patients. On the contrary, the Montreal Workshop^[3] was only focused on developing a global definition and classification of GERD, but did not specifically address the issue of management.

Due to the particular design of our study, we had the opportunity to compare the subjective response to short term high dose PPI in the 2 populations of NERD and EE patients. We found an overall better response in the latter; this is, to our knowledge, a new observation, and is in keeping with the already established concept that NERD patients as a group responds less well to PPI compared with EE patients^[19]. We further extended this observation by showing that NERD patients with typical symptoms, on average, show a smaller decrease in heartburn intensity also during 3-6 mo maintenance therapy with PPI compared with EE patients. We want to emphasize the point that the relatively lower symptom response rate to PPI treatment in NERD patients was not due in our study, as pointed out in the study by Dean *et al*^[19], to the “contamination” of the NERD group by patients with functional heartburn, since our study design avoided this bias. Thus, it appears that the overall therapeutic efficacy of PPI is truly decreased in NERD as opposed to EE patients.

As far as the second aim is concerned, we have

observed that the great majority of patients can be maintained symptom-free by halving the PPI dose after 15 d and 3 mo, respectively: 229/261 (87.7%) of EE and 162/202 (80.2%) of NERD patients were heartburn-free after 3 mo, respectively and 192/219 (87.7%) of EE and 134/179 (74.9%) of NERD patients after 6 mo. Again, it seems that the symptomatic response to PPI treatment is lower in NERD patients as compared to EE also during a maintenance regimen. On the other hand, this finding implies that the step-down therapy could be successfully proposed for the majority of both EE patients and NERD patients following a positive PPI test, even after a short (3-mo) period.

Finally, our study clearly confirms previous data that suggest that quality of life is greatly reduced by GERD symptoms (compared to the general population) independently of the presence or absence of esophagitis^[20]. Interestingly enough, even the relatively short treatment period with PPIs in the present study (6.5 mo) was able to completely restore the quality of life in our patients, or even to improve it to levels above those showed by the general population.

In conclusion, this study is to our knowledge the first one to prospectively address relevant issues in the management of GERD outside a frame of therapeutic randomized, controlled trials. In patients with NERD or erosive esophagitis, a short period of high dose PPI (the so-called PPI test) is a valuable tool for diagnosing suspected GERD symptoms as being acid-related, and thus for selecting those patients who will benefit from PPI therapy. In the further management of these patients, 2 consecutive reductions in PPI dose are able to keep the vast majority of patients asymptomatic and to fully restore their quality of life. The overall response to PPI therapy is lower in NERD patients than in EE patients.

COMMENTS

Background

“The Genval Workshop Report” reviewed the existing evidence concerning gastroesophageal reflux disease (GERD). The study aimed to test these recommendations and to assess the usefulness of a short trial of proton pump inhibitor (PPI) in the initial management and maintenance treatment on GERD patients.

Research frontiers

The PPI test consists of measuring the symptomatic response to a high dose PPI treatment administered for 1 to 2 wk in patients with GERD symptoms and with erosive esophagitis (EE) or without it (so-called NERD). The rationale for using short-term, high dose PPI administration as a diagnostic tool is based on the strong effect of PPIs in inhibiting gastric acid secretion, healing EE and improving GERD symptoms.

Innovations and breakthroughs

This study is, to our knowledge, the first to prospectively address relevant issues in the management of GERD outside a frame of therapeutic randomized, controlled trials. In patients with NERD or EE, the PPI test is a valuable tool for diagnosing suspected GERD symptoms as being acid-related and thus for selecting those patients who will benefit from PPI therapy. In the further management of these patients, 2 consecutive reductions of PPI dose are able to keep the vast majority asymptomatic and to fully restore their quality of life. The overall response to PPI therapy is lower in NERD patients than in EE patients.

Peer review

The study is one of the first trying to prospectively address relevant issues in the management of GERD outside a frame of therapeutic RCTs.

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Interleukin-24 is correlated with differentiation and lymph node numbers in rectal cancer

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strong intensity. VEGF expression was found in 64 out of 90 patients. Negative and weak intensities of IL-24 expression were classified as negative expression for analysis. IL-24 expression was significantly reduced in poorly differentiated tumors in comparison with well or moderately differentiated tumors ($P = 0.004$), N2b to earlier N stages ($P = 0.016$), and stage IIIc to stage III a or IIIb ($P = 0.028$). The number of involved lymph nodes was also significantly reduced in IL-24-positive patients in comparison with IL-24-negative ones. There was no correlation between VEGF expression and patient characteristics. Expression of IL-24 and VEGF was not correlated with survival, but N stage and stages were significantly correlated with survival.

CONCLUSION: IL-24 expression was significantly correlated with histological differentiation, and inversely correlated with the degree of lymph node involvement in stage III rectal cancer.

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Abstract

AIM: To assess the significance of interleukin (IL)-24 and vascular endothelial growth factor (VEGF) expression in lymph-node-positive rectal cancer.

METHODS: Between 1998 and 2005, 90 rectal adenocarcinoma patients with lymph node involvement were enrolled. All patients received radical surgery and postoperative pelvic chemoradiotherapy of 50.4-54.0 Gy. Chemotherapy of 5-fluorouracil and leucovorin or levamisole was given intravenously during the first and last week of radiotherapy, and then monthly for about 6 mo. Expression of IL-24 and VEGF was evaluated by immunohistochemical staining of surgical specimens, and their relations with patient characteristics and survival were analyzed. The median follow-up of surviving patients was 73 mo (range: 52-122 mo).

RESULTS: IL-24 expression was found in 81 out of 90 patients; 31 showed weak intensity and 50 showed

Key words: Interleukin-24; Rectal cancer; Lymph node; Histological differentiation; Vascular endothelial growth factor

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Choi Y, Roh MS, Hong YS, Lee HS, Hur WJ. Interleukin-24 is correlated with differentiation and lymph node numbers in rectal cancer. *World J Gastroenterol* 2011; 17(9): 1167-1173 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i9/1167.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i9.1167>

INTRODUCTION

Treatment outcomes of rectal cancer have been improved with the use of adequate combination therapies

and the identification of effective targets, but relapses are still frequent in advanced-stage patients. The survival rate of patients whose tumors are confined to the rectal wall at diagnosis (stage I and II) is > 75%, but those rates are reduced to 30%-60% in higher-stage patients, according to the degree of penetration into the wall, and lymph node involvement^[1,2]. In order to yield more credibility and power to a study, we analyzed stage III patients for the identification of new therapeutic targets.

The melanoma differentiation associated gene-7, later renamed as interleukin (IL)-24, was identified by subtraction hybridization from human melanoma cells stimulated with interferon- β and mezerein^[3]. The expression of IL-24 is inversely related to human melanoma progression. That is, it is highest in melanocytes and lowest in metastatic melanomas^[3,4]. Transfection of IL-24 into melanoma cells reduces growth without a similar effect on normal cells^[3], and this antiproliferative activity of IL-24 has also been detected in a variety of cancer cells, such as breast, prostate, cervix, colorectal, lung, and nasopharynx carcinomas, as well as glioblastoma^[5-11]. In addition to the antiproliferative effect in cancer cell lines, favorable survival has been marginally identified in high-IL-24-expressing non small cell lung cancer (NSCLC) patients^[12]. From a subset analysis, high IL-24 expression has been revealed as a significant prognostic factor in adenocarcinoma patients. Whether high IL-24 expression also has a significant impact on the survival of patients with types of cancers other than NSCLC is unknown.

Multiple anticancer mechanisms of IL-24 have been reported, including cancer-specific apoptosis induction, cell cycle regulation, an ability to inhibit angiogenesis, potent bystander antitumor activity, and a capacity to enhance the sensitivity of tumor cells to radiation and chemotherapy^[13]. Among them, new vessel formation is required for tumor growth and metastasis^[14]. Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors^[15]. Expression of VEGF is proportional to the degree of carcinogenesis of the colorectum, ranging from 0% in dysplastic adenomas, to 62% in mucosal carcinomas, and 100% in sub-mucosal carcinomas^[16]. However, the prognostic significance of VEGF expression in rectal cancers has been inconclusive so far^[17-21]. Therefore, we also analyzed VEGF expression in rectal cancer patients.

We carried out the first analysis of the correlation between IL-24 expression and prognostic features in rectal cancer patients. To resolve the unanswered question of the effect of VEGF expression on the survival of rectal cancer patients, while limiting biases related to treatment methods and patient heterogeneity, we restricted the analysis to rectal cancer patients with lymph node metastasis who were treated at a single institution.

MATERIALS AND METHODS

Patients

In this retrospective study, we reviewed 96 rectal adenocarcinoma patients with pathologic lymph node involvement,

Table 1 Patients characteristics

	n (%)
Age (yr)	
Median	59
Range	34 - 77
Sex	
Male	47 (52.2)
Female	43 (47.8)
Histologic differentiation	
Well	46 (51.1)
Moderately	34 (37.8)
Poorly	10 (11.1)
T stage	
T1	1 (1.1)
T2	8 (8.9)
T3	79 (87.8)
T4	2 (2.2)
N stage	
N1a	24 (26.7)
N1b	26 (28.9)
N2a	16 (17.8)
N2b	24 (26.7)
Stage	
III a	8 (8.9)
III b	57 (63.3)
III c	25 (27.8)
VEGF expression	
Negative	26 (28.9)
Positive	64 (71.1)
Intensity of IL-24 expression	
Negative	9 (10.0)
Weak	31 (34.4)
Strong	50 (55.6)

IL: Interleukin; VEGF: Vascular endothelial growth factor.

who had consecutively undergone radical surgery and post-operative chemoradiotherapy at Dong-a University Hospital, Busan, South Korea between 1998 and 2005. The analysis of these patients was approved by the Institutional Review Board. Ninety patients were included for analysis, while six patients were excluded; two for lack of surgical specimens, and four due to liver metastasis at diagnosis, familial adenomatosis polyposis, adenosquamous cell carcinoma, and mucinous adenocarcinoma, respectively (Table 1).

Thirty-three patients underwent abdominoperineal resection, and 57 underwent low anterior resection. Patients received postoperative chemoradiotherapy from the fourth to sixth week after radical surgery. Patients were positioned in a prone position on a belly board for radiotherapy. Tumor beds were boosted up to 50.4-54 Gy (1.8 Gy, once daily) after 45 Gy pelvic irradiation with 15 MV X-ray using a three-field technique. Two cycles of 5-fluorouracil with levamisole or leucovorin were concurrently given for radiotherapy, and maintenance chemotherapy was done thereafter for about 6 mo. Patients were followed up at 3-mo intervals for 2 years, 4-mo intervals for the next 2 years, and then every 6 mo. The median follow-up period of the surviving patients was 73 mo (range: 52-122 mo). The seventh edition of the American Joint Committee on Cancer TNM staging system (2010) was used for patient analysis.

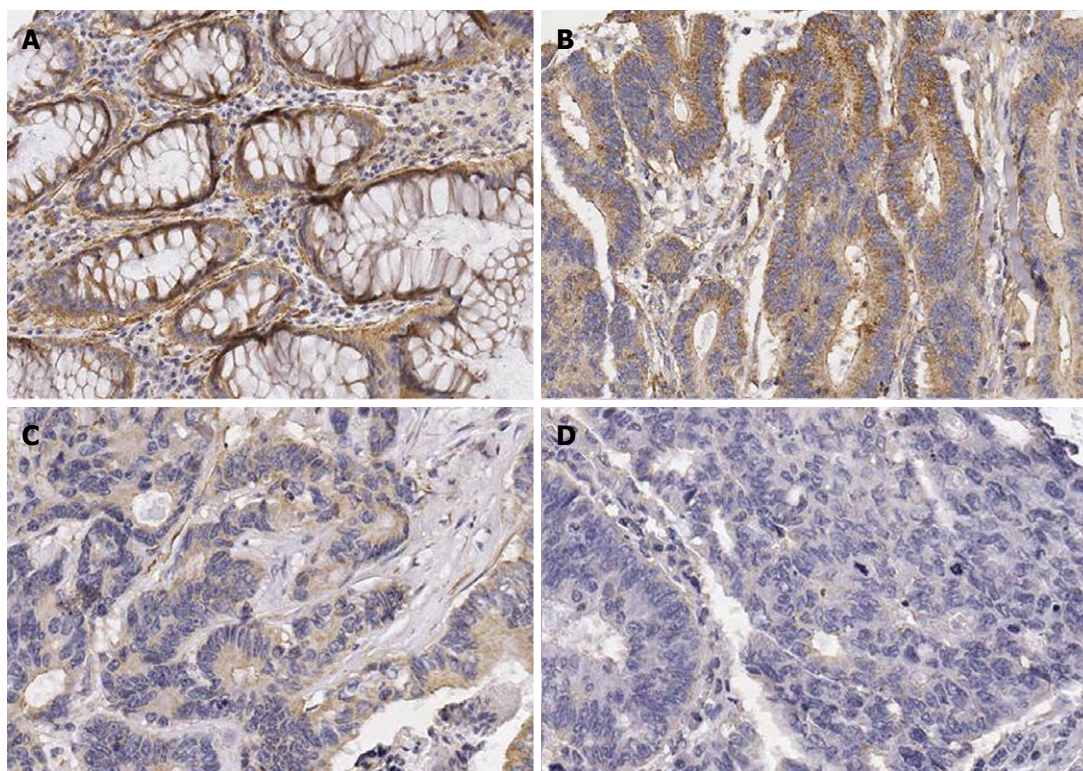


Figure 1 Immunohistochemical staining of interleukin-24 in rectal tissue (200 ×). A: In the normal rectal mucosa tissue, the non-neoplastic glandular epithelial cells were strongly positive for IL-24; B: In rectal cancer, a well-differentiated adenocarcinoma showed strong positive expression of IL-24; C: A moderately differentiated adenocarcinoma showed weak positive expression of IL-24; D: A poorly differentiated adenocarcinoma showed negative expression of IL-24. IL-24: Interleukin-24.

Immunohistochemical staining

The immunohistochemical studies for IL-24 and VEGF were performed on formalin-fixed, paraffin-embedded, 4- μ m-thick tissue sections, using the avidin-biotin-peroxidase complex method. The primary antibodies were a goat polyclonal antibody against IL-24 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1:200 dilution and a rabbit polyclonal antibody against VEGF, which recognized the 121, 165 and 189 isoform (Santa Cruz Biotechnology) at a 1:100 dilution. Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed with a series of graded alcohol solutions. To enhance the immunoreactivity, microwave antigen retrieval was performed at 750 W for 30 min in Tris EDTA (pH 9.0). After the endogenous peroxidase activity was blocked with 5% hydrogen peroxidase for 10 min, incubation with the primary antibody was performed for 1 h at room temperature. An EnvisionTMChemTM Detection Kit (DakoCytomation, Carpinteria, CA, USA) was used for the secondary antibody at room temperature for 30 min. After the tissue samples were washed in Tris-buffered saline for 10 min, 3, 3'-diaminobenzidine was used as a chromogen, and then Mayer's hematoxylin counterstain was applied.

Evaluation of IL-24 expression: IL-24-positive samples were defined as those showing a cytoplasmic staining pattern of the lesional tissue. The staining intensity of IL-24 was graded as follows: 0, negative; 1, weak; 2, strong staining comparable to that seen in a positive control (adjacent

normal glands of colonic mucosa) (Figure 1). The negative intensity stood for no stained cells. Weak intensity was allotted when the staining intensity was weaker than that of adjacent normal mucosa or < 5% of cells were stained, and strong intensity when the staining intensity was stronger than that of normal mucosa.

Evaluation of VEGF expression: Immunostaining of VEGF was considered to be positive if unequivocal staining of the membrane or cytoplasm was seen in > 10% of the tumor cells on the slides of the largest section of the tumor.

Statistical analysis

Survival was calculated from the date of surgery for rectal adenocarcinoma. The Kaplan-Meier method was used for survival analysis, and a log rank test was used for survival difference analysis. Correlation between patient characteristics and IL-24 or VEGF expression was evaluated by Fisher's exact test. The number of involved lymph nodes according to IL-24 intensity or expression states was analyzed by one-way ANOVA and *t* test, respectively. Differences were considered significant at *P* < 0.05. Statistical analyses were carried out with SPSS version 18 (Chicago, IL, USA).

RESULTS

Expression of IL-24 and VEGF

IL-24 expression was found in 81 out of 90 patients: nine

Table 2 Correlation of interleukin-24 and vascular endothelial growth factor expressions with patients' characteristics in lymph node positive rectal cancer patients

	IL-24			VEGF		
	Negative ¹ (%)	Positive ¹ (%)	P value	Negative (%)	Positive (%)	P value
Differentiation			0.009			0.634
Well	19 (41.3)	27 (58.7)		12 (26.1)	34 (73.9)	
Moderate	12 (35.3)	22 (64.7)		10 (29.4)	24 (70.6)	
Poor	9 (90.0)	1 (10.0)	0.004 (poor <i>vs</i> others)	4 (40.0)	6 (60.0)	
N stage			0.055			0.463
N1a	9 (37.5)	15 (62.5)		4 (16.7)	20 (83.3)	
N1b	11 (42.3)	15 (57.7)		8 (30.8)	18 (69.2)	
N2a	4 (25.0)	12 (75.0)		6 (37.5)	10 (62.5)	
N2b	16 (66.7)	8 (33.3)	0.016 (N1~N2a <i>vs</i> N2b)	8 (33.3)	16 (66.7)	
Stage			0.055			0.164
III a	3 (37.5)	5 (62.5)		0 (0.0)	8 (100.0)	
III b	22 (37.3)	37 (62.7)		18 (30.5)	41 (69.5)	
III c	15 (65.2)	8 (34.8)	0.028 (III a, III b <i>vs</i> III c)	8 (34.8)	15 (65.2)	

¹Negative (*n* = 9) and weak (*n* = 31) intensities were classified as negative expression, and strong intensity (*n* = 50) as positive one. IL: Interleukin; VEGF: Vascular endothelial growth factor.

negative, 31 weak intensity, and 50 strong intensity. Most cancer cells belonged to the strong intensity group were stained diffusely, so the proportion of immunoreactions was not analyzed. VEGF expression was observed in 64 out of 90 patients. The staining intensity of VEGF was strong in most stained cells, so the difference in terms of intensity of immunoreaction was not assessed.

Correlation between IL-24 or VEGF expression and clinicopathological factors

IL-24 expression was weaker in poorly differentiated tumors compared to well or moderately differentiated tumors. When the negative and weak intensities of IL-24 expression were categorized as negative expression for analysis (Table 2), IL-24 expression was significantly reduced in poorly differentiated tumors compared to well or moderately differentiated tumors, N2b to earlier N stages, and stage III c to stage III a or III b. These significant findings were maintained when another cut-off value of IL-24 was used for analysis (data not shown). There was no significant association between VEGF expression and patients' characteristics. This non-significance was sustained when other cut-off values of VEGF positivity were applied (data not shown).

Correlation between IL-24 expression and number of involved lymph nodes

IL-24 expression was inversely proportional to the N stages, so we compared the IL-24 expression status with the number of lymph nodes. The mean numbers of involved lymph nodes in the patients with negative, weak, and strong intensities of IL-24 expression were 12.11 ± 13.878 , 5.48 ± 5.253 , and 3.70 ± 3.346 , respectively (*P* < 0.05) (Table 3, Figure 2). The numbers of involved lymph nodes in patients with weak and strong intensities were not different after a multiple comparison test using the Tukey B method.

Table 3 Correlation of interleukin-24 expressions with the number of lymph nodes in the node positive rectal cancer patients

	<i>n</i>	No. of lymph nodes	P value
Intensity of IL-24 expression			0.001
Negative	9	12.11 ± 13.878	
Weak	31	5.48 ± 5.253	
Strong	50	3.70 ± 3.346	
IL-24 expression			0.012
Negative (negative & weak intensity)	40	6.98 ± 8.282	
Positive	50	3.70 ± 3.346	
IL-24 expression			0.000
Negative	9	12.11 ± 13.878	
Positive (weak & strong intensity)	81	4.38 ± 4.238	

IL: Interleukin.

Survival analysis

There were significant differences in disease-specific survival (DSS), disease-free survival (DFS), local-recurrence-free survival (LRFS), and distant-metastasis-free survival (DMFS) with regard to N stages (Table 4). DSS, DFS and DMFS were different according to stages. A significant difference in LRFS was found according to histological differentiation. The expressions of IL-24 and VEGF had no effect on survival.

DISCUSSION

The stage has been known as the most important prognostic factor in colorectal cancer patients until now. The seventh AJCC TNM classification (2010) subdivides N stages according to the number of involved lymph nodes^[22]. N1a is metastasis in one regional node, N1b in two or three, N2a in four to six, and N2b in seven or more. In this study, patients were distributed throughout

Table 4 Survival analysis in the lymph node metastatic rectal cancer patients

	<i>n</i>	5-yr disease specific survival	<i>P</i> value	5-yr disease free survival	<i>P</i> value	5-yr local recurrence free survival	<i>P</i> value	5-yr distant metastasis free survival	<i>P</i> value
Age (yr)									
Less than 60	48	60.4	0.963	51.8	0.918	60.3	0.683	57	0.483
60 or older	42	60.7		53.1		59.5		66.3	
Sex									
Male	47	60.3	0.742	49.2	0.528	57.4	0.903	60.3	0.760
Female	43	60.8		55.8		62.8		62.6	
Differentiation									
Well	46	74.9	0.060	59.3	0.333	73.8	0.008	64.7	0.459
Moderately	34	48.5		45.7		50		59.8	
Poorly	10	33.3		44.4		30		55.6	
T stage									
T1	1	100	0.441	100	0.427	100	0.385	100	0.298
T2	8	87.5		87.5		75		87.5	
T3	79	57.6		48.2		58.2		57.3	
T4	2	50		50		50		100	
N stage									
N1a	24	82.6	0.004	73.7	0.003	74.8	0.001	77	0.015
N1b	26	59.4		55.7		69.2		68.5	
N2a	16	68.8		50		68.8		56.3	
N2b	24	34.2		29.3		29.2		40.8	
Stage									
IIIa	8	100	0.002	100	0.002	100	0.230	100	0.009
IIIb	59	64.6		54.1		80.1		63	
IIIc	23	35.8		30.7		64.5		42.7	
VEGF expression									
Negative	26	53.1	0.801	50	0.851	73.3	0.629	64.1	0.791
Positive	64	61.9		53.5		81.1		60.5	
IL-24 expression									
Negative	40	59.2	0.759	50.7	0.890	60	0.496	60.7	0.849
Positive	50	34.7		53.9		59.9		61.6	

VEGF: Vascular endothelial growth factor; IL-24: Interleukin-24.

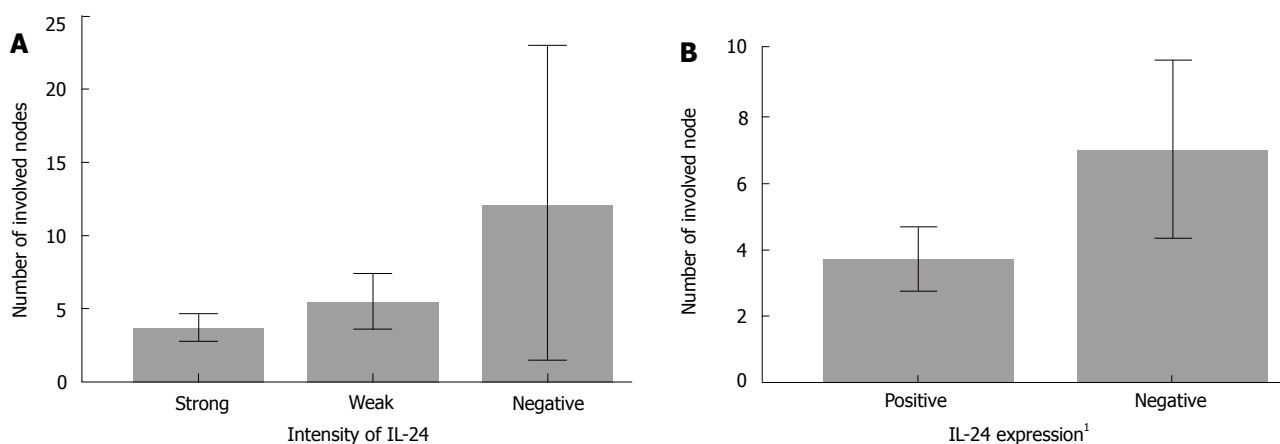


Figure 2 The number of involved lymph nodes according to interleukin-24 intensity (A) and expression status (B) in node-positive rectal cancer patients. ¹Negative (*n* = 9) and weak (*n* = 31) intensities were classified as negative expression, and strong intensity (*n* = 50) as positive. IL: Interleukin.

the N stages. That is, the proportions of the patient study group with cancer in the N1a, N1b, N2a and N2b stages were 26%, 29%, 18% and 26%, respectively. However, patients were not distributed evenly in terms of T stages: 79% were in stage T3. Therefore, to gain reasonable results from the T stage analysis was not possible, unlike the N stage analysis. The higher stages correlated significantly with poorer outcomes in terms of DSS, DFS, LRFS and

DMFS (*P* < 0.05). In addition, higher N stages also had poorer survival rates compared to lower ones. The N stages and stages from the seventh AJCC staging system seem to be applicable, although the subgroup patient numbers were not large in our analysis.

IL-24 promotes growth suppression and induces apoptosis in a broad array of human cancers, after forced expression by means of a plasmid or a replication-competent

adenovirus, but it does not induce growth suppressive or toxic effects in normal cells^[23]. To the best of our knowledge, the clinical significance of IL-24 expression in rectal cancer patients has not been assessed. This is believed to be the first study to analyze the association between IL-24 expression and patient prognostic features in lymph-node-involved rectal cancer patients.

IL-24 expression was weaker in the poorly differentiated tumors, N2b stage, and stage IIIc compared to well or moderately differentiated tumors, N1 or N2a stage, and stage IIIa or IIIb, respectively (Table 2). Moreover, the number of involved lymph nodes was significantly lower in patients with IL-24 expression compared to those without (Table 3). Even though IL-24 expression showed a significant correlation with these prognostic features, the status of IL-24 expression did not affect survival. This discrepancy may in part be explained by an inadequate patient number in the poor prognostic subgroups, and undetermined appropriate cut-off values for IL-24 positivity. Therefore, further studies with larger numbers of patients are necessary in order to develop an adequate grading system of IL-24 expression, and to verify whether IL-24 expression has a prognostic value.

The clinical significance of VEGF expression in rectal cancer is still open to debate. Several studies have insisted that increased VEGF expression is associated with poor prognosis, but some studies have shown that VEGF expression is not related to survival. Casinu *et al*^[21] have claimed from their analysis of lymph-node-positive rectal cancer patients that patients with VEGF-positive tumors have lower event-free survival rates and more frequent distant metastases. However, Bertolini *et al*^[18] have found from their study of locally advanced rectal cancer patients that VEGF expression obtained from pretreatment and post-chemoradiotherapy specimens does not show any significant correlation with DFS and overall survival (OS). In the study of Soumaoro *et al*^[24], OS was worse in colorectal cancer patients with VEGF expression, but this prognostic independence disappeared after multivariate analysis. Our study used unhampered surgical specimens without exposure to any chemoradiotherapy for immunohistochemical staining. VEGF expression was found in 64 out of 90 lymph-node-involved rectal cancer patients. There was no significant survival difference in the rectal cancer patients with regard to VEGF expression in spite of applying several cut-off values for VEGF expression. To verify the influence of VEGF expression on survival in advanced rectal cancer patients, further studies with large numbers of patients are required.

VEGF expression has been reported to be significantly correlated with tumor size, lymph node metastasis, lymphatic invasion, and TNM stage in colorectal cancer patients^[24,25]. It is also significantly associated with lymph node involvement in patients with locally advanced rectal cancer^[26]. However, these associations with VEGF expression were not found in present study.

In addition to the VEGF analysis with tumor tissues, soluble VEGF in the serum or plasma of patients has also been investigated. Werther *et al*^[27] have reported that

preoperative soluble VEGF is of independent prognostic value in patients with colon cancer, but not in those with rectal cancer. Tsai *et al*^[28] have found that patients with plasma VEGF elevation have worse DFS than those without plasma VEGF elevation in lymph-node negative colorectal cancer, but not in lymph-node-positive patients. Therefore, further studies are necessary to assess the role of soluble VEGF in rectal cancer patients.

In conclusion, we observed that IL-24 expression had a significant inverse relationship with N stage, overall stage, and the number of involved lymph nodes, but the status of IL-24 expression did not affect survival. Therefore, further studies with larger numbers of patients are needed in order to verify whether IL-24 expression has a prognostic value.

COMMENTS

Background

The survival rate of rectal cancer patients has been improved with the addition of chemoradiotherapy to surgery. However, that is still inadequate in stage III patients. Therefore, much endeavor is needed to increase the outcome of those through identification of new therapeutic targets. Anticancer activity of interleukin (IL)-24 has been reported in various cancer cells, but it is not known whether IL-24 has clinical importance in rectal cancer patients. In addition, vascular endothelial growth factor (VEGF) is considered to be essential in tumorigenesis, but its prognostic significance is still inconclusive in rectal cancer patients.

Research frontiers

Favorable survival is marginally identified in high-IL-24-expressing non-small cell lung cancer (NSCLC) patients. From a subset analysis, high IL-24 expression has been revealed as a significant prognostic factor in adenocarcinoma patients. However, whether high IL-24 expression has a significant impact on the survival of patients with types of cancers other than NSCLC is unknown.

Innovations and breakthroughs

Selective anticancer effects of IL-24 have been reported *in vitro* and *in vivo* without significant toxic effects on normal cells. These interesting properties may make IL-24 a candidate therapeutic target.

Applications

From this study, correlation of IL-24 expression with histological differentiation and the degree of lymph node involvement in rectal cancer patients was found, but IL-24 expression was not significantly correlated with survival. Therefore, further studies with larger numbers of patients are required in order to assess the prognostic value of IL-24 expression in rectal cancer patients.

Terminology

The melanoma differentiation associated gene-7 was identified by subtraction hybridization from human melanoma cells stimulated with interferon- β and mezerein, and was renamed later as IL-24. Multiple anticancer properties have been identified in a variety of cancer cells without injury to normal cells. Therefore, IL-24 has been emerging as an interesting candidate treatment target in many cancers.

Peer review

Choi *et al* studied immunohistochemically the clinicopathological significance of IL-24 expression in rectal carcinoma. The experiments were conducted appropriately and the results were reasonable.

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Long-term efficacy of infliximab maintenance therapy for perianal Crohn's disease

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Abstract

AIM: To assess the long-term efficacy of seton drainage with infliximab maintenance therapy in treatment of stricture for perianal Crohn's disease (CD).

METHODS: Sixty-two patients with perianal CD who required surgical treatment with or without infliximab between September 2000 and April 2010 were identified from our clinic's database. The activities of the perianal lesions were evaluated using the modified perianal CD activity index (mPDAI) score. The primary endpoint was a clinical response at 12-15 wk after surgery as a short-term efficacy. Secondary endpoints were recurrence as reflected in the mPDAI score, defined as increased points in every major element. The clinical responses were classified as completely healed (mPDAI = 0),

partially improved (mPDAI score decreased more than 4 points), and failure or recurrence (mPDAI score increased or decreased less than 3 points).

RESULTS: There were 43 males and 19 females, of whom 26 were consecutively treated with infliximab after surgery as maintenance therapy. Complete healing was not seen. Failure was seen in 10/36 (27.8%) patients without infliximab and 4/26 (15.4%) patients with infliximab ($P = 0.25$). Partial improvement was seen in 26/36 (72.2%) patients without infliximab and 22/26 (88.5%) patients with infliximab ($P = 0.25$). Short-term improvement was achieved in 48/62 (77.4%) patients. Although the mPDAI score improved significantly with surgery regardless of infliximab, it decreased more from baseline in patients with infliximab (50.0%) than in those without infliximab (28.6%), ($P = 0.003$). In the long-term, recurrence rates were low regardless of infliximab in patients without anorectal stricture. In patients with anorectal stricture, cumulative recurrence incidences increased gradually and exceeded 40% at 5 years regardless of infliximab. No efficacy of infliximab treatment was found ($P = 0.97$). Although the cumulative rate of ostomy creation was also low in patients without stricture and high in patients with stricture, no protective efficacy was found with infliximab treatment ($P = 0.6$ without stricture, $P = 0.22$ with stricture).

CONCLUSION: Infliximab treatment was demonstrated to have short-term efficacy for perianal lesions. Long-term benefit with infliximab was not proven, at least in patients with anorectal stricture.

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Key words: Crohn's disease; Perianal fistula; Infliximab; Anorectal stricture; Long-term efficacy

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INTRODUCTION

In recent years, infliximab (IFX), an IgG1 murine-human chimeric monoclonal antibody against tumor necrosis factor- α , has been recognized to be an effective treatment for active perianal fistula with Crohn's disease (CD)^[1]. However, in the ACCENT II trial, the closure of fistulas after the initial three IFX infusions occurred in 63% of patients at week 14, but decreased to 36% at week 54 with every 8 wk maintenance therapy and a high abscess formation rate (15%) was also seen^[2].

The placement of setons and drains prior to IFX therapy is generally recognized as the best combination therapy, and it allows for control of fistula healing and prevention of abscess formation^[3]. However, the efficacy of IFX with surgical treatment in perianal CD is controversial, and mainly short-term efficacy has been suggested^[4,5]. Moreover, most lesions of perianal CD are complicated by anorectal stricture that could affect the high recurrence rate and the risk for further ostomy creation. Although a further, large and long-term prospective study is needed for proper evaluation, the aim of this study was to assess the short and long-term efficacy of seton drainage in relation to IFX maintenance therapy with or without anorectal stricture for perianal CD retrospectively in advance of a longer duration prospective study.

MATERIALS AND METHODS

Patients

The clinical records of 131 patients with a perianal CD lesion who required surgical treatment between September 1995 and April 2010 were reviewed retrospectively. The surgical indications for all patients were limited to only a perianal lesion. Of these, 62 patients were identified from our out patients clinical records that were appreciable in detail of symptoms for assigning a modified perianal CD activity index (mPDAI) score, and these demographics and relevant medical details were recorded retrospectively^[6].

Patients on concomitant therapy that included corticosteroid and immunosuppressive agents were excluded from this study. Patients who did not use these agents postoperatively or used them after recurrence were included. Most patients were treated with oral antibiotics, including metronidazole, ciprofloxacin, or cefcapene pivoxil hydrochloride hydrate, before surgery as conservative treatment and after surgery as combination therapy. All patients were given a second-generation cefem intraoperatively.

Surgical procedure

Fistulas were classified by the method described by Parks *et al.*^[7]. At surgery, fistula tracts were thoroughly curetted, the abscess cavities were sufficiently drained and rubbed with a surgical spoon. Soft silastic or Teflon-coated vessel tape was inserted along the fistula tracts and tied loosely to facilitate drainage where appropriate as seton drainage. Anal dilation with the index finger was also performed in patients with stricture at surgery. Setons were removed in the outpatient clinic if the infection resolved on MRI findings and left in place if there were continuing signs of infection. The patients with incidental lesions as superficial fistulas were treated with conventional fistulotomy and excluded from this study.

Infliximab maintenance therapy

Initial IFX infusions (5 mg/kg) were administered within 2 wk from seton drainage (0 wk), and consecutive infusions were administered 2 wk and 6 wk later. This was continued as maintenance therapy every 8 wk. The patients who were given episodic infusions or could not be maintained on consecutive infusions due to adverse effects were not included in this study. Anal dilatation was performed in all patients with anorectal stricture preceding initial IFX infusion.

Assessments

The activities of perianal lesions were evaluated using the perianal CD activity index (PDAI)^[6]. The PDAI score is a 5-point index that detects changes in perianal status. The five major elements were the degrees of discharge, pain or restriction of living activity, restriction of sexual activity, type of perianal disease, and induration. Scores ranged from 0 to 20, with higher scores indicating more severe disease activity. Since the mPDAIs were reviewed from clinical records retrospectively in this study, the element of sexual function was not included, and the score ranged from 0 to 16.

The primary endpoint was a clinical response at 12-15 wk after surgery. The effective response was defined as at least a one point or greater reduction in every major element from baseline at week 12 as a short-term efficacy (a decrease of more than 4 points on the mPDAI score). Secondary endpoints were recurrence as reflected in the mPDAI score, defined as increased points in every major element (an increase of more than 4 points). The clinical responses were classified as completely healed (mPDAI = 0), partially improved (mPDAI score decreased more than 4 points), and failure (mPDAI score increased or decreased less than 3 points). Anorectal stricture was defined as the forefinger could not pass through during digital examination due to fibrotic changes.

Statistical analysis

Unless otherwise indicated, all numerical data are expressed as the median and range. Differences in patient's characteristics and mPDAI improvement rates with or without IFX were analyzed using the χ^2 test or the Mann-Whitney *U*-test. Evaluation of the change in mPDAI score

Table 1 Patients' characteristics *n* (%)

	Surgery + infliximab (<i>n</i> = 26)	Surgery alone (<i>n</i> = 36)	<i>P</i> value
Male:Female	16:10	27:9	0.26
Age at initial surgery (median years and range)	27.5 (16-55)	27.5 (16-41)	0.73
Duration from onset of perianal lesion (median month and range)	45 (2.5-275.5)	120 (26.5-339.5)	< 0.01
Duration from initial surgery for perianal lesion (median month and range)	28.2 (0.57-62.4)	74.5 (7.4-218.9)	< 0.01
Types of fistula			
Intrasphincteric	7 (26.9)	4 (11.1)	0.11
Transsphincteric	12 (46.2)	16 (44.4)	0.89
Suprasphincteric	4 (15.4)	8 (22.2)	0.5
Extrasphincteric	3 (11.5)	8 (22.2)	0.28
Anorectal stricture	19 (73.1)	21 (58.3)	0.23

Data are numbers with percentage in parentheses unless otherwise indicated.

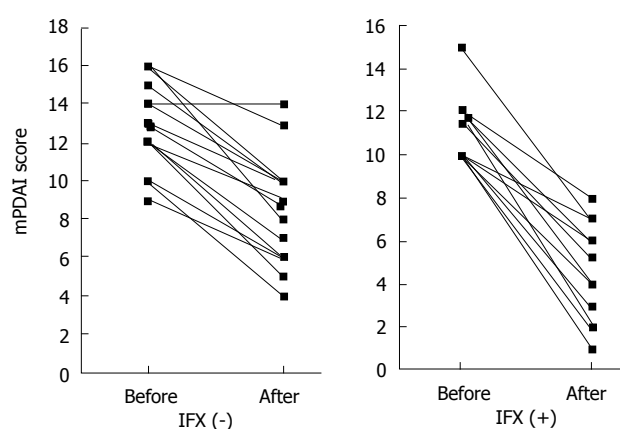


Figure 1 Individual modified perianal Crohn's disease activity index scores in patients before surgery and 12-15 wk after surgery with or without infliximab. The modified perianal Crohn's disease activity index (mPDAI) scores improved significantly with surgery regardless of infliximab (IFX) treatment: from 13.5 (9-16) before surgery to 9.5 (4-14) after surgery without IFX; from 11 (8-15) before surgery to 6 (1-10) with IFX ($P < 0.01$).

was analyzed using Wilcoxon's signed rank test. The cumulative maintained response rates or ostomy creation rates were compared using a log-rank test with Kaplan-Meier analysis. All statistical analyses were performed with SPSS for Windows, version 11.0.1. Two-tailed P values less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Patients demographics

Sixty-two patients who underwent surgery for perianal CD could be followed up at our outpatient clinic consecutively. There were 43 males and 19 females, and their median age at surgery was 27.0 (12-58) years. Of these, 26 patients were treated with IFX after surgery as maintenance therapy from April 2004. Patients' characteristics are shown in Table 1. There was a high frequency of patients with anorectal stricture with or without IFX. Adverse events occurred in 7 patients, with infusion reactions as minor events. No major events related to IFX were seen in this series.

Short-term efficacies

Complete healing was not seen in any patients. Failure was seen in 10/36 (27.8%) patients without IFX and 4/26 (15.4%) patients with IFX ($P = 0.25$). Partial improvement was seen in 26/36 (72.2%) patients without IFX and 22/26 (88.5%) patients with IFX ($P = 0.25$). Short-term improvement was achieved in 48/62 (77.4%) patients. The mPDAI scores improved significantly with surgery regardless of IFX treatment: from 13.5 (9-16) before surgery to 9.5 (4-14) after surgery without IFX; from 11 (8-15) before surgery to 6 (1-10) with IFX ($P < 0.01$) (Figure 1). The rates of improving mPDAI score from baseline were greater in patients with IFX - 50.0% (0-90%) - than in those without IFX -28.6% (0%-60%); ($P = 0.003$) (Figure 2).

Long-term efficacies

In the long-term, recurrences were low regardless of IFX in patients without anorectal stricture (not significant, $P = 0.4$) (Figure 3). However, in patients with stricture, cumulative recurrence incidences increased gradually and exceeded 40% at 5 years in both groups (Figure 4). IFX treatment was not found to be effective ($P = 0.97$). Although the cumulative rate of ostomy creation was also low in patients without stricture and high in patients with stricture, no protective efficacy was found with IFX treatment ($P = 0.6$ without stricture, $P = 0.22$ with stricture) (Figures 5 and 6). The patients' present statuses are shown in Table 2. Of these, 12 patients required abdominoperineal resection (APR), and 14 patients required Hartmann's procedure. There were no significant differences in rates and duration from initial surgery to ostomy creation with or without IFX treatment.

Two patients without IFX treatment were diagnosed as having carcinoma in anorectal fistula and required APR. They had an 8.1- and 10.2-year history of perianal CD, respectively. Both cases demonstrated highly advanced disease and recurred within 2 years after APR.

DISCUSSION

CD is a chronic inflammatory bowel disease of unknown etiology, characterized by fissuring ulcers and segmental

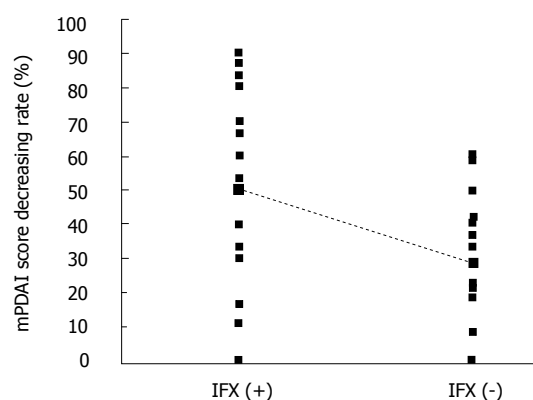


Figure 2 Decreasing rate of the modified perianal Crohn's disease activity index score after surgery in regards to infliximab. The squares represent the median value and the circles represent range plots. The rate of reduction in modified perianal Crohn's disease activity index (mPDAI) score from baseline was significantly greater in patients with infliximab (IFX) -50.0% (0%-90%) - than in those without IFX -28.6% (0%-60%); ($P = 0.003$).

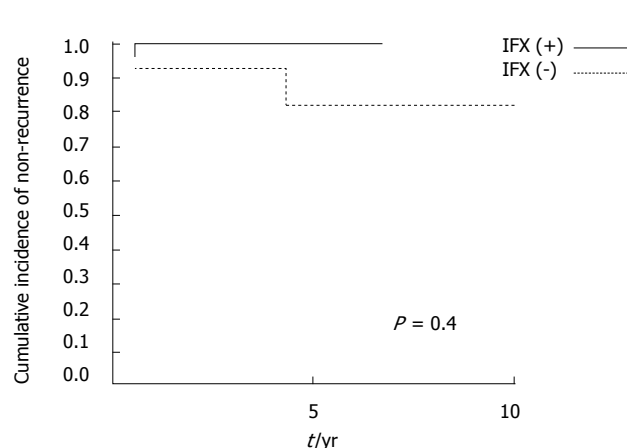


Figure 3 Cumulative incidence of maintaining non-recurrent status in patients without anorectal stricture. The cumulative incidence of fistula recurrence was 0%/5 years with infliximab (IFX) ($n = 7$) and 17.5%/10 years without IFX ($n = 15$), respectively.

Table 2 Present status of surgical treatment

	IFX (-)	IFX (+)	<i>P</i> value
APR	9/36	3/26	0.19
Duration from initial surgery to APR, median yr (range)	2.5 (0-4.3)	3.9 (1.0-4.1)	0.79
Hartmann's procedure	10/36	4/26	0.25
Duration from initial surgery to Hartmann, median yr (range)	2.3 (0-7.0)	0.7 (0-2.9)	0.22

APR: Abdominoperineal resection; IFX : Infliximab.

transmural inflammation of the gastrointestinal tract. Perianal fistulas are a frequent manifestation of CD, and the prevalence ranges from 20% to 50%^[8]. Medical approaches, with metronidazole, ciprofloxacin, azathioprine, or 6-mercaptopurine, and surgical approaches have been involved in combination therapy^[9-11]. These therapies have been suggested to have a short-term benefit. Furthermore, management with loose setons,

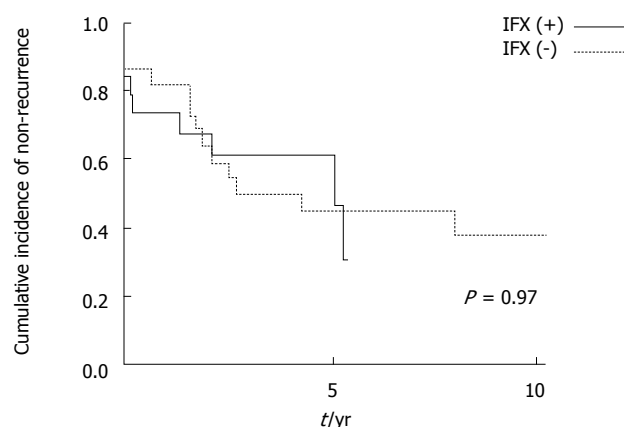


Figure 4 Cumulative incidence of maintaining non-recurrent status in patients with anorectal stricture. The cumulative incidence of fistula recurrence was 53.9%/5 years with infliximab (IFX) ($n = 19$) and 55.0%/5 years and 62.5%/10 years without IFX ($n = 21$), respectively.

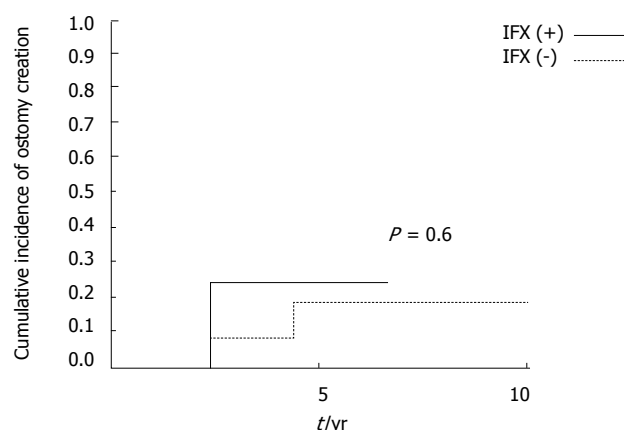


Figure 5 Cumulative incidence of ostomy creation in patients without anorectal stricture. The cumulative incidence of ostomy creation was 25.0%/5 years with infliximab (IFX) ($n = 7$) and 18.5%/5 years without IFX ($n = 15$), respectively.

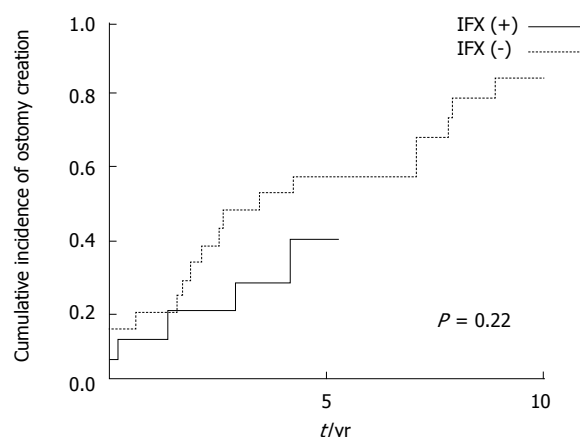


Figure 6 Cumulative incidence of ostomy creation in patients with anorectal stricture. The cumulative incidence of ostomy creation was 38.9%/5 years with infliximab (IFX) ($n = 19$) and 56.5%/5 years and 83.7%/10 years without IFX ($n = 21$), respectively.

advancement flaps, fibrin glue, or collagen plugs has been reported to have a short-term benefit^[12]. Though

the conventional fistulotomy is generally avoided for recurrence and fecal incontinence, surgical treatment with loose setons has shown good results^[13,14]. However, although the ideal goal for treatment of perianal CD lesion is fistula closure and prevention of recurrence, diversion with ostomy creation or APR might often be necessary in patients with refractory lesions.

Recently, IFX treatment has become well established. However, its efficacy for perianal CD has been controversial. Gaertner *et al*^[4] reported that surgery for perianal CD resulted in complete healing in approximately 60% of patients regardless of the use of IFX. However, the time to healing was shorter and the healing rate was improved with surgery plus IFX. Hyder *et al*^[5] also showed the efficacy of IFX in combination with surgery in 85% of cases as measured by PDAI. Although both studies evaluated the short-term efficacy of IFX plus surgery, the medium- or long-term results are less impressive. Also in Japan, IFX in combination with surgery has been recognized as the one of the main treatments for perianal CD, although the results are controversial. Although there are many variables and confounding factors that were not controlled in this retrospective study such as concomitant antibiotics administration, surgery plus IFX treatment was demonstrated to have a short-term benefit compared to surgery alone in improving the rate of PDAI. It is considered that IFX has additional effects which include a greater decrease in the degree of discharge and a reduced healing period though the pain, restriction of living activity and indurations could be improved a little with surgery alone. However, a long-term benefit with maintenance IFX was not proven, at least in patients with anorectal stricture who were excluded from the ACCENT II study, even with the anal dilatation in this series^[2]. Though the surgery with IFX treatment had no efficacy for preventing the recurrence and the comparison of ostomy creation was not significant, the *p* value was 0.08 in regard to ostomy creation in this study. The efficacies of avoidance or time extension for further ostomy creation should be considered in a further long-term and large prospective randomized trial.

On the other hand, prolonged inflammation in a perianal fistula will cause another problem. Although the causative relationship between anorectal fistula and CD has yet to be determined, the epithelial regeneration and hyperplasia are predisposed to malignant changes, and perianal fistula might lead to carcinogenesis due to constant regeneration^[15]. The carcinomas complicating CD have strikingly similar clinicopathologic features to ulcerative colitis with chronic inflammation. The increased risk of carcinoma in areas of prolonged inflammation, such as perianal lesions, has generally been recognized, and surveillance colonoscopy has been recommended for carcinoma screening in CD^[16,17]. However, such surveillance would be difficult for patients with severe pain and stricture of the anorectal CD lesion. In general, sudden changes in clinical symptoms, such as increased discharge, severe pain, or progressive stricture, could raise the suspicion of malignancies. Unfortunately, these malignancies are almost all advanced carcinomas when these symptoms change. Lad *et al*^[18] sug-

gested that contrast-enhanced MRI would be helpful for the diagnosis of carcinoma, but subsequent biopsy would be necessary. Finally, examination under anesthesia may be needed for accurate and early diagnosis, though there is a possibility of missing the diagnosis of occult carcinoma.

In Japan, the incidence of prolonged perianal CD has increased and the condition is associated with a risk of carcinogenesis due to long-standing inflammation^[19]. While the numbers of CD patients with carcinomas are increasing, and the establishment of an effective surveillance protocol is desired, it will be difficult to develop for the above reasons. Furthermore, Sjö Dahl *et al*^[20] suggested that the rectal remnant should be considered a risk factor for carcinogenesis. Prophylactic proctectomy may be justified in patients with persistent or recurrent perianal CD. Even if the long-term efficacy of IFX maintenance therapy is proven, it will be necessary to consider whether the prolonged anorectal CD lesion increases carcinogenesis.

In conclusion, surgery with IFX treatment was demonstrated to have short-term efficacy. However, a long-term benefit with maintenance IFX was not proven, at least in patients with anorectal stricture. Maintenance IFX therapy for patients with anorectal stricture may be contraindicated, even if anal dilatation is performed. Although a long-term prospective randomized trial is needed, surgery with IFX treatment had less efficacy for decreasing the recurrence rate and prophylactic proctectomy may be justified in patients with persistent or recurrent perianal CD, especially in patients with anorectal stricture, for early resolution of occult carcinogenesis.

COMMENTS

Background

In recent years, infliximab (IFX), an IgG1 murine-human chimeric monoclonal antibody against tumor necrosis factor- α , has been recognized to be an effective treatment for active perianal fistula with Crohn's disease (CD).

Research frontiers

In the ACCENT II trial, the closure of fistulas after the initial three IFX infusions occurred in 63% of patients at week 14, but closure decreased to 36% at week 54 with every 8 wk maintenance therapy and there was a high abscess formation rate (15%). The placement of setons and drains prior to IFX therapy is generally recognized as the best combination therapy, and it allows for control of fistula healing and prevention of abscess formation. However, the efficacy of IFX with surgical treatment in perianal CD is controversial, and mainly short-term efficacy has been suggested. Perianal lesions with stricture are recognized to have high recurrence potential, and this is thought to be a contraindication for combination therapy. The aim of this study was to assess the short and long-term efficacy of seton drainage in relation to IFX maintenance therapy with or without anorectal stricture for perianal CD.

Innovations and breakthroughs

The management with loose setons, advancement flaps, fibrin glue, or collagen plugs for perianal CD has been reported to have a short-term benefit. Though the conventional fistulotomy is generally avoided for recurrence and fecal incontinence, surgical treatment with loose setons has shown good results. However, although the ideal goal for treatment of perianal CD lesion is fistula closure and prevention of recurrence, diversion with ostomy creation or abdominoperineal resection may often be necessary in patients with refractory lesions. This study was designed to access data on the long-term efficacy of combination therapy. Although a further, large and long-term prospective study is needed for proper evaluation, the aim of this study was to assess retrospectively the short and long-term efficacy of seton drainage in relation to IFX combination therapy in advance of a prospective study that might take place over a longer period.

Applications

Sixty-two patients were identified from our out patients clinical records that were appreciable in detail of symptoms for assigning a modified perianal CD activity index (mPDAI) score, and the demographics and relevant medical details were recorded retrospectively. To evaluate the efficacies of combination therapy, the authors reviewed the clinical records in regard to anorectal stricture.

Terminology

The activities of perianal lesions were evaluated using the perianal CD activity index (PDAI). The PDAI score is a 5-point index that detects changes in perianal status. The five major elements were the degrees of discharge, pain or restriction of living activity, restriction of sexual activity, type of perianal disease, and induration. Scores ranged from 0 to 20, with higher scores indicating more severe disease activity. Since the mPDAIs were reviewed retrospectively from clinical records in this study, the element of sexual function was not included, and the score ranged from 0 to 16.

Peer review

Surgery with IFX treatment was demonstrated to have short-term efficacy. However, a long-term benefit with maintenance IFX was not proven, at least in patients with anorectal stricture. Maintenance IFX therapy for patient with anorectal stricture may have to be contraindicated, even if anal dilatation is performed. Prophylactic proctectomy may be justified in patients with persistent or recurrent perianal CD. Even if the long-term efficacy of IFX maintenance therapy is proven, it will be necessary to consider whether the prolonged anorectal CD lesion increases carcinogenesis.

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Prognostic factors of T4 gastric cancer patients undergoing potentially curative resection

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Abstract

AIM: To investigate the prognostic factors of T4 gastric cancer patients without distant metastasis who could undergo potentially curative resection.

METHODS: We retrospectively analyzed the clinical data of 71 consecutive patients diagnosed with T4 gastric cancer and who underwent curative gastrectomy at our institutions. The clinicopathological factors that could be associated with overall survival were evaluated. The cumulative survival was determined by the Kaplan-Meier method, and univariate comparisons between the groups were performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazard model and a step-wise procedure.

RESULTS: The study patients comprised 53 men (74.6%) and 18 women (25.4%) aged 39-89 years (mean, 68.9 years). Nineteen patients (26.8%) had postoperative morbidity: pancreatic fistula developed in 6 patients (8.5%) and was the most frequent complication, followed by anastomosis stricture in 5 patients (7.0%). During the follow-up period, 28 patients (39.4%)

died because of gastric cancer recurrence, and 3 (4.2%) died because of another disease or accident. For all patients, the estimated overall survival was 34.1% at 5 years. Univariate analyses identified the following statistically significant prognostic factors in T4 gastric cancer patients who underwent potentially curative resection: peritoneal washing cytology ($P < 0.01$), number of metastatic lymph nodes ($P < 0.05$), and venous invasion ($P < 0.05$). In multivariate analyses, only peritoneal washing cytology was identified as an independent prognostic factor (HR = 3.62, 95% CI = 1.37-9.57) for long-term survival.

CONCLUSION: Positive peritoneal washing cytology was the only independent poor prognostic factor for T4 gastric cancer patients who could be treated with potentially curative resection.

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Key words: Gastric cancer; T4; Prognostic factors; Peritoneal cytology; Venous invasion

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Fukuda N, Sugiyama Y, Wada J. Prognostic factors of T4 gastric cancer patients undergoing potentially curative resection. *World J Gastroenterol* 2011; 17(9): 1180-1184 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i9/1180.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i9.1180>

INTRODUCTION

Although the incidence of gastric cancer has declined worldwide, this disease remains the second leading cause of cancer death because patients with an advanced form of gastric carcinoma still have a poor prognosis^[1,2]. Depth of invasion, lymph node metastasis, or tumor di-

ameter are believed to be independent prognostic factors of gastric carcinoma^[3,4]. Locally advanced gastric cancer defined as T4 in which the tumor perforates serosa (T4a) or invades adjacent structures (T4b)^[5] often has a poor prognosis due to simultaneous distant metastasis such as peritoneal seeding, liver metastasis, and/or distant lymph node involvement. Even though distant metastasis is not apparent in T4 gastric cancer, curative surgery cannot always be performed because such cases sometimes show marked invasion to adjacent structures. Moreover, curative gastrectomy with combined resection of invaded adjacent organs has a reportedly high incidence of post-operative morbidity and mortality^[6,7]. In fact, the overall survival rate for locally advanced gastric cancer patients is under 20% and is approximately 30% for those who can undergo surgical resection^[8].

Nevertheless, a certain number of patients with locally advanced gastric carcinoma could survive curative gastrectomy and progress satisfactorily without tumor recurrence. In this study, we retrospectively studied surgical outcomes and prognostic factors for T4 advanced gastric carcinoma treated with potentially curative resection.

MATERIALS AND METHODS

From 2001 to 2009, 452 gastric cancer patients underwent surgical treatment at our institutions. Of these, 71 patients (15.7%) diagnosed with histological T4^[5] gastric carcinoma and treated with potentially curative resection were selected for this study. All patients underwent D2gastrectomy. Surgery was considered potentially curative [tumor-nodes-metastases (TNM)-R0] or to be resection with curative intent when there was no gross residual tumor after surgery and the resection margins were histologically free from cancer cells. Patients with metastatic disease who had undergone palliative resection were excluded. The study patients comprised 53 men (74.6%) and 18 women (25.4%) aged 39-89 years (mean, 68.9 years). Sixty patients were included in the stage T4a group and 11 cases in the stage T4b group, according to TNM classification^[5]. On histological examination, it was found that T4b gastric carcinomas exhibited invasions to the transverse colon in 5 patients, the pancreas in 3 patients, the diaphragm in 2 patients, and the liver in 1 patient. Of the 71 patients, 7 had tumors located in the upper third of the stomach, 28 had tumors in the middle third of the stomach, 21 had tumors in the lower third of the stomach, and 15 had tumors occupying the entire stomach. The tumor diameter ranged from 20 to 205 mm (mean, 84 mm). Proximal gastrectomy, distal gastrectomy, and total gastrectomy were performed in 3 patients (4.2%), 35 patients (49.3%), and 33 patients (46.5%), respectively. All the surgical procedures were based on the policy of curative resection, which meant complete removal of cancer tissue regardless of combined multi-organ resection with no residual tumor macroscopically.

Data regarding the patients' clinicopathological features, surgical outcomes including morbidity and mortality, and follow-up data were obtained from a clinical database. Histological classification and staging were principally

based on the seventh edition of the International Union Against Cancer (UICC) TNM classification^[5]. We evaluated clinicopathological factors of T4 gastric cancer patients that could be associated with overall survival. These parameters were age, gender, tumor diameter, histological type, lymph node metastasis, metastatic lymph node ratio (MLR), lymphatic invasion (ly), venous invasion (v), and peritoneal washing cytology (CY). For statistical analysis, the patients were grouped into 2 categories with respect to age [≤ 68 years or > 68 years (mean value)], tumor diameter [≤ 84 mm or > 84 mm (mean value)], histological type (differentiated or undifferentiated), number of metastatic lymph nodes (N0 or N1 *vs* N2 or N3)^[5], MLR [≤ 0.27 or > 0.27 (mean value)], and peritoneal washing cytology (CY0 or CY1)^[5]. Similarly, the patients were divided into 2 groups with respect to lymphatic invasion (ly0 or ly1 *vs* ly2 or ly3) and venous invasion (v0 or v1 *vs* v2 or v3) according to the Japanese Gastric Cancer Association (JGCA) system^[9]. Post-operative morbidity and mortality were defined as operation-related complications or death that occurred within 30 days after surgery.

The observation period ended on July 31, 2010. The median follow-up duration from the date of surgery was 24 mo (range, 1-89 mo). Fifty patients (70.5%) were given post-operative adjuvant chemotherapy using S-1 for 29 patients, UFT for 7 patients, paclitaxel for 7 patients, and others for 7 patients. The cumulative survival was determined by the Kaplan-Meier method, and univariate comparisons between the groups were performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazard model and a step-wise procedure. *P* value differences less than 0.05 were considered significant.

RESULTS

Sixty-one patients (85.9%) had lymph node metastasis, 9 (12.7%) had N1, 18 (25.4%) had N2, and 34 (47.9%) had N3 disease. Differentiated tumors were histologically revealed in 31 patients and undifferentiated tumors were seen in 40 patients. The degree of lymphatic invasion according to the JGCA system^[9] were 0.0%, 23.9%, 45.1%, and 31.0% for ly0, ly1, ly2, and ly3, respectively. The degree of venous invasion according to the JGCA system^[9] were 32.4%, 42.3%, 23.9%, and 1.4% for v0, v1, v2, and v3, respectively. Twenty-seven patients (38.0%) were positive for peritoneal washing cytology. Patient characteristics are presented in Table 1. Nineteen patients (26.8%) had postoperative morbidity. Pancreatic fistula occurred in 6 patients (8.5%) and was the most frequent complication, followed by anastomosis stenosis in 5 patients (7.0%). Three patients (4.2%) died of post-operative complications: 2 were due to multi-organ failure associated with pancreatic fistula, and 1 was due to acute gangrenous cholecystitis combined with peritonitis. These complications are listed in Table 2. Thirty-one patients (43.7%) died during the follow-up period. Of these, 28 were related to recurrence of gastric cancer, and 3 were due to another disease or accident. The estimated overall survival at 5 years and the median survival time (MST) for all patients

Table 1 Patient characteristics

Variables	n (%)
Age	
≤ 68	31 (43.7)
> 68	40 (56.3)
Gender	
Male	53 (74.6)
Female	18 (25.4)
Tumor location	
Upper 1/3	7 (9.9)
Middle 1/3	28 (39.4)
Lower 1/3	21 (29.6)
Tumor size (mean, mm)	20-205 (84)
Type of gastrectomy	
Proximal	3 (4.2)
Distal	35 (49.3)
Total	33 (46.5)
Lymph node involvement	
Positive	61 (85.9)
Negative	10 (14.1)
Histological type	
Differentiated	31 (43.7)
Undifferentiated	40 (56.3)

Table 2 Postoperative complications

	Patients (n = 71)	%
Morbidity	19	26.8
Pancreatic fistula	6	8.5
Anastomosis stricture	5	7.0
Anastomosis leakage	3	4.2
Cholecystitis	3	4.2
Abdominal abscess	2	2.8
Ileus	1	1.4
Mortality	3	4.2

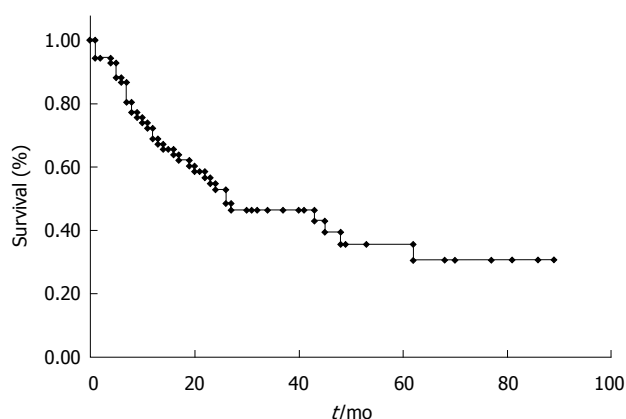


Figure 1 Kaplan-Meier survival curve of T4 gastric cancer patients.

were 34.1% (Figure 1) and 19 mo, respectively.

The clinicopathological records of the 71 patients and the 5-year survival rates are shown in Table 3. The statistically significant prognostic factors were peritoneal washing cytology ($P < 0.01$), number of metastatic lymph nodes ($P < 0.05$), and venous invasion ($P < 0.05$). The 5-year overall survival rate of the patients with positive peritoneal wash-

Table 3 Clinicopathological factors and univariate survival analyses

	n	5-yr survival (%)	P
Age (mean: 68.9)			
≤ 68	31	45.5	0.415 (NS)
> 68	40	26.2	
Gender			
Male	53	31.0	0.510 (NS)
Female	18	50.1	
Tumor diameter (mm, mean: 84)			
≤ 84	39	34.4	0.213 (NS)
> 84	32	33.9	
Histological type			
Differentiated	31	18.6	0.565 (NS)
Undifferentiated	40	40.7	
Number of lymph node meta			
N0 or N1	19	67.4	< 0.05
N2 or N3	52	23.8	
MLR (mean: 0.27)			
≤ 0.27	46	38.9	0.083 (NS)
> 0.27	25	20.7	
Lymphatic invasion			
ly0 or ly1	17	49.3	0.453 (NS)
ly2 or ly3	54	29.7	
Venous invasion			
v0 or v1	53	45.7	< 0.05
v2 or v3	18	9.7	
Peritoneal washing cytology			
Negative (CY0)	44	47.6	< 0.01
Positive (CY1)	27	15.2	

NS: Not significant.

Table 4 Multivariate analysis

	Hazard ratio	95% CI	P
Number of LN meta	1.3104	0.4075-4.2137	0.6501
Venous invasion	0.9642	0.3335-2.7882	0.9464
Peritoneal washing cytology	3.6262	1.3743-9.5683	< 0.01

CI: Confidence interval.

ing cytology was 15.2%, which was significantly decreased compared to patients with negative peritoneal washing cytology (47.6%). The 5-year overall survival rate of patients with N2 or N3 was 23.8%, which was significantly poorer than patients with N0 or N1 (67.4%). Similarly, the 5-year overall survival rate of patients with v2 or v3 was 9.7 %, which was significantly decreased compared to patients with v0 or v1 (45.7%). The tumor diameter, degree of lymphatic invasion, and histological classification were not significant prognostic factors according to the results of the univariate analysis. In multivariate analysis, only peritoneal washing cytology was identified as an independent prognostic factor (HR = 3.62, 95% CI = 1.37-9.57) for long-term survival (Table 4).

DISCUSSION

Owing to the progression of surgical techniques and the

standardization of curative R0 resection, the prognosis of the patients with gastric cancer has been improved in recent years. Nevertheless, patients with advanced gastric carcinoma, especially serosa invading locally advanced tumor diagnosed as T4 in TNM classification^[5], still have a poor prognosis^[10]. The poor prognosis associated with T4 advanced gastric cancer may result from the presence of incurable factors including distant lymph node involvement, peritoneal metastasis, and hematogenous metastasis such as liver metastasis^[11]. If a patient with T4 gastric carcinoma does not have the incurable factors mentioned above, a relatively better survival can be expected when curative surgery regardless of en-block multi-organ resection is achieved. Various T4 gastric carcinoma prognostic factors have been reported in the literature. Kunisaki *et al*^[4] reported that tumor diameter (> 100 mm) and lymph node metastases (more than 7) are poor prognostic factors in T4 gastric cancer patients and concluded that curative surgery with multi-organ resection is indicated for patients with few metastatic lymph nodes (6 or less) and a relatively small tumor diameter (≤ 100 mm). Similarly, several reports suggested that tumor size in gastric cancer is a significant prognostic factor, and large gastric cancers with a diameter > 80 mm have more aggressive behavior and frequent peritoneal recurrences^[12,13]. However, our study revealed that the tumor size was not a significant prognostic factor in T4 gastric carcinoma patients who could undergo potentially curative resection. The divergent conclusions of these reports^[4,12,13] with ours might be explained by different patient populations.

Our study was limited to patients with T4 gastric carcinoma without distant metastasis and who were treated with potentially curative resection, whereas other studies^[4,12,13] included patients with distant metastasis. Therefore, tumor size may not be a significant prognostic factor in T4 gastric carcinoma, if the patient does not have distant metastasis and can be treated with curative resection.

Lymph node metastasis is a commonly reported prognostic factor for poor outcome in patients with T4 gastric carcinoma^[4,11,14]. Saito *et al*^[14] reported that infiltrative type and lymph node metastasis were independent poor prognostic factors in curatively resected patients with T4 gastric carcinoma, and stated that multi-organ resection does not seem to be effective even when curative resection is performed in infiltrating tumors with lymph node metastasis. Jeong *et al*^[11] revealed that lymph node metastasis (greater than pN3) was an independent poor prognostic factor for patients with T4 gastric carcinoma who underwent curative surgery, and concluded that curative resection does not seem to be effective in patients with extensive lymph node metastasis (more than N3). In our study, although patients with more extensive lymph node metastasis (N2 or N3) had a significantly poorer prognosis compared to patients in whom lymph node metastasis was limited (N0 or N1) according to the results of univariate analysis, multivariate analysis revealed that lymph node metastasis was not an independent prognostic factor for T4 gastric cancer patients who underwent potentially curative resection. Although the degree of lymph node metastasis influences surgical

outcomes in patients with T4 gastric carcinoma, a relatively good prognosis can be expected with curative R0 resection followed by adjuvant chemotherapy even if the patient has extensive lymph node metastasis (N2 or N3).

In this study, positive peritoneal washing cytology was identified as the only independent prognostic factor for T4 gastric cancer patients who underwent potentially curative resection. Several reports^[15-21] have emphasized the prognostic significance of intra-peritoneal free cancer cells for potentially curable serosa-invaded gastric carcinoma. Intra-peritoneal free cancer cells which may be exfoliated mainly from the serosal surface of the stomach penetrated by the primary tumor, are closely related to peritoneal dissemination^[18]. Therefore, detection of intra-peritoneal free cancer cells that might have already seeded at the time of operation but cannot be found macroscopically is a key point for influencing the prognosis of T4 gastric cancer patients and for adjuvant treatment planning for those patients. Euanorasetr *et al*^[17] reported that all patients with positive peritoneal washing cytology developed peritoneal recurrence, with no patient surviving more than 5 years, and that the sensitivity of peritoneal washing cytology in predicting peritoneal recurrence was only 61% regardless of its high specificity (100%). In addition, the sensitivity of peritoneal washing cytology was previously reported as relatively low, ranging from 14% to 70%^[16,22-25]. The relatively high false-negative rate might arise from technical flaws such as incomplete sampling during the lavage process^[17]. Recently, the real-time quantitative polymerase chain reaction (PCR) technique has made it possible to detect the presence of only a few cancer cells in the abdominal cavity and this technique is more sensitive than traditional peritoneal lavage cytology^[26,27]. Katsuragi *et al*^[18] reported that the prognosis of patients with isolated tumor cells in the peritoneal lavage fluid detected by PCR-based identification was significantly poorer than the prognosis for PCR-negative patients in T4 gastric cancer. Therefore, detection of intra-peritoneal free cancer cells should be the most important and useful way to infer surgical outcome and prognosis of T4 gastric cancer patients. According to the results, T4 gastric cancer patients with positive peritoneal washing cytology might be treated in the same way as for the patients with peritoneal metastasis. More aggressive adjuvant chemotherapy such as S-1 plus cisplatin^[28] or DCF^[29] should be indicated for patients with T4 gastric cancer with positive peritoneal washing cytology that could undergo potentially curative resection to improve prognosis.

COMMENTS

Background

Although the incidence of gastric cancer has declined particularly in Western countries, the disease remains the fourth most common cancer and continues to be the second leading cause of cancer death worldwide. The therapeutic strategy for advanced gastric carcinoma, such as T4 locally advanced gastric carcinoma, is to improve the prognosis of all gastric cancer patients, since surgical results for early stage gastric carcinoma are satisfactory.

Innovations and breakthroughs

In this study, patients included were limited to T4 advanced gastric carcinoma without distant metastasis who could be treated with potentially curative resec-

tion. Various clinicopathological factors including peritoneal washing cytology that could be associated with overall survival of T4 gastric cancer patient were evaluated on univariate analysis using the Kaplan-Meier method and on multivariate analysis using a Cox proportional hazard model and a step-wise procedure.

Applications

Aggressive adjuvant chemotherapy should be indicated for the patients with T4 gastric carcinoma with positive peritoneal washing cytology to improve the prognosis, even if the tumor can be resected without no residual tumor macroscopically. Thus, identification of effective adjuvant chemotherapy for advanced gastric carcinoma with positive peritoneal cytology for patients who could undergo potentially curative resection will be the problem in the near future.

Peer review

This is an interesting work that underlines the prognostic value of peritoneal cytology in curatively resected T4 gastric carcinomas. The text is well-organized and the key points are clearly described.

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Diagnostic value of antigenemia assay for cytomegalovirus gastrointestinal disease in immunocompromised patients

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(CMV) antigenemia assay for the diagnosis of CMV gastrointestinal disease (GID).

METHODS: One hundred and thirty immunocompromised patients were enrolled in this study. Patients with a history of anti-CMV treatment and who had not undergone examination using the antigenemia assay were excluded. CMV-GID was defined as the detection of large cells with intranuclear inclusions alone or associated with granular cytoplasmic inclusions by biopsy. Biopsy sections were stained with hematoxylin and eosin and immunohistochemically stained with anti-CMV. We evaluated the association between CMV-GID and patient characteristics (symptoms, underlying disease, medication, leukocyte counts, and antigenemia assay). All patients were checked with an human immunodeficiency virus (HIV) antibody test before endoscopic examination. White blood cell (WBC) counts were obtained from medical records within 1 wk of endoscopy. Leukopenia was defined as a total WBC count < 5000 cells/mm³. For HIV patients, we also checked CD4+ counts from medical records.

RESULTS: A total of 99 patients were retrospectively selected for analysis. Of the immunocompromised patients, 19 had malignant disease, 18 had autoimmune disease, 19 had disorders of biochemical homeostasis, three had undergone transplantation, and 45 had HIV infection. A total of 50 patients had received immunosuppressive therapy. No patients had inflammatory bowel disease. Fifty-five patients were diagnosed as having CMV-GID. Univariate analysis indicated an association between HIV infection, leukopenia, and positive antigenemia and CMV-GID ($P < 0.05$). Multivariate analysis using logistic regression revealed that HIV infection and positive antigenemia were the only independent factors related to CMV-GID ($P < 0.01$). The sensitivity, specificity, positive predictive value, and negative predictive value of antigenemia for CMV-GID were 65.4%, 93.6%, 91.9%, and 71.0%, respectively. In a subgroup analysis

Abstract

AIM: To investigate the utility of the cytomegalovirus

sis, patients with leukopenia displayed low sensitivity and high specificity. Minimal differences in accuracy were seen among patients with or without leukopenia. HIV-infected patients displayed low sensitivity and high specificity. Accuracy barely differed between HIV-positive and -negative patients. In HIV-infected patients, CD4 count < 50 cells/ μ L resulted in low sensitivity and high specificity. Differences in accuracy among patients were minor, regardless of CD4 count. In patients who had undergone both quantitative real-time polymerase chain reaction (PCR) and antigenemia assay, real-time PCR was slightly more accurate in terms of sensitivity than the antigenemia assay; however, this difference was not statistically significant ($P = 0.312$).

CONCLUSION: If the antigenemia test is positive, endoscopic lesions are acceptable for the diagnosis of CMV-GID without biopsy. The accuracy is not affected by HIV infection and leukopenia. Either PCR or the antigenemia assay are valid.

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Key words: Cytomegalovirus; Gastrointestinal disease; Antigenemia assay; Real-time polymerase chain reaction; Human immunodeficiency virus infection

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INTRODUCTION

As the number of patients with immune deficiency has been increasing dramatically in recent years, the number of patients with cytomegalovirus (CMV) disease has also been increasing. CMV gastrointestinal disease (CMV-GID) frequently occurs in immunocompromised patients, particularly among those with human immunodeficiency virus (HIV) infection, transplantation, autoimmune diseases, or secondary immunodeficiency^[1-8]. CMV-GID has also been described following the use of steroids, immunosuppressants, or cancer chemotherapy^[1,2]. In immunocompromised patients, CMV-GID in the absence of therapy is a major cause of morbidity and mortality due to events such as massive bleeding or perforation. Therefore, diagnosis at an early stage is essential^[1,2,9-12]. However, diagnosis of this infection is difficult because of wide variations in symptoms and endoscopic features depending on the infected organs^[1,2].

Although the utility of various diagnostic tests for

CMV-GID has been reported, the best approach is to conform the presence of CMV by histological analysis, including immunological staining by endoscopy^[1-3,5,13,14]. Endoscopic examination is generally tolerated, but tissue biopsy can possibly lead to hemorrhage or perforation after endoscopic examination^[10,11,15]. Endoscopists therefore hesitate to perform biopsy when deep, large, and bleeding ulcerous lesions are encountered. Patients receiving anti-thrombotic drugs or with thrombocytopenia also require careful consideration before biopsy.

On many occasions in recent years, noninvasive methods such as the CMV blood antigenemia assay have been applied instead of biopsy to avoid adverse effects^[3,16-22]. However, few reports have examined the diagnostic value of the CMV antigenemia assay for CMV-GID, and the clinical utility of this method in immunodeficiency remains unclear^[3,20-22]. Moreover, the CMV antigenemia assay requires sufficient granulocytes, and leukopenia and low CD4+ counts in patients with HIV infection could thus be expected to influence assay accuracy^[3]. However, no reports have yet clarified this issue.

The aims of this study were to clarify the utility of the CMV antigenemia assay for diagnosing suspected CMV-GID, and to evaluate the accuracy of this assay under different clinical settings.

MATERIALS AND METHODS

Patient selection

One hundred and thirty immunocompromised patients with endoscopic findings who had undergone biopsy were enrolled in this study at the National Center for Global Health and Medicine (NCGM) from January 2002 to September 2009. Patients with a history of treatment with anti-CMV therapy were excluded, as were cases not examined using the CMV antigenemia assay test within 1 wk of endoscopy. Written informed consent was obtained from all patients prior to endoscopy and biopsy. All study protocols were approved by the ethics committee of NCGM.

Immunocompromised patients

Immunocompromised patients are associated with secondary immune deficiency, particularly HIV infection, hematopoietic stem cell transplantation, autoimmune diseases, malignancy, disorders of biochemical homeostasis, and use of steroids, immunosuppressants, or cancer chemotherapy.

Underlying autoimmune diseases included Rheumatoid arthritis, Systemic lupus erythematosus, Still's disease, Behcet's disease, Polymyositis, and Dermatomyositis. Diabetes mellitus, renal insufficiency/dialysis, and hepatic cirrhosis were included among the disorders of biochemical homeostasis. All patients were checked with an HIV antibody test before endoscopic examination.

Clinical manifestations

Gastrointestinal symptoms were collected from medical records written by the doctor who interviewed each per-

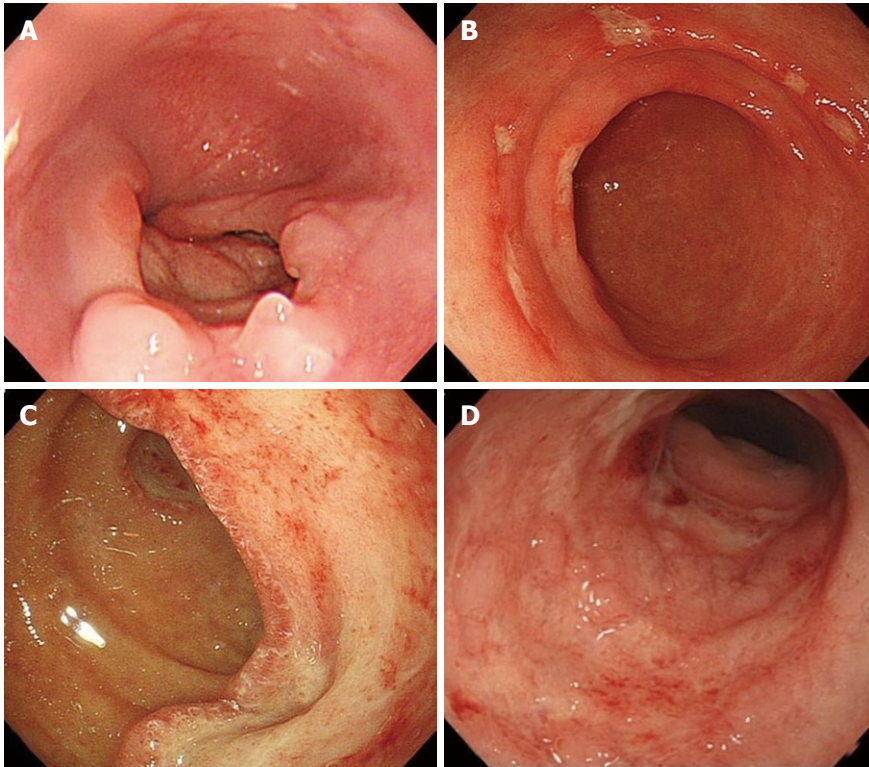


Figure 1 Endoscopic features in cytomegalovirus gastrointestinal disease. A: Deep, punched-out ulcer in the esophagus; B: Multiple, shallow ulcers in the gastric antrum; C: Large, deep ulcer in the duodenum; D: Multiple erosions and edematous mucosa with ulcer in the sigmoid colon.

son face-to-face before endoscopy. Those without records were treated as symptom free. Gastrointestinal symptoms included compromised odynophagia, epigastralgia, nausea, lower abdominal pain, diarrhea, and hematochezia. White blood cell (WBC) counts were obtained from medical records within 1 wk of endoscopy. Leukopenia was defined as a total WBC count < 5000 cells/mm³. For HIV patients, we also checked CD4+ counts from medical records.

Antigenemia assay and quantitative real-time polymerase chain reaction

Antigenemia assay using C10/C11 monoclonal antibodies (Mitsubishi Chemical Medience, Tokyo, Japan) was performed as previously reported^[16,19,20]. A positive result for the CMV antigenemia assay was defined as ≥ 1 CMV-positive cell per 150 000 granulocytes applied.

A total of 47 patients underwent additional examination with real-time polymerase chain reaction (PCR), performed basically as previously reported^[3,23,24]. The minimum detection level was 200 copies/mL of plasma. A positive result for real-time CMV PCR was defined as > 200 copies/mL.

Diagnosis of CMV-GID

CMV-GID was suspected based on endoscopic findings, such as patchy erythema, edematous mucosa, multiple erosions, and ulcers (Figure 1)^[25,26]. Biopsy was therefore performed when such endoscopic findings were encountered. CMV-GID was defined as the detection of large

cells with intranuclear inclusions alone or associated with granular cytoplasmic inclusions by histological testing of biopsy specimens^[1]. Biopsy sections were stained with hematoxylin and eosin, and immunohistochemically stained with anti-CMV (Figure 2). The results were considered positive when the above-mentioned cells showed marked brown coloration in both nuclei and cytoplasm.

Statistical analysis

We divided patients into two groups based on the presence or absence of CMV-GID. Patient characteristics and clinical findings were then compared between groups. Fisher's exact test was used to compare frequencies for patient characteristics and clinical findings, and Mann-Whitney *U* test was used for comparing age and CD4 counts. To identify clinical factors independently associated with a diagnosis of CMV-GID, stepwise logistic regression modeling was used. Sensitivity, specificity, and positive and negative predictive values of CMV antigenemia for diagnosing CMV-GID were calculated. The difference in accuracy between CMV real-time PCR and CMV antigenemia assay was compared according to the area under the curve (AUC). Values of $P < 0.05$ were considered significant. All statistical analyses were performed using Stata software (version 10, Stata Co., USA).

RESULTS

Clinical features

We excluded 10 patients who had received anti-CMV

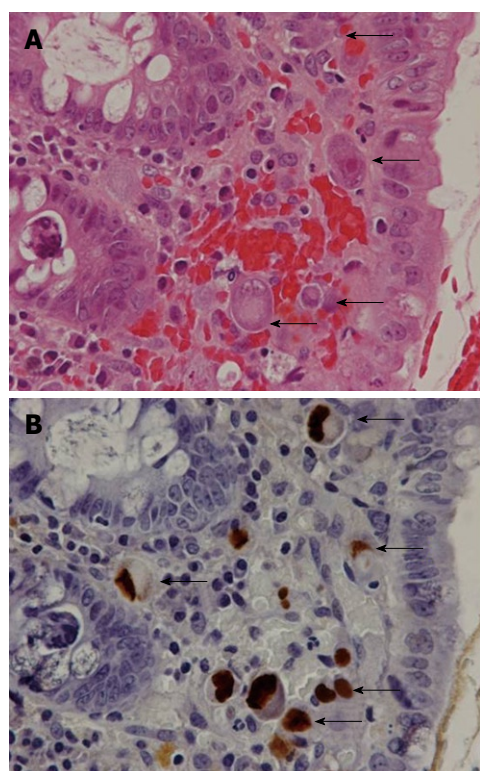


Figure 2 Pathological features in cytomegalovirus gastrointestinal disease. A: Large cells with intranuclear inclusions or associated with granular cytoplasmic inclusions (hematoxylin and eosin stain); B: Cytomegalovirus (CMV)-infected cells (arrows) show brown coloration in both nuclei and cytoplasm (immunohistochemical staining with anti-CMV).

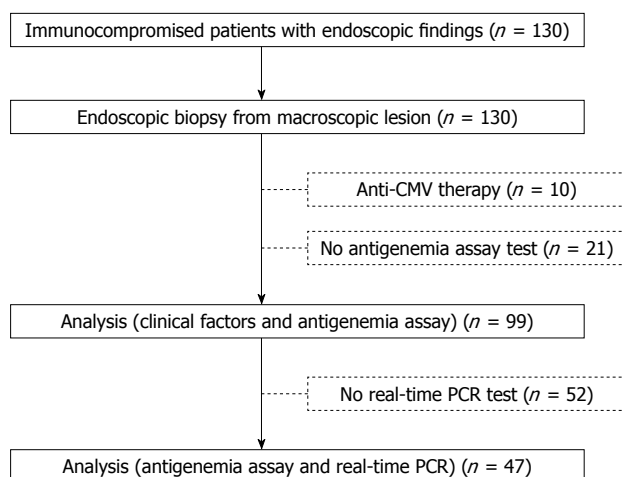


Figure 3 Study design. CMV: Cytomegalovirus; PCR: Polymerase chain reaction.

treatment, along with 21 patients who had not been examined using the CMV antigenemia assay. Thus, a total of 99 patients were retrospectively selected for analysis (Figure 3). Of the immunocompromised patients, 19 (19.1%) had malignant disease, 18 (18.1%) had autoimmune disease, 19 (19.1%) had disorders of biochemical homeostasis, three (3%) had undergone transplantation, and 45 (45.5%) had HIV infection. A total of 50 patients (50.1%) had received immunosuppressive therapy. No

Table 1 Clinical factors for cytomegalovirus gastrointestinal disease (univariate analysis)

	CMV-GID (n = 52)	Non-CMV-GID (n = 47)	P-value
Age (yr, mean \pm SD)	46.8 \pm 16.2	56.6 \pm 17.8	0.050
Male sex	30	41	0.098
Immunodeficiency disease			
HIV infection	33	12	< 0.001
Malignancy	9	10	0.617
Solid cancer	1	3	
Hematological cancer	8	7	
Autoimmune disease	7	11	0.200
Disorders of biochemical homeostasis	8	11	0.312
Chronic renal failure	1	2	
Liver cirrhosis	0	2	
Diabetes mellitus	7	7	
Transplantation	1	2	
Immunosuppressive therapy	25	25	0.611
Steroids	22	19	
Immunosuppressants	8	4	
Chemotherapy	4	4	
Positive CMV antigenemia	34	3	< 0.001
Leukopenia	35	21	0.023
With gastrointestinal symptoms	34	34	0.456

HIV: Human immunodeficiency virus; CMV: Cytomegalovirus; GID: Gastrointestinal disease.

patients had inflammatory bowel disease (IBD). Fifty-five patients were histologically diagnosed with CMV-GID. Univariate analysis (Table 1) identified HIV infection ($P < 0.001$), leukopenia ($P = 0.023$), and positive CMV antigenemia assay ($P < 0.001$) as being associated with CMV-GID. Multivariate analysis revealed HIV infection [odds ratio (OR), 6.57; 95% CI: 2.1-20.2, $P = 0.001$] and positive CMV antigenemia assay (OR, 33.3; 95% CI: 8.1-136.2, $P < 0.001$) as the only factors independently correlated with CMV-GID.

HIV-infected patients included 44 men (97.8%) and their mean age was 42.1 years (range, 25-74 years). Median CD4 count was 57 (interquartile range, 17-111). Patients with CMV-GID showed significantly lower CD4 counts than those without CMV-GID (median CD4 count; CMV-GID *vs* non-CMV-GID: 24 *vs* 150, $P < 0.001$).

Accuracy of CMV antigenemia assay for diagnosing CMV-GID

A positive CMV antigenemia assay showed low sensitivity and high specificity (Table 2). In a subgroup analysis, patients with leukopenia displayed low sensitivity and high specificity. Minimal differences in accuracy were seen among patients with or without leukopenia. HIV-infected patients displayed low sensitivity and high specificity. Accuracy barely differed between HIV-positive and -negative patients. In HIV-infected patients, CD4 count < 50 cells/ μ L resulted in low sensitivity and high specificity. Differences in accuracy among patients were minor, regardless of CD4 count.

In patients who had undergone both quantitative real-

Table 2 Diagnostic accuracy of cytomegalovirus antigenemia for detecting cytomegalovirus gastrointestinal disease

Subgroups	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
All patients (<i>n</i> = 99)	65.40% (55.4-74.9)	93.60% (87.3-97.7)	91.90% (84.7-96.4)	71.00% (60.7-79.4)
Patients with leukopenia (<i>n</i> = 56)	68.60% (54.0-79.7)	100% (93.6-100)	100% (93.6-100)	65.60% (52.2-78.2)
Patients without leukopenia (<i>n</i> = 43)	58.80% (42.1-73.0)	88.50% (74.9-96.1)	76.90% (61.4-88.2)	76.70% (61.4-88.2)
HIV-infected patients (<i>n</i> = 45)	63.60% (48.8-78.1)	100% (92.2-100)	100% (92.2-100)	50.00% (35.8-66.3)
Non-HIV-infected patients (<i>n</i> = 54)	68.40% (54.5-80.5)	91.40% (79.7-96.9)	81.30% (68.6-90.7)	84.20% (70.7-92.1)
HIV-infected patients with CD4 count < 50 (<i>n</i> = 22)	61.90% (40.7-82.8)	100% (84.6-100)	100% (84.6-100)	11.10% (1.12-29.2)
HIV-infected patients with CD4 count ≥ 50 (<i>n</i> = 23)	66.70% (42.7-83.6)	100% (85.2-100)	100% (85.2-100)	73.30% (51.6-89.8)

HIV: Human immunodeficiency virus; PPV: Positive predictive value; NPV: Negative predictive value.

Table 3 Comparison of diagnostic accuracy for detecting cytomegalovirus gastrointestinal disease between antigenemia assay and quantitative real-time polymerase chain reaction (*n* = 47)

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
CMV real-time PCR	73.00% (57.4-84.4)	100% (92.5-100)	100% (92.5-100)	50.00% (36.1-65.9)
CMV antigenemia assay	64.90% (50.7-79.1)	100% (92.5-100)	100% (92.5-100)	43.50% (28.3-57.8)

CMV: Cytomegalovirus; PPV: Positive predictive value; NPV: Negative predictive value; PCR: Polymerase chain reaction.

time PCR and antigenemia assay (Table 3), real-time PCR was slightly more accurate in terms of sensitivity than the antigenemia assay; however, this difference was not statistically significant ($P = 0.312$).

DISCUSSION

CMV-GID is a major cause of morbidity and mortality in immunocompromised patients; therefore, diagnosis at an early stage is essential^[1,2,5,8,9]. However, clinical diagnosis of this disease can be difficult, as physicians need to consider various underlying diseases and clinical presentations. Patients at high risk of CMV-GID have been reported as those with HIV infection or undergoing steroid therapy or cancer therapy^[1]. The present study identified HIV infection as one of the independent factors in secondary immunodeficiency diseases. This is because the number of eligible subjects was small and included immunocompromised patients while excluding immunocompetent patients.

Among the various clinical manifestations, a positive CMV antigenemia assay was found to be a useful factor for diagnosing CMV-GID. The CMV antigenemia assay is one of the most widely used methods for detecting reactivation of CMV infection, but few studies have examined the diagnostic value for CMV-GID^[3,21,22]. Our findings demonstrated 65% sensitivity and 94% specificity of the CMV antigenemia assay for diagnosing CMV-GID. Mori *et al*^[3] reported that only four of 19 patients (21%) developed a positive CMV antigenemia assay before developing CMV-GID; however, all 19 patients subsequently tested positive for CMV antigenemia after diagnosis of CMV-GID. There is a possibility that patients with CMV-GID will develop a positive CMV antigenemia assay at follow-up, but our study did not assess this process after diagnosis of CMV-GID. Fica *et al*^[21] also reported that the CMV antigenemia assay result was positive for 18 of 31

patients (58%) with CMV end-organ disease, with CMV-GID (71%) as the most frequent cause. However, these studies were limited in that the number of subjects was small and the specificity of the CMV antigenemia assay was unknown. Jang *et al*^[22] recently reported that the sensitivity and specificity of the CMV antigenemia assay for diagnosing CMV-GID were 54% and 88%, respectively, in patients with secondary immunodeficiency disease. The reports mentioned above showed that the CMV antigenemia assay has low sensitivity for the diagnosis of CMV-GID, which is consistent with our results.

It has been reported that sufficient granulocytes are essential in evaluating CMV using the antigenemia assay. Previous studies using the antigenemia assay to diagnose CMV-GID have reported that most of the patients were transplant recipients and were mostly HIV-negative^[3,21,22]. No studies have compared the assay among groups of HIV-positive/-negative patients and among groups with or without leukopenia. In patients with HIV infection, most cases of CMV-GID have known to occur with CD4 counts < 50 cells/ μL ^[2,4]. However, whether the accuracy of the antigenemia assay is affected by the immunosuppressed state has not been elucidated. We suspected that such different groups would show differences in the accuracy of CMV antigenemia assay, but found little difference. This suggests that our results are applicable to these different groups in clinical practice.

Besides the CMV antigenemia assay, quantitative real-time PCR is also used for detecting reactivation of CMV infection, and is considered more useful for predicting CMV disease than the CMV antigenemia assay^[23,24]. In our study, quantitative real-time PCR and CMV antigenemia assay were performed simultaneously on 47 patients. The PCR method showed a tendency toward slightly higher sensitivity, but no significant differences were evident. In Japan, the CMV PCR method has not been widely used in

clinical practice because of the higher costs compared to the antigenemia assay. We thus do not recommend use of PCR methods in the sub-diagnosis of CMV-GID, as the antigenemia assay is just as valid.

One limitation of this study was the single-center, retrospective nature of the investigation. A significant difference might not have been confirmed among independent factors due to the small number of patients. Further studies of more patients are needed. Another limitation is the verification bias, which is dependent on the physician's decision to perform the antigenemia assay.

The diagnosis of CMV-GID is considered as the gold standard for identifying CMV cells in tissue samples from endoscopic biopsy^[1,2,13]. Various endoscopic findings are present in CMV-GID, such as ulcer and mucosal inflammation^[25,26]; however, physicians may not perform a biopsy in cases only showing mucosal inflammation without ulcer. Even in cases of severe ulceration that is deep or bleeding, physicians may hesitate to perform a biopsy. In such cases, a diagnosis of CMV-GID may not be reached. Our results suggest that the CMV antigenemia assay is useful for the sub-diagnosis of CMV-GID in immunocompromised patients with endoscopic findings. Considering the high specificity of the test, the use of this method before endoscopy could potentially avoid complications due to biopsy. Positive antigenemia is also useful for evaluating improvements in CMV-GID after anti-CMV treatment. However, the low sensitivity means that if the antigenemia assay yields negative results, biopsy and immunohistochemical staining of specimens with anti-CMV will be required for diagnosis. Negative antigenemia assay results may require a repeat examination at a different time^[3]. Moreover, the use of different non-invasive methods such as quantitative PCR should be considered.

In conclusion, the CMV antigenemia assay is highly useful for diagnosing CMV-GID. If the antigenemia assay provides positive results, the presence of endoscopic lesions should allow diagnosis of CMV-GID without biopsy. The accuracy of the test is unaffected by the presence of HIV infection or leukopenia.

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COMMENTS

Background

Cytomegalovirus (CMV) gastrointestinal disease (GID) is a major cause of morbidity and mortality in immunocompromised patients; therefore, diagnosis at an early stage is essential. However, clinical diagnosis of this disease can be difficult, as physicians need to consider various underlying diseases and clinical presentations.

Research frontiers

The diagnosis of CMV-GID requires an endoscopic biopsy, which is invasive and may lead to complications. While the CMV antigenemia assay is one of the

most widely used methods for detecting reactivation of CMV infection, few studies have examined its diagnostic value for CMV-GID. In this study, the authors demonstrate that the CMV antigenemia assay was highly useful for diagnosing CMV-GID.

Innovations and breakthroughs

There were no studies of diagnosis on CMV-GID related factors using multivariate analysis. In this study, among the various clinical manifestations, human immunodeficiency virus (HIV) infection and positive CMV antigenemia assay were found to be a useful factors for diagnosing CMV-GID by multivariate analysis. As for accuracy of CMV antigenemia for diagnosing CMV-GID, recent reports have highlighted that the sensitivity and specificity were 54% and 88%, respectively, in patients with secondary immunodeficiency disease. However, no studies have compared the assay among groups of HIV-positive/-negative patients and among groups with or without leukopenia. In this study, the sensitivity, specificity, positive predictive value, and negative predictive value of antigenemia for CMV-GID were 65.4%, 93.6%, 91.9%, and 71.0%, respectively. In addition, its accuracy was not affected by the presence of HIV infection and leukopenia. These results are very useful for diagnosing CMV-GID by clinical physicians.

Applications

Considering the high specificity of the test, use of this method before endoscopy could potentially avoid complications due to biopsy. However, the low sensitivity means that if the antigenemia assay yields negative results, biopsy and immunohistochemical staining of specimens with anti-CMV will be required for diagnosis. Negative antigenemia assay results may require repeat examination at a different time. Moreover, the use of different non-invasive methods such as quantitative polymerase chain reaction should be considered.

Peer review

This paper is interesting and it could be valuable for other researchers.

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Strong expression of CD133 is associated with increased cholangiocarcinoma progression

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However, the CD133⁺ cells had a higher invasive ability compared with CD133⁻ cells.

CONCLUSION: CD133⁺ cells play an important role in the invasiveness of cholangiocarcinoma. Targeting of the CD133⁺ cells may be a useful approach to improve treatment against cholangiocarcinoma.

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Key words: CD133; Cholangiocarcinoma; Immunohistochemistry; Invasion; Metastasis

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Leelawat K, Thongtawee T, Narong S, Subwongcharoen S, Treepongkaruna S. Strong expression of CD133 is associated with increased cholangiocarcinoma progression. *World J Gastroenterol* 2011; 17(9): 1192-1198 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i9/1192.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i9.1192>

Abstract

AIM: To determine the role of CD133 in cholangiocarcinoma progression.

METHODS: CD133 protein expression was evaluated by immunohistochemistry in 34 cholangiocarcinoma specimens. In addition, proliferation, chemoresistance and invasive properties of CD133-enriched (CD133⁺) and CD133-depleted (CD133⁻) RMCCA1 cholangiocarcinoma cells were studied and compared.

RESULTS: Strong CD133 expression was observed in 67.6% (23/34) of the cholangiocarcinoma specimens. Strong expression of CD133 was significantly associated with nodal metastasis ($P = 0.009$) and positive surgical margin status ($P = 0.011$). In the *in vitro* study, both the CD133⁺ and CD133⁻ cells had similar proliferation abilities and resistance to chemotherapeutic drugs.

INTRODUCTION

Cholangiocarcinoma is known as one of the most aggressive malignant tumors associated with local invasiveness and a high rate of metastasis. It is also known to be one of the most common causes of cancer death in Thailand^[1]. Three-year survival rates of 35% to 50% are achieved only in a subset of patients who have negative histological margins at the time of surgery^[2-4]. Palliative therapeutic approaches, consisting of percutaneous and endoscopic biliary drainage, have usually been used for these patients because there is no effective chemo-

therapeutic treatment for this type of cancer. Therefore, identification of the molecules involved in cholangiocarcinoma cell progression is crucial for the development of novel drug treatments for this disease.

CD133, a human homologue of a mouse Prominin-1, is a 5-transmembrane cell-surface glycoprotein^[5]. It has been detected in an enrichment of hematopoietic stem cells derived from fetal liver or bone marrow neuroepithelial cells, embryonic epithelial cells and adult immature epithelial cells^[5]. Furthermore, CD133 has been successfully used for the identification of cancer stem cell (CSC) niches in glioblastoma^[6], colon cancer^[7] and other solid carcinomas^[8]. Recently, CD133 was identified in many kinds of cancer specimens, including hepatocellular carcinoma^[9] and colon cancer^[10], and was associated with poor prognoses. To understand the roles of CD133 in cholangiocarcinoma cells, the characteristics of CD133⁺ and CD133⁻ cholangiocarcinoma cells must be investigated; such studies have not been carried out with CD133⁺ cholangiocarcinoma cells. In the present study, we investigated the clinicopathological significance of CD133 expression in human cholangiocarcinoma specimens. In addition, we studied the cell proliferation, chemoresistance and invasiveness of CD133⁺ and CD133⁻ cholangiocarcinoma cells from the RMCCA1 cholangiocarcinoma cell line.

MATERIALS AND METHODS

Human cholangiocarcinoma tissue samples

The cholangiocarcinoma tissue samples analyzed in this study were obtained from cholangiocarcinoma patients who underwent a surgical resection at Rajavithi Hospital in Bangkok, Thailand from 2008 to 2010. The study was approved by the ethics committee of Rajavithi Hospital.

Immunohistochemical staining

Paraffin wax sections of cholangiocarcinoma specimens were dewaxed in xylene, and transferred to alcohol. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol, and the sections were boiled in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven (750 W) for antigen retrieval. Nonspecific binding was blocked by incubating with 3% normal horse serum for 20 min. Sections were incubated overnight at 4°C with a 1/1000 dilution of mouse monoclonal antibody for CD133. Biotinylated rabbit anti-mouse IgM (Dako, Glostrup, Denmark) was applied to the sections, followed by an avidin-biotin-peroxidase conjugate (ABC Elite; Vector Laboratories, Burlingame, California, USA) for 30 min at room temperature. The immunohistochemical reaction was developed with freshly prepared reagents from a Histofine streptavidin-biotin complex peroxidase (SAB-PO) kit (Nichirei Inc., Tokyo, Japan). The immunohistochemical reactions were then visualized under high power magnification ($\times 400$) using an Olympus BH2 microscope (field width, 0.5 mm) and scored into 3 categories based on the percentage of positively stained cells, as follows: (1) negative, $< 5\%$; (2) weak, $5\%-50\%$; and (3) strong $> 50\%$.

Cell culture and materials

The human cholangiocarcinoma RMCCA1 cells^[11] were routinely grown in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO₂ humidified atmosphere. For experiments, cells were grown in Ham's F12 medium supplemented with 1% FBS.

Enrichment of CD133⁺ cholangiocarcinoma cells

Cells were harvested by treatment with 0.25% trypsin (Gibco, LA, USA) in the logarithmic phase of growth followed by centrifugation at 300 g for 5 min. The cells were resuspended in 100 μ L buffer (phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin, 2 mmol/L ethylenediaminetetraacetic acid). Single cells were magnetically labeled with anti-CD133 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 4°C for 30 min and applied to a prepared MS Column (Miltenyi Biotec, Bergisch Gladbach, Germany). CD133⁻ cells were collected in the flow-through of the column; CD133⁺ cells bound to the beads were flushed out by applying the plunger supplied with the column. The percentage of CD133-expressing cells in the original cell populations, the flow-through and the flushed-out fractions was analyzed by fluorescence-activated cell sorting (FACS) with fluorescein isothiocyanate (FITC)-conjugated anti-CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained at a concentration of 1×10^6 cells per 90 μ L buffer and 10 μ L antibody at 4°C for 25 min before FACS analysis.

Cell proliferation assay

For proliferation assays, cells were seeded into 96-well culture plates at a density of 10000 cells per well. For cancer chemoresistance studies, cells were treated with vehicle (PBS) or 5-50 μ mol/L cisplatin. Cells were then incubated for 48 hours before applying the water soluble tetrazolium salts (WST)-1 cell proliferation assay reagent (Roche Diagnostics, Laval, Quebec, Canada) according to the manufacturer's recommendations. The degree of cell proliferation was assessed by determining the $A_{450\text{ nm}}$ of the cell culture medium after addition of WST-1 for 2 h. Results are reported as the percentage of inhibition of cell proliferation, where the optical density measured for vehicle-treated cells was considered to represent 100% proliferation.

Cell invasion assay

The invasiveness of cholangiocarcinoma cells was assayed in a 24-well Biocoat Matrigel invasion chamber (8 μ m; Becton Dickinson, Bioscience, Bedford, MA, USA). There were 50000 cells seeded in the upper chamber. The bottom chamber contained 10% FBS. After 24 h of incubation, the invading cells at the lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with crystal violet and counted in 5 random 200 \times power fields under a light microscope.

Statistical analysis

Statistical analysis of the association between clinicopatho-

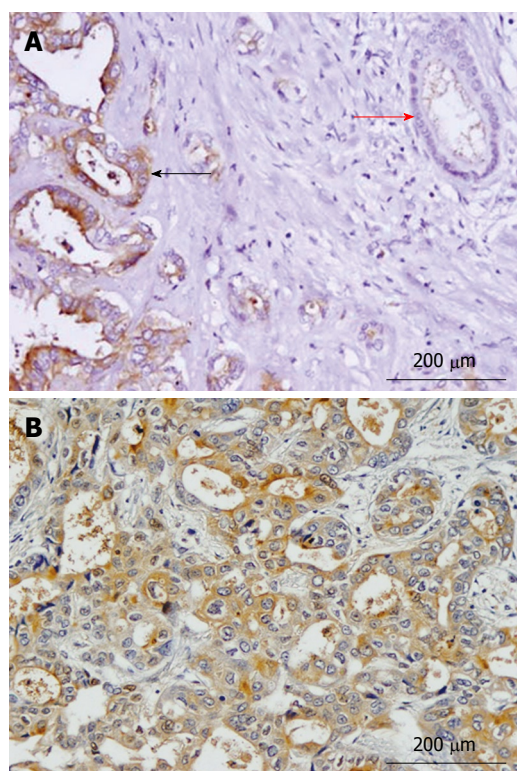


Figure 1 Representative immunohistochemical staining for CD133 in cholangiocarcinoma specimens. A: Normal bile duct cells (red arrow) demonstrated negative staining, whereas cholangiocarcinoma cells had strong cytoplasmic staining (black arrow); B: Moderately differentiated cholangiocarcinoma with overexpression of CD133. Positive staining was observed in the cytoplasm of cholangiocarcinoma cells (200 × magnification).

logical findings and the expression of CD133 was performed by means of the χ^2 test or Fisher's exact test. The Kaplan-Meier method was used to estimate survival as a function of time, and the survival differences were analyzed by log-rank test. The Cox regression model was used for multivariate analysis of prognostic factors. The experiments in the cell proliferation and invasiveness assays were all performed in triplicate, and each result is reported as the mean with standard deviation. Data were compared using the Student *t*-test. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Expression of CD133 in paraffin-embedded cholangiocarcinoma samples

Among the 34 cholangiocarcinoma specimens, 17 specimens were derived from the patients who received extended right hepatectomy, 7 specimens were derived from the patients who received left hepatectomy and 10 specimens were derived from the tissue biopsy of unresectable cholangiocarcinoma patients. Expression of CD133 was detected by immunohistochemistry in these 34 paraffin-embedded cholangiocarcinoma specimens. In non-cancerous bile duct tissues, the immunohistochemical signal for CD133 was negative. In cancerous tissues, specific CD133 signals were localized mainly in the cell membrane and cy-

Table 1 Relation between clinicopathological features and immunohistochemical staining of CD133 in cholangiocarcinoma specimens

Variable	Total (<i>n</i> = 34)	CD133 expression		<i>P</i> -value
		Weak	Strong	
Age				
< 60	18	7	11	0.477
> 60	16	4	12	
Sex				
Male	22	8	14	0.705
Female	12	3	9	
Tumor differentiation				
Well	17	7	10	0.465
Moderate/poor	17	4	13	
Node				
Negative	16	9	7	0.009 ¹
Positive	18	2	16	
Distant metastasis				
Negative	24	10	14	0.113
Positive	10	1	9	
Surgical resection margin ²				
R0	20	10	10	0.011 ¹
R1, R2	14	1	13	
Location of tumor				
Perihilar	12	3	9	0.705
Intrahepatic	22	8	14	
Neurovascular invasion ³				
Positive	15	8	7	0.210
Negative	9	2	7	

¹Statistically significant; ²Surgical resection margin: R0 = negative resection margin, R1 = microscopic positive resection margin and R2 = macroscopic positive resection margin; ³To study the presence of neurovascular invasion, we excluded 10 specimens derived from tissue biopsy.

toplasm of cholangiocarcinoma cells (Figure 1). We found that all cholangiocarcinoma specimens (34/34) exhibited signals indicating CD133 expression (> 5% of cell staining). Applying the criteria for intensity of immunohistochemical staining for CD133, strong expression (> 50% of cell staining) of CD133 was noted in 67.6% (23/34) of cholangiocarcinoma specimens. In addition, we demonstrated that 75% (9/12) of perihilar and 63.6% (14/22) of intrahepatic cholangiocarcinoma samples strongly expressed CD133.

When comparing clinicopathological variables, nodal metastasis (*P* = 0.009) and positive surgical margin status (*P* = 0.011) were more frequent in the CD133 strong expression group compared with the CD133 weak expression group (Table 1).

CD133 expression and patient survival

Univariate analysis revealed that the median survival time was 11 mo in patients with strong CD133 expression and 14 mo in patients with weak expression (*P* = 0.23). We also found that the median survival time in patients with a negative surgical margin was significantly better than in patients with a positive surgical margin (15 mo *vs* 8 mo, *P* = 0.009) (Figure 2). In a multivariate Cox regression analysis, only surgical margin status was indicated as an independent risk factor for survival (*P* = 0.04, Table 2).

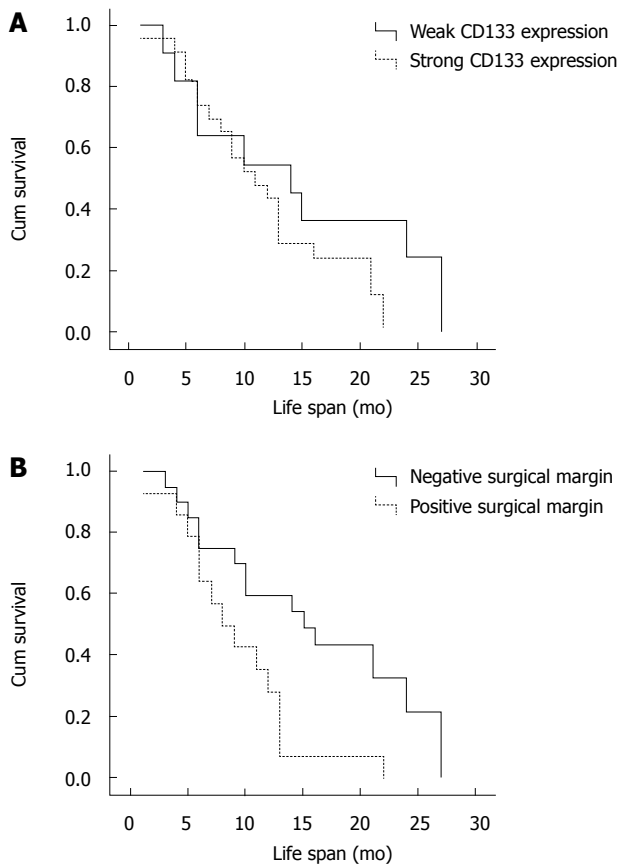


Figure 2 Kaplan-Meier cumulative overall survival curves. A: Survival curves of patients with strong and weak CD133 expression ($P = 0.23$); B: Survival curves of patients with positive and negative surgical margin status ($P = 0.009$).

Enrichment of CD133⁺ cholangiocarcinoma cells

To study the characteristics of CD133⁺ cholangiocarcinoma cells, we enriched the CD133⁺ and CD133⁻ cells from RMCCA1 cholangiocarcinoma cell lines using magnetic cell sorting technology. The percentage of CD133⁺ cells in CD133-enriched cells was 91.9%, and the percentage of CD133⁺ cells in the CD133-depleted cells was 11.2% (Figure 3A). As revealed by morphological studies, no cell morphology differences were observed between CD133⁺ and CD133⁻ RMCCA1 cells (Figure 3B).

Proliferation of CD133⁺ and CD133⁻ cholangiocarcinoma cells

Because previous reports demonstrated that CD133⁺ cells have higher proliferative potential than CD133⁻ cells^[12], we investigated the rate of cell proliferation in CD133⁺ and CD133⁻ RMCCA1 cells cultured for 3 days. The results showed that there was no statistically significant difference in cell proliferation between CD133⁺ and CD133⁻ RMCCA1 cells (Figure 4A).

Chemoresistance of CD133⁺ cholangiocarcinoma cells

Previous studies have proposed that CD133⁺ enrichment of cancer cells is a source of chemotherapy resistance^[13]. Therefore, we investigated the role of CD133 in cholangiocarcinoma cell resistance to chemotherapeutic drugs. CD133⁺ and CD133⁻ RMCCA1 cells were treated with

Table 2 Survival analysis on clinicopathological parameters by Cox's multivariate model

Variables	P-value	Hazard ratio	95% CI	
			Lower limit	Upper limit
Tumor differentiation	0.57	0.77	0.32	1.86
Lymph node metastasis	0.81	1.15	0.36	3.66
Distant metastasis	0.97	1.03	0.22	4.53
Neurovascular invasion	0.33	1.48	0.67	3.26
CD133 expression	0.35	0.53	0.14	2.02
Surgical resection status	0.04 ¹	4.09	1.06	15.72

¹Statistically significant; CI: Confidence interval.

0-50 $\mu\text{mol/L}$ cisplatin for 2 d before cell proliferation assays were performed. The percentage of cell proliferation was set to 100% when cells were treated with vehicle (PBS). Both CD133⁺ and CD133⁻ cells displayed similar degrees of resistance to chemotherapeutic drugs. There was no statistically significant difference in cell proliferation between CD133⁺ and CD133⁻ RMCCA1 cells (Figure 4B).

Cell invasiveness of CD133⁺ cholangiocarcinoma cells

Because the expression of CD133 in cholangiocarcinoma specimens is significantly correlated with the lymph node metastatic status, we performed an *in vitro* invasion assay. When the percentage of CD133⁺ RMCCA1 cell invasion was set at 100%, the percentage of CD133⁻ RMCCA1 cell invasion was $58.3\% \pm 19.91\%$. CD133⁺ RMCCA1 cells showed significantly higher invasive ability than CD133⁻ RMCCA1 cells ($P < 0.001$, Figure 5).

DISCUSSION

In this study, we performed immunohistochemical staining to detect the expression of CD133 in cholangiocarcinoma specimens. We demonstrated that all of the cholangiocarcinoma specimens expressed CD133, and 67.6% of the cholangiocarcinoma specimens demonstrated strong expression of CD133. In addition, strong expression of CD133 was significantly associated with lymph node metastasis and surgical margin status. It is well known that lymph node metastasis and surgical margin status are the major prognostic factors for cholangiocarcinoma^[3,4]. These findings are consistent with a previous study, which demonstrated that cholangiocarcinoma that highly expressed CD133 tended to be related to higher incidences of metastasis and to worse prognoses^[14]. In this study, the multivariate Cox regression analysis demonstrated that surgical margin status is an independent prognostic predictor in our patients. Although we found that patients with high expression of CD133 had a lower median survival time than patients with low expression of CD133 in cholangiocarcinoma specimens, this did not reach the statistical difference. We suggest that this could be attributable to the small sample size of our study. A further study which includes a larger cohort of cholangiocarcinoma patients should be performed before using CD133 as a

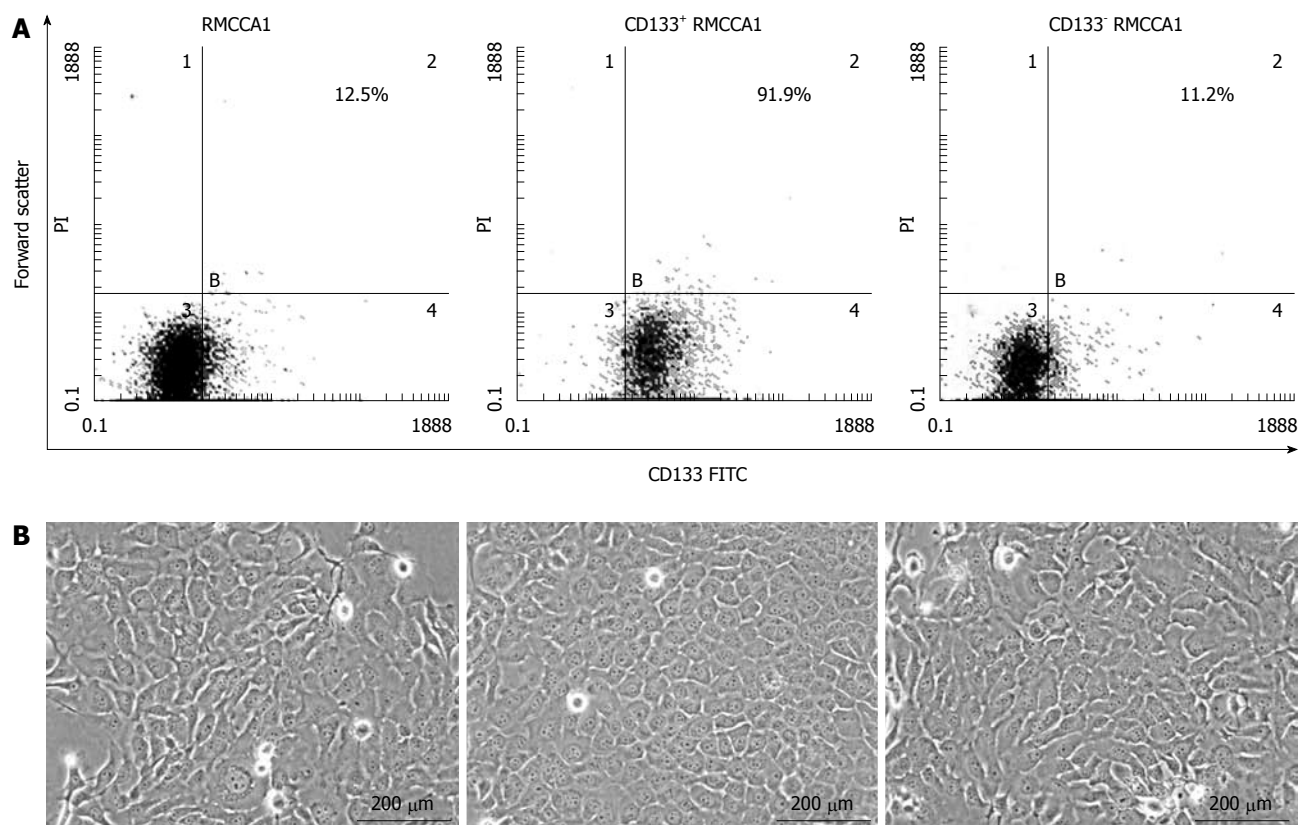


Figure 3 Isolation of CD133⁺ and CD133⁻ cholangiocarcinoma cells. A: The percentage of CD133⁺ expressing cells in the original RMCCA1 cell populations, the flushed-out fractions (CD133⁺ RMCCA1 cells) and the flow-through (CD133⁻ RMCCA1 cells) were analyzed by fluorescence-activated cell sorting; B: The morphology of original, CD133⁺ and CD133⁻ RMCCA1 cells is demonstrated under a phase contrast microscope at 200 × magnification. FITC: Fluorescein isothiocyanate.

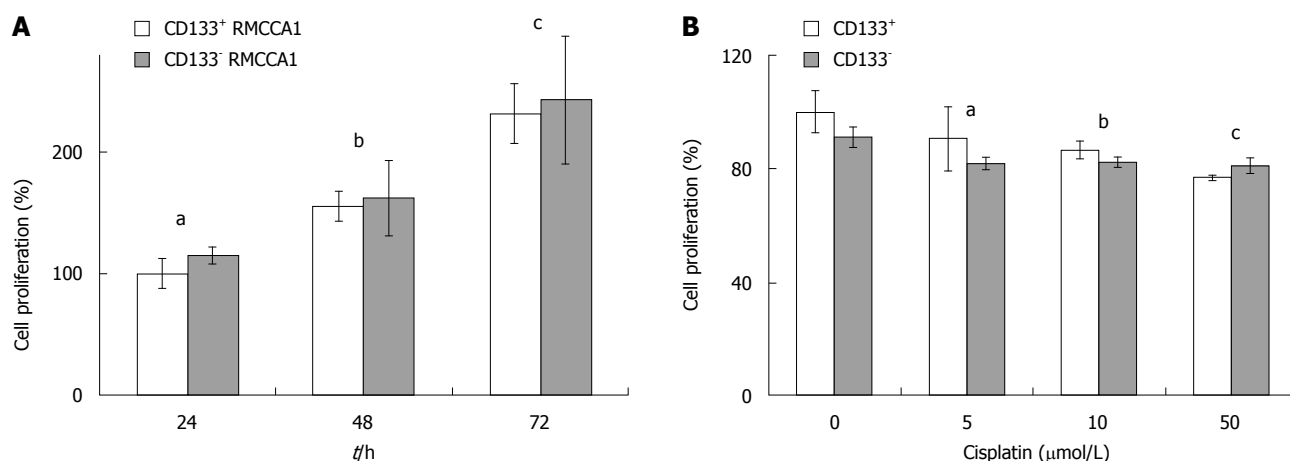


Figure 4 Cell proliferation assays of CD133⁺ and CD133⁻ RMCCA1 cells. A: The proliferation rates of CD133⁺ and CD133⁻ cholangiocarcinoma cells. CD133⁺ and CD133⁻ RMCCA1 cells were cultured for 24–72 h. A cell proliferation assay was performed using WST-1. Results are reported as a percentage of cell proliferation, where the optical density values from CD133⁺ cells cultured for 24 h are set as 100% of proliferation. Data is represented as the mean ± SD of 3 independent experiments. (^a*P* = 0.095, ^b*P* = 0.578, ^c*P* = 0.544 vs the same time of culturing); B: Effect of cisplatin on cholangiocarcinoma cells. CD133⁺ and CD133⁻ RMCCA1 cells were treated with cisplatin at various concentrations (0, 5, 10 and 50 μmol/L) for 2 d. Effects on cell proliferation were measured by WST-1. Results are reported as percentage of cell proliferation, where the optical density values from vehicle-treated cells (0 μmol/L cisplatin) are set as 100% proliferation. Data is presented as the mean ± SD of 3 independent experiments. (^a*P* = 0.060, ^b*P* = 0.056, ^c*P* = 0.053 vs the same concentration of cisplatin). WST: Water soluble tetrazolium salts.

prognostic marker for cholangiocarcinoma.

Previous studies have demonstrated that CD133⁺ cells exhibit more aggressive behavior, including increased cell proliferation^[12] and invasive abilities^[15,16]. Here, we studied the characteristics of CD133 in cholangiocarcinoma cell lines using cell proliferation and invasion assays. Our

results suggest that CD133⁺ RMCCA1 cells have higher invasive activity than CD133⁻ RMCCA1 cells. A previous study indicated that C-X-C chemokine receptor type 4 (CXCR4) is markedly expressed in CD133⁺ pancreatic cancer cells and may be responsible for the increased invasive ability of cells cocultured with pancreatic stromal

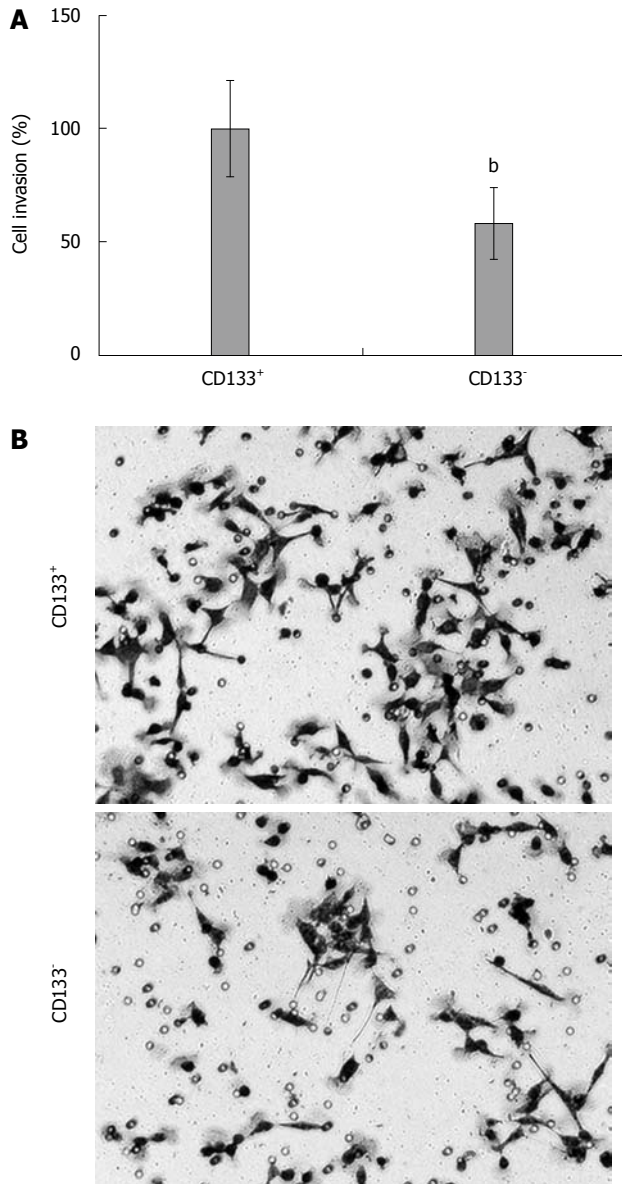


Figure 5 Invasion assays of CD133⁺ and CD133⁻ RMCCA1 cells. A: Invasion assays of CD133⁺ and CD133⁻ RMCCA1 cells were performed using the Biocoat Matrigel invasion chamber. Results are reported as the percentage of cell invasion, where the number of cell invasion from CD133⁺ RMCCA1 cells is set as 100% cell invasion. Data is represented as the mean \pm SD of three independent experiments (^b $P < 0.001$); B: The morphology of CD133⁺ and CD133⁻ RMCCA1 cell invasion is demonstrated under a phase contrast microscope at 200 \times magnification.

cells, which express stromal derived factor-1 (CXCL12), the ligand for CXCR4^[15]. Further studies focusing on the molecular mechanisms that enhance CD133⁺ cholangiocarcinoma cell invasiveness should be performed in the future.

A previous study indicated that enriched CD133⁺ lung cancer cells were resistant to cisplatin treatment^[13]. In contrast, Meng *et al.*^[17] demonstrated that both CD133⁺ and CD133⁻ A549 lung cancer cells exhibited similar degrees of resistance to chemotherapeutic drugs. Our data are consistent with those of Meng *et al.*^[17]: CD133⁺ and CD133⁻ cells exhibited similar cell proliferation abilities and resistance to chemotherapeutic drugs^[18]. We previous-

ly demonstrated that activation of the phosphatidylinositol 3-kinase (PI3K) pathway in cholangiocarcinoma cells protects the cells from cytotoxicity induced by platinum-based chemotherapy. In addition, we also found that there was no difference between Akt and mTOR (mammalian target of rapamycin) activation in CD133⁺ and CD133⁻ cholangiocarcinoma cells (data not shown). These results indicate that the proliferative ability and resistance to chemotherapeutic drugs in cholangiocarcinoma cells are not unique to CD133⁺ cells.

In conclusion, our findings indicate that CD133⁺ cells have higher invasive abilities than CD133⁻ cells. CD133 expression can be found in cholangiocarcinoma, and it significantly correlates with lymph node metastasis and surgical margin status. CD133 may be a potential prognostic factor of cholangiocarcinoma.

COMMENTS

Background

Cholangiocarcinoma is a malignancy arising from the biliary tract. Cholangiocarcinoma is one of the most common causes of cancer death in Thailand. A recent study suggested that expression of CD133 in cholangiocarcinoma specimens is associated with the severity of this disease.

Research frontiers

CD133⁺ cholangiocarcinoma cells have a high ability to invade through Matrigel. In addition, expression of CD133 in cholangiocarcinoma specimens is associated with cholangiocarcinoma progression.

Innovations and breakthroughs

This is believed to be the first report to show that, in cholangiocarcinoma cell lines with high CD133 expression, the neoplastic behavior is more aggressive than in CD133⁻ cells.

Applications

Strong CD133 expression was observed in 67.6% of the cholangiocarcinoma specimens and was significantly associated with nodal metastasis and positive surgical margin status. Therefore, the authors suggest that CD133⁺ cells play an important role in the invasiveness and progression of cholangiocarcinoma. Targeting of the CD133⁺ cholangiocarcinoma cells may be a useful approach to improve therapies directed against cholangiocarcinoma.

Terminology

CD133, a human homologue of a mouse prominin-1, is a 5-transmembrane cell-surface glycoprotein. It has been detected in an enrichment of hematopoietic stem cells derived from fetal liver or bone marrow neuroepithelial cells, embryonic epithelial cells and adult immature epithelial cells.

Peer review

This study provides evidence that high CD133 expression in cholangiocarcinoma cells may be used as a marker for targeted cancer therapy. This was a well performed and clearly presented study.

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Prevalence and risk factors of asymptomatic peptic ulcer disease in Taiwan

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Abstract

AIM: To investigate the prevalence and risk factors of asymptomatic peptic ulcer disease (PUD) in a general Taiwanese population.

METHODS: From January to August 2008, consecutive asymptomatic subjects undergoing a routine health check-up were evaluated by upper gastrointestinal endoscopy. Gastroduodenal mucosal breaks were carefully assessed, and a complete medical history and demographic data were obtained from each patient. Logistic regression analysis was conducted to identify indepen-

dent risk factors for asymptomatic PUD.

RESULTS: Of the 572 asymptomatic subjects, 54 (9.4%) were diagnosed as having PUD. The prevalence of gastric ulcer, duodenal ulcer and both gastric and duodenal ulcers were 4.7%, 3.9%, and 0.9%, respectively. Multivariate analysis revealed that prior history of PUD [odds ratio (OR), 2.0, 95% CI: 1.3-2.9], high body mass index [body mass index (BMI) 25-30: OR, 1.5, 95% CI: 1.0-2.2; BMI > 30 kg/m²: OR, 3.6, 95% CI: 1.5-8.7] and current smoker (OR, 2.6, 95% CI: 1.6-4.4) were independent predictors of asymptomatic PUD. In contrast, high education level was a negative predictor of PUD (years of education 10-12: OR, 0.5, 95% CI: 0.3-0.8; years of education > 12: OR, 0.6, 95% CI: 0.3-0.9).

CONCLUSION: The prevalence of PUD in asymptomatic subjects is 9.4% in Taiwan. Prior history of PUD, low education level, a high BMI and current smoker are independent risk factors for developing asymptomatic PUD.

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Key words: Asymptomatic; Endoscopy; Health check-up; Peptic ulcer disease

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INTRODUCTION

Peptic ulcer disease (PUD) is a common disease of the

digestive system^[1]. The self-reported PUD prevalence among people aged 18 years and older in the United States was 10.3% in 1989^[2], compared with 14% among people aged 20 to 81 years in Hong Kong^[3].

The pathogenesis of symptom development in PUD is unclear. A few studies have revealed that several factors, such as acid, inflammation, or muscle spasm, may be related to the pathogenesis of ulcer pain^[4,5]. A study in Taiwan revealed that 67% of PUD patients had no remarkable symptoms^[6]. Some patients with PUD are asymptomatic until life-threatening complications (e.g. hemorrhage, perforation) develop^[7]. Previous studies found that the majority of patients who died from PUD usually had no ulcer symptoms until the final fatal illness^[8]. Therefore, non-symptom producing ulcers remain a clinical challenge.

Currently, the long-term consequences of silent PUD remain unclear, however, hemorrhage, perforation, and stricture are well known complications of PUD^[9,10]. Therefore, identification and follow up of these asymptomatic patients is essential. Nonetheless, the risk factors for asymptomatic PUD still remain unclear. The aims of this study were to investigate the prevalence and risk factors of asymptomatic PUD in Taiwan.

MATERIALS AND METHODS

Subjects

From January to August 2008, consecutive asymptomatic subjects undergoing a routine endoscopy during a health check-up were invited to participate in this study. The eligible subjects were free of reflux and dyspeptic symptoms such as heartburn, regurgitation, dysphagia, epigastric pain, epigastric fullness, nausea and vomiting in the previous month. Exclusion criteria were (1) use of proton pump inhibitors, histamine-2 receptor antagonists, sucralfate, prostaglandin analogs and antacids within the previous month; (2) use of prokinetic or anticholinergic agents; and (3) serious medical illness.

Study design

A complete history and physical examination were performed for every subject undergoing a health check-up. All subjects were carefully interviewed regarding the presence of reflux or dyspeptic symptoms in the previous month, and subjects who responded negatively were classified as asymptomatic subjects and enrolled in this study. Endoscopies were performed by three experienced endoscopists (Hsu PI, Cheng LC, and Yu HC) using an Olympus GIF XV10 and GIF XQ200 (Olympus Corp., Tokyo, Japan) after the subjects had fasted overnight. The endoscopists were blinded as to whether the patients they were examining were symptomatic or asymptomatic. The patients were carefully examined for gastroduodenal mucosal breaks, and the length of these breaks was measured by opening a pair of biopsy forceps of known span in front of the breaks. Peptic ulcer disease was defined as a circumscribed mucosal break 5 mm or more in diameter, with a well defined ulcer crater^[11].

To assess the relationships between clinical charac-

Table 1 Demographics and endoscopic findings of asymptomatic subjects undergoing a routine health check-up (*n* = 572) *n* (%)

Clinical characteristics	
Age (yr)	
mean \pm SD	51.5 \pm 12.9
< 45	206 (36.0)
45-60	235 (41.1)
> 60	131 (22.9)
Gender	
Men	372 (65.0)
Women	200 (35.0)
Body mass index (kg/m ²)	
mean \pm SD	24.3 \pm 3.5
< 25	321 (55.6)
25-30	225 (38.8)
> 30	26 (4.6)
Endoscopic findings	
Peptic ulcer	54 (9.4)
Gastric ulcer	27 (4.7)
Duodenal ulcer	22 (3.9)
Gastric ulcer and duodenal ulcer	5 (0.9)

teristics and asymptomatic PUD, the following data were recorded for each subject: age; gender; educational status; prior history of PUD; consumption of tobacco, alcohol, coffee, tea, spicy foods or betel nut, and use of non-steroidal anti-inflammatory drugs (NSAIDs) within 4 wk of endoscopy. All variables were categorized for data analyses.

Statistical analysis

The χ^2 test or Fisher's exact test was employed to investigate the relationships between the rate of PUD and clinical characteristics. These variables included the following: gender; age (< 45, 45-60, or > 60 years); educational status (< 10, 10-12, or > 12 years); body mass index [body mass index (BMI): < 25, 25-30, or > 30 kg/m²]; NSAID use (yes or no); history of PUD (yes or no); smoking status (no, former smoker, current smoker); consumption of alcohol, coffee, tea or spicy foods (no, \leq 3 times per week, or > 3 times per week); betel nut habit (yes or no). A *P* value less than 0.05 was considered significant. Significant variables revealed by univariate analysis were subsequently assessed by a stepwise logistic regression method to identify independent clinical factors predicting the presence of PUD in asymptomatic subjects.

RESULTS

Patient demographics and endoscopic characteristics

From January to August 2008, 572 asymptomatic subjects (mean age, 51.5 \pm 12.9 years; age range, 18-87 years; male/female, 372/200) were recruited into this study. Of these patients, 54 (9.4%) had PUD (Table 1). The cases of PUD consisted of 27 gastric ulcer (4.7%), 22 duodenal ulcer (3.9%) and 5 had both gastric ulcer and duodenal ulcer (0.9%).

Comparison of socio-demographic and lifestyle factors in subjects with and without peptic ulcer disease

Table 2 shows the relationships between clinical character-

Table 2 Comparison of socio-demographic and lifestyle factors in asymptomatic subjects with and without peptic ulcer disease *n* (%)

Principal parameter	Non-peptic ulcer	Peptic ulcer	<i>P</i> value
Sex			< 0.001
Men	330 (63.7)	42 (77.8)	
Women	188 (36.3)	12 (22.2)	
Age (yr)			0.082
< 45	199 (38.4)	16 (29.6)	
45-60	211 (40.7)	23 (42.6)	
> 60	108 (20.9)	15 (27.8)	
Education (yr)			0.045
< 10	118 (22.8)	18 (33.3)	
10-12	225 (43.4)	20 (37.0)	
> 12	175 (33.8)	16 (29.7)	
BMI (kg/m ²)			< 0.001
< 25	315 (60.8)	25 (46.3)	
25-30	189 (36.5)	25 (46.3)	
> 30	14 (2.7)	4 (7.4)	
NSAID use			0.628
No	471 (90.9)	50 (92.6)	
Yes	47 (9.1)	4 (7.4)	
Peptic ulcer history			< 0.005
No	370 (71.4)	30 (55.6)	
Yes	148 (28.6)	24 (44.4)	
Smoking status			< 0.001
No	359 (69.3)	27 (50.0)	
Former smoker	75 (14.5)	8 (14.8)	
Current smoker	84 (16.2)	19 (35.2)	
Alcohol drinking			0.137
No	379 (73.2)	36 (66.7)	
≤ 3 times per week	88 (17.0)	13 (24.1)	
> 3 times per week	51 (9.8)	5 (9.2)	
Coffee drinking			0.739
No	299 (57.7)	31 (57.4)	
≤ 3 times per week	100 (19.3)	12 (22.2)	
> 3 times per week	119 (23.0)	11 (20.4)	
Tea drinking			0.209
No	222 (42.9)	19 (35.2)	
≤ 3 times per week	99 (19.1)	11 (20.4)	
> 3 times per week	197 (38.0)	24 (44.4)	
Spicy foods consumption			0.147
No	321 (62.0)	29 (53.7)	
≤ 3 times per week	121 (23.4)	14 (25.9)	
> 3 times per week	76 (14.6)	11 (20.4)	
Betel nut use			0.016
No	506 (97.7)	50 (92.6)	
Yes	12 (2.3)	4 (7.4)	

BMI: Body mass index, indicating weight in kg divided by body surface area; NSAID: Non-steroid anti-inflammatory drug.

istics and the presence of PUD. The subjects with PUD were less educated than those without ulcer. Additionally, male gender, prior history of PUD, current smoker, and betel nut chewing were significantly higher in the PUD group than in the non-PUD group ($P < 0.001$, < 0.005 , < 0.001 , 0.016 , respectively). Furthermore, the PUD group had higher BMI than the non-PUD group. However, the two groups had similar age, alcohol, coffee, tea, and spicy food consumption and NSAID use.

Independent factors influencing the presence of peptic ulcer disease in asymptomatic subjects

Multivariate analysis with stepwise logistic regression

Table 3 Independent risk factors for the presence of peptic ulcer disease in asymptomatic subjects¹

Risk factors	Coefficient	SE	OR (95% CI)	<i>P</i> value
Peptic ulcer history	0.686	0.199	1.99 (1.34-2.93)	0.001
Education (yr)				
10-12	-0.704	0.247	0.49 (0.31-0.80)	0.004
> 12	-0.562	0.265	0.57 (0.34-0.96)	0.034
Current smoker	0.960	0.263	2.61 (1.56-4.38)	< 0.001
BMI (kg/m ²)				
> 25	0.404	0.203	1.49 (1.01-2.23)	0.046
> 30	1.292	0.443	3.64 (1.53-8.68)	0.004

OR: Odds ratio; BMI: Body mass index, indicating weight in kg divided by body surface area. ¹Analysis for sex, education level, BMI, peptic ulcer history, smoking and betel nut consumption; CI: Confidence interval.

showed that history of PUD [odds ratio (OR), 1.99; 95% CI: 1.34-2.93], low education level (years of education 10-12: OR, 0.49; 95% CI: 0.31-0.80; years of education > 12: OR, 0.57; 95% CI: 0.34-0.96), high BMI (BMI 25-30 kg/m²: OR, 1.49; 95% CI: 1.01-2.23; BMI > 30 kg/m²: OR, 3.64; 95% CI: 1.53-8.68) and current smoker (OR, 2.61; 95% CI: 1.56-4.38) were independent factors predicting the development of PUD (Table 3).

DISCUSSION

The clinical presentation of peptic ulcer patients is variable. Some patients with PUD are asymptomatic until life-threatening complications develop. This study demonstrated that the prevalence of asymptomatic PUD in Taiwan was 9.4%. Further analysis showed that prior history of PUD, high BMI, low education level and current smoker were independent predictors for developing asymptomatic PUD.

This study revealed a strong positive association between BMI and asymptomatic PUD. These associations remained robust even after adjusting for several important potential factors, including age, gender and lifestyle. Furthermore, a significant dose-response relationship between BMI and PUD was noted. The odds ratio of BMI 25-30 kg/m² (overweight) and BMI > 30 kg/m² (obesity) were 1.5 and 3.6, respectively. Previous studies indicated that higher BMI, considered as overweight or obese, revealed associations with more severe symptoms of reflux esophagitis^[12,13] and PPI treatment response to achieve sustained symptomatic response in reflux esophagitis^[14]. Little data exist on the association between obesity and PUD. The mechanism by which obesity increases the risk of asymptomatic PUD is unknown. Possible explanations include the increased intra-abdominal pressure and higher acid secretion rates in obese subjects^[15]. Wisén *et al* reported that obese (nondiabetic) patients were found to have higher gastric acid secretion than non-obese patients using the gastric acid secretion test after modified sham feeding^[16]. Moreover, obese subjects appear to be more sensitive to acid in the upper gastrointestinal tract than non-obese subjects^[17]. In addition, studies have demonstrated that obese rats and humans have higher "somatic" pain thresholds because obese patients have a higher plasma

level of endorphins^[18,19].

The present study also demonstrated that subjects with low education level have a higher risk of asymptomatic PUD. It has been well documented that PUD is associated with low socioeconomic status^[20]. That association may be explained by a higher risk of *Helicobacter pylori* (*H. pylori*) infection among less educated groups, probably due to poor standards of hygiene^[21]. Psychological stress, risk lifestyle behaviors, and hard physical work may be other important risk factors for PUD in low education populations^[22].

NSAIDs are known to be associated with gastrointestinal injury, including erosions, ulceration and hemorrhage. During long-term administration, 60%-94% of NSAIDs users have been reported to show mucosal damage and 15%-31% to have evidence of frank gastric ulcer^[23,24]. In this study, NSAID use was not identified as a risk factor for the development of asymptomatic peptic ulcer. The reasons for the lack of association between NSAIDs and asymptomatic peptic ulcer could be due to short-term use of NSAIDs in our patients, the use of selective cyclooxygenase-2 inhibitors in some subjects or a small number of cases (type II error).

Certain lifestyle factors such as consumption of tobacco, alcohol, tea, coffee, betel nut and spicy foods are believed to stimulate gastric acid secretion, however, the findings of previous cross-sectional epidemiological studies have been inconsistent^[25,26]. The current cross-sectional study revealed that only current smoking independently predicted the development of PUD in asymptomatic subjects. A Japanese study of men aged 45 years and older revealed that current smokers were at higher risk of both gastric (OR, 3.4, 95% CI: 2.4- 4.7) and duodenal ulcers (OR, 3.0, 95% CI: 1.9- 4.7), compared with nonsmokers^[27]. However, another study failed to confirm these findings for PUD risk in smokers compared with nonsmokers^[28]. Recent studies have suggested that tobacco smoking causes peptic ulcer only if *H. pylori* infection is present^[29]. A prospective cohort study in Denmark showed that tobacco smoking remained an independent risk factor for PUD despite controlling for *H. pylori* infection status^[30]. Hence, we believe that ulcer patients should be advised to cease smoking. In this study, the specific aim was to investigate the prevalence and risk factors of asymptomatic PUD in Taiwan. It is necessary to further examine the risk factors for peptic ulcer in symptomatic subjects and to investigate the differences in risk factors for PUD between symptomatic and asymptomatic subjects in the future.

Despite its contributions, this study had certain limitations. First, self-selection bias of the population in this trial was possible, because all enrolled subjects had undergone self-paid health examinations and likely had a better economic status than the general population. Second, the studied subjects may differ from subjects in a primary care hospital because our hospital is a tertiary care center. Third, the status of *H. pylori* infection was not routinely examined. Its prevalence might differ between the non-peptic ulcer group and the peptic ulcer group. Nevertheless, most asymptomatic subjects had not received an ex-

amination for *H. pylori* infection before their routine upper gastrointestinal endoscopy and did not know their *H. pylori* status. The major aim of this study was to identify the clinical factors predicting peptic ulcer development prior to endoscopic examination. The findings may be useful for identifying asymptomatic subjects at high risk for the development of peptic ulcer.

In conclusion, the prevalence of PUD in asymptomatic subjects is 9.4% in Taiwan. Prior history of PUD, low education level, a high BMI and current smoker are independent risk factors for developing asymptomatic peptic ulcer.

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COMMENTS

Background

Peptic ulcer disease (PUD) is a common disease of the digestive system and the pathogenesis of symptom development in PUD is unclear. Some patients with PUD are asymptomatic until life-threatening complications (e.g. hemorrhage, perforation) develop. The aims of this study were to investigate the prevalence and risk factors of asymptomatic PUD in Taiwan.

Research frontiers

Currently, the long-term consequences of silent PUD remain unclear, however, hemorrhage, perforation, and stricture are well known complications of PUD. Therefore, identification and follow up of these asymptomatic patients is essential. Nonetheless, the risk factors for asymptomatic PUD still remain unclear.

Innovations and breakthroughs

Helicobacter pylori (*H. pylori*) infection, smoking and non-steroidal anti-inflammatory drugs use are known risk factors of symptomatic PUD but few studies to date have reported the risk factors for asymptomatic PUD. In this study, the authors observed that prior history of PUD, low education level, a high body mass index and current smoker are independent risk factors for developing asymptomatic PUD.

Applications

The status of *H. pylori* infection was not routinely examined and the prevalence of *H. pylori* status might differ between the non-peptic ulcer group and the peptic ulcer group. Nevertheless, the major aim of this study was to identify the clinical factors predicting peptic ulcer development prior to endoscopic examination, and most of the asymptomatic subjects did not receive any tests for *H. pylori* infection.

Terminology

PUD was defined as a circumscribed mucosal break 5 mm or more in diameter, with a well defined ulcer crater. All subjects were carefully interviewed regarding the presence of reflux or dyspeptic symptoms in the previous month, and subjects who responded negatively were classified as asymptomatic subjects and enrolled in this study.

Peer review

The study was well performed and very interesting. However, it contains some serious problems which have to be answered by the authors. The reviewer considered that the comparisons should be also required between the asymptomatic peptic ulcer disease group and the symptomatic peptic ulcer disease group to investigate risk factors of asymptomatic peptic ulcer disease.

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Coffee drinking and pancreatic cancer risk: A meta-analysis of cohort studies

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Abstract

AIM: To quantitatively assess the relationship between coffee consumption and incidence of pancreatic cancer in a meta-analysis of cohort studies.

METHODS: We searched MEDLINE, EMBASE, Science Citation Index Expanded and bibliographies of retrieved articles. Studies were included if they reported relative risks (RRs) and corresponding 95% CIs of pancreatic cancer with respect to frequency of coffee intake. We performed random-effects meta-analyses and meta-regressions of study-specific incremental estimates to determine the risk of pancreatic cancer associated with a 1 cup/d increment in coffee consumption.

RESULTS: Fourteen studies met the inclusion criteria, which included 671080 individuals (1496 cancer events) with an average follow-up of 14.9 years. Compared with individuals who did not drink or seldom drank coffee per day, the pooled RR of pancreatic cancer was 0.82 (95% CI: 0.69-0.95) for regular coffee drinkers, 0.86 (0.76-0.96) for low to moderate coffee drinkers, and 0.68 (0.51-0.84) for high drinkers. In subgroup analyses, we noted that, coffee drinking was associated with

a reduced risk of pancreatic cancer in men, while this association was not seen in women. These associations were also similar in studies from North America, Europe, and the Asia-Pacific region.

CONCLUSION: Findings from this meta-analysis suggest that there is an inverse relationship between coffee drinking and risk of pancreatic cancer.

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Key words: Coffee; Cohort study; Meta-analysis; Pancreatic neoplasm

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INTRODUCTION

Coffee is one of the most widely consumed beverages in the world, with a yearly world average consumption of 1.1 kg per capita, which reaches 4.5 kg in industrialized countries^[1]. More recently, coffee consumption has been associated with a reduction in the risk of several chronic diseases, including type 2 diabetes mellitus, Parkinson's disease and liver disease^[2-4]. Of these associations, the relationship between coffee drinking and cancer risk is of great interest.

Coffee consumption may exert an anticarcinogenic effect in some organs. For example, a reduction in cholesterol, bile acid, and neutral sterol secretion in the colon is a

direct effect of coffee consumption as is increased colonic motility, which can reduce exposure of epithelium to carcinogens^[5]. The components of coffee which have received attention are caffeine (a purine alkaloid), cafestol (a diterpene), kahweol (another diterpene), and chlorogenic acid (a dietary phenol). Cafestol and kahweol, which are active ingredients in the coffee oil, decrease mutagenesis and tumorigenesis in animal models. Diterpenes, found in coffee, reduce genotoxicity of carcinogens and lower DNA adduct formation. Caffeic acid and chlorogenic acid are antioxidants and have been reported to decrease DNA methylation^[6]. More subtly, caffeine itself appears to be protective-affecting cell cycle, proliferation, and apoptosis^[7,8].

Pancreatic cancer is one of the most aggressive and treatment-refractory malignancies in humans. Given that there is no screening test for the early detection of this cancer and no effective treatment to prolong survival time, primary prevention appears to be the most important way of reducing pancreatic cancer mortality. Over the last 4 decades, a number of epidemiologic studies have estimated the association between coffee consumption and pancreatic cancer occurrence. However, the results of these studies were inconsistent. Data from case-control studies may be subject to recall bias with respect to coffee consumption and selection bias with respect to the control group. Additional prospective cohort studies excluding those biases would be more useful for assessing coffee-cancer associations. We, therefore, systematically reviewed and performed a meta-analysis of prospective cohort studies to quantitatively assess the association between coffee intake and pancreatic cancer risk in humans. Because of the high consumption of coffee, even small effects on pancreatic cancer occurrence could have a large impact on public health.

MATERIALS AND METHODS

Literature search

We searched the electronic databases MEDLINE (1966 to August 2010), EMBASE (1985 to August 2010), and Science Citation Index Expanded (1945 to August 2010), using the Medical Subject Heading (MeSH) term coffee combined with pancreatic cancer or pancreatic neoplasm or pancreatic carcinoma. Furthermore, we reviewed reference lists of retrieved articles to search for more studies. Articles published in any language were included.

Inclusion and exclusion criteria

For inclusion, studies had to fulfill the following criteria: have a prospective cohort design; report relative risks (RRs) or hazard ratios and their corresponding 95% CIs (or data to calculate them) of pancreatic cancer relating to every category of coffee intake; and provide the frequency of coffee consumption. Studies were excluded if: case-control design was used; mixed beverage was reported, in which the effect of coffee could not be separated; only surrogate nutrients of coffee were reported; no categories of coffee intake were reported that could not allow for adequate

classification of intake. If multiple published reports from the same study cohort were available, we included only the one with the most detailed information for both outcome and coffee consumption.

Data extraction

Data were extracted independently by two investigators (Yu and Dong) according to the meta-analysis of observation studies in epidemiology guidelines^[9], and discrepancies were resolved by discussion with a third investigator (Zou). For each study, the following information was extracted: first author's last name; year of publication; country of origin; follow-up period; number of subjects and cases; age at baseline; category amounts of coffee intake; outcome assessment; RRs or hazard ratios of pancreatic cancer and corresponding 95% CIs for every category of coffee intake; and covariates adjusted in the statistical analysis.

Statistical analysis

The measures of interest were the RR and the corresponding 95% CIs for included cohort studies. When RRs were not available in the published article, they were computed from the exposure distributions. Because various studies used different measurement units for coffee consumption, we converted these into cups per day as a standard measure. If coffee consumption was indicated by milliliter, we assumed 125 mL as approximately equivalent to 1 cup.

We computed the summary RR for coffee drinkers *vs* nondrinkers and for different levels of consumption by giving each study-specific RR a weight that was proportional to its precision (i.e. the inverse of the variance derived, when necessary, from the reported 95% CIs). To estimate the summary RR for various levels of coffee consumption, we first calculated the study-specific estimate separately for low to moderate consumption and high consumption.

Statistical heterogeneity among studies was estimated using Q and I^2 statistics. For the Q statistic, heterogeneity was considered present when $P < 0.1$. We pooled the study-specific estimates using both the fixed effect model and the random effect model proposed by DerSimonian and Laird; when a significant heterogeneity was found, the random effect model results were presented. A sensitivity analysis was also conducted, in which one study at a time was removed and the rest analyzed to estimate whether the results could have been affected markedly by a single study.

For dose-response analysis, we used the method proposed by Greenland *et al.*^[10] to estimate study-specific slopes from the correlated natural logarithm of the RR across categories of coffee consumption, assigning to each class the dose corresponding to the midpoint of upper and lower boundaries. The highest, open-ended category was assumed to have the same amplitude of consumption as the preceding category^[11]. Then the summary RR for pancreatic cancer risk with a 1 cup/d increment in

coffee consumption was obtained by pooling the study-specific slopes, using the inverse of the corresponding variances as weights.

Finally, publication bias was evaluated through funnel plot visual analysis and with the Begg's and Egger's tests. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with STATA (version 9.0; Stata Co., College Station, TX, USA).

RESULTS

Using the predefined search strategy, we identified 14 prospective cohort studies (Figure 1), including 671 080 participants and 1496 incident cases of pancreatic cancer with an average follow-up of 14.9 years, which were eligible for inclusion in the meta-analysis^[12-25]. The characteristics of the included studies are summarized in Table 1. Initial agreement between the two reviewers on whether a study was eligible for inclusion occurred in 48/50 manuscripts (96%; $\kappa = 0.92$). Of the 14 cohorts included in the meta-analysis, 4 were conducted in Europe (Norway, Sweden and Finland), 6 in North America (the United States), and 4 in Asia (Japan).

Figure 2A shows the estimated RRs for coffee drinkers *vs* non/lowest drinkers from the cohort studies. The summary RR of pancreatic cancer from all combined studies was 0.82 (95% CI: 0.69-0.95). There was significant heterogeneity across the studies ($Q = 21.88$, $P = 0.057$, $I^2 = 40.6\%$). Figure 2B and C give the RRs according to low to moderate and high coffee consumption from various studies. The summary RR was 0.86 (95% CI: 0.76-0.96) for low to moderate coffee consumption, with no heterogeneity between studies ($Q = 16.12$, $P = 0.186$, $I^2 = 25.6\%$). The summary RR for high consumption of coffee was 0.68 (95% CI: 0.51-0.84), also with no heterogeneity between studies ($Q = 7.82$, $P = 0.729$, $I^2 = 0\%$). The summary RR for an increment of 1 cup of coffee per day was 0.96 (95% CI: 0.90-1.02) for all studies combined, but was statistically insignificant.

Various sources of heterogeneity likely exist due to international differences in coffee consumption (e.g. coffee type, serving size, or brewing method) in this analysis. To examine the magnitude of the combined RR in each stratum and its respective test of heterogeneity, we conducted subgroup analyses by gender and geographic regions. The summary RR was 0.73 (95% CI: 0.63-0.84) for men and 0.82 (95% CI: 0.52-1.11) for women when combining all studies. There was no heterogeneity for men ($Q = 9.01$, $P = 0.252$, $I^2 = 22.3\%$) and a significant heterogeneity for women ($Q = 12.42$, $P = 0.029$, $I^2 = 59.7\%$).

Associations were also similar in studies from North America, Europe and the Asia-Pacific region. The RR was 0.81 (95% CI: 0.67-0.95) when considering the 6 studies conducted in North America, 0.86 (95% CI: 0.50-1.22) for the 4 studies from Europe, and 0.76 (95% CI: 0.52-0.99) for the 4 Asian studies. No significant differences by sex were found.

There was no indication of publication bias from either

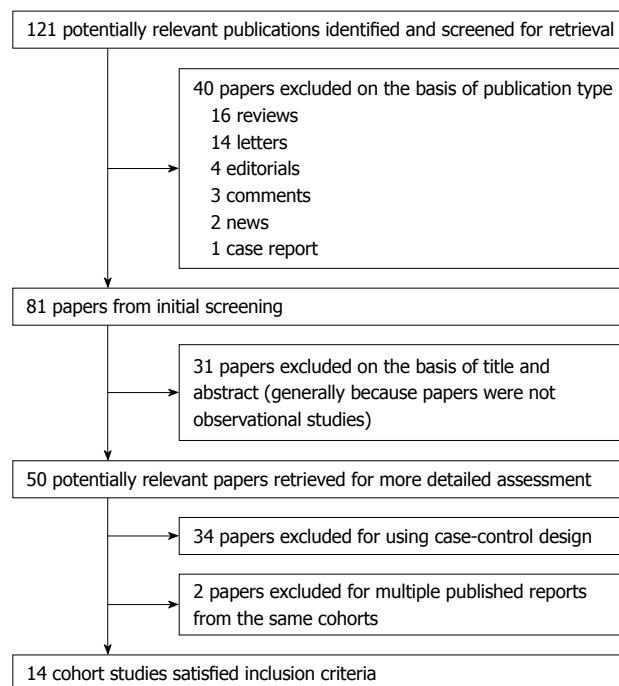


Figure 1 Flow diagram of search strategy and study selection.

visualization of the funnel plot or Egger's ($P = 0.735$) and Begg's ($P = 0.381$) (Figure 3) tests. A sensitivity analysis, in which one study was removed at a time, was performed to evaluate the stability of the results. This analysis confirmed the stability of our results.

DISCUSSION

Coffee consumption, as a major and frequent dietary exposure in diverse cultures around the globe, has been shown to be associated with pancreatic cancer in epidemiological studies. However, there is no comprehensive, up-to-date overview of the entirety of the substantial body of epidemiologic evidence. To address this need, we quantitatively assessed the relationship between coffee intake and incidence of pancreatic cancer in a meta-analysis of cohort studies.

Coffee can potentially impact the etiology of cancer of various sites along multiple pathways, ranging from carcinogenesis to cellular apoptosis. A number of *in vitro* studies suggest that caffeine can influence carcinogenesis through inhibition of DNA repair, and induction of mitotic events before DNA replication is completed^[26-28]. Porta *et al*^[29] argues that in exocrine pancreatic cancer, caffeine, other coffee compounds, or other correlates of coffee drinking could modulate Ki-ras activation by interfering with DNA repair, cell-cycle checkpoints, and apoptosis. However, for most cancer sites, there is a significant amount of evidence to show that there is no detrimental effect following the consumption of up to 6 cups of coffee per day in relation to cancer occurrence. Through the meta-analysis of cohort studies, we found that compared with individuals who did not drink or seldom drank

Table 1 Summary characteristics of studies included in the meta-analysis

Study	Country	Follow-up period	Study subjects	No. of cases	Coffee consumption	Relative risk (95% CI)	Adjustments
Snowdon <i>et al</i> ^[12] 1984	United States	1960-1980	23 912 Aged ≥ 30 yr	71	< 1 cup/d 1 cup/d ≥ 2 cups/d	1.00 (reference) 1.7 (0.9-3.3) 0.8 (0.4-1.6)	Age, sex
Jacobsen <i>et al</i> ^[13] 1986	Norway	1967-1978	16 555 13 664 male 2891 female	63	≤ 2 cups/d 3-4 cups/d 5-6 cups/d ≥ 7 cups/d	1.00 (reference) 1.22 (0.66-2.35) 0.53 (0.21-1.26) 0.62 (0.18-1.75)	Sex, age and residence (men for cigarette smoking)
Nomura <i>et al</i> ^[14] 1986	Japan	1965-1983	7355 male	21	0 cup/d 1-2 cups/d 3-4 cups/d ≥ 5 cups/d	1.00 (reference) 0.83 (0.16-5.34) 1.39 (0.32-8.31) 1.27 (0.27-7.84)	Age, years of smoking, number of cigarettes smoked per day, smoking status at exam, and past smoking status
Hiatt <i>et al</i> ^[15] 1988	United States	1978-1984	122 894	49	< 1 cup/d 1-3 cups/d > 4 cups/d	0.4 (0.1-4.3) 1.2 (0.3-4.4) 0.8 (0.2-4.6)	Age, sex, ethnic origin, blood glucose levels, consumption of alcohol, tea
Zheng <i>et al</i> ^[16] 1993	United States	1966-1986	17 633 male Aged ≥ 35 yr	57	< 3 cups/d 3-4 cups/d 5-6 cups/d ≥ 7 cups/d	1.00 (reference) 0.6 (0.3-1.2) 0.7 (0.4-1.6) 0.9 (0.3-2.4)	Age, smoking index, alcohol index
Shibata <i>et al</i> ^[17] 1994	United States	1981-1990	13 979	63	< 1 cup/d 1 cup/d 2-3 cups/d ≥ 4 cups/d	1.00 (reference) 1.82 (0.75-4.43) 1.67 (0.74-3.77) 0.88 (0.28-2.80)	Sex, age and cigarette smoking
Stensvold <i>et al</i> ^[18] 1994	Norway	1977-1990	42 973 21 735 male 21 238 female Aged 35-54 yr	41 26 M 15 F	≤ 2 cups/d 3-4 cups/d 5-6 cups/d ≥ 7 cups/d	1.00 (reference) 2.76 (0.63-25.21) 3.09 (0.72-27.87) 2.71 (0.59-25.15)	Age, cigarette smoking, county of residence
Zheng <i>et al</i> ^[19] 1996	United States	1986-1993	35 369 female Aged 55-69 yr	66	Never/monthly Weekly-3 cups/d ≥ 4 cups/d	1.00 (reference) 1.82 (0.87-3.82) 2.15 (1.01-4.07)	Age, education, smoking status, pack-years of smoking, physical activity, all fruit and vegetable intake, total energy intake, waist/hip ratio, family history of cancer, prior history of blood transfusion
Michaud <i>et al</i> ^[20] 2001	United States	1986-1998 1980-1996	136 593 47 794 male Aged 40-75 yr 88 799 female Aged 30-55 yr	288 130 M 158 F	None < 1 cup/d 1 cup/d 2-3 cups/d > 3 cups/d	1.00 (reference) 0.94 (0.65-1.36) 0.60 (0.38-0.94) 0.88 (0.65-1.21) 0.62 (0.27-1.43)	Age in 5-yr categories, pack-years of smoking, body mass index, history of diabetes mellitus, history of cholecystectomy, energy intake, and period
Isaksson <i>et al</i> ^[21] 2002	Sweden	1961-1997	21 884 9680 male 12 204 female Aged 36-75 yr	131	0-2 cups/d 3-6 cups/d ≥ 7 cups/d	1.00 (reference) 0.91 (0.60-1.38) 0.39 (0.17-0.89)	Sex, age, cigarette smoking
Lin <i>et al</i> ^[22] 2002	Japan	1988-1997	99 527 44 646 male 54 881 female Aged 40-79 yr	225	Nondrinkers 1-2 cups/mo 1-4 cups/wk 1 cup/d 2-3 cups/d ≥ 4 cups/d		Age, cigarette smoking in pack-years
Stolzenberg-Solomon <i>et al</i> ^[23] 2002	Finland	1985-1997	27 111 male Aged 50-69 yr	163	≤ 321.4 g/d > 321.4-≤ 450.0 g/d > 450.0-≤ 624.9 g/d > 624.9-≤ 878.6 g/d > 878.6 g/d	1.00 (reference) 1.48 (0.89-2.46) 1.12 (0.61-2.03) 1.72 (1.01-2.86) 0.95 (0.54-1.68)	Age, years of smoking
Khan <i>et al</i> ^[24] 2004	Japan	1984-2002	3158 1524 male 1634 female Aged ≥ 40 yr	25 12 M 13 F	≤ several times/mo ≥ several times/wk	1.00 (reference) 0.38 (0.01-1.05)	Age, sex, health education, health examination, health status, smoking
Luo <i>et al</i> ^[25] 2007	Japan	1990-2003	102 137 48 783 male 53 354 female Aged 40-69 yr	233 135 M 98 F	Rarely 1-2 cups/wk 3-4 cups/wk 1-2 cups/d ≥ 3 cups/d	1.00 (reference) 1.0 (0.7-1.4) 1.1 (0.7-1.7) 0.9 (0.6-1.3) 0.8 (0.4-1.3)	Body mass index, frequency of sports, smoking status, alcohol intake, history of diabetes, history of cholelithiasis, study area, age and tea consumption

coffee per day, the pooled RR of pancreatic cancer was 0.82 (95% CI: 0.69-0.95) for regular coffee drinkers, 0.86

(0.76-0.96) for low to moderate coffee drinkers, and 0.68 (0.51-0.84) for high drinkers. Overall, an increase in con-

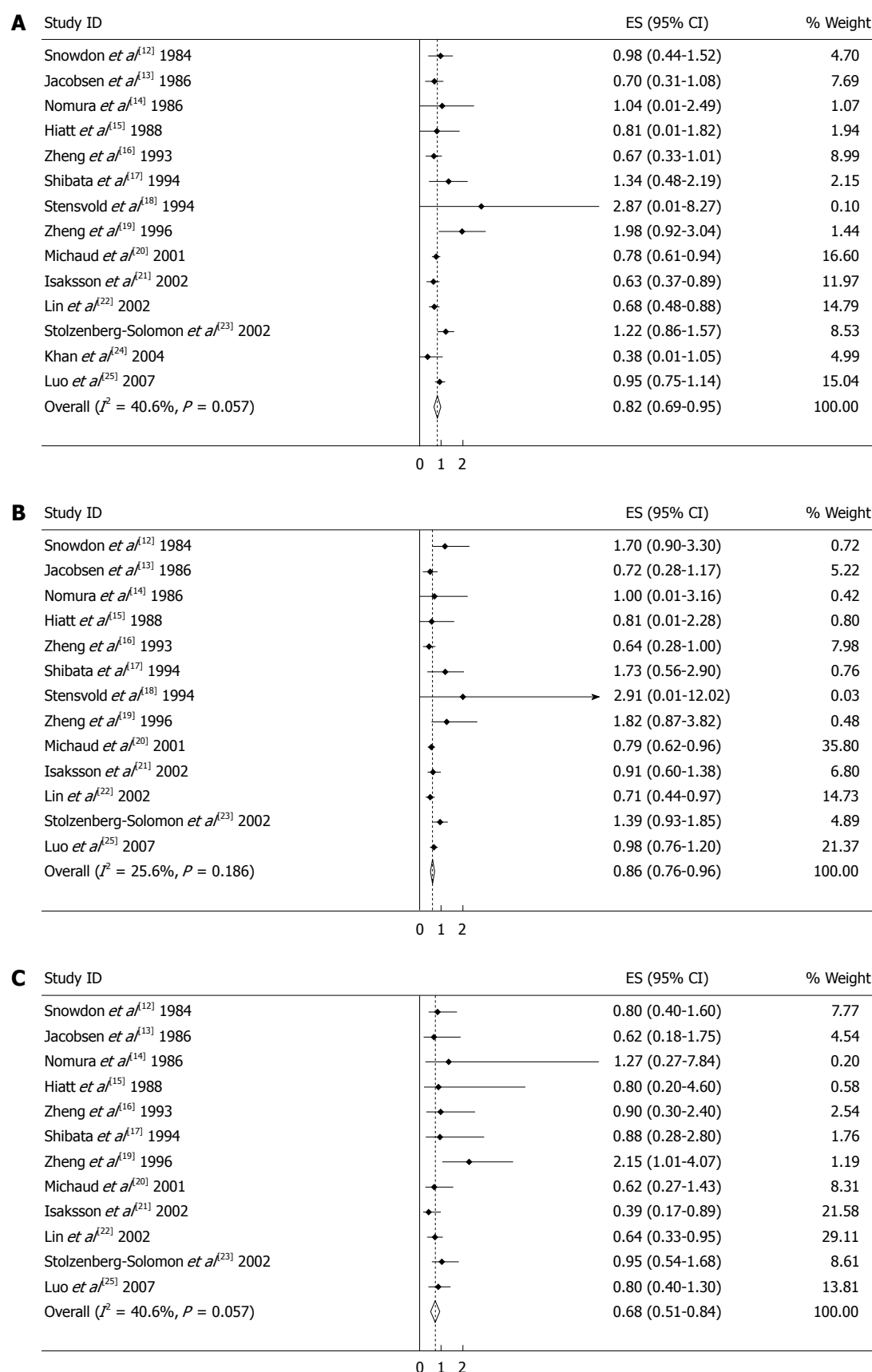


Figure 2 Summary relative risks of pancreatic cancer for coffee drinkers vs non/lowest drinkers from included studies (A), low to moderate coffee drinkers vs non/lowest drinkers from included studies (B) and high coffee drinkers vs non/lowest drinkers from included studies (C). Weights are from random effect analysis. Squares represent study-specific relative risk estimates (size of the square reflects the study-specific statistical weight, that is, the inverse of the variance); horizontal lines represent 95% CIs; diamonds represent summary relative risk estimates with corresponding 95% CIs.

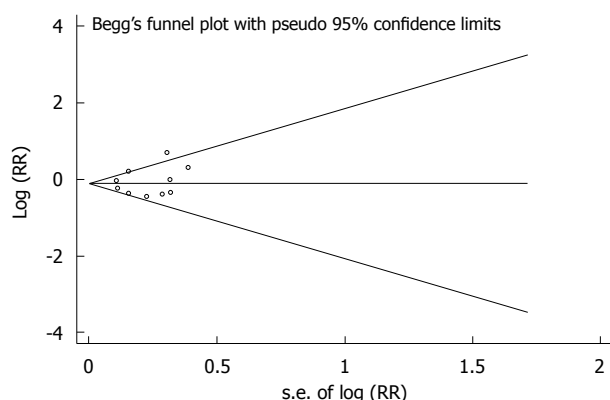


Figure 3 Publication bias in the studies. Begg's funnel plot indicating no publication bias in the studies included in this meta-analysis. No indication of publication bias was noted from both visualization of funnel plot and Egger's test. RR: Relative risk.

sumption of 1 cup of coffee per day was associated with a 4% reduced risk of pancreatic cancer (RR, 0.96; 95% CI: 0.90-1.02). Thus, the evidence presented above suggests that coffee intake might prevent pancreatic cancer occurrence in humans.

Over the past two decades, many studies have been carried out on coffee and pancreatic cancer following the early warning in the early 1980s that coffee consumption was related to pancreatic cancer risk. Some ecological^[30], case-control^[31], and cohort^[20,22] studies carried out in the USA, Canada, Europe and Asia investigated the relationship between coffee consumption and the risk of pancreatic cancer. In general, these investigations yielded inconsistent results, with a meta-analysis on 25 case-control studies giving a summary effect estimate of 1.04 (95% CI: 1.00-1.07) and a summary RR of 1.00 (0.94-1.07) per 1 cup/d for 10 cohort studies^[5]. Since the WCRF report, Luo *et al.*^[25] studied the association between drinking coffee and the risk of pancreatic cancer in a large population-based cohort study in Japan. Among 102 137 participants followed for an average of 11 years in which 233 incident cases of pancreatic cancer were identified, there was no increased risk of pancreatic cancer with coffee intake. A reduced risk was apparent among men who drank at least 3 cups of coffee per day compared with those who did not drink any or only rarely drank coffee. After a pooled analysis of 14 cohort studies, we found that there was a reverse association between coffee consumption and the risk of pancreatic cancer.

Some limitations of this meta-analysis should be acknowledged. First, as in all observational studies of diet and disease, the possibility of bias and confounding can not be excluded. However, cohort studies, which are less susceptible to bias because of the prospective design, also showed an inverse association between coffee consumption and risk of pancreatic cancer, suggesting that the finding is not likely attributable to recall and selection bias. Individual studies may have failed to adjust for potential known or unknown confounders. Second, our results are likely to be affected by the misclassification of

coffee consumption. Coffee exposure is mostly assessed in relation to the number of cups of coffee consumed daily, weekly or monthly. However, most of the studies included in our meta-analysis did not provide information on coffee type, serving size, or brewing method. Serving sizes and brewing methods for coffee can vary substantially within and between countries. Standard coffee cups are larger in the United States than in Europe or Japan, and the difference in the strength of the coffee brewed may compensate for the different serving size between countries^[32]. Third, we extracted the risk estimates that reflected the greatest degree of the control potential confounders, because it was hard to obtain raw data from each study to conduct standardized adjustments. Therefore, it is probable that the results based on the adjustment for different confounders were different from those based on standardized adjustments. Finally, only published studies were included in our meta-analysis. Therefore, publication bias may have occurred although no publication bias was indicated from both visualization of the funnel plot and Egger's test.

In summary, there is substantial evidence from both laboratory and animal studies on the favorable influence of coffee on the risk of pancreatic cancer. Although well designed studies, in particular randomized clinical studies among high risk populations, are needed to provide valuable insights into coffee consumption and the risk of pancreatic cancer, our meta-analysis which included 14 prospective cohort studies confirmed that coffee consumption is inversely associated with the risk of pancreatic cancer.

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COMMENTS

Background

Coffee consumption, as a major and frequent dietary exposure in diverse cultures around the globe, has been shown to be associated with pancreatic cancer in epidemiological studies. However, there is no comprehensive, up-to-date overview of the entirety of the substantial body of epidemiologic evidence.

Research frontiers

Over the past two decades, many studies have been carried out on coffee and pancreatic cancer. Some ecological, case-control, and cohort studies carried out in the USA, Canada, Europe and Asia investigated the relationship between coffee consumption and the risk of pancreatic cancer. However, these investigations yielded inconsistent results, with a meta-analysis on 25 case-control studies giving a summary effect estimate of 1.04 and a summary relative risk (RR) of 1.00 per 1 cup/d for 10 cohort studies.

Innovations and breakthroughs

Findings from this meta-analysis suggested that, compared with individuals who did not drink or seldom drank coffee per day, the pooled RR of pancreatic cancer was 0.82 for regular coffee drinkers, 0.86 for low to moderate coffee drinkers, and 0.68 for high drinkers. Coffee drinking was associated with a reduced risk of pancreatic cancer for men, while this association was not seen in women.

Applications

Coffee consumption may reduce pancreatic cancer incidence and it has a

consistent preventive effect on some type of cancers. These epidemiological observations should provide information for the exploration of biological mechanisms involved in the inverse relationship between coffee drinking and risk of pancreatic cancer.

Terminology

Roasted coffee is a complex mixture of more than one thousand chemicals. Many of these constituents could potentially alter cancer risk through several biological mechanisms. The anticarcinogenic components in coffee which have received attention are caffeine, cafestol, kahweol, polyphenols, caffeic acid and chlorogenic acid.

Peer review

Dr. Dong *et al* showed that coffee consumption may reduce the pancreatic cancer incidence, using meta-analysis. The results are very interesting.

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p53 codon 72 polymorphism and liver cancer susceptibility: A meta-analysis of epidemiologic studies

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Abstract

AIM: To evaluate the association between p53 codon 72 polymorphism and liver cancer risk by means of meta-analysis.

METHODS: Two investigators independently searched the Medline, Embase and Chinese Biomedicine databases. Summary odds ratios and 95% CI for p53 codon 72 polymorphism and liver cancer were calculated in fixed-effects model (Mantel-Haenszel method) and random-effects model (DerSimonian and Laird method) when appropriate.

RESULTS: This meta-analysis included 1115 liver cancer cases and 1778 controls. The combined results based on all studies showed that there was a statistically significant link between Pro/Pro genotype and liver cancer, but not between Arg/Arg or Pro/Arg genotype and liver cancer. When stratifying for race, similar results were obtained, i.e. patients with liver cancer had a significantly higher frequency of Pro/Pro genotype than non-cancer patients among Asians. After stratifying the

various studies by control source, gender, family history of liver cancer and chronic hepatitis virus infection, we found that (1) patients among hospital-based studies had a significantly higher frequency of Pro/Pro and a significantly lower frequency of Arg/Arg genotype than individuals without cancer; (2) female patients with liver cancer had a significantly lower frequency of Arg/Arg and a higher frequency of Pro/Arg+Pro/Pro genotypes than female individuals without cancer; (3) subgroup analyses for family history of liver cancer did not reveal any significant association between p53 codon 72 polymorphism and liver cancer development; and (4) patients with negative hepatitis virus infection had a significantly higher frequency of Pro/Pro and a significantly lower frequency of Arg/Arg genotype than individuals without cancer.

CONCLUSION: This meta-analysis suggests that the p53 codon 72 polymorphism may be associated with liver cancer among Asians.

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Key words: Liver cancer; p53 codon 72; Gene polymorphism; Meta-analysis

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Chen X, Liu F, Li B, Wei YG, Yan LN, Wen TF. p53 codon 72 polymorphism and liver cancer susceptibility: A meta-analysis of epidemiologic studies. *World J Gastroenterol* 2011; 17(9): 1211-1218 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i9/1211.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i9.1211>

INTRODUCTION

Primary liver cancer is the sixth most common cancer in

the world and the third most common cause of cancer mortality^[1]. A 2005 analysis of the worldwide incidence of and mortality from cancer showed that 626 000 cases of liver cancer occurred in 2002, 82% of which are from developing countries and that 598 000 patients die annually of this disease^[1]. China alone accounts for 55% liver cancer death worldwide. Moreover, the 5-year survival rate was 8% in the United States during 1988-2001^[2], 9% in Europe during 1995-1999^[3], and 5% in developing countries in 2002^[1]. The major etiologies of hepatocellular carcinoma (HCC) include infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), cigarette smoking, alcohol drinking and aflatoxin B1 (AFB₁) exposure^[4-9]. However, not all individuals with exposure to the risk factors develop cancer even after a long-term follow-up. The pathogenesis of human HCC is a multistage process with the involvement of a series of genes, including oncogenes and tumor suppressor genes.

The p53 tumor suppressor gene, located on chromosome 17p13, is of critical importance for the regulation of cell cycle and maintenance of genomic integrity. Loss of p53 function has been suggested to be a critical step in multistage hepatocarcinogenesis^[10]. A specific p53 mutation at codon 249 in exon 7 was associated with AFB₁-induced HCC in certain areas of high AFB₁ contamination^[11]. The wild-type p53 gene exhibits a polymorphism at codon 72 in exon 4, with a single nucleotide change that causes a substitution of proline for arginine (Arg72Pro)^[12]. The polymorphism occurs in the proline-rich domain of p53 protein, which is necessary for the protein to fully induce apoptosis. It is found that in cell lines containing inducible versions of alleles encoding the Pro and Arg variants, the Arg variant induces apoptosis more markedly than the Pro variant^[13]. In other words, the two polymorphic variants of p53 are functionally distinct, and these differences may influence cancer risk. The polymorphism consists of a single base pair change of either arginine or proline which creates 3 distinct genotypes: homozygous for arginine (Arg/Arg), homozygous for proline (Pro/Pro) and a heterozygote (Pro/Arg)^[14]. p53 codon 72 polymorphisms have been reported to be associated with cancers of the lung^[15], esophagus^[16], stomach^[17], colorectum^[18], breast^[19], bladder^[20] and cervix^[21].

In recent years, a number of case-control studies were conducted to investigate the association between p53 codon 72 polymorphism and liver cancer susceptibility in humans. But these studies reported conflicting results. No quantitative summary of the evidence has ever been performed. The purpose of this meta-analysis was to quantitatively summarize the evidence for such a relationship.

MATERIALS AND METHODS

Literature search strategy

Search was applied to the following electronic databases: Medline (from 1966 to September 2010), Embase (from 1950 to September 2010) and Chinese Biomedicine databases (from 1979 to September 2010). The following

key words were used: "p53" or "codon 72", "liver" or "hepatocellular", "carcinoma" or "cancer" or "tumor". The search was without restriction in language, but with restriction in the studies conducted in human subjects. The reference lists of reviews and retrieved articles were hand searched at the same time. We did not include abstracts or unpublished reports. If more than one article was published by the same author using the same case series, we selected the study with the largest series.

Inclusion and exclusion criteria

We reviewed abstracts of all citations and retrieved studies. The following criteria were used to include published studies: (1) evaluating the association between p53 codon 72 polymorphism and liver cancer; (2) case-control study; and (3) sufficient genotype data were presented to calculate the odds ratio (OR) with confidence interval (CI). Major reasons for exclusion of studies were: (1) no control; (2) duplicate; and (3) no usable data reported.

Data extraction

All data were extracted independently by two reviewers (Chen X and Liu F) according to the prespecified selection criteria. Disagreement was resolved by discussion. The following data were extracted: the last name of the first author, study design, publication year, statistical methods, ethnicity of the population, genotyping methods, number of liver cancer cases and controls studied and results of studies.

Statistical analysis

The statistical analysis was conducted using STATA 8.2 (Stata Corp LP, College Station, TX, USA), $P < 0.05$ was considered statistically significant. Dichotomous data were presented as OR with 95% CI. Statistical heterogeneity was measured using the Q statistic test ($P < 0.10$ was considered statistically significant heterogeneity)^[22]. Either a random-effects model (DerSimonian-Laird method^[23]) or fixed-effects model (Mantel-Haenszel method^[24]) was used to calculate pooled effect estimates in the presence or absence of heterogeneity, respectively. To establish the effect of clinical heterogeneity among the studies on the conclusions of this meta-analysis, subgroup analyses were conducted based on race, study design, gender, chronic hepatitis virus status and family history of liver cancer patients. Several methods were used to assess the potential for publication bias. Visual inspection of funnel plot asymmetry was conducted. The Begg's rank correlation method^[25] and the Egger's weighted regression method^[26] were used to statistically assess publication bias. $P < 0.05$ was considered statistically significant.

RESULTS

Study characteristics

There were 2248 papers relevant to the search words. Through screening the title and reading the abstract and

Table 1 Characteristics of studies included in the meta-analysis

First author	Design	Yr	Country	Ethnicity	Case/control				Genotyping	HWE
					<i>n</i>	Arg/Arg	Arg/Pro	Pro/Pro		
Yu <i>et al</i> ^[38]	HCC	1999	China	Asian	80/328	28/112	35/141	17/75	PCR-RFLP	0.02
Anzola <i>et al</i> ^[36]	HCC	2003	Spain	Caucasian	97/111	46/65	47/42	4/4	PCR-SSCP	0.38
Leverì <i>et al</i> ^[35]	PCC	2004	Italy	Caucasian	86/254	46/122	33/113	7/19	PCR-RFLP	0.30
Zhu <i>et al</i> ^[31]	HCC	2005	China	Asian	469/567	135/197	252/284	82/86	PCR-RFLP	0.32
Ezzikouri <i>et al</i> ^[30]	PCC	2007	Morocco	Caucasian	96/222	52/129	31/79	13/14	PCR-RFLP	0.69
Yoon <i>et al</i> ^[29]	HCC	2008	Korea	Asian	287/296	110/124	111/135	66/37	PCR-RFLP	0.98

HCC: Hospital-based case-control; PCC: Population-based case-control; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; PCR-SSCP: Single strand conformation polymorphism analysis of polymerase chain reaction products; HWE: Hardy-Weinberg equilibrium of genotypes in control group. χ^2 -test is used, if $P > 0.05$, frequencies of genotypes in control group was in Hardy-Weinberg equilibrium.

Table 2 Meta-analysis of p53 codon 72 polymorphism and liver cancer, odds ratio (95% CI)

Subgroups	Arg/Arg	<i>P</i> value	Pro/Pro	<i>P</i> value	Pro/Arg	<i>P</i> value	Pro/Arg + Pro/Pro	<i>P</i> value
Race								
Asian	0.83 (0.68-1.00)	0.543	1.35 (1.06-1.71)	0.048	1.00 (0.83-1.20)	0.120	NA	NA
Caucasian	0.90 (0.67-1.20)	0.199	1.56 (0.91-2.69)	0.420	0.99 (0.73-1.33)	0.160	NA	NA
Gender								
Male	0.72 (0.47-1.09)	0.023	NA	NA	NA	NA	1.39 (0.91-2.12)	0.023
Female	0.49 (0.26-0.94)	0.862	NA	NA	NA	NA	2.03 (1.07-3.85)	0.862
Control source								
HCC	0.80 (0.67-0.96)	0.575	1.34 (1.06-1.70)	0.106	1.04 (0.88-1.24)	0.094	NA	NA
PCC	1.03 (0.73-1.45)	0.280	1.65 (0.92-2.79)	0.220	0.82 (0.57-1.17)	0.772	NA	NA
Family history								
Yes	0.32 (0.07-1.48)	0.667	NA	NA	NA	NA	3.08 (0.67-14.08)	0.667
No	0.72 (0.28-1.81)	0.013	NA	NA	NA	NA	1.39 (0.55-3.53)	0.013
Hepatitis virus infection								
Positive	1.08 (0.75-1.56)	0.980	0.90 (0.56-1.44)	0.459	0.99 (0.70-1.40)	0.550	NA	NA
Negative	0.55 (0.32-0.94)	0.204	2.07 (1.29-3.30)	0.373	1.19 (0.83-1.71)	0.338	NA	NA

NA: Due to lack of data, meta-analyses cannot be performed. HCC: Hospital based case-control studies; PCC: Population based case-control studies. *P* value for heterogeneity. If $P < 0.10$, random effect model was used; otherwise, fixed effect model was used.

the entire article, 12 cohort studies were identified^[27-38]. Six of them were excluded (four studies reported duplicate data^[31-34] and three are not related to liver cancer^[27,28,37]). As a result, six studies^[29-31,35,36,38] were selected, including 1115 liver cancer cases and 1778 controls. These studies were carried out in China, Spain, Italy, Morocco and Korea. Characteristics of the studies included in the meta-analysis are presented in Table 1.

Quantitative data synthesis

The combined results based on all studies showed that there was a statistically significant link between Pro/Pro genotype and liver cancer (OR = 1.38, 95% CI: 1.11-1.72, $P = 0.004$), but not between Arg/Arg or Pro/Arg and liver cancer (Arg/Arg, OR = 0.85, 95% CI: 0.72-1.00; Pro/Arg, OR = 0.99, 95% CI: 0.85-1.16). When stratifying for race, similar results were obtained, i.e. patients with liver cancer had a significantly higher frequency of Pro/Pro (OR = 1.35, 95% CI: 1.06-1.71, $P = 0.014$) genotype than non-cancer patients among Asians (Figure 1).

When stratifying by control source, we found that patients among hospital-based studies had a significantly higher frequency of Pro/Pro (OR = 1.34, 95%

CI: 1.06-1.70, $P = 0.014$) and a significantly lower frequency of Arg/Arg (OR = 0.80, 95% CI: 0.67-0.96, $P = 0.018$) genotype than patients without cancer, but not in population-based studies. When stratifying for gender, we found that female patients with liver cancer had a significantly lower frequency of Arg/Arg (OR = 0.49, 95% CI: 0.26-0.94, $P = 0.031$) and a higher frequency of Pro/Arg+Pro/Pro (OR = 2.03, 95% CI: 1.07-3.85, $P = 0.031$) genotypes than female individuals without cancer. When we stratified the various studies by family history of liver cancer, no statistically significant results were observed for all the analyses. When stratifying by chronic hepatitis virus status, we found that patients with negative hepatitis virus infection had a significantly higher frequency of Pro/Pro (OR = 2.07, 95% CI: 1.29-3.30, $P = 0.002$) and a significantly lower frequency of Arg/Arg (OR = 0.55, 95% CI: 0.32-0.94, $P = 0.028$) genotype than individuals without cancer, but not in patients with positive hepatitis virus infection (Table 2).

Heterogeneity and publication bias

No statistically significant heterogeneity was observed among trials for all the analyses with the Q statistic (Arg/

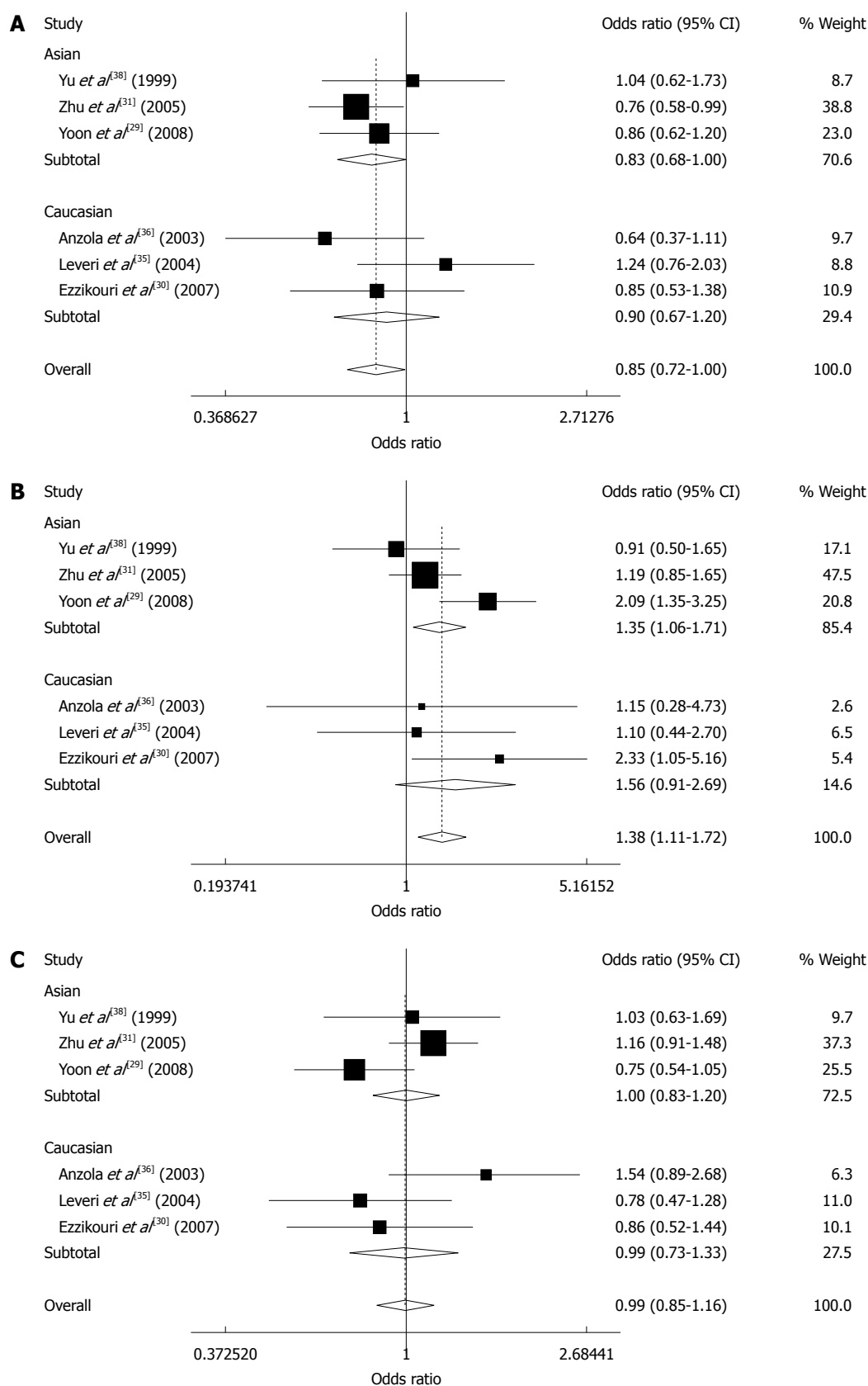


Figure 1 Meta-analysis of p53 codon 72 Arg/Arg (A), Pro/Pro (B) and Pro/Arg (C) and liver cancer risk.

Arg $P = 0.458$; Pro/Pro $P = 0.152$; Pro/Arg $P = 0.161$). In addition, L'Abbe plots did not show evidence of heterogeneity (Figure 2A). Review of funnel plots could

not rule out the potential for publication bias for all the analyses. Publication bias was not evident when the Begg rank correlation method (Arg/Arg $P = 1.00$; Pro/Pro P

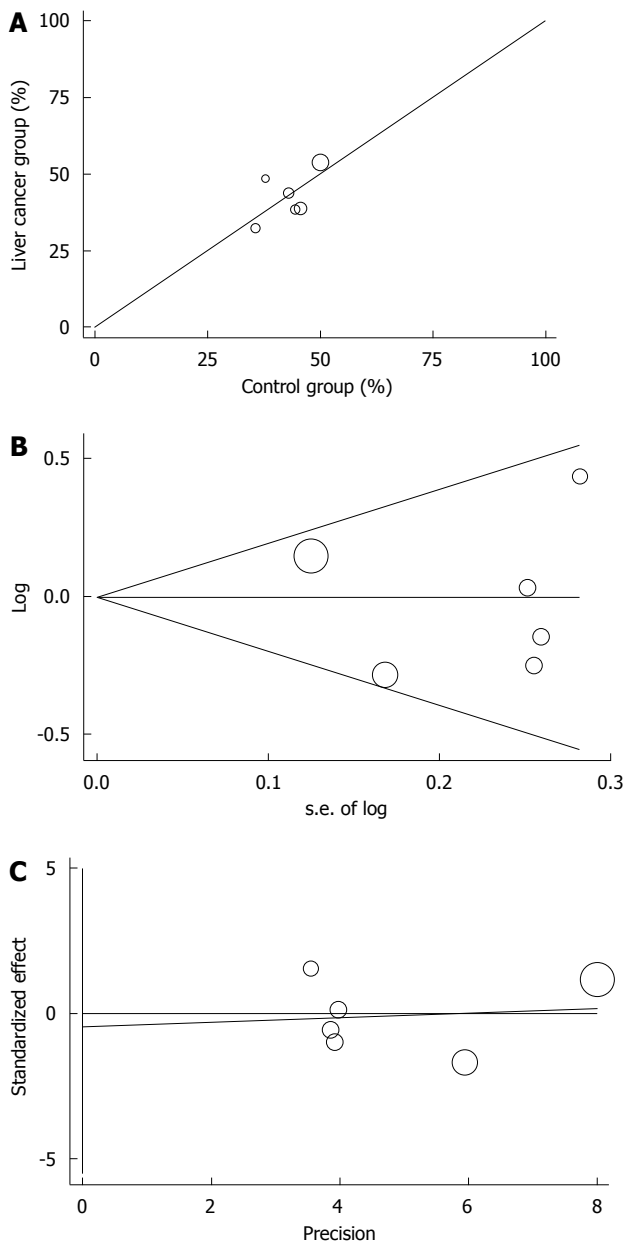


Figure 2 L'Abbe plots (A), Begg's funnel plot (B) and Egger's publication bias plot (C) of p53 codon 72 polymorphism and liver cancer risk.

= 1.00; Pro/Arg $P = 0.707$) and the Egger weighted regression method (Arg/Arg $P = 0.440$; Pro/Pro $P = 0.995$; Pro/Arg $P = 0.818$) were used (Figure 2B and C).

DISCUSSION

Although many environmental factors are found to correlate with the tumorigenesis of liver cancer^[4-9], the risk factors still need to be further elucidated. It has been recognized that the most important risk factor for the development of HCC is cirrhosis^[39]. Chronic infections with HBV and HCV are the most frequent causes of cirrhosis worldwide. A large number of cohort and case-control studies have shown that alcohol consumption causes liver cirrhosis and is an independent risk factor for primary

liver cancer^[6,40,41]. Epidemiological studies reported elevated HCC risks associated with exposure to aflatoxins after adjustment for HBV exposure^[42]. Cigarette smoking has been causally associated with the risk of HCC^[6,43]. However, there are a portion of patients without known risk factors who eventually developed liver cancer^[44]. Previous studies had shown an interaction of environmental factors and genetic predisposition in the development of liver cancer^[31,38]. Therefore, genetic predisposition may contribute to the process of tumorigenesis.

A genetic predisposition to liver cancer has been suggested by many studies^[45-47]. Recent studies suggest that single nucleotide polymorphism may be related to the tumorigenesis of liver cancer^[48,49]. The p53 gene and its encoded protein controls cell cycle, cell growth and apoptosis, which has a common polymorphism at codon 72 of exon 4 that encodes either Pro or Arg. Until recently, a number of studies have been conducted to find the relationship between p53 codon 72 polymorphism and liver cancer risk. Most of these studies were based on small sample sizes. Moreover, there are still some conflicting results. As a powerful statistical method, meta-analysis can provide a quantitative approach for pooling the results of different researches on the same topic, and for estimating and explaining their diversity^[50,51].

We found that Pro/Pro genotype had a 1.38-fold statistically significant increased risk of liver cancer in this meta-analysis. When stratifying for race, patients with liver cancer had a significantly higher frequency of Pro/Pro genotype than individuals without cancer among Asians. When stratifying the various studies by control source, gender, family history of liver cancer and chronic hepatitis virus infection, we found that (1) patients in hospital-based studies had a significantly higher frequency of Pro/Pro and a significantly lower frequency of Arg/Arg genotype than patients without cancer; (2) female patients with liver cancer had a significantly lower frequency of Arg/Arg and a higher frequency of Pro/Arg+Pro/Pro genotypes than female individuals without cancer; (3) subgroup analyses for family history of liver cancer did not reveal any significant association between p53 codon 72 polymorphism and liver cancer development; and (4) patients with negative hepatitis virus infection had a significantly higher frequency of Pro/Pro and a significantly lower frequency of Arg/Arg genotype than individuals without cancer.

A number of studies have shown significant differences in the biochemical properties of the p53 protein, depending on the particular polymorphic form. It has been shown that the Arg/Arg and Pro/Pro variants differ in binding activity, transcriptional activation, apoptosis induction and cell cycle arrest^[13,52]. The p53 Arg variant induces apoptosis faster and more efficiently than the p53 Pro variant^[13]. One explanation of such higher apoptotic potential is the greater ability of the Arg variant to localize to the mitochondria; this localization is accompanied by the proapoptotic release of cytochrome C into the cytosol^[13]. In addition, p53 Arg72 is more active

than p53 Pro72 in the induction of apoptosis through a transcription-dependant pathway. Pim *et al.*^[53] also found that the Arg72 form of p53 is significantly more efficient than the Pro72 form in inducing apoptosis. In contrast, the Pro72 form appears to induce a higher level of G1 arrest than the Arg72 form. These data indicate that the two polymorphic variants of p53 are functionally distinct, and these differences may influence cancer risk. From our meta-analyses, we found that patients with liver cancer had a significantly higher frequency of Pro/Pro than non-cancer patients ($P = 0.004$), which can be explained by the points of view mentioned above.

Another major finding of this study was the different associations of p53 codon 72 gene polymorphism with the risk of liver cancer based on race. In fact, race-specific variation in the distribution of genotypes in the p53 codon 72 polymorphism has been demonstrated^[54]. Because race may be related to the disease, either through common risk factors or other genes in linkage disequilibrium with p53, confounding by race, or population stratification, may lead to result bias in studies conducted on ethnically diverse populations that did not account for possible confounding^[55]. In this subgroup analysis, the frequency of Pro/Pro genotype showed distinct differences among Asians and Caucasians. The pooled OR associated with p53 codon 72 gene polymorphism was statistically significant among Asians, but not in Caucasians. The discrepancy might be due to genetic background and/or environmental exposure differences.

Results of meta-analyses often depend on control selection procedures^[56]. Arg/Arg and Pro/Pro genotype frequency might be different between the two control sources (hospital-based and population-based) (Table 1). In subgroup analysis stratified by the different study designs, the hospital-based controls resulted in a significantly stronger association between p53 Arg72 Pro polymorphism and development of liver cancer than population-based controls.

It is widely accepted that family history of liver cancer and chronic hepatitis virus infection are obvious risk factors for development of liver cancer. By pooling the available data that evaluated associations and interaction between p53 Arg72 Pro genotype and family history of liver cancer risk, the p53 Arg72 Pro genotype was not found to be associated with increased risk of liver cancer in those either with or without family history of liver cancer. Interestingly, we found that patients with negative hepatitis virus infection were at higher risk for liver cancer than patients with positive hepatitis virus infection. One explanation for the preferentially increased liver cancer risk of the p53 Arg72Pro polymorphism among hepatitis virus-negative but not hepatitis virus-positive subjects, is that the effect of the Pro allele may be concealed by chronic HBV infection since the relative risk of HCC among chronic HBV carriers is 10-200-folds higher than among non-carriers^[57,58]. Moreover, we demonstrated that there is an association between p53 Arg72Pro and enhanced risk of liver cancer in female patients. Such differences between

men and women have already been reported in colorectal cancer, which were explained by exogenous hormones intake^[18]. However, because of the limited study sample size, these results should be interpreted with caution.

However, there are still some limitations in this meta-analysis: (1) only published studies were included in the meta-analysis; therefore, publication bias may have occurred, even though the use of a statistical test did not show it; (2) these results should be interpreted with caution because the population from five countries and controls were not uniform; (3) the number of cases and controls in the included studies was low; and (4) meta-analysis is a retrospective research that is subject to the methodological limitations. In order to minimize the bias, we developed a detailed protocol before initiating the study, and performed a meticulous search for published studies using explicit methods for study selection, data extraction and data analysis. Nevertheless, our results still should be interpreted with caution.

In conclusion, this meta-analysis suggests that the p53 codon 72 polymorphism may be associated with liver cancer, and that difference in genotype distribution may be associated with race, gender and chronic hepatitis virus status of patients. Due to limited number of cases in this analysis, it is critical that larger and well-designed multicenter studies based on the same ethnic group are needed to confirm our results.

COMMENTS

Background

The wild-type p53 gene exhibits a polymorphism at codon 72 in exon 4, with a single nucleotide change that causes a substitution of proline for arginine (Arg72Pro). This change has been implicated as a risk factor for liver cancer, but individual studies have been inconclusive or controversial. The aim of this meta-analysis was to clarify the effect of p53 Arg72Pro polymorphism on the risk of liver cancer.

Research frontiers

There have been many studies on the association between p53 genetic polymorphism Arg72Pro and liver cancer risk, but no meta-analysis has been conducted.

Innovations and breakthroughs

To the best of our knowledge, this is the first systematic review that has investigated the association between p53 codon 72 polymorphisms and liver cancer. This meta-analysis revealed that the p53 codon 72 polymorphism may be associated with liver cancer among Asians.

Applications

It can be seen from this paper that p53 polymorphism Arg72Pro could alter the susceptibility to liver cancer. It suggests that, even a common variant in the functional region of a definitively meaningful gene had an effect on human diseases, such as cancer.

Terminology

Meta-analysis is a means of increasing the effective sample size under investigation through the pooling of data from individual association studies, thus enhancing the statistical power of the analysis.

Peer review

The authors analyzed the association between p53 codon 72 polymorphism and liver cancer susceptibility in humans through this meta-analysis, and quantitatively summarized the evidence for such a relationship. They found that patients with liver cancer had a significantly higher frequency of Pro/Pro than non-cancer patients among Asians. Female patients with liver cancer had a significantly lower frequency of Arg/Arg and a higher frequency of Pro/Arg+Pro/Pro than female individuals without cancer.

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Metastasis-associated protein 1 induces VEGF-C and facilitates lymphangiogenesis in colorectal cancer

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Abstract

AIM: To study the correlation between high metastasis-associated protein 1 (MTA1) expression and lymphangiogenesis in colorectal cancer (CRC) and its role in production of vascular endothelial growth factor-C (VEGF-C).

METHODS: Impact of high MTA1 and VEGF-C expression levels on disease progression and lymphovascular

density (LVD, D2-40-immunolabeled) in 81 cases of human CRC was evaluated by immunohistochemistry. VEGF-C mRNA and protein expressions in human LoVo and HCT116 cell lines were detected by real-time polymerase chain reaction and Western blotting, respectively, with a stable expression vector or siRNA.

RESULTS: The elevated MTA1 and VEGF-C expression levels were correlated with lymph node metastasis and Dukes stages ($P < 0.05$). Additionally, high MTA1 expression level was correlated with a large tumor size ($P < 0.05$). A significant correlation was found between MTA1 and VEGF-C protein expressions in tumor cells ($r = 0.371$, $P < 0.05$). Similar to the VEGF-C expression level, high MTA1 expression level was correlated with high LVD in CRC ($P < 0.05$). Furthermore, over-expression of MTA1 significantly enhanced the VEGF-C mRNA and protein expression levels, whereas siRNAs - knocked down MTA1 decreased the VEGF-C expression level.

CONCLUSION: MTA1, as a regulator of tumor-associated lymphangiogenesis, promotes lymphangiogenesis in CRC by mediating the VEGF-C expression.

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Key words: Metastasis-associated protein 1; Vascular endothelial growth factor-C; Lymphangiogenesis; Colorectal cancer

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INTRODUCTION

Lymphatic metastasis is one of the most important metastatic routes of epithelial cancer including colorectal cancer (CRC)^[1]. Excessive formation of new lymphatics (lymphangiogenesis) in CRC is a key process for lymphatic metastasis, and lymphatic vessel density (LVD) is an indicator for lymphangiogenesis^[1-3]. Metastasis-associated protein 1 (MTA1) is expressed in a wide range of epithelial cancers and plays a crucial role in tumor metastasis^[4]. MTA1 has been established as the only single gene showing consistently increased in lymph node metastases of head and neck squamous cell carcinoma^[5]. A large number of clinicopathological studies show that elevated MTA1 is correlated with lymph node metastasis of epithelial cancer^[4,6-9]. These data indicate that MTA1 is involved in lymphatic metastasis of epithelial cancer. However, whether the pro-metastatic effects of MTA1 are mediated by promoting lymphangiogenesis remains unknown.

MTA1, as a part of the nucleosome remodeling and deacetylation complex (NuRD), promotes metastasis of cancer by regulating many tumor-associated genes^[4,9]. For example, MTA1 physically binds to hypoxia-inducible factor-1 α (HIF-1 α) and increases its transcriptional activity, leading to enhanced expression of vascular endothelial growth factor (VEGF)-A which is associated with metastasis^[10,11]. VEGF-C is another member of the VEGF family and the major regulatory factor for tumor lymphangiogenesis^[1,2]. Similar to VEGF-A, VEGF-C is also directly induced by HIF-1 α ^[12-14], and therefore, is a potential target molecule of MTA1. Thus, whether MTA1 can promote lymphangiogenesis by up-regulating the expression of VEGF-C in CRC is unknown and worthy of further study.

In this study, the expression of MTA1 was correlated with VEGF-C in CRC specimens. MTA1 was related with high LVD, indicating that MTA1 promotes lymphangiogenesis and thus induces metastasis of tumor. Furthermore, MTA1 mediated VEGF-C expression, suggesting MTA1 can promote lymphangiogenesis by increasing the VEGF-C expression level in CRC.

MATERIALS AND METHODS

Colorectal cancer samples

Eighty-one CRC tissue specimens, provided by Department of Pathology, First Affiliated Hospital of Jinan University, were fixed in 10% buffered formalin for 24-48 h, embedded in paraffin wax, and then cut into sections which were stained with hematoxylin and eosin and reviewed twice by two experienced pathologists to verify the diagnosis, histological grade and type, based on the system of the Union for International Cancer Control (UICC). A synopsis of the clinicopathological parameters is provided in Table 1.

Immunohistochemical staining

The sections of CRC tissue specimen were stained with immunohistochemistry (IHC) using a streptavidin-peroxidase

Table 1 Correlation of metastasis-associated protein 1 and vascular endothelial growth factor-C immunohistochemistry with clinicopathological parameters of patients

Parameters of patients	n	MTA1			VEGF-C		
		High	Low	P value	High	Low	P value
Age							
≤ 60	34	11	23	0.805	16	18	0.552
> 60	47	14	33		19	28	
Gender							
Male	45	17	28	0.132	21	24	0.483
Female	36	8	28		14	22	
Tumor Size							
≤ 5 cm	46	10	36	0.042	18	28	0.395
> 5 cm	35	15	20		17	18	
Location							
Colon	47	16	31	0.467	18	29	0.294
Rectum	34	9	25		17	17	
Depth of invasion							
T1 + T2	15	3	12	0.313	4	11	0.152
T3 + T4	66	22	44		31	35	
Differentiation							
Well, moderately	59	15	44	0.083	24	35	0.451
Poorly	22	10	12		11	11	
Dukes stages							
A + B	41	8	33	0.004	10	31	0.001
C + D	40	17	23		25	15	
Lymph node metastasis							
Yes	40	17	23	0.025	24	16	0.003
No	41	8	33		11	30	

MTA1: Metastasis-associated protein 1; VEGF-C: Vascular endothelial growth factor-C.

technique (Beijing Zhong Shan Golden Bridge Biological Technology Co., Ltd., China). Briefly, the sections were incubated in methanol/H₂O₂ for 30 min to inhibit the endogenous peroxidase activity, washed with PBS for 5 min and blocked with normal goat serum for 20 min at room temperature. The sections were incubated with antibodies against MTA1 (sc-9446; Santa Cruz Biotechnology Inc., CA, USA) or VEGF-C (sc-7133; Santa Cruz Biotechnology Inc., CA, USA) overnight at 4°C, then with biotinylated secondary antibody for 1 h at room temperature and avidin-conjugated peroxidase for 45 min at room temperature. The sections were washed three times with PBS between each step. Peroxidase was stained with diaminobenzidine (1 mg/mL) and H₂O₂ for 5 min and washed with tap water for 10 min. The sections were counterstained with hematoxylin for 1 min. PBS was used as a negative control instead of primary antibody.

Evaluation of immunostaining

Two investigators who were blinded to the patient outcomes and all clinicopathological findings examined the stainings independently. Positive expression of MTA1 and VEGF-C was detected by estimating the staining intensity and the percentage of tumor cells showing specific immunoreactivity. Samples with 10% tumor cells were defined as positive. The intensity score was defined as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong

staining), respectively, as previously described^[15,16]. Tumors with a score > 2 (moderate and strong expression) showed a high expression level of MTA1 and VEGF-C^[15,16].

Assessment of LVD

LVD in CRC tissue sections that were single-stained for D2-40 was quantitatively analyzed as previously described^[13,17]. Tumoral LVD located at the periphery of tissue, within 2 mm of the tumor and adjacent to the invasive front was assessed. Five areas with most lymphatic regions ("hot spots") were chosen by light microscopy at 40 × magnification. LVD was assessed by counting all stained vessels at 200 × magnification. The assessed mean number of lymphatics was determined and expressed as LVD^[17,18]. The LVD was scored and counted by two investigators independently who were blinded to clinical information about the patients.

Cell culture

Human CRC cell lines, HCT116 and LoVo, purchased from Center of Experimental Animals, Sun Yat-Sen University (Guangzhou, China), were cultured in Dulbecco's modified Eagle's medium (Life Technologies Corporation, CA, USA) containing 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum in a humidified atmosphere containing 5 mL CO₂ at 37°C.

RNA interference

Two 21-nucleotide MTA1 siRNAs (si-MTA1#1 and si-MTA1#2) were designed to target the sequences 5'-GGAGAAUCGAGGAGCUACA-3' and 5'-GCAUCAUUGAGUACUACUACA-3' of MTA1 mRNA (NCBI accession number: NM_004689.3). BLAST searches indicated that the targeted regions had no significant homology with any other genes. A non-targeting siRNA was used as a negative control (NC, 5'-UUCUCCGAACGUGUCACGUTT-3') in all siRNA transfection experiments. All siRNAs, purchased from Shanghai GenePharma Co., Ltd (Shanghai, China) were transfected twice at 24-h intervals with Oligofectamine reagent (Life Technologies Corporation, CA, USA) according to its manufacturer's protocol^[19].

Plasmid and transfection

Plasmid MTA1-EGFP-C1 (a gift from Professor Chen Anman at Huazhong University of Science and Technology, China)^[20] and plasmid EGFP-C1 vector (as a blank control) was transfected into LoVo cells with Oligofectamine reagent (Life Technologies Corporation, CA, USA) according to its manufacturer's protocol. Medium was supplemented with 400-800 µg/mL G418 (Life Technologies Corporation, CA, USA) to screen for positive cells, then with 400 µg/mL G418 after two weeks. Positive cell clones were isolated by ring chining and further expanded for genetic and functional characterization. Expression of MTA1 was detected by real-time polymerase chain reaction (PCR) and Western blotting, respectively.

Real-time PCR

MTA1 and VEGF-C mRNA was analyzed by real-time PCR as previously described^[19]. Briefly, total RNA was extracted from cells using TRIzol (Life Technologies Corporation, CA, USA). First strand cDNA was synthesized from mRNA using a Primescript™ RT reagent kit (TaKaRa, Tokyo, Japan). Real-time PCR was carried out using the SYBR Premix ExTaq™ (TaKaRa, Tokyo, Japan) according to its manufacturer's instructions. The input was normalized by GAPDH mRNA. Gene-specific primers are as follows: 5'-AGCTACGAGCAGCACAACGGGGT-3' (forward primer for mta1) and 5'-CACGCTTGGTTTCCGAGGAT-3' (reverse primer, 289 bp), 5'-AACCTCATGTGTGTCCGTC-3' (forward primer for vegf-c) and 5'-TGGCAAACTGATTGTTACTGG-3' (reverse primer, 156 bp), 5'-ACAGTCCATGCCATCACTGCC-3' (forward primer for GAPDH) and 5'-GCCTGCTTCACACCTTCTTG-3' (reverse primer, 266 bp). Experiments were performed in triplicate, with the results represented as mean ± SE.

Western blotting

MTA1 and VEGF-C protein expression was detected by Western blotting as previously described^[21]. Briefly, the cells were lysed in a RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, and 50 mM TrisCl, pH = 7.5) containing protease inhibitors. After separated by SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes and subjected to immunoblotting with antibodies against MTA1 (Santa Cruz Biochemistry, CA, USA), VEGF-C (Santa Cruz Biochemistry, CA, USA) and tubulin (Sigma-Aldrich, Inc., MI, USA) at 4°C overnight. After washed, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using the ECL chemiluminescence system (Thermo Fisher Scientific Inc., MA, USA).

Statistical analysis

Statistical analysis was carried out using the SPSS 13.0 for Windows. Pearson's χ^2 test was used to examine the relation between immunostaining scores of two different markers within different clinicopathological subgroups. Spearman's rank-order correlation was used to test immunostaining scores of the significant association between MTA1 and VEGF-C. Student's *t*-test was used to determine differences in two data sets, including the effect of LVD and siRNA treatment or gene transfection on gene expression. All tests were two-tailed and *P* < 0.05 was considered statistically significant.

RESULTS

Immunohistochemistry for MTA1 and VEGF-C expression in CRC tissue specimens

The expression of MTA1 and VEGF-C protein was detected in CRC tissue samples with IHC staining and the correlation between their expression and clinicopathological parameters was further analyzed. The MTA1 immu-

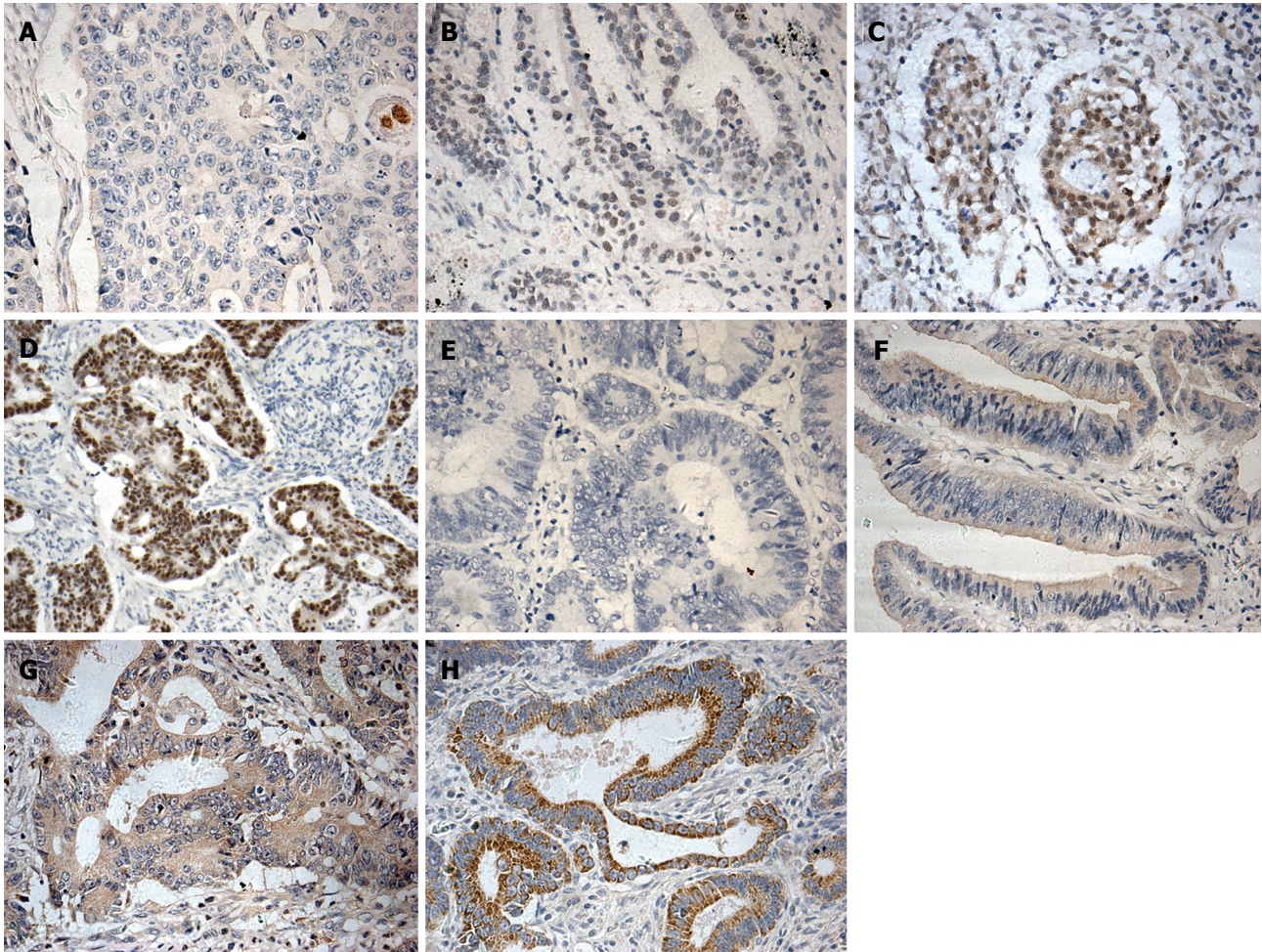


Figure 1 Immunohistochemical labeling for metastasis-associated protein 1, vascular endothelial growth factor-C in colorectal cancer. Metastasis-associated protein 1 (MTA1) (A-D) and VEGF-C (E-H) expressions are indicated as score 0 (A and E), score 1 (B and F), score 2 (C and G), and score 3 (D and H), respectively. MTA1 showing extremely weak staining of cytoplasm and intense staining of nuclei, while VEGF-C showing staining of cytoplasm of tumor cells ($\times 200$).

noreactivity was detected primarily in nuclei and weakly in cytoplasm of tumor cells with with IHC staining (Figure 1A-D), whereas positive VEGF-C staining was observed in cytoplasm (Figure 1E-H). However, faint staining of VEGF-C was occasionally observed in normal epithelial cells and stromal components showed, particularly in adjacent stromal endothelial cells, which is consistent with the reported findings^[16,22,23].

The correlation between the MTA1 and VEGF-C expression and the clinicopathologic parameters is shown in Table 1. High MTA1 expression level was observed in 25 (30.8%) of the 81 tumor tissue samples, while high VEGF-C expression level was found in 35 (43.2%) of the 81 tumor tissue samples. The MTA1 and VEGF-C expressions were correlated with lymph node metastasis and Dukes stages ($P < 0.05$). Additionally, the high MTA1 expression level was correlated with a large tumor size ($P < 0.05$). Neither MTA1 nor VEGF-C was correlated with tumor location, differentiation, infiltration, and sex or age of the patients.

A significant correlation was found between MTA1 and VEGF-C protein expressions in tumor cells ($r = 0.371$, $P < 0.05$, Table 2), indicating that the high MTA1 expression level can facilitate lymphatic metastasis of CRC.

Table 2 Correlation between expression levels of metastasis-associated protein 1 and vascular endothelial growth factor-C in human colorectal cancer

MTA1 ^a	VEGF-C ^a			
	Score 0	Score 1	Score 2	Score 3
Score 0	10 ¹	8	5	2
Score 1	8	13	6	4
Score 2	4	2	8	1
Score 3	0	1	3	6

^a $P = 0.001$, $r = 0.371$, Spearman's coefficient of correlation. ¹Number of cases. MTA1: Metastasis-associated protein 1; VEGF-C: Vascular endothelial growth factor-C.

Correlation between high MTA1 and VEGF-C expression level and LVD in CRC

It has been reported that high peritumoral LVD is an independent risk factor for lymphangiogenesis^[3,24]. Immunostaining of D2-40 is a specific marker for evaluation of lymphatic invasion and lymphatic microvessel density in human cancers^[3,24,25]. To further determine whether MTA1 plays a role in lymphangiogenesis, the correlation between the

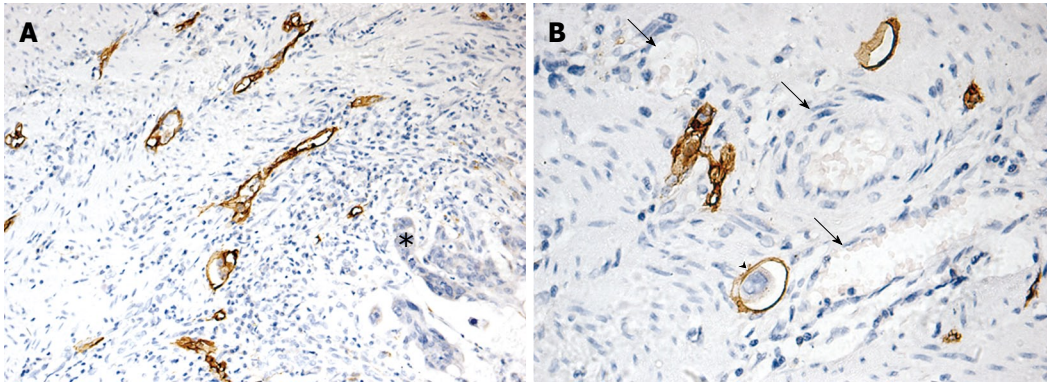


Figure 2 Morphological features of D2-40 positive lymphatic vessels in colorectal cancer. A: Positive D2-40 stained lymphatic vessels with thin walls and irregular shapes in peritumoral area (asterisk, $\times 100$); B: Positive D2-40 stained lymphatic vessel containing tumor emboli within tumor mass (arrow head) and D2-40-negative erythrocytes (black arrows, $\times 200$).

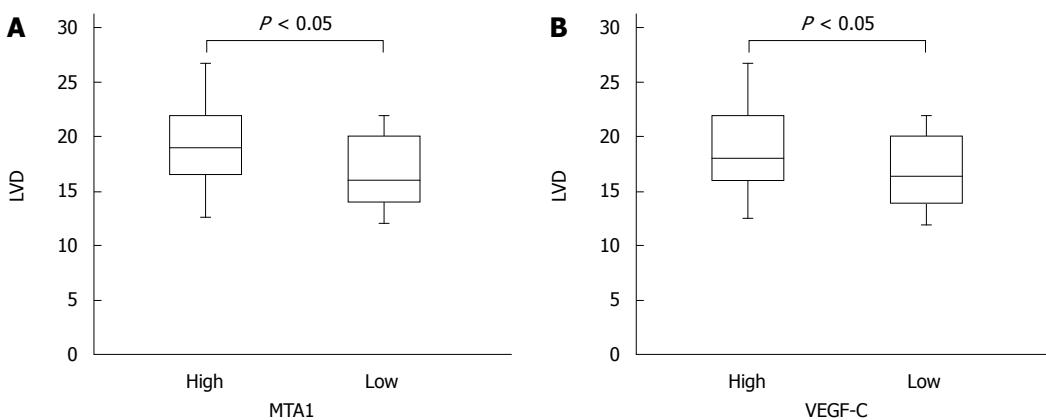


Figure 3 Correlation between metastasis-associated protein 1 or vascular endothelial growth factor-C expression and lymphovascular density. Analysis of subgroups with low and high metastasis-associated protein 1 (MTA1) and VEGF-C are presented by box bars. Tumors with higher MTA1 and VEGF-C expression showed significantly higher microvessel density than tumors with lower expression. LVD: Lymphovascular density. VEGF-C: Vascular endothelial growth factor-C.

expression of MTA1 and the quantity of D2-40 positive lymphatic vessels was analyzed in this study. The lymphatic vessels were lined with a single layer of D2-40 positive endothelial cells, while the adjacent blood vessels containing erythrocytes showed negative staining. Lymphatic vessels were distributed unevenly throughout the sections, and mostly located in the peritumoral area (Figure 2).

The LVD was 9–31 with a mean of 17.76. The mean LVD was 19.92 and 19.60, respectively, in patients with a high MTA-1 and VEGF-C expression level, and 16.37 and 16.80, respectively, in those with a low, MTA-1 and VEGF-C expression level ($P < 0.05$), indicating that MTA1 also promotes lymphangiogenesis of CRC as VEGF-C. The correlation between MTA1 and VEGF-C expression and LVD is shown in Figure 3.

MTA1 regulates the expression of VEGF-C in CRC cell lines

After a positive relation was established between MTA1 and VEGF-C expressions in CRC tissue specimens, whether MTA1 mediates VEGF-C expression in CRC cell lines was studied. HCT-116 cells with a high MTA1 level and LoVo cells with a low MTA1 level were used in our next experiments as previously described^[26,27]. The

expression of MTA1 and VEGF-C was detected in these two cell lines by real-time PCR and Western blotting, respectively. The MTA1 and VEGF-C mRNA and protein expression levels were significantly higher in HCT-116 cells than in LoVo cells (Figure 4A).

To determine whether MTA1 is responsible for the expression of VEGF-C, the MTA1 stable expression vector was used to mimic the MTA1 protein expression in LoVo cells, showing that the MTA1 stable expression vector significantly increased the MTA1 protein expression, while the VEGF-C protein expression level was remarkably up-regulated by the MTA1 stable expression vector compared to the mock and control vector (Figure 4B, left panel). Furthermore, over-expression of the MTA1 stable vector enhanced the VEGF-C mRNA expression level compared to the mock and control vector (Figure 4B, right panel), suggesting that MTA1 is required for VEGF-C expression.

To further specifically confirm the regulation of VEGF-C by MTA1, siRNA-mediated knockdown of MTA1 was employed. HCT116 cells were transfected with two siRNAs specific for MTA1. MTA1-targeted siRNAs specifically suppressed the MTA1 protein expression, while the level of an unrelated gene (like Tubulin) was unaffected.

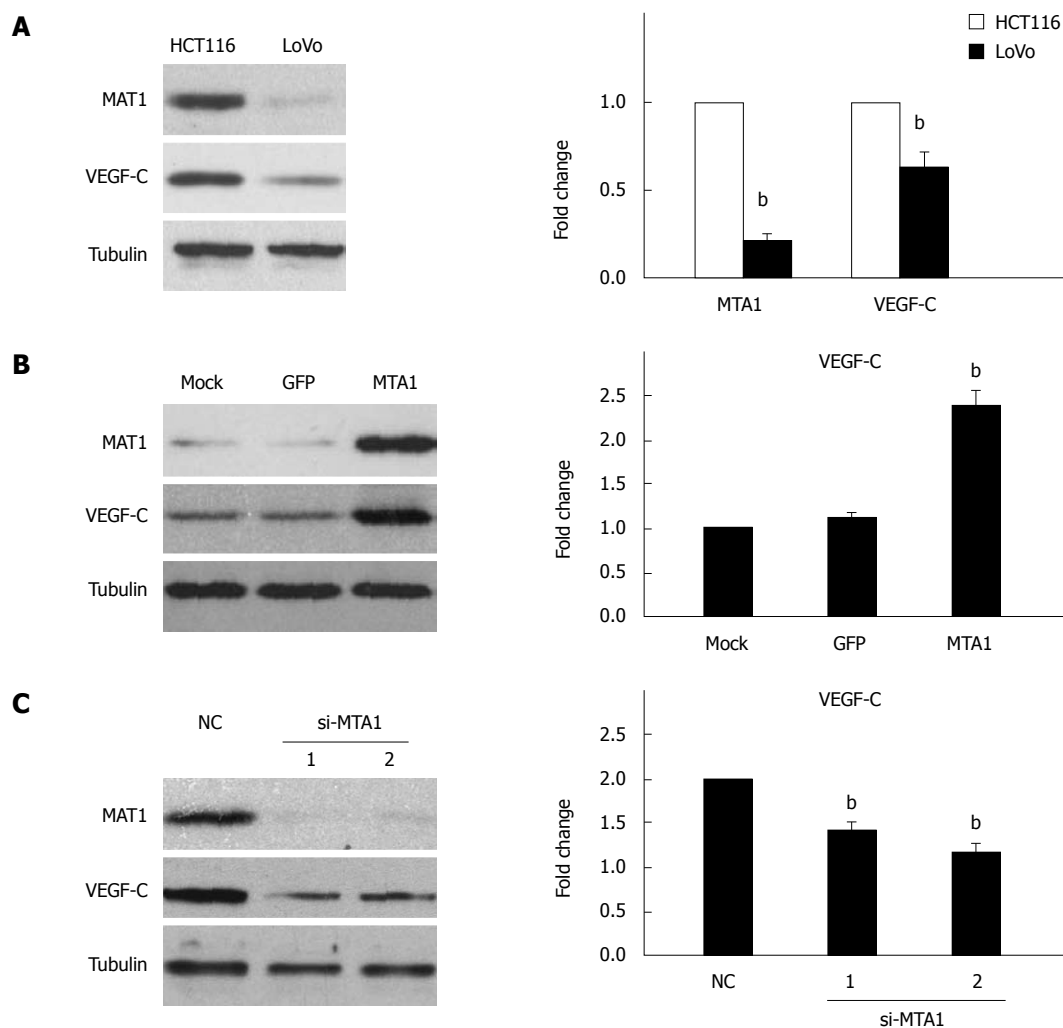


Figure 4 Metastasis-associated protein 1 regulates the expression of vascular endothelial growth factor-C in colorectal cancer cell lines. Western blotting (left panel) and real-time PCR (right panel) showing different expressions of metastasis-associated protein 1 (MTA1) and VEGF-C in HCT116 and LoVo cell lines (A), increased VEGF-C expression in LoVo cells due to overexpression of MTA1 (B), and decreased VEGF-C expression in HCT116 cells due to knocked down MTA1 (C). 1 and 2 indicate two different si-MTA1 oligonucleotide as showed in "Materials and Methods". All data represent three independent experiments \pm SE, with $n = 3$ and $^bP < 0.01$. VEGF-C: Vascular endothelial growth factor-C.

ed, verifying that the siRNAs have a high selectivity and efficacy (Figure 4C, left panel). Furthermore, the silencing of MTA1 endogenous expression significantly decreased the VEGF-C protein expression, whereas the RNA interference technique had no significant effect on the VEGF-C expression as revealed by the negative control siRNA (NC), (Figure 4C, left panel), indicating that MTA1 is necessary for VEGF-C expression. Furthermore, knockdown of MTA1 expression decreased the VEGF-C mRNA expression level (Figure 4C, right panel), suggesting that MTA1 mediates VEGF-C expression in CRC cell lines.

DISCUSSION

This study investigated whether the expression of VEGF-C regulated by MTA1 affects the lymphangiogenesis of colon cancer. IHC studies in CRC specimens and mechanistic studies with genetic manipulation in colon cancer cell lines, demonstrated that MTA1-mediated expression of VEGF-C

could facilitate the lymphangiogenesis of colon cancer.

MTA1 is associated with progression to the metastatic state in many epithelial cancers, including carcinomas of breast^[6,28], prostate^[22], and esophagus^[7], as well as head and neck squamous cell carcinoma^[5]. However, the relation between MTA1 and CRC metastasis is not clear. It has been showed that the expression of MTA1 mRNA in CRC is significantly correlated to the depth of invasion and lymph node metastasis^[29]. It was reported that the expression level of MTA1 mRNA is higher in colorectal cancer tissue than in its adjacent normal tissue^[30]. In this study, the MTA1 protein expression in CRC tissue specimens was evaluated with IHC staining, showing that the MTA1 expression level was significantly higher in primary CRC tumors with lymph node metastases than in those without lymph node metastases, which is consistent with the reported data^[29,30].

Hematogenous and lymphatic metastases are the two main forms of tumor metastasis to distant organs. Jang *et al.*^[31] examined MTA1 protein expression and microves-

sel density (MVD) in breast cancer tissue specimens and showed that MTA1 overexpression is significantly correlated with high MVD. The proangiogenic effects of MTA1 have also been observed in hepatocellular carcinoma^[32] and prostate cancer^[22]. In this study, the over-expression of MTA1 was significantly associated with the increased tumor LVD, indicating that MTA1 facilitates tumor metastasis by up-regulating angiogenesis and lymphangiogenesis.

The presence of VEGF-C, an independent predictor of poor survival, is associated with lymphangiogenesis^[17,23]. In this study, the expression of VEGF-C was correlated with lymphatic metastases and greater LVD in CRC, which is consistent with the reported findings^[1,23,27]. More importantly, the lymphangiogenesis up-regulated by MTA1 was related to the induction of VEGF-C, the expression of MTA1 in CRC tissue specimens was associated with that of VEGF-C, over-expression of MTA1 increased the expression level of VEGF-C, and knock-down of MTA1 reduced it in CRC cell lines, indicating that MTA1 mediates the induction of VEGF-C during lymphangiogenesis of CRC.

Although we demonstrated MTA1 could mediate VEGF-C expression, its precise mechanism remains to be defined. MTA1 regulates the expression of target genes by inhibiting or promoting their transcriptional activity. For example, MTA1 has a strong transcription repressing activity on estrogen receptor- α ^[33] and BRCA1^[34] (breast cancer type 1 susceptibility protein) by forming a complex with NuRD. However, other studies demonstrate that MTA1 stimulates the transcriptional activity of several gene promoters by interacting with RNA polymerase II^[35,36]. Moreover, the expression of MTA1 can be strongly induced under hypoxic conditions in breast cancer cell lines, and MTA1 over-expression increases the transcriptional activity and stability of HIF-1 α protein^[10,11]. Hypoxia is one of the most powerful inducers of lymphangiogenesis, and VEGF-C is a target molecule of HIF-1 α ^[13,14,37,38]. Therefore, MTA1 can promote VEGF-C expression through the HIF-1 α pathways. The precise mechanism involved in MTA1 targeting of VEGF-C should be further studied.

In conclusion, MTA1 plays a role in lymphangiogenesis of human CRC by up-regulating VEGF-C, a key regulator of lymphangiogenic factors, thus providing a novel mechanism underlying the relation between the high MTA1 level, cancer metastasis and poor outcome. Therefore, MTA1 is a potentially novel target for the treatment of lymphatic metastasis of human CRC.

COMMENTS

Background

Excessive formation of new lymphatics (lymphangiogenesis) in colorectal cancer (CRC) facilitates metastasis of cancer cells to lymph nodes and distant organs. Metastasis-associated protein 1 (MTA1) is expressed in a wide range of epithelial cancers including CRC, and plays a crucial role in their metastasis. Vascular endothelial growth factor-C (VEGF-C) is the major regulatory factor for CRC lymphangiogenesis.

Research frontiers

MTA1 is involved in regulation of VEGF family during metastasis. Whether MTA1 promotes lymphangiogenesis by up-regulating the expression of VEGF-C

in CRC needs to be further studied.

Innovations and breakthroughs

This is the first study to demonstrate that MTA1 plays a role in lymphangiogenesis by up-regulating VEGF-C, thus providing a novel mechanism of MTA1 underlying the promotion of CRC metastasis.

Applications

The findings in this study are of value in explanation of the mechanism of CRC lymphangiogenesis. MTA1 may be used as a new potential target for the treatment of lymphatic metastasis of human CRC, although further animal experiments are required to confirm its activity in promoting lymphangiogenesis of CRC.

Peer review

It is a well written manuscript with promising results that may be the basis of forthcoming new research in CRC biology and therapy. The correlation between MTA1 and VEGF-C is of great clinical importance, as new therapeutic targets may be identified based on the results of this study.

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TP53 Arg72Pro polymorphism is associated with esophageal cancer risk: A meta-analysis

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genotypes for Arg/Arg + Arg/Pro vs Pro/Pro (OR = 0.73, 95% CI: 0.57-0.94, $P = 0.014$). Subgroup analyses according to the source of controls and the specimens used for determining *TP53* Arg72Pro genotypes or sample size showed that significantly reduced risk was observed only in studies which have population-based controls (Arg/Arg vs Pro/Pro: OR = 0.56, 95% CI: 0.47-0.66, $P < 0.001$), and use white blood cells or normal tissue to assess *TP53* genotypes of cases (Arg/Arg vs Pro/Pro: OR = 0.56, 95% CI: 0.47-0.65, $P < 0.001$) or include at least 200 subjects (Arg/Arg vs Pro/Pro: OR = 0.56, 95% CI: 0.47-0.65, $P < 0.001$). Analysis restricted to well-designed studies also supported the significantly decreased risk of EC (Arg/Arg vs Pro/Pro: OR = 0.54, 95% CI: 0.46-0.64, $P < 0.001$).

CONCLUSION: *TP53* Arg72 carriers are significantly associated with decreased EC risk. Nevertheless, more well-designed studies are needed to confirm our findings.

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Key words: *TP53*; Codon 72; Polymorphism; Esophageal cancer; Meta-analysis

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Abstract

AIM: To investigate the association between *TP53* Arg72Pro polymorphism and esophageal cancer (EC) risk using meta-analysis.

METHODS: All eligible studies published before March 1, 2010 were selected by searching PubMed using keywords "p53" or "*TP53*", "polymorphism" or "variation", "esophageal" and "cancer" or "carcinoma". Crude odds ratios (ORs) with 95% confidence intervals (CIs) were assessed for EC risk associated with *TP53* Arg72Pro polymorphism using fixed- and random-effects models.

RESULTS: Nine case-control studies involving 5545 subjects were included in this meta-analysis. Significantly reduced risk of EC was associated with *TP53*

INTRODUCTION

Esophageal cancer (EC) is the eighth most common can-

cer and sixth most deadly cancer worldwide. China and southern and eastern Africa are the relatively high risk areas^[1,2]. There are two main forms of EC histologically: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EA). ESCC constitutes the majority (over 90%) of EC, but the incidence rates of EA have sharply increased in many Western countries recently^[3-5]. The development of EC is a multifactorial process associated with a variety of risk factors. The two major risk factors of EC are tobacco smoking and alcohol drinking^[6-8]. Inherited predisposition may also explain the high rates of EC^[9].

TP53 is a major regulator of the cell response to stress and serves as a tumor suppressor by inducing cell cycle arrest or apoptosis^[10]. Inactivation of the TP53 signaling pathway has been seen in most human cancers^[11]. Previously, polymorphisms of TP53 have been reported to be the possible risk factors for some kinds of tumors^[12]. The most common polymorphism of TP53 is at the 72nd amino acid residue, with an arginine (Arg) to proline (Pro) change because of a G→C transverse^[13]. Differences in the biochemical or biological characteristics of these wild-type TP53 variants have been reported^[14]. The Arg72 variant can better induce apoptosis than the Pro72 variant, indicating that the two polymorphic variants of TP53 are functionally distinct, which may influence the cancer risk or treatment^[15].

A number of studies have reported the role of TP53 Arg72Pro polymorphism in cancers such as cervical cancer^[16], lung cancer^[17], breast cancer^[18], and gastric cancer^[19], but little is known about the association of TP53 polymorphism with EC. In recent years, several studies focused on the association between TP53 Arg72Pro polymorphism and EC susceptibility, with inconsistent results^[20-29]. Hence, we performed a meta-analysis of all eligible studies to estimate the association between TP53 polymorphism and the risk of EC.

MATERIALS AND METHODS

Publication search

We searched the articles using the terms “p53” or “TP53”, “polymorphism” or “variation”, “esophageal” and “cancer” or “carcinoma” in Medline database utilizing the PubMed engine, and all eligible studies were published before March 1, 2010. We evaluated all associated publications to retrieve the most eligible literatures. Their reference lists were hand-searched to find other relevant publications. Articles were limited to English language papers.

Inclusion and exclusion criteria

The following inclusion criteria were used to select literatures for the meta-analysis: (1) published in peer-reviewed journals; (2) articles about TP53 Arg72Pro polymorphism and risk of EC; and (3) containing useful genotype frequencies. The exclusion criteria were: (1) none-case-control studies; (2) control population including malignant tumor patients; (3) the genotype frequen-

cies of control group departing from Hardy-Weinberg equilibrium (HWE); and (4) duplicated publications.

Data extraction

Two investigators (Jiang and Yao) reviewed and extracted information from all eligible publications independently, according to the inclusion and exclusion criteria listed above. An agreement was reached by discussion between the two reviewers whenever there was a conflict. The following items were collected from each study: first author's surname, year of publication, country of origin, ethnicity, source of controls, specimens used for assessment of TP53 Arg72Pro genotypes, total number of cases and controls as well as numbers of cases and controls with Arg/Arg, Arg/Pro and Pro/Pro genotypes, respectively.

Statistic analysis

Crude odds ratios (ORs) with 95% confidence intervals (CIs) were used to assess the association between TP53 Arg72Pro polymorphism and EC risk. The pooled ORs were performed for homozygote comparison (Arg/Arg *vs* Pro/Pro), dominant model (Arg/Arg + Arg/Pro *vs* Pro/Pro), and recessive model (Arg/Arg *vs* Arg/Pro + Pro/Pro), respectively. Stratified analyses were performed based on the source of controls, the specimens used for determining TP53 Arg72Pro genotypes and sample size (cases and controls in total). A Chi-square-based *Q*-test was performed to check the heterogeneity^[30]. If $P \geq 0.1$ was obtained in the heterogeneity test, ORs were pooled according to the fixed-effects model (the Mantel-Haenszel model)^[31], otherwise the random-effects model (the DerSimonian and Laird model) was used^[32]. One-way sensitivity analyses were performed to evaluate the stability of the meta-analysis results^[33]. The potential publication bias was estimated using Egger's linear regression test by visual inspection of the Funnel plot. $P < 0.05$ was considered statistically significant in publication bias^[34]. If publication bias existed, the Duval and Tweedie non-parametric “trim and fill” method was used to adjust for it^[35]. All statistical tests were performed with the software STATA version 10.0 (Stata Corporation, College station, TX).

RESULTS

Study characteristics

Eighteen studies were identified through literature search and selection based on the inclusion criteria. By the extraction of data, seven articles which are not case-control studies and one review article were excluded. Among the remaining 10 studies, one study^[29] was excluded due to the genotype frequencies of controls deviated from HWE. In one study^[24], two groups of controls (a high risk population and a low risk population) were used. However, the genotype frequencies of the low-risk population controls deviated from HWE. Therefore, only the high-risk population controls of this study were included in the final analysis.

The characteristics of nine eligible case-control studies are summarized in Table 1. The sample size of the 9

Table 1 Main characteristics of included studies in the meta-analysis

First author (yr)	Country	Ethnicity	Source of controls	Specimens	Sample size (case/control)
Lee ^[20] (2000)	China (Taiwan)	Asian	Hospital	White blood cells	90/254
Peixoto ^[21] (2001)	China	Asian	Population	Exfoliated esophageal cells	32/57
Hamajima ^[22] (2002)	Japan	Asian	Hospital	White blood cells	102/241
Li ^[23] (2002)	China	Asian	Population/blood donors	Tumor tissue	62/131
Hu ^[24] (2003)	China	Asian	Population	White blood cells	120/130
Vos ^[25] (2003)	South Africa	African	Unknown	Tumor tissue, White blood cells	73/115
Hong ^[26] (2005)	China	Asian	Population	Normal Esophageal tissue	758/1420
Cai ^[27] (2006)	China	Asian	Population	White blood cells	204/389
Shao ^[28] (2008)	China	Asian	Population	White blood cells	673/694

Table 2 Distribution of *TP53* Arg72Pro genotypes among esophageal cancer cases and controls included in the meta-analysis *n* (%)

First author (yr)	Cases			Controls		
	Arg/Arg	Arg/Pro	Pro/Pro	Arg/Arg	Arg/Pro	Pro/Pro
Lee ^[20] (2000)	20 (22.2)	46 (51.1)	24 (26.7)	94 (37)	116 (45.7)	44 (17.3)
Peixoto ^[21] (2001)	8 (25)	13 (40.6)	11 (34.4)	9 (15.8)	24 (42.1)	24 (42.1)
Hamajima ^[22] (2002)	37 (36.3)	51 (50)	14 (13.7)	91 (37.8)	107 (44.4)	43 (17.8)
Li ^[23] (2002)	27 (43.5)	21 (33.9)	14 (22.6)	29 (22.1)	67 (51.1)	35 (26.7)
Hu ^[24] (2003)	29 (24.2)	60 (50)	32 (26.7)	38 (29.2)	68 (52.3)	24 (18.5)
Vos ^[25] (2003)	26 (35.6)	42 (57.5)	5 (6.8)	37 (32.2)	62 (53.9)	16 (13.9)
Hong ^[26] (2005)	199 (26.3)	340 (44.9)	219 (28.9)	425 (29.9)	731 (51.5)	264 (18.6)
Cai ^[27] (2006)	41 (20.1)	89 (43.6)	74 (36.3)	117 (30.1)	178 (45.8)	94 (24.2)
Shao ^[28] (2008)	163 (24.2)	306 (45.5)	204 (30.3)	195 (28.1)	366 (52.7)	133 (19.2)

Arg: Arginine; Pro: Proline.

studies ranged from 89 to 2178. In total, 2114 EC cases and 3431 controls were included in the meta-analysis. Distribution of *TP53* genotype frequencies among EC cases and controls of the nine studies are shown in Table 2. The frequencies of heterozygote genotype among the cases of the studies using the specimens of exfoliated esophageal cells^[21] or tumor tissues^[23] were obviously lower than those of other studies. In studies with at least 200 samples, there was not a wide variation of Arg72 and Pro72 allele frequencies among controls, with the Arg72 allele frequencies ranging from 53% to 60%^[20,22,24,26-28]. But in studies with less than 200 samples, the control groups represented diverse frequencies of the Arg72 allele, which were 37%^[21], 48%^[23] and 59%^[25], respectively.

Meta-analysis results

When all the eligible studies were pooled into the meta-analysis, evidence was found in an association between significantly decreased EC risk and the variant genotypes of *TP53* in the dominant model (OR = 0.73, 95% CI: 0.57-0.94, *P* = 0.014, Table 3). However, significant inter-study heterogeneity existed in all genetic models (Table 3). In order to figure out the main reasons of the heterogeneity among studies and obtain exact consequence on the relationship between *TP53* Arg72Pro polymorphism and EC susceptibility, stratified analyses were then performed.

In stratified analysis according to the source of controls, significant association between reduced EC risk and *TP53* genotypes was found solely in subgroup of studies

with population-based controls in all genetic models (homozygote comparison: OR = 0.56, 95% CI: 0.47-0.66, *P* < 0.001; dominant model: OR = 0.57, 95% CI: 0.50-0.66, *P* < 0.001; recessive model: OR = 0.80, 95% CI: 0.70-0.92, *P* = 0.001; Table 3). Significantly increased EC risk, however, was observed in the subgroup of a study with different source of controls selected from population and blood donors in homozygote comparison (OR = 2.33, 95% CI: 1.03-5.24, *P* = 0.041) and recessive model (OR = 2.71, 95% CI: 1.42-5.02, *P* = 0.003, Table 3). No evidence of association was observed in studies without clear presentation of hospital-based controls or the source of controls (Table 3).

We divided the included studies into four subgroups according to the specimens used. As a result, significantly reduced EC risk was found only in subgroups where white blood cells or normal tissue were used to determine *TP53* genotypes in different genetic models (homozygote comparison: OR = 0.56, 95% CI: 0.47-0.65, *P* < 0.001; dominant model: OR = 0.58, 95% CI: 0.51-0.67, *P* < 0.001; recessive model: OR = 0.78, 95% CI: 0.68-0.88, *P* < 0.001; Table 3). Nevertheless, significantly excessive risk of EC was observed in the subgroup of a study using tumor tissue to extract genomic DNA for genotyping *TP53* by homozygote comparison and recessive model (Table 3). This study also has different sources of controls. No significant association was observed in the studies using mixed specimens of white blood cells and tumor tissues or exfoliated esophageal cells to assess *TP53* genotypes (Table 3).

We also stratified the included studies into two sub-

Table 3 Results of meta-analysis for *TP53* Arg72Pro polymorphism and esophageal cancer risk

Study groups	<i>n</i> ¹	Sample size (case/control)	Arg/Arg vs Pro/Pro			Arg/Arg + Arg/Pro vs Pro/Pro			Arg/Arg vs Arg/Pro + Pro/Pro		
			OR (95% CI)	<i>P</i> ²	<i>P</i> ³	OR (95% CI)	<i>P</i> ²	<i>P</i> ³	OR (95% CI)	<i>P</i> ²	<i>P</i> ³
Total	9	2114/3431	0.76 (0.54-1.07) ⁴	0.114	0.001	0.73 (0.57-0.94) ⁴	0.014	0.009	0.89 (0.69-1.13) ⁴	0.334	0.004
Source of controls											
Population	5	1787/2690	0.56 (0.47-0.66)	< 0.001	0.273	0.57 (0.50-0.66)	< 0.001	0.404	0.80 (0.70-0.92)	0.001	0.324
Population/blood donors	1	62/131	2.33 (1.03-5.24)	0.041	-	1.25 (0.61-2.54)	0.538	-	2.71 (1.42-5.20)	0.003	-
Hospital	2	119/495	0.70 (0.22-2.18) ⁴	0.533	0.022	0.80 (0.37-2.04) ⁴	0.754	0.051	0.69 (0.36-1.31) ⁴	0.252	0.08
Unknown	1	73/115	2.25 (0.73-6.91)	0.157	-	2.20 (0.77-6.28)	0.142	-	1.17 (0.63-2.16)	0.626	-
Specimen of cases											
White blood cells or normal tissue	6	1947/3128	0.56 (0.47-0.65)	< 0.001	0.233	0.58 (0.51-0.67)	< 0.001	0.219	0.78 (0.68-0.88)	< 0.001	0.321
White blood cells/tumor tissue	1	73/115	2.25 (0.73-6.91)	0.157	-	2.20 (0.77-6.28)	0.142	-	1.17 (0.63-2.16)	0.626	-
Tumor tissue	1	62/131	2.33 (1.03-5.24)	0.041	-	1.25 (0.61-2.54)	0.538	-	2.71 (1.42-5.20)	0.003	-
Exfoliated esophageal cells	1	32/57	1.94 (0.59-6.38)	0.275	-	1.39 (0.56-3.41)	0.474	-	1.78 (0.61-5.19)	0.292	-
Sample size											
≥ 200 subjects	6	1947/3128	0.56 (0.47-0.65)	< 0.001	0.233	0.58 (0.51-0.67)	< 0.001	0.219	0.78 (0.68-0.88)	< 0.001	0.321
< 200 subjects	3	167/303	2.21 (1.24-3.93)	0.007	0.969	1.47 (0.90-2.40)	0.121	0.677	1.73 (1.15-2.61)	0.009	0.182

¹Number of comparisons; ²*P* value for the association; ³*P* value for the heterogeneity; ⁴Random effects model was used when *P* value for heterogeneity test < 0.1, otherwise, fixed-effects model was used. Arg: Arginine; Pro: Proline; OR: Odds ratio.

groups by sample size. One included studies with at least 200 participants, and the other included studies with less than 200 participants. Interestingly, studies in the former subgroup also used white blood cells or normal tissue as the specimens to assess *TP53* genotypes, and significant association between reduced EC risk and *TP53* genotypes was observed in all genetic models (Table 3). However, in the latter subgroup, significantly increased EC risk was found in homozygote comparison (OR = 2.21, 95% CI: 1.24-3.93, *P* = 0.007) and recessive model (OR = 1.73, 95% CI: 1.15-2.61, *P* = 0.009).

We performed the analysis only in well-designed studies with population-based controls with at least 200 participants using white blood cells or normal tissue to determine *TP53* genotypes. Significantly decreased risk of EC was found in all genetic models (homozygote comparison: OR = 0.54, 95% CI: 0.46-0.64, *P* < 0.001; dominant model: OR = 0.56, 95% CI: 0.49-0.65, *P* < 0.001; recessive model: OR = 0.79, 95% CI: 0.69-0.91, *P* = 0.001; Figure 1).

Sensitivity analysis

A single study involved in the meta-analysis was deleted each time to reflect the effects of individual data-set on the pooled ORs, and most of the corresponding pooled ORs were not materially altered (data not shown).

Publication bias

Both Begg's funnel plot and Egger's test were performed to assess the publication bias of literatures. Begg's funnel plots did not reveal any evidence of obvious asymmetry except for heterozygote comparison and dominant model in the overall meta-analysis (figures not shown). The Egger's test results suggested that publication bias was evident in heterozygote comparison (*P* = 0.003) and dominant model (*P* = 0.004), but not evident in homozygote comparison (*P* = 0.058) and recessive model (*P* = 0.389). The Duval and Tweedie non-parametric "trim and fill" method was used to adjust for publication bias. Meta-analysis with

and without using "trim and fill" method did not draw different conclusions (data not shown), indicating that our results were statistically robust.

DISCUSSION

Since the identification of *TP53* Arg72Pro polymorphism^[13], a number of studies^[20-29] have investigated the genetic effect of this polymorphism on EC susceptibility, but the results are inconclusive. This led us to undertake the present meta-analysis, which could quantify all the available data and might help us to distinguish the true from the false, to explore a more robust estimate of the effect of this polymorphism on EC risk. The main finding of our meta-analysis with 9 published studies including 2114 cases and 3431 controls is that *TP53* Arg72 carriers are significantly associated with decreased EC risk, and the results of increased risk or no effect of this polymorphism on EC may be due to methodological errors such as selection bias, inappropriate specimens used for genotype assessment, or limited statistical power.

We found that the distribution of *TP53* Arg72Pro genotypes in controls deviated from HWE in the study by Yang *et al.*^[29], although it has a relatively large sample size including 435 cases and 550 controls. Yang *et al.*^[29] reported that *TP53* Arg/Arg genotype was associated with significantly increased EC risk (OR = 6.48, 95% CI: 4.65-9.03), which is contrary to our results of meta-analysis. It is well known that deviation from HWE may be due to genetic reasons including non-random mating, or the alleles reflecting recent mutations that have not reached equilibrium, as well as methodological reasons including biased selection of subjects from the population, or genotyping errors^[36,37]. In despite of the reasons of disequilibrium, the results of genetic association studies might be spurious if the controls were not in HWE^[38,39]. In order to guarantee the criteria for the eligible studies, only studies with controls in HWE were included in this meta-analysis.

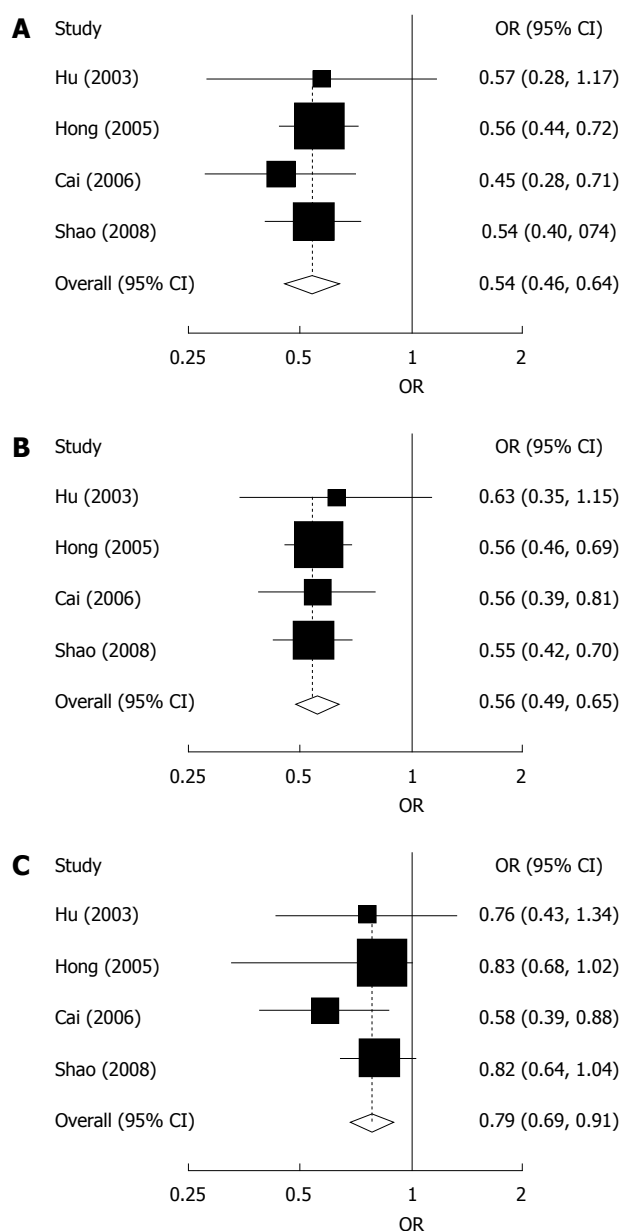


Figure 1 Forest plots for the relationship between *TP53* Arg72Pro polymorphism and esophageal cancer risk in studies with population-based controls with at least 200 participants, using white blood cells or normal tissues to determine *TP53* genotypes. A: Homozygote comparison; B: Dominant model; C: Recessive model. The first authors' surname and year of publication are given in the left part of the figure. The size of the black square corresponding to each study is proportional to the sample size. The centre of each square represents the odds ratio (OR) and the horizontal line shows the corresponding 95% CI. The pooled OR was obtained using fixed-effects model and is represented by hollow diamond, where its centre indicates the OR and its ends correspond to the 95% CI. Arg: Arginine; Pro: Proline.

In the present study, statistically significant inter-study heterogeneity of genotype effect was detected in different genetic models when all the eligible studies were pooled into the meta-analysis. Pooling despite the presence of heterogeneity may yield the mean of varying effect sizes, but the biological interpretation of such a mean and its clinical application would be very difficult^[40,41]. Therefore, it is important to explore the source of heterogeneity rather than obtaining a potentially meaningless pooled

summary measure^[42]. In order to identify the source of heterogeneity and ascertain the exact genetic effect of *TP53* Arg72Pro polymorphism on EC risk, we stratified the studies according to the source of controls, the specimens used for assessment of *TP53* genotypes and sample size. We found that the heterogeneity was remarkably decreased when the studies were divided according to the specimens used and sample size, indicating that the two factors may contribute to the observed heterogeneity.

In two of the nine included studies where the specimens used for assessment of *TP53* genotype were exfoliated esophageal cells^[21] or tumor tissues^[23], the frequencies of heterozygote genotype were obviously lower than those of other studies. This indicates that loss of heterozygosity (LOH) may exist and the distribution of *TP53* genotypes in these cases may not be the same as that in normal tissue or cells. Generally, spurious results may be obtained from genetic association studies with inappropriate material for determining genotypes^[16]. In our meta-analysis, significant association between *TP53* Arg72Pro polymorphism and reduced EC risk was not observed in subgroups using inappropriate material to determine *TP53* genotypes but only in the subgroups using white blood cells or normal tissues. Consequently, in genetic association studies, DNA from white blood cells or normal tissues should be used for determining genetic polymorphism, but not tumor tissue or exfoliated cells, in which LOH is a frequent event^[43,44].

Lacking sufficient statistical power is an unnegligible problem in genetic association studies detecting the possible risk for the polymorphism^[45]. It is likely that most genetic polymorphisms represent modest effects on disease susceptibility. An adequately powered study to detect single genetic associations would typically require a relatively large sample size, depending on the prevalence of the implicated polymorphism and the exact OR. Some of the eligible studies for our meta-analysis had a very small sample size and may have limited statistical power to detect a slight effect or may have generated a fluctuated risk estimate^[46,47]. Carefully conducted meta-analysis of these data is essential to clarify whether these associations are true or not. Through stratified analysis, we found significantly increased EC risk associated with *TP53* genotypes in subgroups of the studies with less than 200 participants, which was contrary to the results in subgroup of the studies with at least 200 participants. Given that all of the studies with a sample size of less than 200 used inappropriate specimens for determining *TP53* genotypes, the results may be unreliable.

Some limitations of this meta-analysis should be addressed. Firstly, publication bias was detected for heterozygote comparison and dominant model in overall meta-analysis. The potential reason may be that results from small studies were more likely to be published if there was positive data reported. Therefore, well-designed studies with large sample size are required. Secondly, in the subgroup analyses by ethnicity, the included studies involved only Asians and Africans. Data concerning other ethnicities such as Caucasians were not found. For Africans, only

one study was conducted, with a small sample size of 73 cases and 115 controls, which has not enough statistical power to find the real association. Thus, additional studies are warranted to evaluate the effect of this functional polymorphism on EC risk in different ethnicities, especially in Africans and Caucasians. Thirdly, lack of original data, including data of genotypes and environmental risk factors, of the included studies limited our further evaluation of potential gene-environment interaction, especially the interaction between human papillomavirus (HPV) infection and TP53 Arg72Pro polymorphism, which was investigated in several studies^[23,48-50]. However, unlike HPV infection in cervical carcinoma, the role of HPV in the etiology of EC remains controversial^[8]. A more precise analysis should be conducted if individual data are available.

Despite some limitations, the results of this meta-analysis still suggest that TP53 Arg72 allele is a protective factor for EC. The significantly reduced EC risk was found only in subgroup analyses of well-designed studies. Therefore, it is necessary to conduct large-sample studies using appropriate materials for assessment of genotypes, as well as homogeneous EC patients and unbiased selected controls. Such studies taking these factors into account may eventually lead to a better and comprehensive understanding of the association between TP53 Arg72pro polymorphism and EC risk.

COMMENTS

Background

Esophageal cancer (EC) is the eighth most common cancer and sixth most deadly cancer worldwide. A common polymorphism of TP53 at the 72nd amino acid residue, with an arginine (Arg) to proline (Pro) change because of a G→C transverse has been implicated as a risk factor for EC, but individual studies have been inconclusive or controversial.

Research frontiers

A number of studies have reported the role of TP53 Arg72Pro polymorphism in cancers such as cervical cancer, lung cancer, breast cancer, and gastric cancer, but the association of TP53 polymorphism with EC is not fully understood.

Innovations and breakthroughs

The present study demonstrated that TP53 Arg72 carriers are significantly associated with decreased EC risk, and suggested that increased risk or no effect of Arg72 variant on EC reported may be due to methodological errors such as selection bias, inappropriate specimens used for genotype assessment, or limited statistical power.

Applications

In this report, the association between TP53 Arg72Pro polymorphism and EC risk was observed, and the Arg72 allele decreased the EC risk, which is meaningful to early diagnosis, prevention and individual-based treatment of EC. Therefore, Arg72Pro polymorphism of the TP53 gene might be a potential therapeutic target for EC.

Terminology

TP53 is a major regulator of the cellular response to stress and serves as a tumor suppressor by inducing cell cycle arrest or apoptosis. Inactivation of the TP53 signaling pathway has been seen in most human cancers and polymorphisms of TP53 have also been reported to be the possible risk factors for some kinds of tumors.

Peer review

This study is an interesting meta-analysis on the association of TP53 ArgPro polymorphism with EC risk. Out of the 9 studies that survived the selection criteria, they found that TP53 Arg72 carriers were significantly associated with decreased EC risk. The authors concluded that previous reports of increased risk or no effect of this polymorphism on EC may be due to methodological er-

rors such as selection bias, inappropriate specimen or limited statistical power and they give guidelines on how to avoid these pitfalls.

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Helicobacter pylori-negative Russell body gastritis: Case report

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as well as immunohistochemical detection. The cells with eosinophilic inclusions stained positive for CD138, CD79a, and κ and lambda light chains, which confirmed plasma cell origin. In particular, κ and lambda light chains showed a polyclonal origin and the patient was negative for immunological dyscrasia. The histological observations were confirmed by ultrastructural examination. The cases reported in the literature associated with *H. pylori* infection have shown regression of plasma cells after eradication of *H. pylori*. Nothing is known about the progression of *H. pylori*-negative cases. The unusual morphological appearance of this type of chronic gastritis should not be misinterpreted during routine examination, and it should be distinguished from other common forms of chronic gastritis. It is mandatory to exclude neoplastic diseases such as gastric carcinoma, lymphoma and plasmacytoma by immunohistochemistry and electron microscopy, which can help with differential diagnosis. The long-term effects of plasma cells hyperactivation are still unknown, because cases of gastric tumor that originated in patients affected by Russell body gastritis have not been described in the literature. We are of the opinion that these patients should be scheduled for endoscopic surveillance.

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Abstract

Russell body gastritis is an unusual form of chronic gastritis characterized by the permeation of lamina propria by numerous plasma cells with eosinophilic cytoplasmic inclusions. Very few cases have been reported in the literature; the majority of which have shown *Helicobacter Pylori* (*H. pylori*) infection, thus suggesting a correlation between plasma cell presence and antigenic stimulation by *H. pylori*. We present a case of Russell body gastritis in a 78-year-old woman who was undergoing esophago-gastroduodenoscopy for epigastric pain. Gastric biopsy of the gastroesophageal junction showed the presence of cells with periodic acid-Schiff-positive hyaline pink bodies. Giemsa staining for *H. pylori* infection was nega-

Key words: Russell body; Gastritis; *Helicobacter Pylori*; Plasma cells; Crystalline inclusions

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INTRODUCTION

Russell body gastritis is an unusual form of chronic gastritis. Its distinctive histological feature is permeation of the lamina propria by plasma cells (called Mott cells) that contain eosinophilic cytoplasmic inclusions (Russell bodies), which displace the nuclei to the periphery of the cell^[1]. Patients generally present with non-specific symptoms, such as epigastric pain, dyspepsia and nausea, which suggests the presence of gastric inflammation. In the majority of the reported cases, concomitant infection with *Helicobacter pylori* (*H. pylori*) has been documented, which suggests plasma cell infiltration as the natural consequence of the infection and antigenic stimulation^[2-6], or immunosuppression as in HIV infection^[7,8] and Epstein-Barr virus-associated carcinoma^[9].

We report a case of Russell body gastritis not associated with *H. pylori* infection as rarely observed previously^[7,10].

CASE REPORT

A 78-year-old female patient was admitted to the Gastroenterology Unit of the Fondazione IRCCS Cà-Granda Ospedale Maggiore Policlinico to undergo esophagogastroduodenoscopy (EGDS) for epigastric pain. EGDS showed a hyperemic gastric mucosa, and biopsies were taken from the antral region and the esophagogastric junction. Histological examination showed moderate chronic gastritis in the antral region with no polymorphonuclear neutrophil activity, glandular atrophy of the gastric mucosa, or intestinal metaplasia, according to Sydney classification system^[11].

The lamina propria of the gastroesophageal junction mucosa showed the presence of cells with hyaline pink bodies that were periodic acid-Schiff (PAS)-positive and PAS-diastase-resistant (Figure 1A and B). No mitotic activity or atypia was observed. Giemsa staining for *H. pylori* infection in the antral and cardiac regions was negative, as was immunohistochemical detection.

The cells with eosinophilic inclusions stained positive for CD138 (Figure 1C), CD79a, and κ and lambda light chains, and negative for cytokeratin pool and leukocyte common antigen. κ and lambda light chains showed a polyclonal origin of plasma cells. Evaluation for immunological dyscrasia was negative. Ultrastructural examination showed the presence of plasma cells with an abundance of round and electron-dense material, up to 5 μ m in diameter, in the rough endoplasmic reticulum (RER) (Figure 1D). These findings were suggestive of a diagnosis of Russell body gastritis.

The patient performed a 13C urea breath test (UBT), which gave a negative result; as did abdominal ultrasonographic examination, chest X-ray, electrocardiographic study and routine biochemical analysis. In the absence of confirmed *H. pylori* infection, the patient was treated with proton pump inhibitors, which led to resolution of epigastric pain, and long-term clinical endoscopic follow-up was scheduled.

DISCUSSION

The first case of Russell body gastritis was described in 1998, when Tazawa and Tsutsumi reported a localized accumulation of plasma cells with Russell bodies in the gastric mucosa, in association with *H. pylori* infection^[3].

Many authors have suggested that chronic antigenic stimulation caused by *H. pylori* infection can result in overproduction of immunoglobulins by plasma cells. In contrast with the majority of case reports that have been positive for *H. pylori*^[1-3,5,6,8], our patient, at the time of examination, was negative by histology and UBT. Although we could not exclude a precedent infection, it is possible to hypothesize a different etiology for Russell body gastritis, as in the case described by Erbersdobler *et al.*^[10], which was associated with ethanol and analgesic abuse and a history of fungal esophagitis. Recently, vacA and cagA *H. pylori* genotypes, which are characterized by increased pathogenicity, have been associated with the development of Russell bodies and Mott cells in the antral mucosa^[12]. However, we think that a direct link between *H. pylori* infection and Mott cells in the gastric mucosa has yet to be demonstrated because the high frequency of *H. pylori* infection in western countries is not associated with an increase in Russell body gastritis, which is still a rare event.

As in our patient, subjects affected by Russell body gastritis are generally women, from 47 to 80 years old (median: 60 years), with non-specific symptoms (abdominal and epigastric pain, dyspepsia and/or nausea) that overlap with those of irritable bowel syndrome, and without specific endoscopic markers. These could be the reasons for the rare diagnosis of Russell body gastritis, which is probably underestimated.

Russell body gastritis must be clearly differentiated from neoplastic diseases, such as signet ring carcinoma, MALToma and plasmacytoma^[3]. Most often, the differential diagnosis is with monoclonal gammopathy of undetermined significance, which develops after chronic antigen stimulation (in the present case, *H. pylori* antigens) in subjects with a genetic predisposition. Immunohistochemistry is essential to exclude a monoclonal origin of these plasma cells^[4]. Absence of nuclear atypia and mitosis, lack of lymphoepithelial lesions, and a polyclonal pattern of the plasma cells are factors that favor diagnosis of Russell body gastritis when negative staining for cytokeratins rules out carcinoma.

The cases reported in literature associated with *H. pylori* infection show that eradication of *H. pylori* leads to regression of the activated plasma cells, with the disappearance of Russell bodies and Mott cells^[6]. Nothing is known about the progression of *H. pylori*-negative cases.

Russell body gastritis is an unusual and rare form of chronic gastritis; it can be associated with *H. pylori* infection, and it can be misinterpreted during ordinary routine examination.

The peculiar presence of Mott cells in the lamina propria of the gastric mucosa needs to be distinguished from other common forms of chronic gastritis, and it is

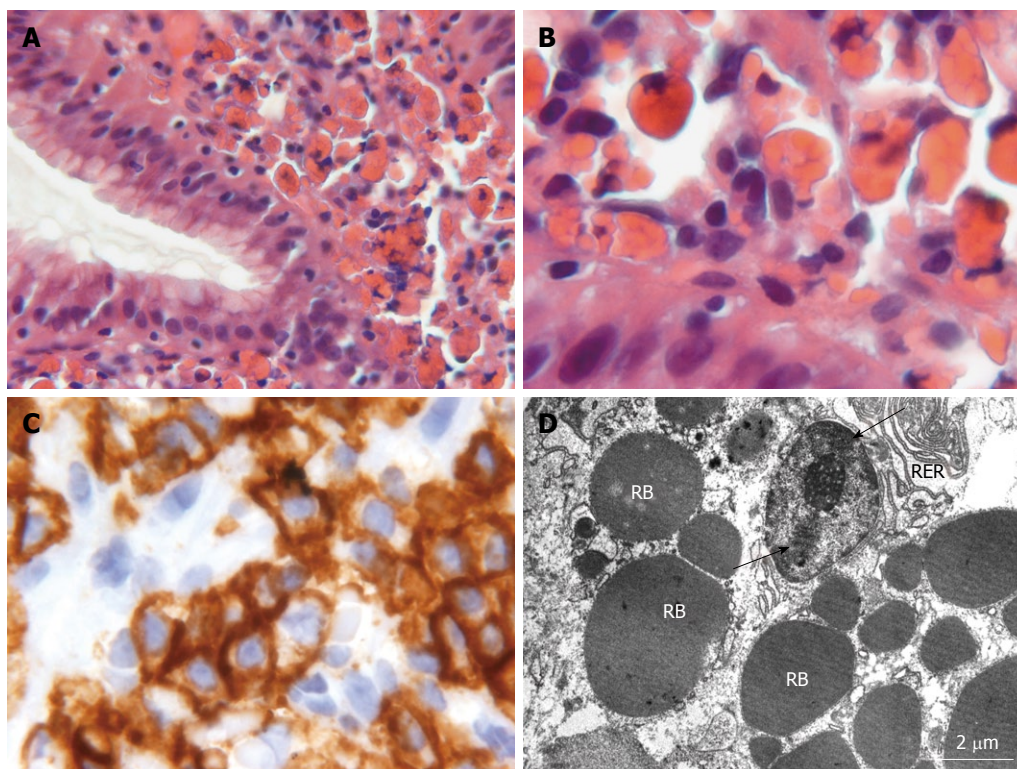


Figure 1 Histological, immunohistochemical and ultrastructural features of Russell body gastritis. A: Gastroesophageal junction sample showing monomorphic cells with eosinophilic inclusions: Russell bodies; B: Higher magnification of plasma cells with crystalline inclusions; C: Immunohistochemical reactivity of plasma cells for CD138 antibody; D: Electron micrograph of a plasma cell with typical condensation pattern of nuclear chromatin (arrows) and well-developed rough endoplasmic reticulum (RER). Several Russell bodies (RB) with 1-5 µm diameter are present in dilated RER cisternae.

mandatory to exclude neoplastic diseases such as gastric carcinoma, lymphoma and plasmacytoma by means of immunohistochemistry and electron microscopy, which can help in the differential diagnosis.

The long-term effects of plasma cell hyperactivation are still unknown. Gastric tumors that originate in patients with Russell body gastritis are not described in the literature; however, since the etiopathogenesis of this entity is not completely understood, we are of the opinion that these patients should be scheduled for endoscopic surveillance.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

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Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Title: Title should be less than 12 words.

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Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 vs 3.61 ± 1.67 , $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

Instructions to authors

uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:....; B:....; C:....; D:....; E:....; F:....; G: ...*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. ^a*P* < 0.05, ^b*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, ^c*P* < 0.05 and ^d*P* < 0.01 are used. A third series of *P* values can be expressed as ^e*P* < 0.05 and ^f*P* < 0.01. Other notes in tables or under illustrations should be expressed as ¹F, ²F, ³F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability^[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references^[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Please provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at <http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed> and <http://www.crossref.org/SimpleTextQuery/>, respectively. The numbers will be used in E-version of this journal.

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Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date,

volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

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Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *ν* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantum numbers can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *grrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindIII*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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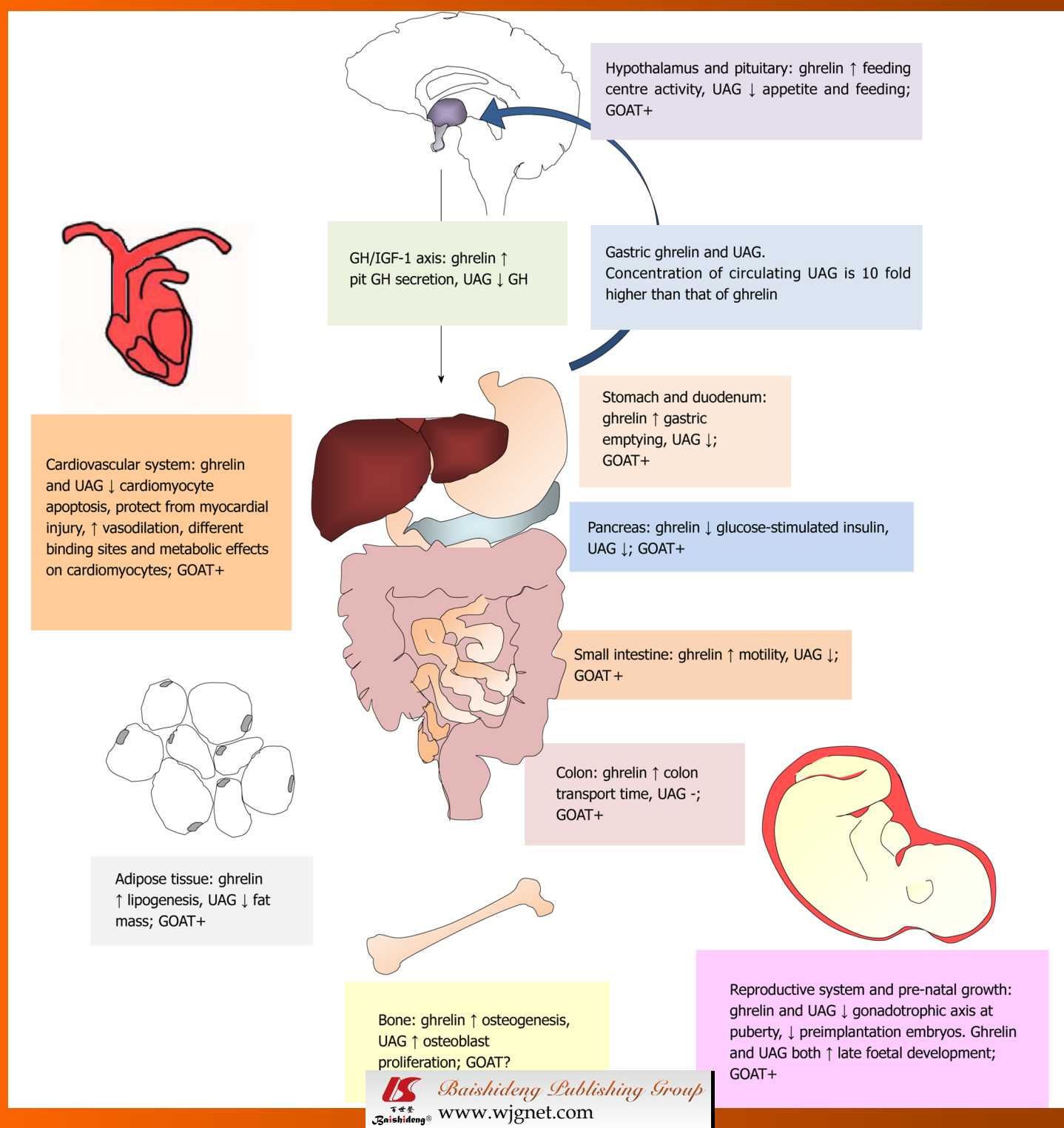
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Contents

Weekly Volume 17 Number 10 March 14, 2011

EDITORIAL

- 1237 Diagnosis and therapy of ascites in liver cirrhosis
Biecker E
- 1249 Endocrine impact of *Helicobacter pylori*: Focus on ghrelin and ghrelin
o-acyltransferase
Jeffery PL, McGuckin MA, Linden SK

REVIEW

- 1261 S100B protein in the gut: The evidence for enteroglial-sustained intestinal
inflammation
Cirillo C, Sarnelli G, Esposito G, Turco F, Steardo L, Cuomo R

ORIGINAL ARTICLE

- 1267 Portal vein thrombosis and arteriportal shunts: Effects on tumor response
after chemoembolization of hepatocellular carcinoma
*Vogl TJ, Nour-Eldin NE, Emad-Eldin S, Naguib NNN, Trojan J, Ackermann H,
Abdelaziz O*
- 1276 Proteome of human colon cancer stem cells: A comparative analysis
Zou J, Yu XF, Bao ZJ, Dong J
- 1286 Treatment of pediatric refractory Crohn's disease with thalidomide
Zheng CF, Xu JH, Huang Y, Leung YK
- 1292 Hepatitis B virus infection: A favorable prognostic factor for intrahepatic
cholangiocarcinoma after resection
Zhou HB, Wang H, Li YQ, Li SX, Wang H, Zhou DX, Tu QQ, Wang Q, Zou SS, Wu MC, Hu HP

BRIEF ARTICLE

- 1304 Does deep sedation impact the results of 48 hours catheterless pH testing?
Korrapati V, Babich JP, Balani A, Grendell JH, Kongara KR
- 1308 Impact of remote ischemic preconditioning on wound healing in small bowel
anastomoses
Holzner PA, Kulemann B, Kuesters S, Timme S, Hoeppner J, Hopt UT, Marjanovic G
- 1317 Vigorous, but differential mononuclear cell response of cirrhotic patients to
bacterial ligands
*Barbero-Becerra VJ, Gutiérrez-Ruiz MC, Maldonado-Bernal C, Téllez-Avila FI,
Alfaro-Lara R, Vargas-Vorácková F*

- 1326** Lower esophageal sphincter relaxation is impaired in older patients with dysphagia
Besanko LK, Burgstad CM, Mountfield R, Andrews JM, Heddle R, Checklin H, Fraser RJL
- 1332** Gastroesophageal reflux disease symptoms: Prevalence, sociodemographics and treatment patterns in the adult Israeli population
Moshkowitz M, Horowitz N, Halpern Z, Santo E
- 1336** Non-sequential narrow band imaging for targeted biopsy and monitoring of gastric intestinal metaplasia
Rerknimitr R, Imraporn B, Klaikeaw N, Ridditid W, Jutaghokiat S, Ponauthai Y, Kongkam P, Kullavanijaya P
- 1343** Biochemically curative surgery for gastrinoma in multiple endocrine neoplasia type 1 patients
Imamura M, Komoto I, Ota S, Hiratsuka T, Kosugi S, Doi R, Awane M, Inoue N
- 1354** Hemi-hepatectomy in pediatric patients using two-surgeon technique and a liver hanging maneuver
Mochizuki K, Eguchi S, Hirose R, Kosaka T, Takatsuki M, Kanematsu T
- 1358** Congenital bronchoesophageal fistula in adults
Zhang BS, Zhou NK, Yu CH
- 1362** Polymorphisms of interleukin-10 promoter are not associated with prognosis of advanced gastric cancer
Liu J, Song B, Wang JL, Li ZJ, Li WH, Wang ZH
- 1368** Efficacy of endoscopic therapy for gastrointestinal bleeding from Dieulafoy's lesion
Cui J, Huang LY, Liu YX, Song B, Yi LZ, Xu N, Zhang B, Wu CR
- 1373** Autoantibodies against MMP-7 as a novel diagnostic biomarker in esophageal squamous cell carcinoma
Zhou JH, Zhang B, Kernstine KH, Zhong L

CASE REPORT

- 1379** Metal stenting to resolve post-photodynamic therapy stricture in early esophageal cancer
Cheon YK

Contents

World Journal of Gastroenterology
Volume 17 Number 10 March 14, 2011

ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

APPENDIX I Meetings
I-VI Instructions to authors

ABOUT COVER Jeffery PL, McGuckin MA, Linden SK. Endocrine impact of *Helicobacter pylori*: Focus on ghrelin and ghrelin *o*-acyltransferase.
World J Gastroenterol 2011; 17(10): 1249-1260
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Diagnosis and therapy of ascites in liver cirrhosis

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Abstract

Ascites is one of the major complications of liver cirrhosis and is associated with a poor prognosis. It is important to distinguish noncirrhotic from cirrhotic causes of ascites to guide therapy in patients with noncirrhotic ascites. Mild to moderate ascites is treated by salt restriction and diuretic therapy. The diuretic of choice is spironolactone. A combination treatment with furosemide might be necessary in patients who do not respond to spironolactone alone. Tense ascites is treated by paracentesis, followed by albumin infusion and diuretic therapy. Treatment options for refractory ascites include repeated paracentesis and transjugular intrahepatic portosystemic shunt placement in patients with a preserved liver function. Potential complications of ascites are spontaneous bacterial peritonitis (SBP) and hepatorenal syndrome (HRS). SBP is diagnosed by an ascitic neutrophil count > 250 cells/mm³ and is treated with antibiotics. Patients who survive a first episode of SBP or with a low protein concentration in the ascitic fluid require an antibiotic prophylaxis. The prognosis of untreated HRS type 1 is grave. Treatment consists of a combination of terlipressin and albumin. Hemodialysis might serve in selected patients as a bridging therapy to liver transplantation. Liver transplantation should be considered in all patients with ascites and liver cirrhosis.

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INTRODUCTION

Ascites is one of the major complications of liver cirrhosis and portal hypertension. Within 10 years of the diagnosis of cirrhosis, more than 50% of patients develop ascites^[1]. The development of ascites is associated with a poor prognosis, with a mortality of 15% at one-year and 44% at five-year follow-up, respectively^[2]. Therefore, patients with ascites should be considered for liver transplantation, preferably before the development of renal dysfunction^[1]. This article will give a concise overview of the diagnosis and treatment of patients with ascites in liver cirrhosis and the management of the most common complications of ascites.

EVALUATION AND DIAGNOSIS

Since 15% of patients with liver cirrhosis develop ascites of non-hepatic origin, the cause of new-onset ascites has to be evaluated in all patients^[3]. The most important diagnostic measure is diagnostic abdominal paracentesis. Paracentesis is considered a safe procedure even in patients with an abnormal prothrombin time, with an overall complication rate of not more than 1%^[4]. More serious complications such as bowel perforation or bleeding into the abdominal cavity occur in less than one in 1000 paracenteses^[5]. Data supporting cut-off values for

coagulation parameters are not available^[6]. One study which included 1100 large-volume paracenteses did not show hemorrhagic complications despite platelet counts as low as 19 g/L and international normalized ratios as high as 8.7^[7]. The routine prophylactic use of fresh frozen plasma or platelets before paracentesis is therefore not recommended^[6].

Ascitic fluid analysis should include total protein concentration, a neutrophil count and inoculation of ascites into blood culture bottles at the bedside. Determination of ascitic protein concentration is necessary to identify patients who are at increased risk for the development of spontaneous bacterial peritonitis (SBP), since a protein concentration below 1.5 g/dL is a risk factor for the development of SBP^[8]. Spontaneous bacterial peritonitis is defined as an ascitic neutrophil count of more than 0.25 g/L (see "treatment of SBP") and inoculation of ascitic fluid in blood culture flasks at the bedside helps to detect bacteria in the ascitic fluid^[9]. Additional test are only necessary in patients in whom other causes of ascites are in the differential diagnosis^[10].

In patients in whom a cause of ascites different from liver cirrhosis is suspected, the determination of the serum-ascites-albumin gradient (SAAG) is useful. The SAAG is ≥ 1.1 g/dL in ascites due to portal hypertension with an accuracy of 97%^[11].

MANAGEMENT OF UNCOMPLICATED ASCITES

Uncomplicated ascites is defined as the absence of complications including refractory ascites, SBP, marked hyponatremia or hepatorenal syndrome (HRS)^[10].

There are no defined criteria as to when treatment of ascites should be initiated. Patients with clinically inapparent ascites usually do not require a specific therapy. It is recommended that patients with clinically evident and symptomatic ascites should be treated.

In patients with alcoholic liver cirrhosis, the most important measure is alcohol abstinence. In the majority of patients with alcoholic liver disease, alcohol abstinence results in an improvement of liver function and ascites^[12]. Also, decompensated cirrhosis due to chronic hepatitis B infection or autoimmune hepatitis often shows a marked improvement in response to antiviral or immunosuppressive treatment, respectively^[13].

Patients with uncomplicated mild or moderate ascites do not require hospitalization and can be treated as outpatients. Patients with ascites have a positive sodium balance, i.e. sodium excretion is low relative to sodium intake. Hence, the mainstay of ascites therapy is sodium restriction and diuretic therapy. Sodium intake should be restricted to 5-6 g/d (83-100 mmol/d NaCl)^[14-16]. A more stringent restriction is not recommended since this diet is distasteful and may worsen the malnutrition that is often present in patients with liver cirrhosis^[16]. A French study showed that a more stringent sodium restriction of 21 mmol/d led to a faster mobilization of ascites in the first 14 d, but revealed

no difference after 90 d^[17]. Another study found no benefit in patients treated with a strict sodium restriction of 50 mmol/d compared to patients with a moderate sodium restriction of 120 mmol/d^[18].

Theoretically, upright posture aggravates sodium retention by an increase of plasma renin activity and has led to the recommendation of bed rest. However, there are no clinical studies that provide evidence that bed rest actually improves ascites^[6].

Therapy of ascites that is based solely on sodium restriction is only applicable in patients with a 24 h sodium excretion of more than 80 mmol (90 mmol dietary intake - 10 mmol loss by sweat and feces) since an adequate sodium excretion is the requirement for a negative sodium balance. Patients with a 24 h sodium excretion less than 80 mmol/24 h need diuretic therapy.

Hyponatremia is a common finding in patients with ascites and liver cirrhosis, but a study including 997 patients with liver cirrhosis found severe hyponatremia (≤ 125 mmol/L) in only 6.9% of patients^[19]. Another study, including 753 patients evaluated for liver transplantation, found hyponatremia of less than 130 mmol/L in 8% of patients and an increase in the risk of death as sodium decreased to between 135 and 120 mmol/L^[20]. Since the total body sodium is not decreased in patients with ascites and hyponatremia (dilution hyponatremia), rapid correction of serum sodium is not indicated but has the risk of severe complications^[21]. Fluid restriction is recommended in patients with severe hyponatremia (120-125 mmol/L)^[6], but clinical studies that have evaluated the efficacy of fluid restriction, or the extent of hyponatremia when fluid restriction should be initiated, are lacking.

MEDICAL THERAPY

The activation of the renin-aldosterone-angiotensin-system in patients with liver cirrhosis causes hyperaldosteronism and increased reabsorption of sodium along the distal tubule^[22]. Therefore, aldosterone antagonists like spironolactone or its active metabolite potassium canrenoate are considered the diuretics of choice^[23]. Patients with mild to moderate ascites are treated with a monotherapy of spironolactone. The starting dose is 100-200 mg/d^[24]. A monotherapy with a loop diuretic like furosemide is less effective compared to spironolactone and is not recommended^[23]. If the response to 200 mg spironolactone within the first two weeks is not sufficient, furosemide with an initial dose of 20-40 mg/d is added. If necessary, the spironolactone dose is increased stepwise up to 400 mg/d and the furosemide dose is increased up to 160 mg/d^[22,23,25].

The daily weight loss in patients with or without peripheral edema should not exceed 1000 g or 500 g, respectively^[26]. A sufficient diuretic effect is achieved when only small amounts of ascites are left and peripheral edema has completely resolved.

It is generally recommended to apply furosemide orally, since intravenous administration bears the risk of azotemia^[27,28]. A combination therapy of spironolactone and

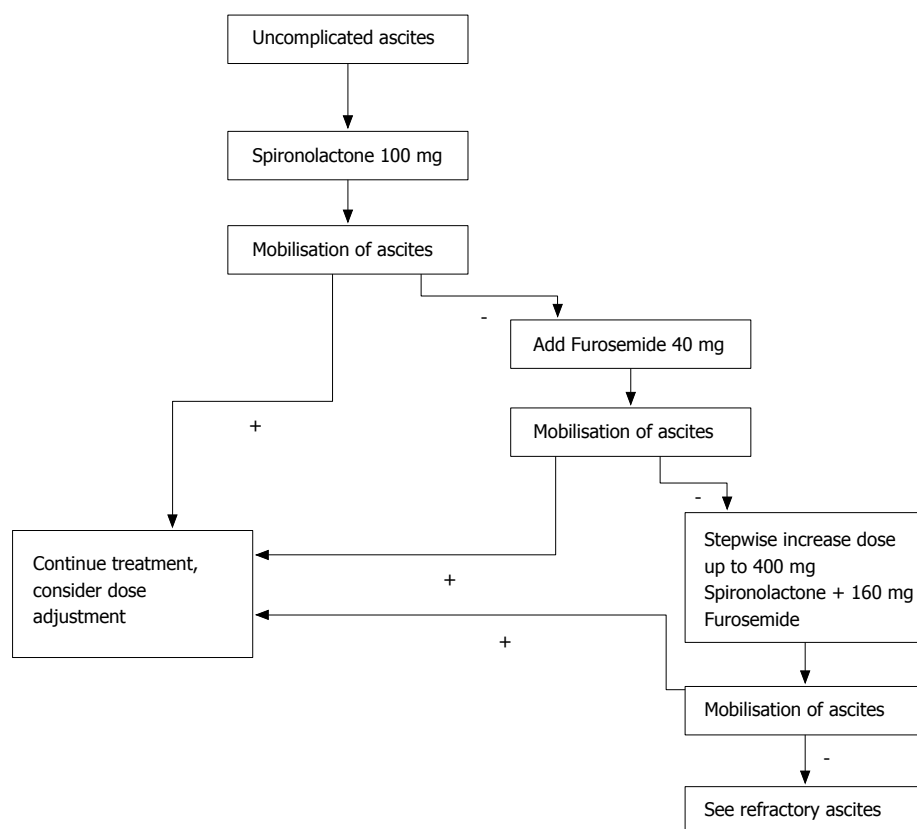


Figure 1 Treatment algorithm for the diuretic treatment of patients with uncomplicated ascites.

furosemide shortens the response time to diuretic therapy and minimizes adverse effects such as hyperkalemia^[29]. Angeli and co-workers compared the sequential therapy with potassium canrenoate and furosemide with the initial combination therapy of these two drugs^[30]. Patients receiving the sequential therapy were treated with an initial dose of 200 mg potassium canrenoate that was increased to 400 mg/d. Non-responders to the initial therapy were treated with 400 mg/d of potassium canrenoate and furosemide at an initial dose of 50 mg/d that was increased to 150 mg/d. Patients receiving the combination therapy were treated with an initial dose of 200 mg/d potassium canrenoate and 50 mg of furosemide that was increased to 400 mg/d and 150 mg/d, respectively. A sufficient treatment response was achieved in both treatment groups. However, there were more adverse effects of diuretic treatment (e.g. hyperkalemia) in the patients receiving the sequential therapy. In contrast to this study, another study found no difference comparing sequential and combination therapy^[31]. Possible explanations for these conflicting results are the different patient populations included in the studies. Angeli *et al*^[30] included patients with recidivant ascites and reduction in the glomerular filtration rate whereas in the study of Santos *et al*^[31], the majority of patients had newly diagnosed ascites.

An established scheme for an initial combination therapy is 100 mg spironolactone and 40 mg furosemide per day given in the morning^[32]. If this dosage is not sufficient, a stepwise increase keeping the spironolactone/fu-

rosemide ratio (e.g. 200 mg spironolactone/80 mg furosemide) is possible. The combination of spironolactone and furosemide lowers the risk of a spironolactone-induced hyperkalemia.

The combination of sodium restriction, spironolactone and furosemide achieves a sufficient therapy of ascites in patients with liver cirrhosis in 90% of cases^[18,23,25]. Figure 1 shows an algorithm for the diuretic treatment of patients with uncomplicated ascites.

Several studies have evaluated the newer loop-diuretic torasemide in patients with liver cirrhosis and ascites^[33-35]. Torasemide was shown to be at least as effective and safe as furosemide and is considered an alternative in the treatment of ascites^[33-35].

Amiloride is an alternative to spironolactone in patients with painful gynecomastia. Amiloride is given in a dose of 10-40 mg/d but is less effective than potassium canrenoate^[36].

Complications of diuretic therapy include hepatic encephalopathy, renal failure, gynecomastia, electrolyte disturbances such as hyponatremia, hypo- or hyperkalemia, as well as muscle cramps^[22,23,30,31,36]. To minimize these complications, it is generally advised to reduce the dosage of diuretic drugs after the mobilization of ascites. Complications of diuretic therapy are most frequent in the first weeks after initiation of therapy^[30].

A common complication of diuretic therapy is hyponatremia. The level of hyponatremia at which diuretic treatment should be stopped is a subject of discussion. It

is generally agreed that diuretics should be paused when the serum sodium is less than 120-125 mmol/L^[6].

THERAPY OF REFRACTORY ASCITES

Refractory ascites is defined as ascites that does not respond to sodium restriction and high-dose diuretic treatment (400 mg/d spironolactone and 160 mg/d furosemide) or that reoccurs rapidly after therapeutic paracentesis^[3]. About 10% of patients with cirrhosis and ascites are considered to have refractory ascites^[6]. The median survival of patients with ascites refractory to medical treatment is approximately six months^[3,37-39].

Possible treatment options for refractory ascites include large volume paracentesis (LVP), transjugular intrahepatic portosystemic shunt (TIPS) and liver transplantation.

Large volume paracentesis

Large volume paracentesis is the treatment of choice for patients with tense ascites. It is considered a safe procedure^[40] and complication rates are not higher than in diagnostic paracentesis^[4,7]. The risk of bleeding is generally low and a relationship between the degree of coagulopathy and the risk of bleeding is not evident^[40]. Hence, there are no data that support the prophylactic administration of pooled platelets and/or fresh frozen plasma prior to paracentesis. Nevertheless, in patients with severe coagulopathy, paracentesis should be performed with caution. Paracentesis is performed under sterile conditions. If ultrasound is available, it should be used to localize the best puncture site to minimize the risk of bowel perforation.

The most important complication following LVP is paracentesis-induced circulatory dysfunction (PICD)^[41-43]. This is caused by a depletion of the effective central blood volume leading to a further stimulation of vasoconstrictor systems. Post-paracentesis circulatory dysfunction is characterized by a deterioration of renal function that can ultimately culminate in hepatorenal syndrome in up to 20% of patients^[42]. A rapid re-accumulation of ascites^[44], hyponatremia, as well as an increase in portal pressure^[45], are additional consequences. PICD is associated with an increase in mortality^[41].

Paracentesis of not more than 5 L can safely be conducted without post-paracentesis colloid infusions and the risk of PICD^[46]. If a paracentesis of more than 5 L is performed, the administration of albumin is advisable^[47]. However, it has to be kept in mind that albumin is costly, and that studies that are large enough to demonstrate decreased survival in patients who are given no plasma expander compared to patients given albumin are lacking^[41]. There are no studies that have evaluated the appropriate dose of albumin after paracentesis. In the available studies, 5 to 10 g of albumin per litre of removed ascites have been given^[41,42,47,48]. Hence, a dose of 6-8 g albumin per litre of removed ascites seems appropriate^[6]. Dextran-70 and polygeline as alternative plasma expanders have been compared to albumin for the prevention of PICD after LVP but have been shown to be less effective^[41]. However,

a benefit in survival in favor of albumin over dextran-70, polygeline and saline was not shown in three trials^[41,49,50]. Albumin should be administered slowly after the completion of paracentesis to reduce the risk of a volume overload.

Large volume paracentesis *per se* does not positively influence renal sodium and water retention. To prevent the re-accumulation of ascites after LVP, sodium restriction and diuretic treatment are necessary^[51].

TIPS

TIPS provides a side-to-side porto-caval shunt. It is usually placed under local anesthesia by transhepatic puncture of the (usually) right main branch of the portal vein using an approach from a hepatic vein. After the connection between the hepatic and portal vein is established, the tract is dilated and a stent is placed^[52].

Contraindications for TIPS in the therapy of recurrent ascites include advanced liver disease (bilirubin > 5 mg/dL), episodic or persistent hepatic encephalopathy, cardiac or respiratory failure and hepatocellular carcinoma^[53-57].

TIPS insertion causes an increase in right atrial and pulmonary artery pressure as well as an increase in cardiac output, a reduction of systemic vascular resistance, a reduction of effective arterial blood volume and, most importantly, a reduction of portal pressure^[52,58-67]. Whereas the effect on renal function (increased sodium excretion and increased glomerular filtration rate) persists, the increase in cardiac output tends to return to pre-TIPS levels^[59-63,67].

Compared to repeated LVP, TIPS is more effective in the therapy of ascites^[53,55,68], but the effect on mortality is less clear. Whereas two studies showed no difference in mortality comparing paracentesis and TIPS^[53,57], another two studies revealed decreased mortality in patients receiving TIPS^[55,56]. A meta-analysis based on individual patient data from four randomized trials showed that TIPS in patients with refractory ascites improved transplant-free survival^[68].

A frequent complication after TIPS insertion is hepatic encephalopathy, which occurs in 30%-50% of patients^[69,70], but seems to be less frequent in carefully selected patients with preserved liver function. Other complications are shunt thrombosis and shunt stenosis^[69,71]. Shunt thrombosis and shunt stenosis were shown to be less frequent in polytetrafluoroethylene (e-PTFE) coated stents compared to non-coated stents in one study^[72]. A retrospective multicenter study showed that the use of e-PTFE covered stents is associated with a higher 2-year survival compared to non-covered stents^[73].

Figure 2 shows an algorithm for the treatment of refractory ascites.

HEPATORENAL SYNDROME

The occurrence of renal failure in patients with advanced liver disease in the absence of an identifiable cause of renal failure is defined as hepatorenal syndrome^[3]. Therefore, it is essential to rule out other possible causes of renal fail-

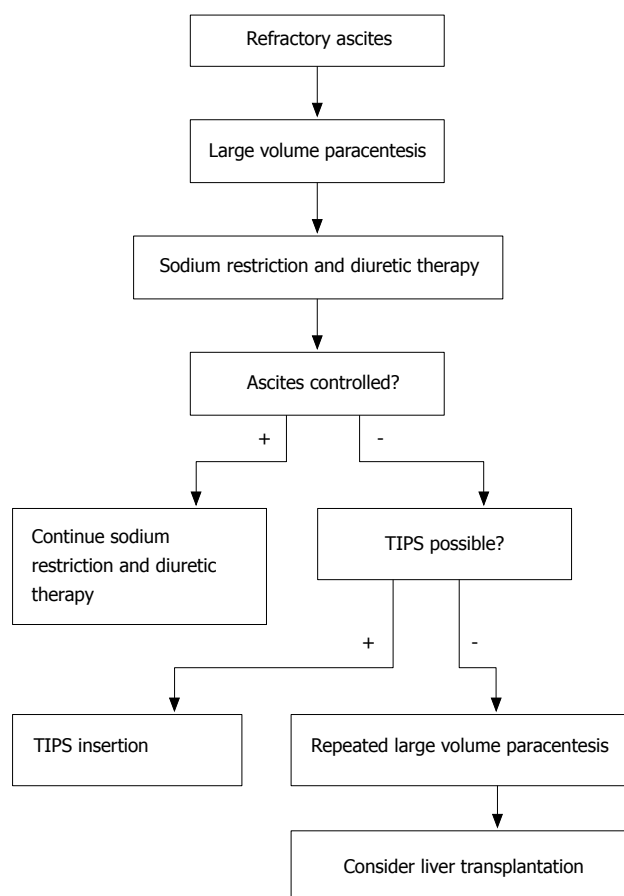


Figure 2 Treatment algorithm for the treatment of patients with refractory ascites. TIPS: Transjugular intrahepatic portosystemic shunt.

ure before the diagnosis of hepatorenal syndrome (HRS) is made. In 1994, the International Ascites Club defined criteria for the diagnosis of hepatorenal syndrome^[3] that were modified in 2007^[74]. The modified criteria include: (1) cirrhotic liver disease with ascites; (2) a serum creatinine > 1.5 mg/dL; (3) no improvement of serum creatinine (decrease to ≤ 1.5 mg/dL) after at least 2 d with diuretic withdrawal and volume expansion with albumin (1 g/kg body weight/d up to 100 g/d); (4) absence of shock; (5) no current or recent treatment with nephrotoxic drugs; and (6) absence of parenchymal kidney disease^[74]. According to the progression of renal failure, two types of HRS are defined: type 1 is rapidly progressive with a doubling of the initial serum creatinine to > 2.5 mg/dL or a 50% reduction of the initial 24-h creatinine clearance to < 20 mL/min in less than 2 wk. Patients with HRS type 2 do not have a rapidly progressive course^[74].

HRS type 1 often develops in temporal relationship with a precipitating factor like infection (e.g. spontaneous bacterial peritonitis)^[75,78] or severe alcoholic hepatitis in patients with advanced cirrhosis. The prognosis of all patients with HRS is poor with a median survival of approximately three months^[79,80]. The prognosis of patients with untreated HRS type 1 is even worse, with a median survival of only one month^[81].

Treatment should be initiated immediately after the diagnosis is made to prevent further deterioration of renal

function. Several treatment options of HRS are available: drug therapy, hemodialysis, TIPS and liver transplantation.

Drug therapy of HRS consists of the application of vasopressors in combination with albumin.

One randomized, controlled study compared the effect of noradrenalin as a continuous intravenous infusion in combination with albumin *vs* terlipressin and albumin in 40 patients with HRS type 1^[82]. The study did not reveal a significant difference in short-term survival between the two groups. Two studies demonstrated that treatment with octreotide alone is not successful^[83,84] but that a combination with the vasopressor midodrine is required. Two other studies investigated the effect of octreotide and midodrine in combination with albumin^[85,86]. One small study compared octreotide 200 μ g subcutaneously three times a day, midodrine up to 12.5 mg/d orally and albumin 10-20 g/d with dopamine plus albumin^[85]. The results in the octreotide/midodrine group were superior to those in the dopamine group. A larger, retrospective study compared a combination treatment with octreotide/midodrine and albumin with a treatment with albumin only^[86]. The mortality rate of the patients treated with the combination of octreotide, midodrine and albumin was lower than the mortality rate of the patients treated with albumin alone (43% *vs* 71%, respectively). More data are available with regard to the vasoconstrictor terlipressin in the treatment of HRS type 1. Treatment with terlipressin is started with an initial dose of 1 mg 4-6 times a day and is given in combination with albumin (1 g/kg body weight on day 1, followed by 40 g/d)^[87]. If a reduction of serum creatinine of at least 25% is not achieved after three days of therapy, the dose is increased to a maximum of 2 mg 4-6 times a day. The treatment is continued until a reduction of serum creatinine below 1.5 mg/dL is achieved. The median response time is around two weeks^[88]. Patients with a better liver function and an early increase in arterial pressure after initiation of treatment have a higher probability of treatment response^[88]. Treatment with terlipressin is successful in 40%-50% of patients^[89,90]. Cardiovascular or ischemic complications are the most important adverse effects of terlipressin treatment^[80,89]. A meta-analysis of randomized trials regarding terlipressin and other vasoactive drugs showed an improved short-term survival for the patients treated with terlipressin^[90].

Two small studies evaluated the effect of terlipressin in patients with HRS type 2^[91,92]. Both studies showed an improvement of renal function with terlipressin treatment^[91,92].

TIPS has been shown to improve renal function in HRS type 1 patients in two studies^[60,93]. However, the results of these studies might be biased since only patients with a maintained liver function underwent TIPS insertion. In addition, TIPS insertion is beneficial in patients with HRS type 2^[53].

Few data are available on the role of hemodialysis in patients with HRS type 1^[94,95]. Hemodialysis seems to be effective, but studies comparing hemodialysis with medical treatment or TIPS are lacking. Hence, hemodialysis remains a therapy option in selected patients with electrolyte

disturbances, severe acidosis or volume overload and as a bridging therapy in patients awaiting liver transplantation.

The treatment option of choice for patients with HRS type 1 and HRS type 2 is liver transplantation^[96]. However, the survival rate of 65% is low compared to other cirrhotic patients who have undergone liver transplantation. In addition, the mortality rate on the waiting list for liver transplantation of patients with HRS is higher than for patients with cirrhosis without HRS. Combined liver-kidney transplantation is usually not necessary. Only patients who have been on hemodialysis for more than 12 wk might be considered for combined liver-kidney transplantation as renal function might irreversibly deteriorate in patients with HRS and long-term hemodialysis^[97,98].

SPONTANEOUS BACTERIAL PERITONITIS

Spontaneous bacterial peritonitis (SBP) is a bacterial infection of the peritoneal cavity in patients with cirrhosis and ascites^[99-101]. All patients with cirrhosis and ascites are at risk of developing SBP. Symptoms are often unspecific and include signs of peritonitis, clinical and laboratory signs of inflammation, deterioration of liver function, gastrointestinal bleeding and hepatic encephalopathy^[102-104]. The prevalence in hospitalized patients is approximately 10% and higher than in outpatients (1.5%-3.5%)^[102,103].

The diagnosis of SBP is based on a positive ascitic fluid bacterial culture and an elevated ascitic fluid neutrophil count in patients without an evident source of infection^[105]. Ascitic bacterial culture is negative in more than 50% of patients with suspected SBP and an elevated neutrophil count^[8,100,101,106]. If bacteria are found in the culture, the most common bacteria include *E. coli* as well as streptococcus species and enterococci^[99-101,106].

A neutrophil count of more than 250 cells/mm³ (0.25 × 10⁹/L) is considered diagnostic of SBP^[107]. An ascitic neutrophil count ≥ 250 cells/mm³ but with negative cultures is termed as culture-negative neutrocytic ascites. Several studies were undertaken to investigate the use of reagent strips ("dipstick testing") designed for the use in urine in the diagnosis of SBP. However, a review of studies comparing different types of reagent strips with cytochemical methods revealed a high rate of false negative results for the reagent strips^[108].

Patients with an ascitic fluid neutrophil count ≥ 250 cells/mm³ and clinical signs of SBP should receive antibiotic treatment. Also, cirrhotic patients with ascites and signs or symptoms of infection or unexplained clinical deterioration should receive treatment regardless of a neutrophil count below 250 cells/mm³, since it is known that bactericides (positive ascitic fluid culture with a neutrophil count below 250 cells/mm³) might precede the neutrophil response^[109].

Treatment should be initiated with a broad-spectrum antibiotic as long as results of bacterial culture are not available. The treatment of choice is a third-generation cephalosporin. Most data are available for cefotaxime. Cefotaxime covers 95% of the causative bacteria including the most common isolates *E. coli*, *Klebsiella pneumoniae*

and pneumococci^[110]. In addition, it reaches high concentrations in the ascitic fluid^[110,111]. In most patients, 5 d of treatment is as effective as 10 d of treatment^[112]. Ceftriaxone was also shown to be effective in the treatment of SBP and is an alternative to cefotaxime^[113]. Amoxicillin/clavulanic acid, given as a sequential intravenous/oral therapy was shown to be as effective as cefotaxime in a small study^[114]. Intravenous ciprofloxacin is similarly effective with respect to survival and SBP resolution rate as treatment with cefotaxime, but costs are higher^[115]. In uncomplicated SBP, oral ofloxacin was shown to be as effective as cefotaxime^[116]. Since patients who have received quinolone prophylaxis against SBP may have developed a quinolone resistant flora, quinolones should not be used in these patients^[6].

Failure of the initial antibiotic treatment should be considered in patients in whom the initial neutrophil count does not decrease below 25% of the pre-treatment value after two days of treatment^[117]. Treatment failure might be due to bacteria resistant to the initial treatment or secondary peritonitis. Under these circumstances, treatment has to be modified according to susceptibility testing (if available) or on an empiric basis.

The addition of intravenous albumin to cefotaxime in the treatment of SBP has been shown to be effective in two studies^[76,118]. One controlled randomized trial compared patients with SBP receiving cefotaxime alone vs patients with cefotaxime plus albumin 1.5 g/kg body weight at diagnosis, followed by 1 g/kg albumin on day 3. The study revealed a decrease in mortality from 29% in the cefotaxime group to 10% in the cefotaxime/albumin group^[76]. The study by Sigal *et al*^[118] found that combination treatment with albumin is particularly effective in patients with an impaired liver and kidney function (bilirubin > 4 mg/dL and creatinine > 1 mg/dL, respectively) but that combination treatment with albumin is not necessary in patients who do not fulfil these criteria.

In patients who promptly respond to antibiotic treatment, a follow-up paracentesis and ascitic fluid analysis is not necessary^[6]. In patients who do not respond to treatment or show a delayed response, a follow-up ascitic fluid analysis is mandatory for further evaluation^[119].

Several subgroups of patients at high risk for the development of SBP have been identified in the past. Risk factors for SBP are ascitic fluid protein concentration < 1.0 g/dL, variceal hemorrhage and a prior episode of SBP^[6]. Several randomized controlled trials have shown a benefit of prophylactic antibiotic treatment in these patients^[120-126].

Variceal bleeding is a major risk factor for the development of SBP, especially in patients with advanced cirrhosis and severe bleeding^[120,126-134]. Antibiotic prophylaxis in patients with variceal hemorrhage has been shown to not only decrease the rate of SBP^[8,101,106] but also decrease the risk for rebleeding^[135] and hospital mortality^[128].

Norfloxacin (400 mg twice daily) for 7 d has been widely used for selective intestinal decontamination in cirrhotic patients with variceal bleeding^[8,101,126]. In patients with gastrointestinal bleeding and advanced cirrhosis,

intravenous ceftriaxone (1 g/d for 7 d) has been shown to be superior to norfloxacin^[121].

Low ascitic fluid protein concentration (< 1.0 g/dL) is a risk factor for the development of SBP^[123,136-138] and prophylactic antibiotic treatment is advisable in these patients. Most data are available for prophylaxis of SBP using norfloxacin^[125,139-142]. One randomized trial in which patients with low ascitic fluid protein concentration (< 1.0 g/dL) or a bilirubin > 2.5 mg/dL were treated with continuous norfloxacin or inpatient-only norfloxacin showed that the incidence of SBP was lower in the continuous treatment group at the expense of more resistant flora when they did develop infection^[141]. These findings were substantiated by another randomized trial comparing daily norfloxacin (400 mg for twelve months) with placebo in patients with low ascitic fluid protein concentration (< 1.5 g/dL) and an impaired liver or kidney function^[139]. The patients in the verum group had a lower incidence of SBP and hepatorenal syndrome as well as a survival advantage (after three months but not after one year) compared to the patients receiving placebo^[139].

Another randomized, double blind, placebo-controlled trial compared ciprofloxacin 500 mg/d for twelve months with placebo in patients with ascitic fluid protein concentration less than 1.5 g/dL and impaired liver function (Child-Pugh score 8.3 ± 1.3 and 8.5 ± 1.5 , in the placebo and ciprofloxacin group, respectively)^[142]. The study revealed a trend towards a lower incidence of SBP in the ciprofloxacin group but the result was not significant. Nevertheless, the 1-year survival was higher in the patients in the ciprofloxacin group^[142]. This might be attributed to the fact that the probability of remaining free of bacterial infections was higher in the ciprofloxacin group^[142].

The overall recurrence rate of SBP in patients surviving the first episode of SBP is approximately 70% in the first year^[106] and survival rates are 30%-50% and 25%-30% in the first and second year after SBP, respectively. Norfloxacin is effective in the secondary prophylaxis of SBP. One randomized, double blind, placebo-controlled multicenter study revealed that prophylactic treatment with 400 mg/d norfloxacin reduced the recurrence rate of SBP from 68% to 20%^[122]. Another trial compared norfloxacin 400 mg/d with rifloxacin 400 mg/wk and did not find a significant difference in the SBP recurrence rate between the two treatment groups^[143]. The effects of trimethoprim-sulfamethoxazole, ciprofloxacin and norfloxacin were assessed in three more studies^[124,125,144]. All three studies revealed a reduced occurrence of SBP in the patients receiving prophylactic treatment. However, the significance of the studies in the setting of secondary prophylaxis is limited since the studies included patients with and without prior episodes of SBP. There are no trials available that have studied for how long secondary prophylaxis should be given.

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Endocrine impact of *Helicobacter pylori*: Focus on ghrelin and ghrelin *o*-acyltransferase

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Abstract

Ghrelin is predominantly produced by the gastric entero-endocrine cell compartment and is octanoylated by the recently discovered ghrelin *o*-acyltransferase (GOAT) before secretion into the bloodstream. This octanoylation is essential for many of the biological properties of ghrelin including appetite stimulation and anti-inflammatory properties as only the acylated form of ghrelin binds to the ghrelin receptor, the growth hormone secretagogue receptor (GHS-R). Given the gastric location of ghrelin production, it is perhaps not surprising that insult to the gastric mucosa affects circulating ghrelin levels in humans. *Helicobacter pylori* (*H. pylori*) infects more than fifty percent of the world's population and once established within the gastric mucosa, can persist for life. Infection is associated with chronic gastritis, gastric atrophy and ulceration, reduced appetite and a lower body mass index (BMI). The large majority of studies investigating levels of circulating ghrelin and ghrelin expression in the stomach in patients with *H. pylori* infection

indicate that the bacterium has a negative impact on ghrelin production and/or secretion. Eradication of infection restores ghrelin, improves appetite and increases BMI in some studies, however, a causative relationship between *H. pylori*-associated serum ghrelin decline and food intake and obesity has not been established. Most studies measure total ghrelin in the circulation although the measurement of the ratio of acyl/total ghrelin gives a clearer indication that the ghrelin acylation process is altered during infection and atrophy. GOAT is essential for the production of biologically-active, acyl ghrelin and the impact of *H. pylori* on GOAT expression and activity will be highly informative in the future.

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Key words: Appetite; Ghrelin; Ghrelin *o*-acyltransferase; *Helicobacter pylori*; Infection; Inflammation; Obesity

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection of the gastric mucosa in humans has been shown to alter the normal gastric physiology. Ghrelin is a 28 amino acid peptide hormone, primarily produced in, and secreted from, the gastric mucosa, that has been demonstrated to be involved in appetite, food intake and energy homeostasis. *H. pylori* colonization has been negatively associated with circulating ghrelin levels although this finding is the subject

of much controversy. The following review seeks to address differences in the published studies regarding the impact of *H. pylori* infection on ghrelin and introduces a putative role for the newly-discovered ghrelin acylating enzyme, ghrelin *o*-acyltransferase (GOAT).

THE GUT AS AN ENDOCRINE ORGAN

In addition to classical endocrine tissues such as the pituitary, thyroid and adrenal glands, peripheral organs are a rich source of hormones, some of which act in an intra-crine/autocrine/paracrine manner. Amongst these organs, the gut is the largest and most diffuse endocrine organ producing an array of peptide hormones important for both enteric and non-enteric physiology. Enteroendocrine cells are distributed throughout the gastrointestinal tract, however, a significant proportion of endocrine hormones involved in appetite regulation are produced and secreted by the gastric mucosa.

The gastric mucosa

In the stomach, the surface epithelium is connected *via* the foveolae and neck region to the deeper gastric glands (Figure 1). Undifferentiated stem cells are located in the neck region and migrate outwards along the foveolar axis differentiating into mucous cells, and inwards differentiating into mucous, parietal, chief and endocrine cells^[1]. Histologically, the stomach is divided into three different regions - cardia, corpus/fundus and antrum/pylorus (Figure 1). The cardia comprises the proximal 0.5-2 cm of the stomach, and is mainly composed of mucus-producing cells and scattered parietal cells. In the corpus and fundus, most cells in the upper part of the glands are acid-secreting parietal cells, whereas the lower half of the glands is dominated by chief (peptic) cells that secrete digestive enzymes (pepsinogen and lipase). Most of the endocrine cells are found in the lower third of the glands. The antrum/pylorus occupies the distal quarter of the stomach, is mainly composed of mucus producing cells, with sparse parietal and endocrine cells located in the connecting neck region, and the endocrine cells are dominated by gastrin-producing (G) cells^[2]. Maintenance of gastric secretory functions is achieved through endocrine and paracrine mediators. Acid secretion is induced by a variety of stimuli and governed by the endocrine cells of the stomach. Gastrin is released from the G cells of the antral mucosa and travels through the blood stream to the corpus where the enterochromaffin-like cells are stimulated to secrete histamine which, in turn, stimulates the parietal cells to secrete acid^[2]. In contrast, somatostatin and prostaglandins, inhibit acid secretion^[2].

The gut-brain axis and ghrelin

Gut-derived regulatory peptide hormones are typically considered to be important in the control of basic enteric physiology including gastric acid secretion, digestion, gut motility and blood flow. Additional gastric hormones have been identified which point to further critical roles for the stomach in controlling appetite and satiety. *Via* interaction with their cognate receptors in the brain, these hormones

form part of an important signalling network often referred to as the gut-brain axis which co-ordinates many of the functions of the gut, including appetite modulation. The most potent orexigenic hormone and the only circulating orexigen described thus far is ghrelin.

Ghrelin is a peptide hormone consisting of 28 amino acids with a hydrophobic octanoyl moiety (or to a lesser extent decanoic acid) esterified to the third residue, serine^[3]. Ghrelin was discovered by "reverse pharmacology" using the endogenous growth hormone secretagogue receptor (GHS-R) which had been considered an orphan G protein-coupled receptor prior to discovery of ghrelin, but is now accepted as the major ghrelin receptor^[4]. Initially characterised as a new component of the growth hormone (GH)/insulin-like growth factor axis, ghrelin was shown to be a potent stimulator of GH secretion from mammalian somatotroph cells after activation of the GHS-R. This activity is reliant upon a complex interaction of signalling pathways incorporating the phospholipase C, cAMP and nitric oxide/cGMP systems^[5].

Human ghrelin is produced predominantly by the P/D₁ enteroendocrine cells of the gastric fundus (known as X/A-like cells in rodents) and the octanoylated mature hormone is secreted into the general circulation *via* the capillary networks of the gastric lamina propria^[6]. Interestingly, whilst ghrelin expression and secretion is greatest in the gastric mucosa, the GHS-R is present at its highest level in the pituitary^[7] and hypothalamic nuclei^[4], leading to speculation that ghrelin and its receptor have evolved to provide a link between peripheral energy homeostasis and GH secretion^[8].

The mechanisms underlying the co- and post-translational modifications of ghrelin have only recently begun to be deciphered. Hydrophobicity conferred by acylation may allow for the bidirectional transport of ghrelin across the blood-brain barrier^[9]. This modification also facilitates the binding of ghrelin to the GHS-R and is essential for GHS-R-mediated ghrelin activity. GOAT is a lipid transferase from the membrane bound *o*-acyl transferase (MBOAT) family of acyl transferases that specifically acylates proghrelin^[10]. Expression of murine GOAT is highest in the stomach, followed by pancreas, small intestine and colon^[11], although in human tissues the pancreas may be the major site of expression^[10]. As anticipated, GOAT expression is enriched in the ghrelin-producing gastric enteroendocrine cells of mice although a small subset of ghrelin immunopositive cells appear to be devoid of GOAT^[12], supporting the hypothesis that unacylated ghrelin has independent biological functions. Furthermore, it is tempting to speculate that unacylated ghrelin secreted from the stomach could be acylated by paracrine GOAT-expressing cells. Mice with genetic disruption of GOAT completely lack circulating acylated ghrelin but display supraphysiological levels of serum unacylated ghrelin, suggesting that GOAT may play a role in the translational control of ghrelin synthesis and/or secretion and activity^[13] or that ghrelin feedback mechanisms are disrupted in the absence of GOAT.

Plasma ghrelin secretion is episodic and concentrations

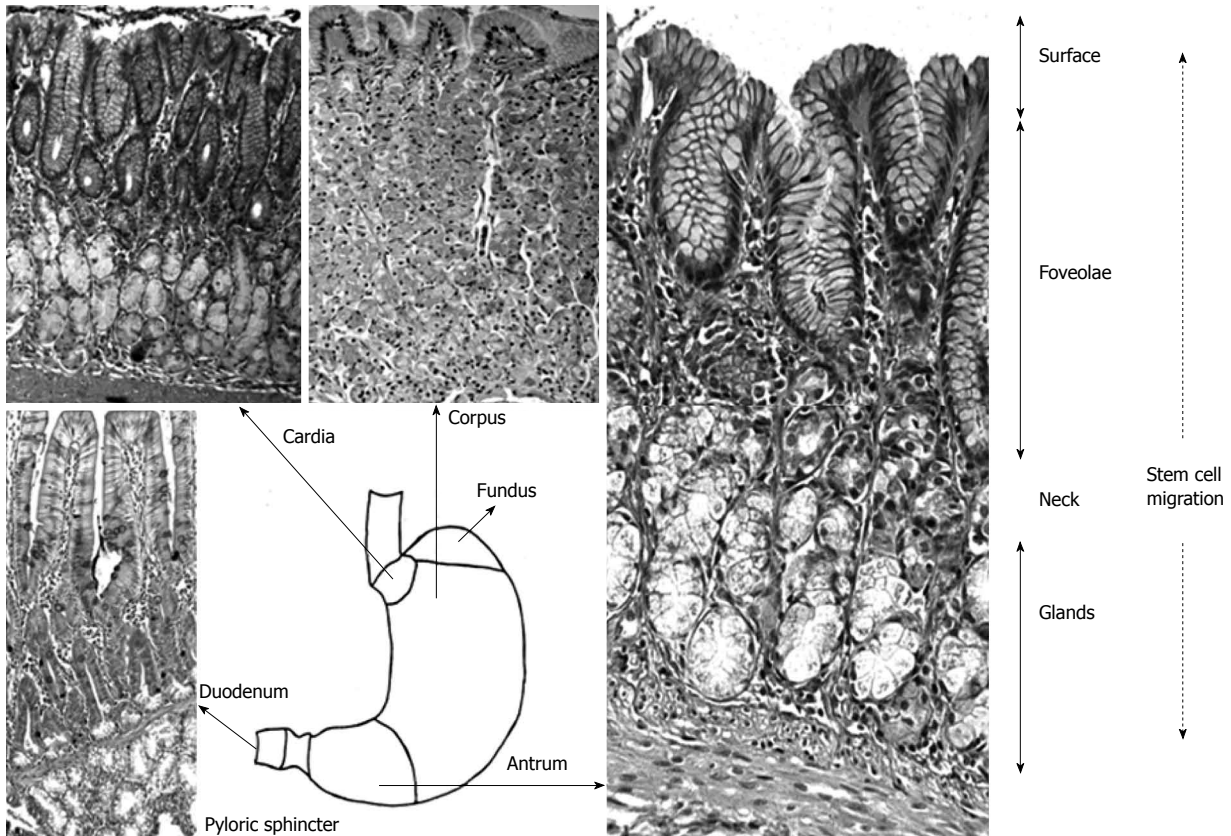


Figure 1 The regions of the stomach. In the stomach, undifferentiated stem cells located in the neck region migrate outwards or inwards specializing into mucous, parietal, chief and endocrine cells. In this figure, the antrum is shown at a three-fold larger magnification than the other images of the stomach. Ghrelin expression has been detected throughout the human gastric mucosa with strongest expression in the oxyntic mucosa.

in adults of healthy body mass index (BMI) are reported to range from 100-200 fmol/mL^[13] with healthy women having higher levels of acyl and total ghrelin than healthy men^[14]. Short-term fasting stimulates ghrelin secretion which may then initiate feeding in humans and rodents; post-prandial ghrelin serum levels are then reduced dramatically^[15]. In obesity, plasma ghrelin is decreased compared to normal counterparts^[16]. This phenomenon has previously been thought to be due to the reduced need for production and secretion of orexigenic ghrelin during periods of caloric excess. Ghrelin targets central nervous system appetite networks on multiple levels with a complex interplay occurring between it and other appetite-modulating peptides including neuropeptide Y, agouti-related protein, pro-opiomelanocortin and leptin^[17]. Whilst it is well established that pharmacological doses of ghrelin profoundly influence food intake, recent studies dissecting the mechanisms of the ghrelin acylation process have challenged the traditional dogma surrounding the endogenous ghrelin/GHS-R system and its role in appetite modulation that has existed since its discovery over ten years ago. Using GOAT transgenic knockout mouse models, Kirchner *et al.*^[13], have proposed that ghrelin and GOAT are primarily lipid-sensing molecules that are highly sensitive to changes in dietary fatty acid intake, specifically medium chain fatty acids and that acyl ghrelin signals to the brain that energy-dense food is available. In this regard, diets rich in octanoate (found in breast milk, coconut milk and

commercial infant formula) will result in an increase in the fatty acid substrate for GOAT and, therefore, a theoretical increase in circulating, acylated ghrelin. Experimental studies have shown that ghrelin influences metabolic substrate utilization in rodents^[18]. Additionally, it appears that GOAT plays a crucial role in this facet of ghrelin activity because the GOAT knockout mouse displays increased fat oxidation and is leaner than wild-type mice when given a high fat diet^[13]. Taken together, these studies advocate a role for ghrelin/GOAT and the GHS-R in sensing and reversing states of energy deficiency and reducing fat utilisation, suggesting that during times of nutrient excess, inhibition of ghrelin activity may reduce hyperphagia, obesity and type 2 diabetes. During prolonged caloric restriction, however, ghrelin activity is essential for maintaining blood glucose levels and preventing death in mice and this protective effect is mediated *via* increased GH secretion^[19].

ACYLATED GHRELIN IS NOT THE ONLY GHRELIN

The ghrelin/GHS-R axis may be expanded to incorporate several ghrelin isoforms that have been identified, including the splice variant des-Gln14-ghrelin and the non-modified des-octanoyl or unacylated ghrelin^[20]. Despite being the most predominant species of ghrelin in serum, unacylated ghrelin cannot bind to and, therefore,

does not activate the GHS-R. Initially thought to be a by-product of bioactive ghrelin degradation, unacylated ghrelin now appears to be an important hormone with an array of biological activities, particularly in the cardiovascular system, bone physiology, reproductive axes and prenatal growth^[20]. Many actions of unacylated ghrelin oppose those of acylated ghrelin. For example, the GH/IGF-1 axis is downregulated in transgenic mice overexpressing the non-modified isoform of ghrelin, resulting in a smaller than normal phenotype^[21], and food intake and gastric emptying are suppressed in mice administered unacylated ghrelin^[22]. It seems likely that these apparently inverse biological properties are the result of alternative receptor activation, and support the notion of the existence of undiscovered non-GHS-R ghrelin receptor(s). The differences and similarities between the isoforms in regards to function in gastrointestinal and selected peripheral tissues is summarised in Figure 2.

Interactions of ghrelin with the anorectic, pro-inflammatory adipokine leptin

It is well documented that ghrelin and the adipokine leptin exert mutually antagonistic regulatory effects on energy balance and appetite at the hypothalamic level. Whereas ghrelin stimulates NPY neurons in the arcuate nucleus, leptin inhibits these neurons and stimulates proopiomelanocortin (POMC) neurons, thereby suppressing food intake. The stomach contributes, in part, to plasma leptin levels, although the majority of leptin production occurs in adipose tissue. Stomach-derived leptin is expressed in the lower half of the gastric fundic glands in an endocrine cell type distinct from ghrelin-producing cells^[23]. The gastric co-expression of these two antagonistic hormones involved in appetite modulation, generation of satiety signals and energy homeostasis again highlights the importance of the stomach as an endocrine organ.

Ghrelin suppresses inflammation in rodent models of disease and in humans

Ghrelin and GHS-R are expressed in immune cells, and manipulation of ghrelin/GHS-R expression and activity affects T cell function. In immune cells, as in the hypothalamus, ghrelin again antagonises leptin. Human T cells stimulated by leptin increase their production of the pro-inflammatory, anorectic cytokines IL-1 β , IL-6 and TNF α as well as displaying increased GHS-R1a expression. Co-treatment with ghrelin inhibits leptin-induced cytokine levels in a dose-dependent manner^[24]. Knockdown of ghrelin in primary human T cells increases Th1 cytokine production and IL-17 secretion suggesting a role for autocrine/paracrine ghrelin in the endogenous restraint of pro-inflammatory cytokine production and release^[25]. Studies demonstrating an *in vivo* anti-inflammatory role for ghrelin are rapidly increasing in number and include mouse models of pancreatitis and colitis^[26]. The therapeutic effect of ghrelin has been attributed to the down-regulation of pro-inflammatory cytokines (including IL-12, IFN- γ , and TNF- α), recruitment of inflammation-suppressing regulatory T cells and increased levels of the

anti-inflammatory cytokine IL-10^[26]. Recently, ghrelin has also been shown to inhibit secretion of pro-inflammatory high-mobility group box 1 (HMGB1; a DNA-binding cytokine that is a critical late mediator of inflammation) from activated macrophages, and treatment with ghrelin reduces serum HMGB1 levels in rodent models of sepsis^[27]. Pro-inflammatory cytokine production in the resident macrophages of the brain and spinal cord (microglia) is also reduced by ghrelin treatment, thereby reducing the severity of experimental autoimmune encephalomyelitis, a model of multiple sclerosis^[28]. In clinical trials, ghrelin has been used with success as an anti-inflammatory agent in cachexic patients with chronic respiratory infection and inflammation. In these patients, ghrelin treatment increased body weight and significantly suppressed inflammation in the lungs by decreasing neutrophil infiltration/accumulation and reducing serum TNF- α ^[29].

Exogenous ghrelin is gastroprotective and prokinetic

Gastric mucosal injury is attenuated by central and peripheral administration of ghrelin in rodent models of gastric and duodenal disease. Ghrelin also accelerates healing in these ulcerogen models by increasing mucosal healing, proliferation and blood flow,^[30] and these factors have been shown to be dependent on an intact GH/IGF axis, endogenous nitric oxide activity and vagal and sensory nerve integrity^[31, 32]. Ghrelin protects gastric mucosal cells from apoptosis induced by *H. pylori* LPS by increasing constitutive nitric oxide synthase activity, reducing caspase-3 and inducible nitric oxide synthase^[33]. Unsurprisingly, given its structural similarity to the classical prokinetic peptide motilin, ghrelin also has a prokinetic effect on gut motility in many species including humans^[34] and the benefits of this are being evaluated in clinical trials in patients with gastrointestinal motility disorders.

Non-infectious gastric disorders disturb ghrelin expression

Non-infectious gastric disorders are known to impact ghrelin secretion from the stomach and subsequent circulating ghrelin levels. Ghrelin-producing endocrine tumours of the stomach induce supra-physiological ghrelin levels^[35] which may result in desensitization of the GHS-R. Recently, methyl donor deficiency (MDD) in pregnant rats was shown to result in reduced circulating ghrelin but not ghrelin gene transcription in the offspring^[36]. The altered polarity of the gastric fundic glands due to the nutritional deficiency in these rats may be responsible for a reversal in normal ghrelin secretion; that is, ghrelin may be secreted into the gastric lumen instead of the normal secretion pattern into the blood. The authors speculated that the reduction in ghrelin could be the cause of the significant intrauterine and post-natal growth restriction associated with MDD.

Autoimmune atrophic gastritis has also been shown to decrease ghrelin production and secretion^[37] and acylation of proghrelin may be disrupted - it is tempting to speculate that this may be due to the effects of inflammation on the activity and/or expression of the more recently discovered GOAT.

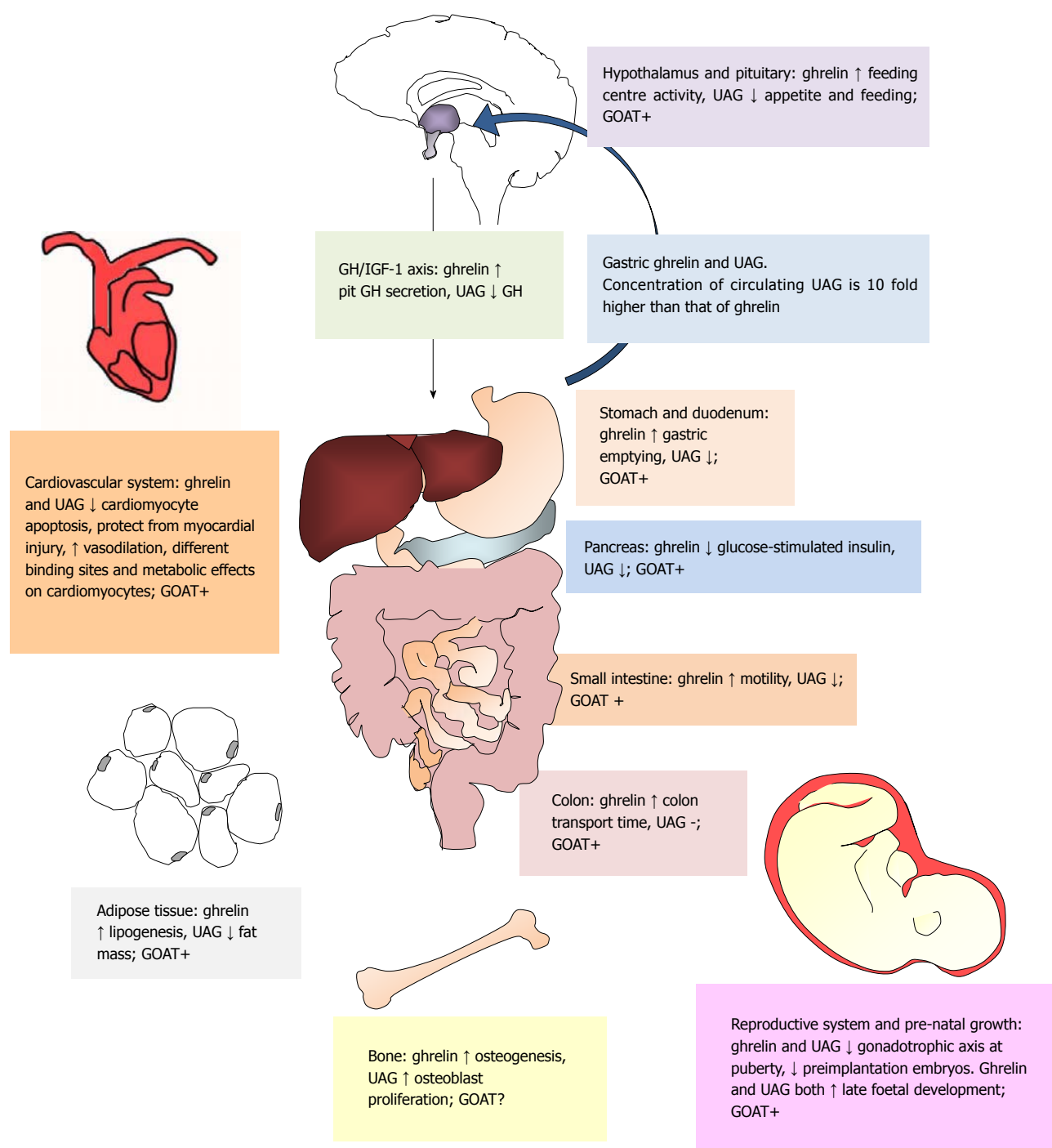


Figure 2 Diagram summarising the similarities and diversities of ghrelin and unacylated ghrelin activities in gastrointestinal and selected peripheral tissues/systems. The expression of ghrelin *o*-acyltransferase (GOAT) in most peripheral tissues suggests that acylation of locally-produced unacylated ghrelin (UAG) is possible. GOAT + indicates tissues that have been found to express mRNA for GOAT. GOAT? indicates that GOAT expression status is unknown. Pit: Pituitary; ↑: Increases or stimulates; ↓: Decreases; -: Effect unknown.

Atrophic gastritis is also associated with chronic *H. pylori* infection in a subset of patients. Of the infectious agents causing gastric pathogenicity, *H. pylori* is the most common one, however, other *Helicobacter* species can cause milder forms of gastritis^[38].

H. PYLORI INFECTION

H. pylori is a Gram-negative, rod/spiral-shaped micro-aer-

ophilic bacterium that causes life long infection. *H. pylori* has strategies that allows its survival and persistence in the inhospitable environment of the stomach. Adherence of *H. pylori* to the mucosal surface supports gain of nutrients and also facilitates entry into epithelial cells and the underlying mucosal tissue as shown *in vivo*^[39,40] and *in vitro*^[41]. Adherence of *H. pylori* is dependent on expression of bacterial attachment proteins (adhesins) and the cognate host oligosaccharides (glycans), displayed by glycoproteins

and glycosphingolipids in gastric epithelium and also by mucins in the gastric mucus layer^[42-44]. The microbe is well adapted to the gastric mucus niche, having long whip-like flagella facilitating locomotion through the mucus layer. In addition, surface-bound urease that catalyzes the hydrolysis of urea to ammonia and carbon dioxide allows the microbe to “neutralize” its microenvironment. Flagella, urease, adhesins, and genes encoding proteins with a predicted function in chemotaxis are essential for *H. pylori* colonization of laboratory animals^[45].

The majority of *H. pylori* infected individuals remain asymptomatic, but 10%-15% develop peptic ulcers, and 3% gastric non-cardia adenocarcinoma^[46]. Since most infected individuals show no clinical symptoms, the pathological process must be influenced by host/environmental factors in addition to the genotype of the infecting strain. Inter-individual factors, such as the type of host structures that *H. pylori* can bind to (blood group dependent), account for some of the variance of pathology as a consequence of infection^[47]. Stress is another factor that may work in conjunction with *H. pylori* or cause ulcers through alternative pathways, as people exposed to disasters as well as prisoners of war have increased occurrence of ulcers, and wound healing is slower in stressed individuals^[48]. The intense humoral and cellular immune responses associated with gastric colonization are usually unable to eradicate *H. pylori* and may play a major role in the morbidity of the disease.

H. pylori infection is one of the most common bacterial infections in the world, colonizing approximately half the population^[49]. Colonization usually occurs before the age of ten^[49], and once established within the gastric mucosa, the bacterium can persist for life, although transient infection occurs in a few individuals^[50].

The classification of *H. pylori* as a human carcinogen is mainly based on results from prospective epidemiological studies showing a relationship between infection with *H. pylori* and subsequent development of gastric carcinoma. Eradication of *H. pylori* has also resulted in reversal of pre-neoplastic conditions^[51], and *H. pylori* eradication in gastric ulcer patients reduces the risk of developing gastric cancer^[52], further supporting an association between *H. pylori* infection and cancer development.

Effect of *H. pylori* infection on the gastric endocrine system

In the majority of infected individuals, *H. pylori* infection is antrum predominant, and acid production by the largely unaffected corpus is enhanced leading to an increased risk of duodenal ulcers. In a minority, infection is corpus predominant, and infection is associated with progressive gastric atrophy and intestinal metaplasia, subsequent hypochlorhydria and an increased risk of gastric cancer^[53,54]. Possibly, the initial infection starts in the antrum, and in individuals with a lower basic acid production, the infection spreads to the corpus, as supported by the observations that relatives of gastric cancer patients have a lower basic acid production than the general population^[55], and that patients with antral predominant *H. pylori* infection

and gastritis developed corpus predominant infection and gastritis after treatment with acid suppressive therapy^[56]. Hypochlorhydria permits infection with other bacteria that may enhance the production of carcinogenic (e.g. N-nitroso) compounds. Thus, impaired control of gastric juice secretion is observed in chronic gastritis due to *H. pylori* infection, but the site/type of infection determines what effect *H. pylori* has on the endocrine system, or the downstream results of the endocrine system such as acid production. Further differences can be attributed to the development of intestinal metaplasia in a subset of individuals with chronic gastritis, and the endocrine-cell population present in the intestinal metaplasia resembles that found in the cryptal region of the normal small intestine^[57]. The literature is biased towards the antrum predominant infection, as these represent the majority of infections, and the effects of corpus predominant infection may therefore be disguised in many studies.

Effects of *H. pylori* on endocrine cells

Colonization of *H. pylori* in the human stomach results in release of chemoattractants such as IL-8, IL1 β and TNF α , which stimulate G cells, and the gastrin cell count is increased in *H. pylori* infected gastric mucosa^[58]. The number of D cells producing somatostatin decreases simultaneously^[59]. Similarly, some species of swine *Helicobacter* alter the number of endocrine cells in gastric mucosa. Some of these alterations, for example an increase in the number of G cells, decrease in the number of D cells and especially an increase in the ratio of G to D cells can be responsible for the development of gastroesophageal ulcers in swine^[60].

The impact of *H. pylori* on circulating plasma ghrelin levels highlights the importance of GOAT

Due to the gastric location of ghrelin-producing cells it is intuitive that ghrelin production, acylation and/or secretion might be compromised by chronic gastritis and atrophy and that this in turn will affect appetite, weight and BMI.

There have been contradictory reports in the literature in regards to the effect of *H. pylori* infection on circulating ghrelin levels and some of these discrepancies may be due to differences in populations (age, race, geography, gender, diet and overall health), extent of disease (for example whether atrophy is present or not) and *H. pylori* strain differences. This is also complicated by the use of different immunoassays to measure ghrelin and by the fact that acylated ghrelin is highly unstable and degrades rapidly to unacylated ghrelin^[61]. The optimum method of plasma ghrelin measurement is a contentious issue - a number of researchers maintain that measurement of total ghrelin is reflective of active ghrelin levels and is an adequate approach. Most studies examining the impact of *H. pylori* have examined total ghrelin levels only and the majority of these studies determined that infection decreases plasma ghrelin levels (Table 1). However, an important theme that emerges from these studies is that plasma total

Table 1 Original studies measuring total ghrelin only (in chronological order)

Subjects	Nationality	Tissue	Total ghrelin in <i>H. pylori</i> infection (or cure)	Ref.
39 Adults F	Turkish	G	-	[78]
10 Adults F + M	UK	P	↑ after <i>H. pylori</i> cure	[79]
68 Adults F + M	Japanese	P	↓	[80]
160 Adults F + M	Japanese	G + P	↓ more so as atrophy increases	[81]
89 Adults F + M	Japanese	P	↓ but did not recover after cure	[82]
225 Adults F + M	Japanese	P	↓ in chronic gastritis	[83]
61 Adults F + M	Japanese	G + P	↓ and associated with virulence strain	[84]
132 Adults F + M	Japanese	P	↓	[85]
100 Adults F + M	Polish	P	↓	[86]
56 Adults F + M	Japanese	P	↓	[87]
146 Children F + M	Polish	P	↓	[88]
134 Adults F + M	Japanese	G + P	↑ or ↓ after cure, depending on BMI. Ghrl mRNA ↑	[89]
62 Adults (> 75 yr) F + M	French	G + P	↓	[90]
120 Adults, 60 children F + M	Polish	P	↓	[91]
63 Adults F + M	Korean	G + P	- no healthy control group	[92]
50 Adults F + M	Turkish	P	- in absence of atrophic gastritis	[93]
85 Prepubertal children F + M	Italian	P	↓ related to severity of gastritis	[69]
256 Adults M	American	G + P	-	[94]
100 Adults F + M	Chinese	P	↓	[95]
22 Adults F + M	Korean	G + P	↑ ghrl mRNA after cure	[96]
341 Adults F + M	Chinese	P	↓ in males only	[97]

F: Female; M: Male; G: Gastric tissue; P: Plasma; Ghrl: Ghrelin; ↑: Increased; ↓: Decreased.

Table 2 Original studies measuring the effect of *H. pylori* infection on plasma acyl and unacylated ghrelin levels

Subjects	Nationality	Tissue	Acyl and total ghrelin in <i>H. pylori</i> infection (or cure)	Ref.
69 Adults F + M	Japanese	P	↓ acyl ghrl in atrophy only	[98]
50 Adults F + M	Italian	P	↑ acyl ghrl and acyl/total ratio in atrophy cf. healthy controls	[63]
220 Adults F + M	Japanese	P	↓ acyl ghrl associated with atrophy and ↑ after cure	[62]

F: Female; M: Male; P: Plasma; Ghrl: Ghrelin; ↑: Increased; ↓: Decreased.

ghrelin is reduced only in the presence of gastric atrophy and that in a subset of these studies, plasma ghrelin levels are negatively correlated with the severity of atrophy^[62,63].

The potentially different, even perhaps inverse, biological roles of acyl- and unacylated ghrelin suggest that the ratio of modified to unmodified ghrelin is highly important. In the context of *H. pylori* infection, few studies have taken this into account and in those that have, the findings are again contradictory (Table 2). In Japanese adults^[62], the acylated ghrelin/total ghrelin ratio as well as plasma acyl ghrelin levels are reduced, whereas in a study conducted on Western males^[63], there was a significant increase in acyl ghrelin and indeed the ratio of acylated ghrelin/total ghrelin which the authors speculate may be due to an endogenous, compensatory increase in the acylation process in response to a loss of total ghrelin secretion. A logical extension of this work would be to assess the level of expression and activity of GOAT during *H. pylori* infection and chronic gastritis and gastric atrophy.

The substrates for GOAT are the prohormone proghrelin and dietary medium chain free fatty acids which are used directly by GOAT. GOAT preferentially acylates proghrelin with octanoic acid, however, other species of acyl ghrelin (including C10:0 ghrelin) are present in the circulation at low levels and even unnatural forms of ghrelin

(C7:0 ghrelin) can be synthesised in mice when they are fed a diet rich in n-heptanoic acid or glyceryl triheptanoate^[13]. This new research suggests another potential reason for the effect of *H. pylori* infection on acyl ghrelin levels in addition to ghrelin endocrine cell destruction by gastric atrophy and or inflammatory cell destruction. Given that infection has been associated with malabsorption due to hypochlorhydria, vomiting, dyspepsia and with increased susceptibility to other enteric pathogens, especially in children^[64], it is tempting to speculate that altered dietary intake or dysregulated absorption of fatty acid substrate for GOAT could alter the ratio of acyl to total ghrelin during *H. pylori* infection. In this regard, a proportion of medium chain triglycerides are absorbed directly in the stomach as well as the small intestine^[65], and chronic gastritis leading to gastric atrophy may impair this absorption and reduce the ability of GOAT to acylate ghrelin. GOAT expressed in the small intestine of humans most likely contributes to systemic acyl ghrelin levels, and duodenal ulceration due to *H. pylori* infection may disrupt acyl production and secretion in this region of the gut. The development of techniques to assess human GOAT expression and function and the use of GOAT transgenic and knockout animal models for *H. pylori* infection experiments will allow researchers to explore this empirically in the near future.

***H. pylori*, malnutrition and growth failure**

Children with chronic gastrointestinal disease, including Crohn's disease, are known to have a higher incidence of growth retardation and pubertal delay when compared to their healthy counterparts^[66]. In a significant proportion of *H. pylori*-positive children, growth impairment, as indicated by a reduced mean height to below the 25th percentile, is evident and may be linked to *H. pylori* associated factors including, but not limited to, dyspepsia, diarrhoea, malnutrition and iron deficiency anaemia^[67]. Due to its potent stimulation of GH release, ghrelin may contribute to postnatal growth in humans. Ghrelin is synthesised and acylated by the placenta and is detected in foetal circulation from week 20-23, indicating that it may also play an important role in pre-natal growth^[68]. In support of this is a study showing that ghrelin deficiency in rat pups due to MDD contributes to severe intrauterine growth retardation^[36]. Whether there is a causative relationship between reduced circulating ghrelin during *H. pylori* infection and growth retardation/delay in infected children has not been adequately tested. Successful early eradication of *H. pylori* in a small study of infected children was associated with increased BMI and lean and fat mass, but not ghrelin, which was actually decreased^[69]. This may suggest that changes in ghrelin levels after *H. pylori* cure are epiphenomenal, however, only total ghrelin was measured in this study and the determination of the ratio of acyl to total ghrelin in cured children would have been more informative.

In contrast to the Pacifico *et al* study in which paediatric subjects cured of *H. pylori* have decreased plasma ghrelin, most studies report an increase in gastric ghrelin expression and secretion into the circulation after *H. pylori* cure in adults^[70,71]. However, in agreement with Pacifico *et al*, a study by Choe *et al*^[70] showed no increase in plasma or tissue ghrelin levels in children post *H. pylori* eradication and indeed no difference in ghrelin expression between *H. pylori* positive and negative children before treatment. It may be that the gastropathology of infected children differs from infected adults resulting in no significant impact on ghrelin-producing cells. Neutrophilic activity is associated with decreased density of ghrelin-producing cells^[71] and the neutrophilic infiltration of the mucosa is less in infected children than it is in adults^[72]. Gastric atrophy, which typically takes more than 10 years of chronic infection to develop, is rarer in children from Western developed countries, although the incidence of *H. pylori* associated atrophy is significantly higher in the Japanese paediatric population^[73]. Even in adults, specific measurement of acyl ghrelin in patients with varying severity of atrophic gastritis showed that atrophy is the defining factor for changes in plasma acyl ghrelin, irrespective of *H. pylori* status^[62].

IS OBESITY A POTENTIAL CONSEQUENCE OF *H. PYLORI* ERADICATION AND DOES GHRELIN HAVE A ROLE?

Human populations have evolved with very high rates of

chronic *H. pylori* infection. Therefore, it is likely that our metabolic rheostats have evolved to work in the context of chronic gastric infection. Indirect evidence such as the lower BMI and adiposity in *H. pylori* infected compared to uninfected populations, coinciding with lower levels of circulating ghrelin in infected individuals that can be increased after eradication, hints at a role for *H. pylori* as an obesity preventing agent. Whether there is a causative relationship between the falling prevalence of *H. pylori* infection and rising obesity rates in developed countries, where fewer children are becoming colonized, is a highly controversial topic. In a large study with 6724 adult patients who comprised a probability sample of the USA population, *H. pylori* seropositivity (either CagA-positive or -negative strains) was not associated with BMI or serum leptin levels, and ghrelin levels were not measured^[74]. Serological methods used in this study may not be as accurate at measuring *H. pylori* activity as other techniques. Nonetheless, there is no strong data to support a protective role of *H. pylori* in the prevention of obesity in developed countries. However, this lack of evidence may be due to the fact that both dietary habits associated with obesity and *H. pylori* infection are more prevalent in groups with low socioeconomic status^[75-77]. Eradication can restore a normal BMI in adults and growth velocity in children, however, this occurs particularly in developing countries and in Japan, where severity of atrophic gastritis is more pronounced. Whether ghrelin and other appetite-regulating peptides have a physiological role in this process is yet to be determined.

CONCLUSION

H. pylori infection affects the gut-brain axis due to its direct effects on gastric mucosa and on the enteroendocrine cell population including ghrelin-producing cells. Ghrelin is anti-inflammatory, anti-apoptotic and wound healing in gastritis, therefore, loss of ghrelin acylation during gastritis may impair healing. The relationships between *H. pylori*, ghrelin and BMI, adiposity and appetite remain speculative, but a greater understanding may give important insights into the pathophysiological processes underlying obesity in Western populations (Figure 3). By measuring gastric GOAT during infection in the future, we can shed more light on the effect of inflammation and infection on acyl ghrelin levels as opposed to the less illuminating total ghrelin levels.

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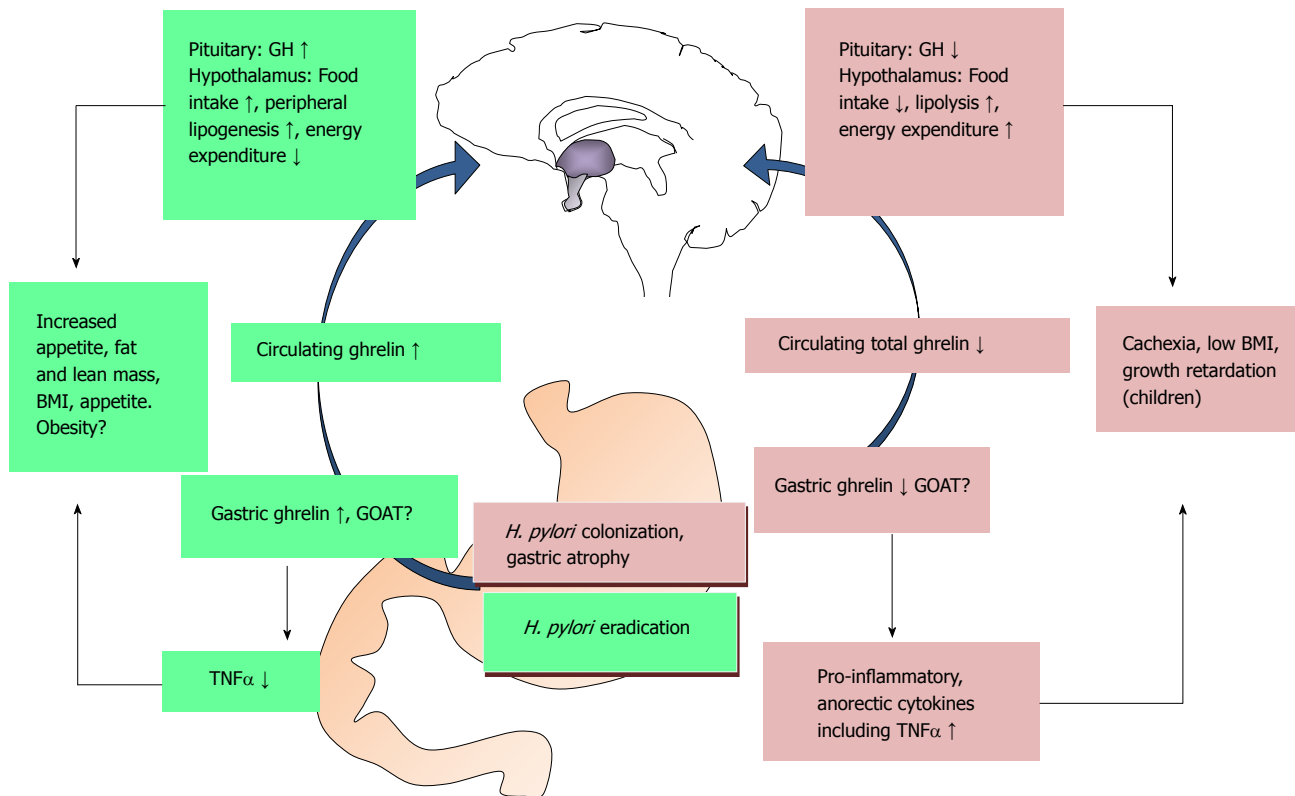


Figure 3 Potential pathways leading to reduced appetite, weight loss and pro-inflammatory state in chronic *Helicobacter pylori* infection, implicating the ghrelin axis. The majority of studies identify reduced gastric and circulating ghrelin in patients with chronic infection and gastric atrophy which may in turn lead to decreased growth hormone (GH) secretion and somatic growth in children. Reduction in ghrelin signalling in the hypothalamic feeding centres during chronic infection may be responsible for the observed reduced body mass index (BMI) in patients; *Helicobacter pylori* (*H. pylori*) eradication and subsequent restoration of ghrelin may reverse this situation. It is speculated that this in turn can contribute to obesity. GOAT? indicates that the effect of *H. pylori* status on GOAT expression is unknown. ↑: Increase; ↓: Decrease. TNF: Tumor necrosis factor.

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S100B protein in the gut: The evidence for enteroglial-sustained intestinal inflammation

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the major features of EGCs and S100B protein occurring in intestinal inflammation deriving from such.

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Abstract

Glial cells in the gut represent the morphological and functional equivalent of astrocytes and microglia in the central nervous system (CNS). In recent years, the role of enteric glial cells (EGCs) has extended from that of simple nutritive support for enteric neurons to that of being pivotal participants in the regulation of inflammatory events in the gut. Similar to the CNS astrocytes, the EGCs physiologically express the S100B protein that exerts either trophic or toxic effects depending on its concentration in the extracellular milieu. In the CNS, S100B overexpression is responsible for the initiation of a gliotic reaction by the release of pro-inflammatory mediators, which may have a deleterious effect on neighboring cells. S100B-mediated pro-inflammatory effects are not limited to the brain: S100B overexpression is associated with the onset and maintenance of inflammation in the human gut too. In this review we describe

INTRODUCTION

Intestinal tissues are innervated by a complex and extensive component known as the enteric nervous system (ENS)^[1]. The ENS is characterized by the presence of neurons and enteric glial cells (EGCs) which are arranged into interconnected ganglia distributed between 2 major plexuses, and they control several gut functions^[2,3]. During the course of time, the traditional view of EGCs has changed from being a mere mechanical support for surrounding neurons to that of a more articulate and complex nature, since they are actively involved in the regulation of homeostasis, motility and inflammatory processes within the gut^[4,5].

Similar to the astrocytes in the central nervous system (CNS), the EGCs release several signaling molecules^[4,5]. Among these, great importance has been given to the better comprehension of the specific glial-derived S100B protein^[6-8]. This protein is a small, diffusible neurotrophin that is situated in the cytoplasm and/or the nucleus of both

nervous and non-nervous tissues^[9,10]. In the brain, S100B has been considered a “Janus face” neurotrophin^[11,12] because it exerts opposite actions depending on its concentration in the extracellular milieu: it has a pro-proliferative and neurogenic effect on astroglia and on serotonergic neurons at nanomolar concentrations, as well as a neuro-degenerative function^[12] at micromolar concentrations, determining dysregulated glial cell proliferation and amplifying neuroinflammation. Similar to the brain, recent studies have suggested the involvement of S100B in inflammatory processes occurring in the gut, highlighting the importance of EGCs as key regulators of gut homeostasis^[13-15].

In this review, we will focus on the role of EGCs and the S100B protein and we will take them into consideration by looking at both experimental animal models and some human diseases for which evidence exists, and in particular their involvement in inflammatory conditions of the human gut where the role of S100B appears to be prominent.

ENTERIC NERVOUS SYSTEM

The gut is characterized by a sequence, starting from the serosa, as follows: subserosa, longitudinal muscle, myenteric plexus, circular muscle, submucosal plexus, muscularis mucosae and mucosa^[2]. The myenteric and submucosal plexuses are characterized by the presence of ganglia which, in turn, contain enteric neurons and EGCs in a ratio of 1:7^[16]. In the ENS, the enteric ganglia are involved in basic gut functions, such as the regulation of peristalsis, secretion and blood flow and the modulation of the immune/inflammatory processes^[17-19]. Several neurotransmitters are involved in the control of all these intestinal functions, such as vasoactive intestinal peptide and nitric oxide (NO)^[20,21]. In particular, NO is produced by the biosynthetic enzyme neuronal NO synthase (NOS), which is expressed in myenteric neurons, and by the inducible form of NOS (iNOS), which is expressed in EGCs^[15].

EGCs: both protective and destructive cells in the gut

EGCs are small cells with a “star-like” appearance^[16] containing intracellular arrays of 10 nm filaments made up of glial fibrillary acidic protein (GFAP)^[16,22-24]. This cell population was first described by Dogiel^[25] using methylene blue staining on full thickness preparations. At present, the S100B protein and GFAP are commonly used as specific markers in order to identify EGCs^[22,26]. More recently, other markers have been proposed for the identification of glial cells in the human gut, especially in whole-mount preparations: Sox8/9/10, a specific nuclear marker^[16]. EGCs release a wide range of factors accounting for the development, survival and differentiation of peripheral neurons^[27]. Traditionally, EGCs have been considered as a mechanical support for enteric neurons, but, in recent years, this restrictive view has changed to one of a more articulate and complex nature, since it has been described that EGCs are involved in the maintenance of intestinal homeostasis^[28-30]. Indeed, EGCs control intestinal epithelial barrier (IEB)

functions, as demonstrated in animal studies in which the ablation of enteroglial network enhances intestinal vascular permeability together with an increase in IEB paracellular permeability^[31-34]. Furthermore, *in vitro* data has shown that EGCs partially decrease IEB permeability *via* the release of S-nitrosoglutathione and the regulation of zonulin-1 and occludin expression^[35,36]. Although the function of glial mediators still have to be identified, it is conceivable that they could be actively involved in the EGCs-mediated effects on barrier functions.

Besides the well documented ‘protective role’, EGCs are activated by means of inflammatory insults and they may directly contribute to an inflammatory condition working as an antigen presenting cell-type promoting a variegated release of cytokine synthesis^[13-15,35-37] in the gut milieu. Therefore, EGCs may act as “receptors” for cytokines and they themselves produce interleukin-6 (IL-6) and IL-1b^[38,39]. Moreover, EGCs express iNOS and L-arginine, the machinery for the time-delayed and micromolar release of NO, one of the most important signaling molecules involved in host-immune defense against viruses and bacteria as well as a well-known pro-inflammatory mediator^[15,40,41].

EGC-SELECTIVELY EXPRESSED PROTEINS

GFAP

Mature EGCs are rich in the intermediate filament protein, GFAP^[42,43]. Its expression is modulated by glial cell differentiation, inflammation and injury^[42], indicating that the level of GFAP accords with the functional state of glial cells.

In animals, two classes of glial cells can be distinguished, namely the GFAP positive (+) and GFAP negative (-) groups, as demonstrated by von Boyen *et al.*^[8]. In the same study, it was suggested that pro-inflammatory cytokines control GFAP+ enteric glia, which, in turn, are involved in the modulation of the integrity of the bowel during inflammation^[8].

In humans, GFAP expression is altered in the mucosa of patients with inflammatory bowel diseases (IBD), as well as ulcerative colitis (UC) and Crohn’s disease^[33].

S100B protein

S100B belongs to the S100 protein family that includes more than 20 EF-hand Ca²⁺-Zn²⁺ binding proteins^[9,10,44-47]. S100B is the homodimer of β subunit^[48]. In the brain, S100B in nanomolar concentrations promotes neuronal survival, neurite outgrowth^[49] and it stimulates astrocytic proliferation^[50], increasing the intracellular free Ca²⁺ levels *in vitro*^[51]. On the other hand, micromolar amounts of S100B protein have been observed in several neuropathologies such as Alzheimer’s disease and Down’s syndrome^[52,53].

In the human gut, among S100 proteins, only the S100B protein is specifically and physiologically expressed

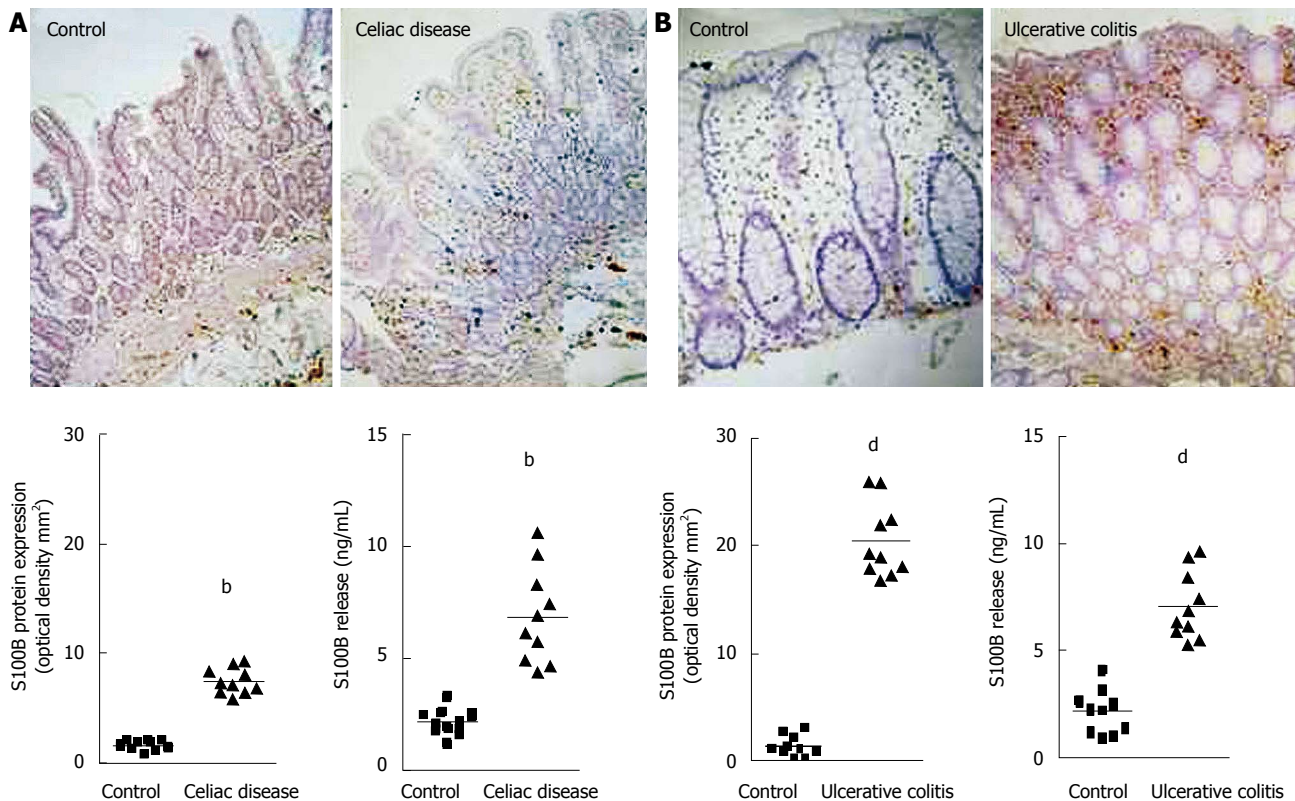


Figure 1 Changes in S100B protein expression during intestinal inflammation. A: Celiac disease^[13]. Immunohistochemistry shows stronger S100B immunopositivity in the duodenal mucosa of patients affected by celiac disease, compared with healthy controls (original magnification, $\times 100$). The graphs represent S100B protein expression (left) and release (right) in healthy controls and patients with celiac disease ($^bP < 0.01$); B: Ulcerative colitis^[14]. Immunohistochemistry shows stronger S100B immunopositivity in the rectal submucosa of patients with ulcerative colitis, compared with healthy controls (original magnification, $\times 100$). The graphs represent S100B protein expression (left) and release (right) in healthy controls and patients with ulcerative colitis ($^dP < 0.01$).

by EGCs^[13-15], while other members, such as S100A8, S100A9 and S100A12 are found in phagocytes and in intestinal epithelial cells in patients affected by IBD^[54,55].

Recent findings have demonstrated that aberrant expression and the release of S100B correlate with the gut inflammatory status^[13,14]. Interestingly, the search for a specific S100B signaling receptor has demonstrated that this protein may accumulate at the RAGE (receptor for advanced glycation end products) site only in micromolar concentrations^[14,56-59]. Such interaction leads to mitogen-activated protein kinase (MAPK) phosphorylation and consequent nuclear factor- κ B (NF- κ B) activation^[13] which, in turn, leads to the transcription of different cytokines and iNOS protein. Thus, S100B can be considered as an easily diffusible pro-inflammatory cytokine which gains access to the extracellular space especially at immune-inflammatory reaction sites in the gut^[13-15,60,61].

EGCs AND S100B IN GUT INFLAMMATION

In humans, recent and increasing data has demonstrated that EGCs and S100B protein are directly involved in gut inflammatory diseases^[13,14,33]. Previous investigations described abnormalities of the enteroglial network in patients with Crohn's disease and UC^[33]. More recently, glial abnor-

malities have been confirmed by 2 separate studies carried out by our group^[13,14]. In particular, we demonstrated that, in patients with celiac disease (CD)^[13] and UC^[14], EGCs participate in the modulation of mucosal NO production *via* S100B overexpression and release. Indeed, in patients with CD, we demonstrated that S100B plays an active role in NO-dependent inflammation^[13]. In particular, increased S100B protein expression and release were observed in the duodenal mucosa of patients with untreated CD, compared to healthy controls (Figure 1A)^[13]. Very interestingly, S100B upregulation was accompanied by enhanced iNOS protein expression and consequent NO release, both representing crucial features in CD^[62].

The relationship between S100B and NO production was confirmed by the demonstration that the administration of exogenous S100B protein to non-inflamed duodenal biopsy specimens from healthy controls, resulted in both iNOS protein expression and NO release, indicating that micromolar concentrations of this protein are able to participate in the inflammatory response of even "healthy duodenum"^[13]. Besides NO production, exogenous S100B mediates a significant increase in lipid peroxidation associated with a marked increase in phosphorylated-p38 MAPK protein expression and with the activation of NF- κ B, in accordance with the previously mentioned studies^[13].

Taken together, these observations represented the

first data in humans suggesting that *via* S100B upregulation, EGCs directly participate in NO-dependent inflammation occurring in CD, and they paved the way by supposing that EGCs are part of complex immunoregulatory effectors since they establish a strategic first defense line against foreign antigens. By means of EGC proliferation, changes in enteroglia architecture have been reported also in patients with IBD^[33,63]. Several studies have shown that EGC markers are differentially altered in Crohn's disease and UC with a decrease in EGC density in Crohn's disease and a gliosis-like phenomenon in UC^[33,63].

In support of these observations, it has recently been confirmed that EGCs directly participate in the chronic mucosal inflammation of patients with UC^[14]. In fact, S100B immunoreactivity significantly increased in the rectal mucosa of these patients when compared to the mucosal S100B expression in healthy controls (Figure 1B)^[14]. This upregulation was associated with the specific stimulation of iNOS and consequent abnormal mucosal NO production, both representing characteristic features of UC^[64,65].

In addition, *via* iNOS expression, exogenous S100B induces a significant and concentration-dependent increase in NO production in the human rectal mucosa of healthy controls *via* RAGE interaction^[14], confirming the ability of EGCs to modulate NO production and the specificity of S100B protein-mediated responses in the human gut.

Further confirming that EGCs are part of the complex system of immunoregulatory effectors in the gut, it has been shown that the addition of pro-inflammatory stimuli to rectal mucosal tissue led to EGC activation, again *via* RAGE involvement^[14] as demonstrated by both S100B upregulation and enhanced NO production. These findings indicate that EGCs are able to recognize inflammatory stimuli and that once activated, they produce and release S100B up to micromolar concentrations, thereby contributing to NO production in the human gut.

CONCLUSION

ECGs as a target for new drugs aimed at inflammatory gut disorder management

In summary, emerging evidence now indicates that EGCs actively participate in the modulation of inflammatory responses in the human gut. Targeting their hyperactivation in the gut in inflammatory disorders may represent a novel approach to diminish tissue damage and to counteract the lack of long-term effectiveness of classical immunosuppressant agents.

Additional studies investigating the relationship between EGCs and immune cells are warranted in order to carry out an in-depth examination of the role of glial cells and glia-derived factors in the modulation of immune/inflammatory responses in the human gut. Preliminary data indicates that EGCs-derived S100B is able to affect peripheral blood and intestinal mucosal immune cell responses *via* RAGE^[66].

The application of this approach may help the future evaluation of the relationships between EGCs and im-

mune cells in order to better understand the pathophysiology of intestinal inflammation and to establish new therapeutic approaches towards the treatment of gut inflammatory disorders.

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Portal vein thrombosis and arterioportal shunts: Effects on tumor response after chemoembolization of hepatocellular carcinoma

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Abstract

AIM: To evaluate the effect of portal vein thrombosis and arterioportal shunts on local tumor response in advanced cases of unresectable hepatocellular carcinoma treated by transarterial chemoembolization.

METHODS: A retrospective study included 39 patients (mean age: 66.4 years, range: 45-79 years, SD: 7) with unresectable hepatocellular carcinoma (HCC) who were treated with repetitive transarterial chemoembolization (TACE) in the period between March 2006 and October 2009. The effect of portal vein thrombosis (PVT) (in 19 out of 39 patients), the presence of arterioportal shunt (APS) (in 7 out of 39), the underlying liver pathology,

Child-Pugh score, initial tumor volume, number of tumors and tumor margin definition on imaging were correlated with the local tumor response after TACE. The initial and end therapy local tumor responses were evaluated according to the response evaluation criteria in solid tumors (RECIST) and magnetic resonance imaging volumetric measurements.

RESULTS: The treatment protocols were well tolerated by all patients with no major complications. Local tumor response for all patients according to RECIST criteria were partial response in one patient (2.6%), stable disease in 34 patients (87.1%), and progressive disease in 4 patients (10.2%). The MR volumetric measurements showed that the PVT, APS, underlying liver pathology and tumor margin definition were statistically significant prognostic factors for the local tumor response ($P = 0.018$, $P = 0.008$, $P = 0.034$ and $P = 0.001$, respectively). The overall 6-, 12- and 18-mo survival rates from the initial TACE were 79.5%, 37.5% and 21%, respectively.

CONCLUSION: TACE may be exploited safely for palliative tumor control in patients with advanced unresectable HCC; however, tumor response is significantly affected by the presence or absence of PVT and APS.

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Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Portal; Shunt; Thrombosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common neoplasms in the world and its incidence is increasing worldwide. It is associated with liver cirrhosis in 80% of cases and it is the leading cause of death among cirrhotic patients^[1]. Liver resection and liver transplantation are curative therapeutic options^[2]. Likewise, percutaneous ablative treatments such as percutaneous ethanol injection (PEI)^[3], radiofrequency ablation^[4], microwave ablation^[5] or MR-guided laser-induced thermotherapy (LITT)^[6] can also be applied as therapeutic options with curative potential. However, curative treatments are applicable in only 30%-40% of cases due to the presence of multifocal tumors or limited hepatic reserve at the time of diagnosis^[7,8]. Transarterial chemoembolization (TACE) was introduced as a palliative local therapeutic option for the treatment of unresectable HCC. The goal of palliation is to control symptoms, improve life quality and prolong survival^[8,9]. Different studies have investigated the predictors of survival after TACE and concluded that the prognosis is multifactorial, including the extent of the tumor at diagnosis and also the extent of liver injury^[8,10,11].

The purpose of this study was to evaluate retrospectively the role of TACE in local tumor control of unresectable HCC and the effect of portal vein thrombosis (PVT) and arteriportal shunts (APS), as well as other associated pathological factors, on the local tumor response in advanced cases.

MATERIALS AND METHODS

Approval of this study was obtained from the institutional review board, and informed consent was obtained from all patients including approval of the protocol of treatment and the anonymous use of the data for research purposes.

Patients

The medical records of all patients who had unresectable hepatocellular carcinoma and who were treated with TACE between March 2006 and October 2009 were retrospectively evaluated. A total of 39 patients (33 males and 6 females) ranging in age from 45-79 years (mean: 66.4 years, SD: 7) with unresectable HCC were treated with repetitive TACE. The total number of sessions was 219, range: 1-17, mean: 5.6 sessions, SD: 3.7. Patient demographics, lesion pathology, treatment, and outcome data, including all histopathology reports and imaging studies, were collected from the electronic medical record archiving system and were subsequently analyzed. Special emphasis was placed on the stage of disease at the time of first embolization. The number, location and size of liver tumors were obtained by reevaluating the original CT

Table 1 Patient and tumor criteria (n)

Total number	39
Age (yr)	Range: 45-79, mean: 66.4, SD: 7
Gender (M:F)	33:6
Underlying liver pathology	
Hepatitis C virus	17
Hepatitis B virus	5
Alcoholic cirrhosis	7
Toxic cirrhosis	2
Cryptogenic cirrhosis	8
Child-Pugh Score	
Child A	27
Child B	12
Tumor number	
Solitary	20
Multiple	19
2 lesions	6
3 lesions	3
4 lesions	1
> 5 lesions	9
Tumor margin definition	
Well defined margin	20
Ill defined margin	19
Portal vein thrombosis	19
Main (partial thrombosis)	3
Main lobar branch	10
Segmental branch	6
A-P shunt	7

and magnetic resonance imaging (MRI) scans. The patient and tumor characteristics are summarized in Table 1.

Inclusion and exclusion criteria

All patients involved in the study were not eligible for surgical or local ablative therapies. The inclusion criteria of the study were: (1) Tumors of any size associated with portal vein thrombosis, either partial thrombosis of the main portal vein or segmental portal vein branch thrombosis; (2) Tumors associated with APS with or without PVT; (3) Large solitary tumors of more than 5 cm in maximal diameter, multinodular or bilobar tumors; and (4) Patients with Child-Pugh Scores between A and B.

The exclusion criteria were: (1) Patients with poor performance status (Karnofsky index < 50%); (2) Nutritional impairment; (3) Presence of ascites; (4) Encephalopathy; (5) High serum total bilirubin level (> 3 mg/dL); (6) Serum albumin level < 2.0 mg/dL; (7) Renal failure (serum creatinine level > 2 mg/dL); (8) Cardiovascular or respiratory failure; (9) Florid infection; (10) Main PV complete thrombosis; and (11) Extrahepatic tumor manifestation.

Management protocol of the patients

The protocol for management was decided by a multidisciplinary team composed of hepatic surgeons, interventional radiologists, hepatologists and medical oncologists. The end point of TACE therapy was defined as stable disease for two successive sessions or disease progression.

TACE technique

TACE was performed with a treatment interval of 4-6 wk. For patients with bilobar disease, the treatment was

performed to control disease in the lobe with higher tumor burden as seen on MRI performed immediately before the procedure; the second lobe was treated in another session. All angiographies were performed on an Axiom Multistar system (Siemens; Erlangen). After the introduction of a 5 French sheath into a femoral artery, an angiographic survey of the abdominal vessels was performed using a 5 F pigtail catheter in the first TACE course. After exclusion of the presence of a right hepatic artery by selective catheterization of the mesenteric artery, indirect portography followed, outlining the portal circulation in the venous phase. Afterwards, a 5 French Cobra catheter was placed in the coeliac trunk and advanced beyond the gastroduodenal artery. If possible, the tip of the catheter was advanced further into segmental arteries adjacent to the tumor.

The embolization suspension, containing 5-10 mg/m² mitomycin C as a chemotherapeutic agent and 1-10 mL Lipiodol, an iodized oil, was administered, followed by injection of 60-180 mg degradable starch microspheres (EmboCept, PharmaCept) for vascular occlusion. The embolization material was injected slowly with fluoroscopic control until stasis of blood flow was observed. Devascularization after embolization was confirmed by an additional angiographic study of the hepatic artery.

Follow up after TACE

TACE procedure was performed on an outpatient basis. After the procedure, patients were observed for 10-12 h to ensure adequate hydration and symptomatic treatment of pain and vomiting. After the observation time, patients who had remained symptom-free were discharged to the care of the referring oncologist. If complications developed, patients were to be readmitted immediately. Complications were evaluated adopting the Society of Interventional Radiology (SIR) criteria^[12]. Major complications were defined as any event that resulted in additional treatment including an increased level of care, hospital stay beyond observation status (including readmission after initial discharge, substantial morbidity and disability and death. All other complications were classified as minor.

Local tumor response

The local tumor response to the treatment protocol was evaluated using the response evaluation criteria in solid tumors (RECIST) and MRI volumetric assessment.

According to RECIST criteria, complete response was defined as the complete disappearance of all recognizable tumor in the liver confirmed at 4 wk after the procedure. Partial response was defined as a reduction of at least 30% in the sum of the longest diameter of the lesions, taking as reference the baseline study, and was confirmed at 4 wk. Stable disease was defined when neither partial response nor progressive disease criteria were met, taking as reference the smallest sum of the longest diameter recorded since treatment started. Progressive disease was defined as the appearance of new lesions or as an increase of at least 20% in the sum of the longest diameter of the lesions, taking as reference the smallest-sum longest diameter recorded since treatment started^[13].

To evaluate the local tumor response using volumetric MRI measurements, tumor volumes were calculated before treatment, one month after the first TACE session and after the last TACE session. Tumor volumes were calculated with the ellipsoidal volume formula: Volume = (length × width × height × 0.523). For assessment of multicentric tumors; in patients with up to 3 focal lesions, the sum of all tumor volumes was calculated. For patients with more than 3 tumors, the sum of the largest 3 tumors was calculated.

The percentage of volume changes before and after treatment was then calculated. The local tumor response was considered as progressive if there was increase in the post-treatment volume (after the last TACE session) compared to the pretreatment volume, and as regressive if there was reduction in the post-treatment volume compared to the initial volume.

Imaging technique

The morphologic tumor response (number, size and volume) was evaluated on MRI in consensus by two senior radiologists. For initial treatment planning, unenhanced and contrast-enhanced [application of 0.1 mmol of gadopentate dimeglumine (Magnevist, Schering, Berlin, Germany) per kilogram body weight] T1-weighted gradient-echo sequences (FLASH-2D) with transversal and sagittal slice orientation (TR/TE: 135/6 ms; FA 80°; FOV 350 mm; matrix 134 × 256; slice thickness 8 mm) MRI studies were carried out for all patients with a conventional 1.5-T system (Magnetom Symphony; Siemens, Erlangen, Germany). Additional non-enhanced T2-weighted turbo-spin-echo (TSE) sequences (TR/TE: 3 800/92 ms; FA 150°; FOV 350 mm; matrix 115 × 256; slice thickness 8 mm) and contrast-enhanced dynamic VIBE sequences (TR/TE: 4.5/1.8 ms; FA 15°; FOV 350 mm; matrix 128 × 256; slice thickness 8 mm) were used for the differentiation of the lesions.

Lipiodol retention in the tumor and the liver parenchyma was verified with non-enhanced CT examinations. In addition, CT allows optimal comparison between results on follow-up images in the subsequent sessions and can efficiently exclude major post-procedure complications such as pancreatitis, hepatic infarction, mesenteric ischemia, and ascites or ectopic embolization. CT was performed 24 h after TACE using the spiral technique (slice thickness, 8 mm) on fourth-generation scanners (Somatom plus or Somatom plus 4, Siemens, Erlangen, Germany).

Statistical evaluation

For assessment of different risk factors, patients were divided into two groups according to the following: (1) Presence or absence of PVT; (2) Presence or absence of APS; (3) Child-Pugh scores (A or B); (4) Number of focal lesions (solitary or multiple); and (5) Definition of the focal lesions (well defined or ill defined margin). Patients were further divided into groups according to the underlying liver pathology (HCV, HBV, alcoholic, toxic and cryptogenic cirrhosis). Data were statistically described in terms of range, mean ± SD, frequencies (number of

Table 2 Response evaluation criteria in solid tumors in different patient groups *n* (%)

	Total	PVT		APS		Child A	Child B	Solitary	Multiple	Well defined	Ill defined
		Yes	No	Yes	No						
No. of patients	39	19	20	7	32	27	12	20	19	20	19
Partial response	1 (2.6)	0	1 (5)	0	1 (3.1)	0	1 (8.3)	0	1 (5.2)	1 (5)	0
Stable disease	34 (87.1)	18 (94.7)	16 (80)	6 (85.7)	28 (87.5)	24 (88.9)	10 (83.3)	18 (90)	16 (84.2)	17 (85)	17 (89.4)
Progressive disease	4 (10.3)	1 (5.3)	3 (15)	1 (14.2)	3 (9.30)	3 (11.1)	1 (8.3)	2 (10)	2 (10.5)	2 (10)	2 (10.5)

PVT: Portal vein thrombosis; APS: Arterioportal shunt.

cases) and relative frequencies (percentages) when appropriate. Comparison of quantitative variables between different groups was done using Wilcoxon Mann Whitney *U* test for independent samples. Comparison of quantitative variables over the study period was done using Wilcoxon matched-pairs test comparisons. A probability value (*P* value) less than 0.05 was considered statistically significant. The influence of variables on prognosis was evaluated by univariate analysis. When the result was statistically significant, multivariate regression analysis was used to analyze the factors influencing the prognosis to avoid any confounding interaction between them.

Survival times were calculated beginning with the dates of the first TACE treatment using the Kaplan-Meier method. The log-rank test was used to determine the significance of the difference between patient survival rates in different patient groups.

RESULTS

The treatment protocols were well tolerated by all patients, with only minor side effects and no major complications. Post-embolization syndrome in the form of nausea and vomiting occurred in 9 patients (23%) and a dull aching upper abdominal pain persisting for 24 h in 11 patients (28.2%). There was no mortality within 30 d from the time of TACE.

Local tumor response using RECIST criteria

Local tumor morphologic evaluations according to RECIST criteria were as follows: no patient achieved complete response, partial response in one patient (2.6%), stable disease in 34 patients (87.1%) and progressive disease in 4 patients (10.3%). The comparisons of the local tumor response according to RECIST criteria in different patient groups are summarized in Table 2.

Local tumor response using volumetric MR imaging assessment

The local tumor response using MR volumetric measurements revealed the following results: tumor volume reduction in 17 patients (43.6%) and tumor volume progression in 22 patients (56.4%). The changes of the tumor volumes after TACE and the local tumor response are summarized in Tables 3 and 4.

The local tumor response evaluated using the percentage of volume changes between the pre- and post-treat-

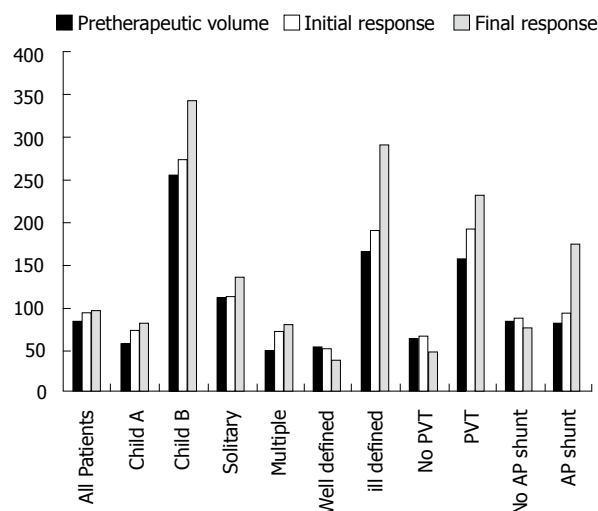


Figure 1 Magnetic resonance imaging volumetric changes of the median tumor volume (mL) in different patient groups. PVT: Portal vein thrombosis; AP: Arterioportal.

ment volumes was statistically significant and influenced by the following factors: the presence or absence of PVT, presence or absence of APS and tumor margin definition ($P = 0.018$, 0.008 and 0.001 , respectively) (using Mann-Whitney-*U*-test), as tumor volume regression was detected more in the absence of PVT and APS and in tumors with well defined margins (Figure 1). Multivariate analysis (using multiple regression test) showed that the tumor margin definition seems a more significant factor compared to the presence of PVT or AP shunts ($P = 0.002$).

The local tumor response was insignificantly affected by the number of focal lesions and Child-Pugh score of the patient ($P = 0.478$ and 0.893 , respectively). Correlation between the underlying liver pathology and the percentage of volume changes between the pre- and post-treatment volumes was statistically significant ($P = 0.034$) (using Kruskal Wallis-test). However, correlation between the different patient groups failed to prove statistical significance (using Conover-Iman-Test). The changes of the median tumor volumes are illustrated in Figure 2. The correlation between the pre- and post-treatment tumor volumes for all patients is illustrated in a scatter gram (Figure 3).

Survival analysis

The overall survival rates calculated from the time of first TACE sessions for all patients at 6, 12 and 18 mo were

Table 3 Magnetic resonance imaging volumetric changes of tumors after transarterial chemoembolization in different patient groups

	Total	PVT		APS		Child A	Child B	Solitary	Multiple	Well defined	Ill defined
		Yes	No	Yes	No						
No. of patients	39	19	20	7	32	27	12	20	19	20	19
Initial tumor volume (mL)											
Range	1.2-1358	1.2-1358	28.8-301.9	28.9-237	1.2-1358	1.1-1355	56.7-1358	1.2-1355	23.4-1358	1.2-301.9	28.8-1358
Mean	227.6	354.5	107	109.2	253.5	162.3	374.5	246	208.2	97.0	365
SD	318.76	418.2	78.5	75.2	345.8	275.8	370.5	317	327.2	60.0	411
Tumor volume 1 mo after the 1st TACE											
Range	1.3-1637.5	1.3-1637.5	15.6-334.8	39-239	1.3-637.5	1.3-1500	23.7-1637.5	1.3-1499.7	15.6-1637.5	1.3-334.9	39-1637.5
Mean	244.5	402.6	94.3	126.5	270.3	159.6	435.6	242.6	246.6	65.3	412
SD	369	480.1	72.8	85.0	402.2	284.7	470.0	334.3	411.9	75.3	473
Tumor volume at the end of treatment											
Range	1.6-2053.4	1.6-2053.4	9.4-403.1	94.5-308	1.6-2053.4	1.6-2053.4	22.5-1637.5	1.6-2053.4	9.4-1637.4	1.6-403.1	83-2053.4
Mean	286.1	486	96.2	188.6	307.5	197.4	485.6	280.8	291.7	74.3	509.1
SD	453.2	583.7	100	72.5	490	391	534.2	452.9	465.8	69.5	568

PVT: Portal vein thrombosis; APS: Arteriportal shunt; TACE: Transarterial chemoembolization

Table 4 Local tumor response using volumetric magnetic resonance imaging analysis *n* (%)

	Total	PVT		APS		Child A	Child B	Solitary	Multiple	Well defined	Ill defined
		Yes	No	Yes	No						
No. of patients	39	19	20	7	32	27	12	20	19	20	19
Tumor volume reduction after last TACE session	17 (43.6)	5 (26.3)	12 (60)	0 (0)	17 (53.1)	13 (48.1)	4 (33.3)	9 (45)	8 (42.1)	13 (65)	4 (21.05)
Tumor volume progression after last TACE session	22 (56.4)	14 (73.7)	8 (40)	7 (100)	15 (46.9)	14 (51.9)	8 (66.7)	11 (55)	11 (57.9)	7 (35)	15 (78.9)

PVT: Portal vein thrombosis; APS: Arteriportal shunt; TACE: Transarterial chemoembolization

79.5%, 37.5% and 21%, respectively (Figure 4). The median survival time was 10 mo. There was an insignificant difference in the survival rates between different patient groups on analysis by log-rank test (PVT: $P = 0.653$, APS: $P = 0.822$, number of lesions: $P = 0.26$, margin definition of lesion: $P = 0.155$ and Child-Pugh score: $P = 0.09$) (Figure 4).

DISCUSSION

Despite the remarkable advancement and availability of novel curative options, a great proportion of HCCs are still not eligible for curative treatment due to advanced tumor stage or poor hepatic functional reserve^[7]. There is no evidenced-based knowledge regarding a standard systemic chemotherapy protocol that improves the overall survival in advanced HCC patients^[14]. Combination chemotherapy using different mixtures of doxorubicin, 5-FU, mitomycin C, bleomycin, cisplatin, interferon (PIAF), gemcitabine and mitoxantrone has been employed in the treatment of advanced HCC. Although some of the combination regimes have shown improved tumor control in phase II studies, most of them fail to demonstrate any survival advantage in randomized phase III studies^[15].

New research trials utilizing sorafenib have demonstrated survival extension in two phase III trials in North America, Europe and in the Asia-Pacific area, which respectively reported a median survival after treatment of 10.7 and 6.5 mo^[16]. However, drug-related adverse effects including diarrhea, hand-foot skin reaction, anorexia, alopecia, weight loss, dry skin, abdominal pain and voice changes have been reported^[17].

Attention has been focused on locoregional approaches for the palliative treatment of HCC in recent years. Although TACE is widely used in the palliative treatment of unresectable HCC, its role remains controversial^[18-20]. It has been shown to provide reasonable survival advantages in two randomized control trials and a meta-analysis^[21].

Different reports have investigated the predictors of survival after TACE for unresectable HCC. These predictors may be related to the tumor burden (number of focal lesions, size of the tumor, tumor/liver volume ratio and PVT), the liver function (Child-Pugh score), health status (constitutional syndrome and Karnofsky index) or may be related to the treatment protocol^[8,10,20,22,23]. In a large scale study of 8510 patients who underwent TACE, multivariate analyses revealed the following variables to be independent predictors of patient prognosis: degree of liver

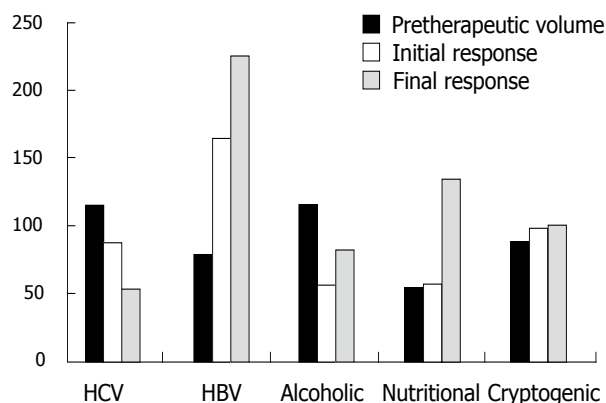


Figure 2 Magnetic resonance imaging volumetric changes of the median tumor volume (mL) according to the underlying liver pathology. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

damage, maximum tumor size, number of lesion(s) and portal vein invasion^[8]. Portal vein invasion showed much higher risks than the other variables.

The endpoint in cancer research is overall survival. Nonetheless, other potential endpoints, such as response rate and time to progression, are currently used. In this study, the predictors of local tumor response after TACE were evaluated, as well as the role of TACE in local tumor control for unresectable advanced HCC. Since volumetric quantification can lead to a different assessment result compared with uni- and bi-dimensional measurement techniques^[24], the local tumor response to the TACE treatment was evaluated using both RECIST criteria and MRI volumetric measurements. According to RECIST criteria, tumor stability was achieved in 34/39 patients (87.1%), and partial response in 1 patient (2.6 %), while progression occurred in 4 patients (10.3%) and, by volumetric measurements, reduction of the tumor volume occurred in 17 patients (43.6%) and progression in (56.4%), taking into consideration the inclusion of advanced pretreatment tumor stage regarding the size, number and portal vein invasion. This may emphasize the role of TACE in local tumor control even in the advanced stages of HCC. Univariate analysis revealed the following 4 variables as significant prognostic factors: PVT, APS, tumor margin definition and underlying liver pathology (Figures 5 and 6). The clinical significance of the presence of PVT, APS and tumor margin ill definition is clearly demonstrated by the progressive increase in the corresponding median tumor volumes at the end of TACE treatment (Figure 1). Multivariate analysis showed that the tumor margin ill definition affects the local tumor response more significantly compared to the presence of PVT or AP shunts.

TACE has been limited in palliative treatment of cases associated with major portal vein (PV) invasion due to the possibility of liver failure following embolization and that it may predispose to hepatic infarction. Transcatheter arterial chemo infusion (TACI) has been an option in such cases^[25]. However, recent studies have shown that TACE using less aggressive embolization can be performed

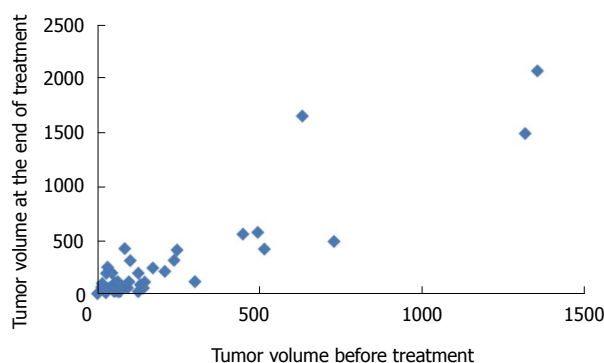


Figure 3 Scatter gram. Correlation between the pre- and post-treatment volume.

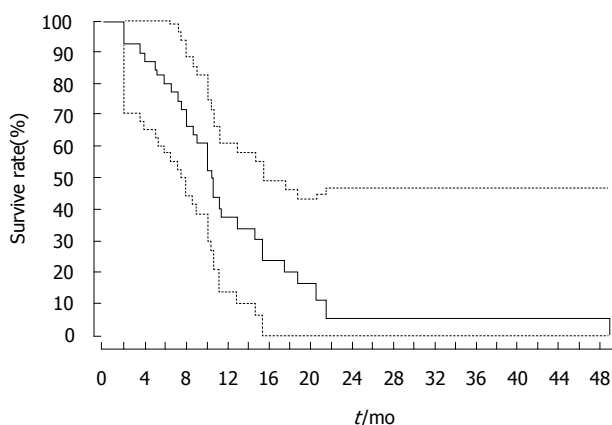


Figure 4 Kaplan-Meier survival curve for all patients showing the overall survival rates calculated from the time of first transarterial chemoembolization sessions. The estimated survival rates at 6, 12 and 18 mo were 79.5%, 37.5% and 21%, respectively, with a median survival time of 10 mo. Kaplan-Meier with confidence $P = 0.95$.

safely in patients with major PV thrombosis with no increase in morbidity or mortality^[26-28]. The current study included patients with partial thrombosis of the main PV or thrombosis of one of its lobar branches or segmental branches. The procedure was tolerated by all patients and none of the patients developed major complications that required prolonged hospitalization. Chung *et al*^[29] explained the prognostic significance of PVT by suggesting that the presence of tumor thrombi in the PV results in extensive tumor spread throughout the liver.

Arterioportal shunts associated with HCC have been reported in about 28.8% to 63.2% of HCCs. Ngan *et al*^[30] explained the poor prognostic clinical significance by suggesting that the shunt flow to the portal vein aggravates the portal venous pressure, which induces life-threatening conditions such as esophageal varix, ascites and hepatic encephalopathy. In this present study 7/39 patients (17.9%) had associated APS, six of these cases were associated with ill defined tumor margin and four cases were associated with PVT. The MR volumetric measurements revealed progression of the tumor in the 7 patients (100%) and the percentage of volume changes between both groups (APS and no APS) were statistically significant. Huang *et al*^[31] explained poor response to TACE by the fact that during

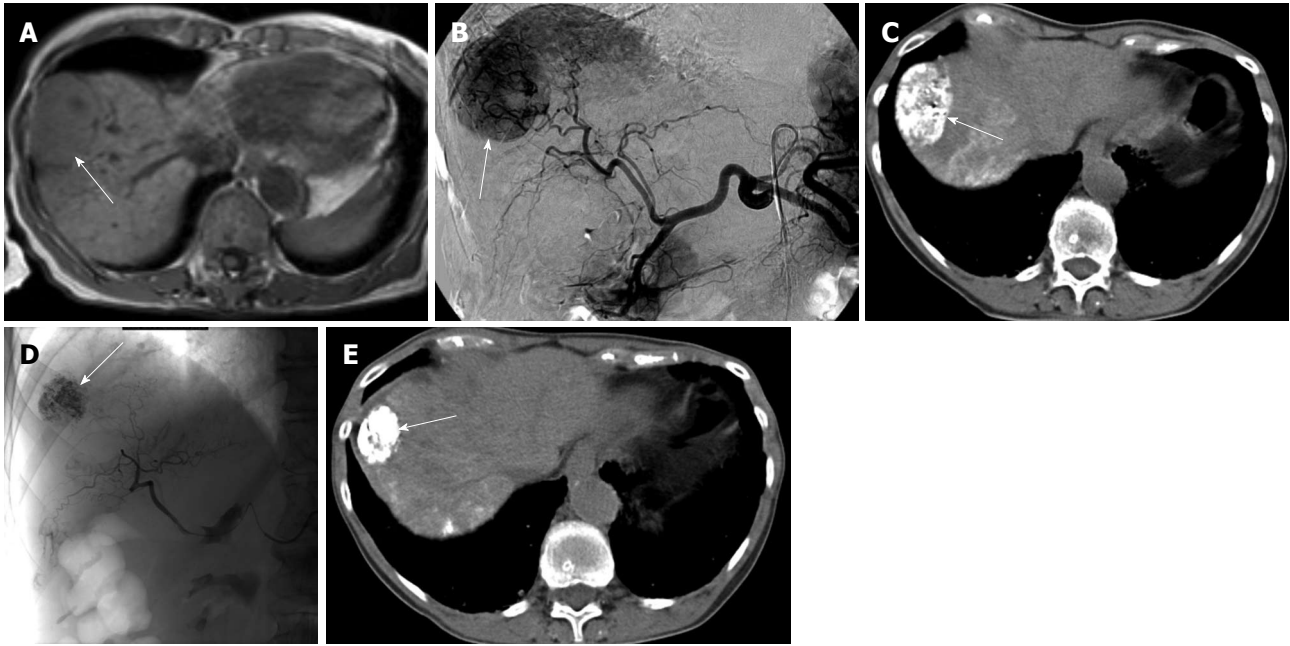


Figure 5 A 76-year-old female patient, hepatitis C virus liver cirrhosis. A: Axial magnetic resonance imaging (MRI) T1 WI showing an isointense subcapsular hepatocellular carcinoma at segment 8 (white arrow); B: Digital subtraction angiography before the 1st transarterial chemoembolization (TACE) showing a large tumor blush at segment 8 (white arrow); C: Computed tomography (CT) after the first embolization showing dense Lipiodol uptake by the tumor (white arrow); D: Hepatic arteriography after the 5th TACE session showing occluded feeding arteries and Lipiodol concentration within the tumor (white arrow); E: CT after the last TACE session showing reduction of the tumor size (> 50%) by MRI volumetry (white arrow).

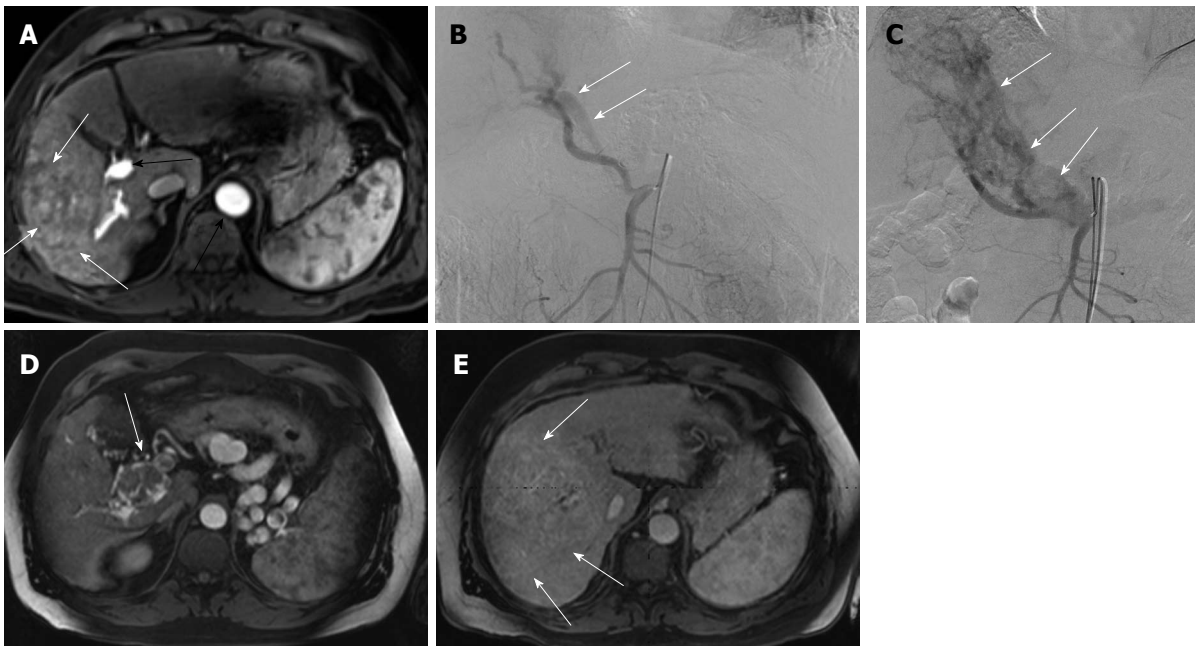


Figure 6 A 58-year-old male patient with alcoholic cirrhosis. A: Axial magnetic resonance imaging (MRI) T1 post contrast weighted image showing an ill defined mass in segments 5 and 6 (white arrows). Note enhancement of the portal vein (black arrow) in the arterial phase (black arrow head at the aorta) denoting an underlying arteriportal shunt; B: Digital subtraction angiography showing opacified portal vein (white arrows) during the arterial phase (APS) (white arrows); C: Digital subtraction angiography during the last (6th) transarterial chemoembolization (TACE) showing the APS with markedly dilated and partially thrombosed portal vein (white arrows); D, E: Axial MRI T1 post contrast after the last TACE showing tumor infiltration of the dilated main portal vein (white arrow in Figure 6D) and progression of the tumor size (> 20%) by MRI volumetry (white arrows in Figure 6E) (white arrows).

TACE, oil emulsion may be diverted into the portal vein branches and delivered to non-tumor hepatic tissue instead of being deposited intratumorally. Although the univariate analysis revealed a statistical significance between the un-

derlying liver pathology and the local tumor response, the multivariate analysis failed to prove the clinical significance, probably because of the sample size and the registered data which are located out of the overlapping area statistically.

Limitations of this study are the retrospective design and the heterogeneous population. However, this study at least illustrates the role of TACE in local tumor control of unresectable HCC, even in advanced cases, and the prognostic factors affecting the tumor response.

In conclusion, TACE may be applied in the palliative treatment of patients with unresectable advanced HCC for local tumor control. The local tumor response to TACE is significantly affected by the presence or absence of portal vein thrombosis, arteriportal shunt, tumor margin definition and the underlying liver pathology.

COMMENTS

Background

To date, there is no evidence-based knowledge regarding a standard systemic chemotherapy protocol that improves the overall survival in advanced hepatic cancer (HCC). Attention has been focused on regional liver approaches for the palliative treatment of HCC in recent years, including transarterial hepatic chemoembolization (TACE). This therapy has been shown to provide reasonable survival advantages in two randomized control trials and a meta-analysis. However, the factors that determine tumor response under the effect of local chemotherapy are still under research and evaluation.

Research frontiers

This research focused on the vascular factors that may affect the tumor response and patient survival in patients undergoing TACE therapy in advanced HCC. Both portal vein thrombosis (PVT) and arteriportal shunts (APS) were found to be statistically significant factors influencing reduced tumor responsiveness to TACE therapy. Other pathological factors which may determine the tumor end response are the underlying liver pathology, Child-Pugh status and tumor margin definition.

Innovations and breakthroughs

The study concludes that TACE may be exploited safely for palliative tumor control in patients with advanced unresectable HCC; however tumor response is significantly affected by the presence or absence of PVT and APS.

Terminology

Transarterial Chemoembolization is a minimally invasive therapeutic procedure which consists of: catheterization of the hepatic artery to inject the chemotherapeutic medications selectively in the feeding vessels of the liver tumor. The therapeutic medications consist of chemotherapeutic agent (which inhibits tumor cell proliferation) in a mixture with occlusive material (to block the feeding vessels of the tumor). Both agents work together to suppress tumor activity and kill tumor cells. Portal vein thrombosis: thrombosis of the major blood vessel of the liver that carries the nutritive materials from the gastrointestinal tract to be metabolized in the liver. The hepatic portal vein is responsible for 75% of blood supply to the liver. Arteriportal shunt: This is an abnormal communication between the hepatic artery and portal vein tributaries.

Peer review

I think this article is well written.

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Proteome of human colon cancer stem cells: A comparative analysis

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Abstract

AIM: To isolate and identify the biological characteristics of human colon cancer stem cells (SW1116 cells) and further study their proteome.

METHODS: SW1116 cells were isolated and cultured with a serum-free medium (SFM). Sphere formation was assayed to observe the formation of colon cancer stem cell spheres. SW1116 cells were inoculated into a serum-containing medium for observing their differentiation characteristics. Proliferation curve and cross-resistance of SW1116 cells to different drugs were detected by MTT. Percentage of SP cells in SW1116 cells was detected with Hoechst33342 staining. Telomerase activity in SW1116 cells was checked by polymerase chain reaction (PCR)-enzyme linked immunosorbent assay. Expressions of stem cell relevant genes and proteins were detected by reverse transcription-PCR and Western blot, respectively. Total protein was isolated from SW1116 cells by two-dimensional gel electrophoresis (2-DE) and

differentially expressed proteins were identified by tandem mass spectrometry (MALDI-TOF/TOF).

RESULTS: The isolated SW1116 cells presented as spheroid and suspension growths in SFM with a strong self-renewal, proliferation, differentiation and drug-resistance ability. The percentage of SP cells in SW1116 cells was 38.9%. The SW1116 cells co-expressed the CD133 and CD29 proteins. The telomerase activity in SW1116 cells was increased. The expressions of different stem cell relevant genes and proteins were detected. The proteomic analysis showed that the 26 protein spots were differently expressed in SW1116 cells and 10 protein spots were identified as ubiquitin fusion-degradation 1-like protein, nuclear chloride channel protein, tubulin β , Raichu404X, stratifin, F-actin capping protein α -1 subunit, eukaryotic translation elongation factor 1 delta isoform 2, hypothetical protein, glyceraldehyde-3-phosphate dehydrogenase and guanine nucleotide binding protein β polypeptide 2-like 1, respectively.

CONCLUSION: SW1116 cells are biologically characterized by self-renewal, proliferation and differentiation, and the differently expressed proteins in SW1116 cells may be essential for isolating cancer stem cells.

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Key words: Proteome; Stem cell; Colon cancer; Isolation; Characterization

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INTRODUCTION

Colon cancer is the leading cause of cancer-related death in developed countries^[1]. Although its incidence has significantly reduced in most Asian countries, the incidence of colorectal cancer in Japan is increasing due to westernized diets. More information on factors influencing the increase, progression, and metastasis of colon cancer will lead to the development of its novel diagnostic and treatment methods. One of these factors is stem cells, but their function in the early stage of cancer is not completely understood.

Stem cells are defined as cells able to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation. They play an important role in the maintenance of organ homeostasis and may be responsible for tumorigenesis and contribute to resistance to cancer therapy^[2]. Since the identification and characterization of cancer stem cells (CSC) in hematological malignancies, an increasing number of studies have described CSC in solid tumors such as ovarian tumor^[3], colon tumor^[4], lung tumor^[5], breast tumor^[6], liver tumor^[7], melanoma^[8] and pancreatic tumor^[9], raising that the cancer stem cell hypothesis can be applied to all neoplastic systems. CSC are more important than other tumor cells because they are capable of self-renewing, differentiating, and maintaining tumor growth and heterogeneity, thus playing an important role in both tumorigenesis and therapeutics. However, research has been hampered by the lack of distinct molecular markers for CSC.

The most recent research findings have extended the criteria for CSC to human colon cancers, suggesting that colon carcinoma is organized in a hierarchical fashion, where only a CD133⁺ small subset of cells with self-renewal properties are able to recapitulate the bulk tumor population^[4,10]. CD133⁺ cells within colon carcinoma can be propagated *in vitro* as spheroid culture retains the tumorigenic capacity under these conditions. The high resistance of CD133⁺ cells to apoptosis induced by chemotherapeutic drugs is additionally consistent with the cancer stem cell hypothesis, whereas the number of CSC is particularly resistant to death-induced signals. In addition to CD133 based identification of colon cancer stem cells, Dalerba and co-workers^[11] have recently reported an alternative protocol for the isolation of human colon CSC by exploiting the surface phenotype EpCAM^{high}/CD44⁺/CD166⁺. In their study, EpCAM and CD44 antigens were selected on the basis of their previously described expression in human breast cancer stem cells. CD166 is known as a mesenchymal stem cell marker and its increased expression in colon cancer is associated with a poor clinical outcome^[12]. In this study, a comparative proteomic analysis of a human colon CSC line was performed to find more specific phenotypic markers for colon cancer.

MATERIALS AND METHODS

Reagents and cell line

Human colon cancer cells (SW1116 cells) were purchased

from Shanghai Institute of Life Science, Chinese Academy of Sciences. TeloTAGGG Telomerase polymerase chain reaction (PCR) ELISAPLUS kit was purchased from Roche Molecular Biochemicals (Basel, Switzerland). Chemiluminescent detection kit was from SuperArray Bioscience (Frederick, MD, USA). Bio-Rad protein assay kit and silver stain plus™ kit were from Bio-Rad (Hercules, CA, USA).

Isolation and culture of human SW1116 cells

Human SW1116 cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco BRL, USA), 1×10^5 U/L penicillin G and 100 mg/L streptomycin in an atmosphere containing 5% CO₂ at 37°C. Adherent SW1116 cells were dissociated to single cell suspensions and seeded in serum-free medium (SFM). After spheres of SW1116 cells were formed, proliferation and differentiation potentials of SW1116 cells were observed. SW1116 cells were isolated and maintained in a SFM (DMEM/F12 medium) containing 20 µg/L human recombinant epidermal growth factor (EGF; Invitrogen, Carlsbad, CA, USA), 20 µg/L human recombinant basic fibroblast growth factor (bFGF; Invitrogen, Carlsbad, CA, USA), 2 mmol/L L-glutamine, 4 U/L insulin, 1×10^5 U/L penicillin G, and 100 mg/L streptomycin.

Sphere formation assay

Primary spheres of SW1116 cells were dissociated to single cell suspensions and inoculated in ultra-low attachment 96-well plates (Corning Life Sciences, Acton, MA) (100 cells per well) supplemented with 200 µL SFM. Then, 25 µL SFM per well was added every 2 d. The number of spheres of SW1116 cells in each well was evaluated 14 d after culture.

Differentiation assay of spheres

Two days after primary culture, SW1116 cells were plated in 24-well culture plates with 10% FBS and cultured with FBS-supplemented medium every two days. Differentiation potentials of SW1116 cells were observed under a microscope.

Cell proliferation assay

SW1116 cells were seeded onto 35 mm Petri dishes at a density of 1×10^4 . Cultured SW1116 cells were stained with trypan blue and counted in triplicate under a microscope for 6 wk.

Chemosensitivity test

SW1116 cells were seeded onto 96-well plates at a density of 5×10^3 per well. Twenty four hours after drugs were added at different concentrations, SW1116 cells were exposed to drugs for 2 d. Then MTT (5 mg/mL) was added and the plates were incubated at 37°C for 4 h before 100 µL 100% dimethyl sulfoxide was added to each well. The optical density of SW1116 cells in each well was detected with a microplate reader (Bio-Rad, Model 550) at a wavelength of 570 nm. The survival time

of SW1116 cells was calculated as OD value of OM- ex-
erted cells/OD value of mocked-cells $\times 100\%$.

Fluorescence-activated cell sorting analysis

Stained SW1116 cells at a concentration of 1×10^6 cells per 100 μL buffer contained PBS at pH 7.2, 0.5% BSA, and 2 mmol/L EDTA. Antibodies against CD133 (anti-CD133-PE, BD Pharmingen, Franklin Lakes, USA) and CD29 (anti-CD29-FITC, Chemicon, Billerica, MA, USA) were used. Antibody was incubated at 4°C for 30 min. FACS analysis was done using a FACS calibur flow cytometer (Becton Dickinson).

Hoechst33342 staining

SW1116 cells were maintained in a SFM containing 5 mg/L Hoechst 33342, cultured in an atmosphere containing 5% CO_2 at 37°C for 90 min, harvested, trypsinized and fixed with 4% methanol for 20 min. SP cells were counted under a fluorescence microscope.

Telomerase assay

To quantitatively detect changes in telomerase activity, SW1116 cells were assayed with a telomerase PCR-enzyme linked immunosorbent assay (ELISA) kit according to its manufacturer's instructions. After PCR-ELISA, telomerase activity was detected using a microplate reader (Bio-Rad, Model 550) and recorded as the absorbance units. Relative telomerase activity (RTA) within different samples was obtained according to the following equation. $\text{RTA} = [(A_s - A_{so})/A_{s,IS}] / [(A_{TS8} - A_{TS8,0})/A_{TS8,IS}] \times 100$ Where A_s is the absorbance of sample, A_{so} is the absorbance of heat- or RNase-treated sample, $A_{s,IS}$ is the absorbance of internal standard (IS) of the sample, A_{TS8} is the absorbance of control template (TS8), $A_{TS8,0}$ is the absorbance of lysis buffer, and $A_{TS8,IS}$ is the absorbance of IS of the TS8.

Reverse transcription-PCR

Total RNA was isolated from SW1116 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following its manufacturer's instructions. First strand cDNA was synthesized using M-MLV for reverse transcription (RT-PCR) in a 24 μL solution containing 2 μL Oligo(dT)18 (500 $\mu\text{g}/\text{mL}$) (Sangon Co., Shanghai, China), 1 μg total RNA, 2 μL dNTP (10 mmol/L) (Sangon Co., Shanghai, China) and 7 μL DEPC water. Then, 5 μL 5 \times first-strand buffer, 6 μL DEPC water, 1 μL RNasin ribonuclease inhibitor (40 U/ μL) (Hua Mei Co., Guangzhou, China) were added and incubated at 42°C for 2 min. One μL M-MLV reverse transcriptase (Hua Mei Co., Guangzhou, China, 200 U) was added, the solution was placed into water bath at 42°C for 50 min and then at 70°C for 15 min. A 50 μL solution containing 1 μL Taq DNA polymerase (Hua Mei Co., Guangzhou, China), 5 μL 10 \times buffer, 1 μL dNTP (10 mmol/L), 2 μL primer (10 $\mu\text{mol}/\text{L}$) and 1 μL cDNA (0.1 $\mu\text{g}/\mu\text{L}$) was used for PCR. PCR amplification was performed in a thermal cycler (Perkin-Elmer Co., USA). The PCR products were analyzed and photographed with a gel documentation system (FR-200, Shanghai Fu Ri Bio Co., China). RT-

Table 1 Sequences of primers for RT-PCR and length of RT-PCR products

Genes	Primer sequences	Products
GAPDH	5'-TTGGTATCGTGGAAGGACTCA-3' 5'-TGTCATCATATTGGCAGGTT-3'	270 bp
CD133	5'-TGGGGCTGCTGTTTATTATTCT-3' 5'-TGCCACAAAACCATAGAAGATG-3'	194 bp
CD29	5'-GGAAAACGCAAAATTGTCAG-3' 5'-TTGGGGTTGCACTCACACAC-3'	600 bp
Mus-1	5'-GGCTTCGTCACITACATGGACCAGGCG-3' 5'-GGAAACTGGTAGGTGTAG-3'	542 bp
ABCG2	5'-GGGTTCTCTTCTTCTGACGACC-3' 5'-TGGTTGTGAGATTGACCAACAGACC-3'	398 bp
TERT	5'-CGGAAGAGTGTCTGAGACAA-3' 5'-GGATGAAGCGGAGTCTGGA-3'	145 bp
Oct-4	5'-GACAACAATGAGAACCTTCAGA-3' 5'-CTGGCGCCGGTTACAGAACCA-3'	218 bp
Sca-1	5'-AACCATATTTGCCTTCCCGTCT-3' 5'-CCAGTGTGCTCCTCCAGTG-3'	135 bp

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Mus-1: Musashi-1.

PCR primers were designed and synthesized by Sangon Co, Shanghai, China (Table 1).

Western blotting

SW1116 cell extract was prepared using a lysing buffer containing 20 mmol/L Tris-HCl at pH 7.5, 0.1% Triton X, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{mL}$ aprotinin, and 10 $\mu\text{g}/\text{mL}$ leupeptin and centrifuged ($12000 \times g$) at 4°C . Total protein concentration was measured by BCA assay. Cellular extracts containing 50 μg total protein were subjected to 10% SDS-PAGE, and then transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA, USA). Blots were probed at 4°C overnight with primary antibodies in 5% milk/TBST. The antibodies used for Western blotting were CD133, CD29, Musashi-1, ABCG2, TERT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA).

Two-DE and silver-staining

Cultured SW1116 cells were harvested, washed with PBS, and lysed in a lysis buffer containing 8 mol/L urea, 4% CHAPS, 40 mmol/L Tris, 65 mmol/L DTT, 2% Bio-Lytes⁺ and centrifuged at $25000 \times g$ for 1 h at 4°C . Protein concentrations were measured by a modified Bradford assay. All samples were stored at -80°C for electrophoresis. Two-DE was performed using the PROTEAN IEF and PROTEAN xi II systems (Bio-Rad, Hercules, CA, USA). Total protein (80 mg) was run in an IEF system using a 17 cm pH 3 - 10 ReadyStrip (Bio-Rad, Hercules, CA, USA). The total Vh was 47000-52000. Following IEF separation, gel strips were equilibrated with buffer I containing 6 mol/L urea, 30% glycerol, 2% SDS, 1% DTT, followed by buffer II (DTT was replaced with 2.5% IAA), each for 15 min. The equilibrated gel strips were placed on top of a 12% T slab gel and

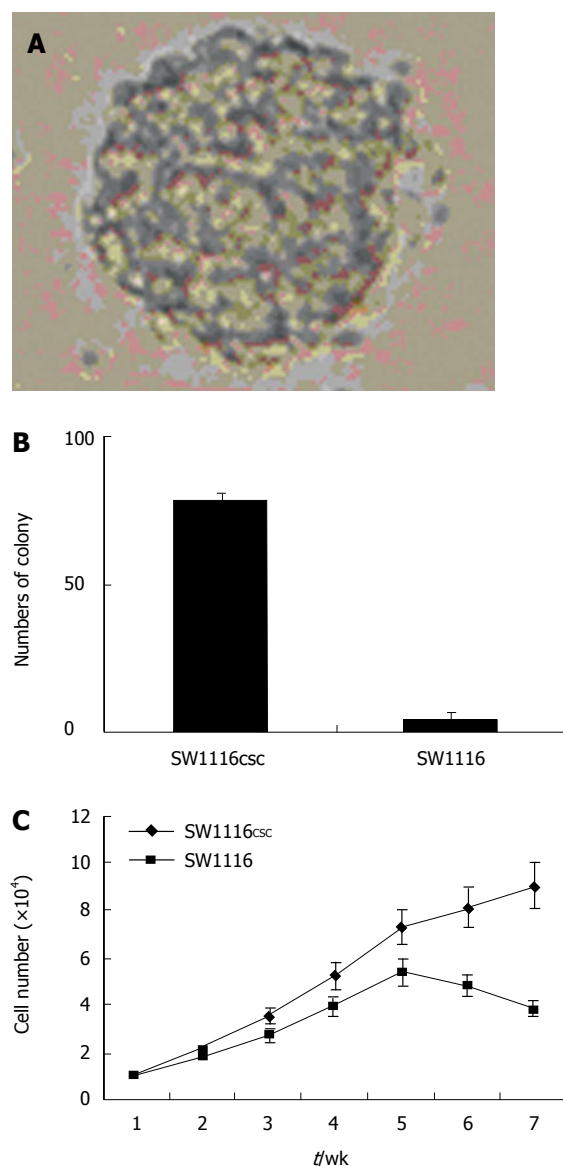


Figure 1 Spheres (A), sphere formation assay (B), and proliferation assay (C) of SW1116 cells.

sealed with 0.5% agarose. SDS-PAGE was performed for 30 min at a constant current of 10 mA and then at 25 mA until bromophenol blue reached the bottom of gels. The separated proteins were visualized with silver diamine-staining. For preparative 2-DE, 400 μ g of total proteins was separated as described above. The total Vh of IEF was 90000-120000. Proteins were detected with modified silver-staining compatible with MS analysis. Experiments (from cell culture to 2-DE) were performed in triplicate.

Image acquisition and statistical analysis

Silver-stained 2-DE gels were scanned at an optical resolution of 84.7 μ m perpixel using a GS-710 imaging densitometer (Bio-Rad, Hercules, CA, USA). Digitized images were analyzed with the PD Quest 7.1 software package (Bio-Rad, Hercules, CA, USA). Following spot detection, a matchset including the three batches of SW1116 cells was built. A reference gel was selected from one of the

SW1116 gels, and unmatched protein spots of the member gels were automatically added to the reference gel. The raw quantity of each spot in a member gel was divided by the total quantity of valid spots in the gel. Quantitative analysis of SW1116 gels was performed by Student's *t*-test.

Protein identification

Protein spots were excised from gels, destained and washed until the gels became clear. The spots were kept in 0.2 mol/L NH_4HCO_3 for 20 min, dried by lyophilization, and digested overnight in 12.5 ng/mL trypsin in 0.1 mol/L NH_4HCO_3 . Peptides were extracted three times with 50% ACN (Fisher, Fair Lawn, New Jersey, USA), 0.1% TFA (Merck, Schuchardt, Hohenbrunn, Germany) and dried in vacuum. The peptide mixture was dissolved in 0.1% TFA and desalted using a C18 ZipTip (Millipore, Bedford, MA). The eluted peptides in 0.1% TFA/50% ACN mixed with an equal volume of 0.1% TFA/30% ACN saturated with CHCA solution were applied onto the target, air-dried and analyzed by Ultraflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF mass spectrometry (MS; Bruker, Bremen, Germany). The extraction voltage was set at 20 kV and the cut-off mass value was set at 500. Tandem mass spectrometry (MS/MS) spectra were used to search protein identity from NCBI human database using Mascot search engine (www.matrixscience.com).

RESULTS

Isolation and growth characterization of SW1116 cells

Serum-free culture condition was used to isolate and maintain SW1116 cells. SFM could maintain an undifferentiated stem cell state, and addition of bFGF and EGF induced the proliferation of multipotent, self-renewing, and expandable colon stem cells. Within 24-48 h of primary culture, a minority fraction of SW1116 cells that demonstrated growth into clonally derived spheres was yielded (Figure 1A). Primary sphere formation assay showed that the frequency of SW1116 cells was $1.9\% \pm 0.3\%$. The majority of the remaining cancer SW1116 cells exhibited adherence, loss of proliferation, and subsequent differentiation, whereas tumor spheres remained non-adherent, continuous proliferation and expansion in tumor cell culture over time. When plated in a medium with 10% FBS, SW1116 cells exhibited adherent growth phenomena. After 72 h, no difference was observed in cellular volume or shape of SW1116 cells.

The self-renewing capacity of SW1116 cell spheres was assayed by dissociating primary tumor spheres, and plating SW1116 cells at serial dilutions down to 1 cell/well. Nearly all the dissociated primary tumor spheres were able to form secondary tumor spheres, exhibiting a self-renewing ability. SW1116 cells at a density of 100 cells/well generated a greater mean number of secondary tumor spheres (87.4 ± 5.1) than that (1.9 ± 0.3) of SW1116 cells (Figure 1B).

Difference was also detected in proliferation rate of SW1116 cells. The number of SW1116 cells was calcu-

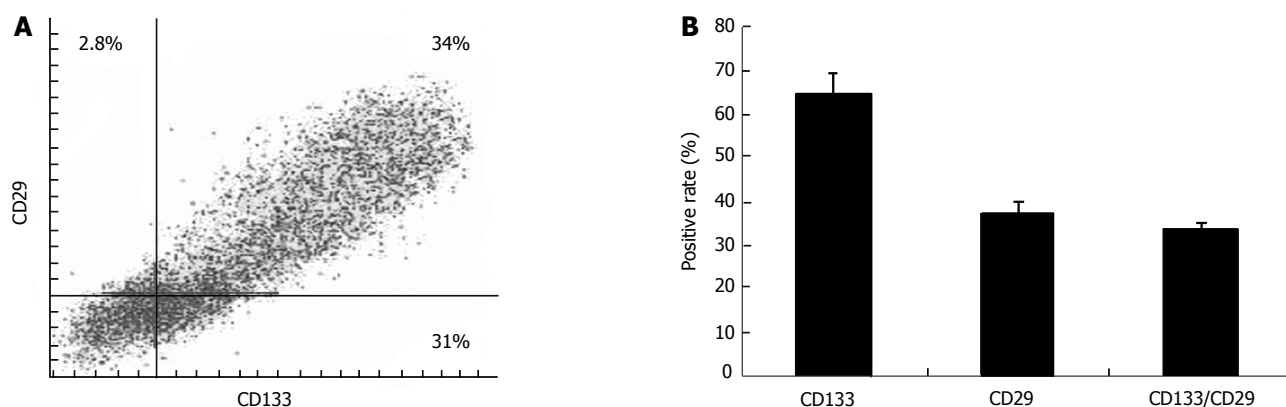


Figure 2 Flow cytometry analysis showing the expressions of CD133 and CD29 (A) and the positive rates of CD133 and CD29 (B) in SW1116 cells.

Table 2 Chemosensitivity of SW1116csc and SW1116 cells to chemotherapeutic drugs (mean \pm SD)

Drugs	IC ₅₀ (mg/L)		Times (fold)
	SW1116csc	SW1116 cells	
Paclitaxel	23.4 \pm 1.8	1.5 \pm 0.2	15.6
Adriamycin	29.7 \pm 2.1	4.4 \pm 0.4	6.8
Etoposide	9.8 \pm 0.3	2.1 \pm 0.2	4.7
Cytarabine	34.2 \pm 2.5	9.6 \pm 0.7	3.6
Fluorouracil	57.7 \pm 3.8	26.5 \pm 1.5	2.2
Cisplatin	11.6 \pm 0.9	7.3 \pm 0.3	1.6
Mitomycin	0.67 \pm 0.04	0.46 \pm 0.03	1.5

IC₅₀: The half maximal inhibitory concentration.

lated during the first 7 wk after seeding. A difference was observed in growth rate of SW1116 cells (Figure 1C). SW1116 cells grew slowly and showed a growth inhibition after 5 wk.

Cross-resistance of SW1116 cells to chemotherapeutic drugs

Chemosensitivity of SW1116csc and SW1116 cells to chemotherapeutic drugs was detected by MTT, which showed that the resistance of SW1116 cells to paclitaxel, adriamycin, etoposide, cytarabine, fluorouracil, cisplatin and mitomycin was 15.6-fold, 6.8-fold, 4.7-fold, 3.6-fold, 2.2-fold, 1.6-fold and 1.5-fold higher than that of SW1116csc (Table 2), indicating that SW1116 cells are more sensitive than SW1116csc to therapeutic drugs.

Identification of CD133 and CD29 expression in SW1116 cells

FACS analysis showed that 65% and 36.8% of SW1116 cells were positive for CD133 and for CD29 protein, respectively. SW1116 cells (34%) co-expressed CD133 and CD29, suggesting that the in vitro propagated spheroid cells can express both markers (Figure 2A and B).

Side population cells in SW1116 cells

After cultured for 30 d, SW1116csc and SW1116 cells were stained with Hoechst 33342 to analyze differences in the SP proportion. The data showed that SW1116csc

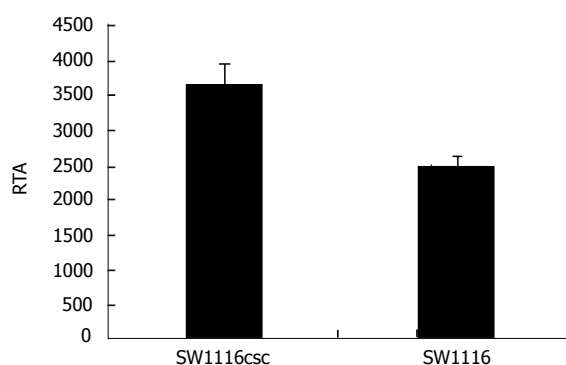


Figure 3 Telomerase activities in SW1116csc and SW1116 cells. RTA: Relative telomerase activities.

contained 38.9% \pm 7.5% of Hoechst 33342-stained dull cells, while SW1116 cells contained only 1.2% \pm 0.3% of Hoechst 33342-stained dull cells.

Telomerase activity of SW1116 cells

Telomerase reactivation is essential for stabilization of telomere length in attaining cellular immortality and telomerase is activated in human CSC. In this study, the RTA of SW1116csc and SW1116 cells was 3674 \pm 287 and 2518 \pm 140, respectively, indicating that the telomerase activity of SW1116csc is higher than that of SW1116 cells (Figure 3, $P < 0.01$).

Expressions of stem cell genes and proteins in SW1116 cells

RT-PCR showed that the expressions of CD133, CD29, Musashi-1, ABCG2, TERT genes increased significantly in SW1116 cells, while no change occurred in expressions of Oct-4 and Sca-1 gene in SW1116csc and SW1116 cells (Figure 4A). Western blot showed that the expressions of CD133, CD29, Musashi-1, ABCG2, and TERT proteins in SW1116csc and SW1116 cells were increased at transcriptional level (Figure 4B).

Proteome differential expression in SW1116csc and SW1116 cells

The silver-stained 2-DE gels of proteomes expressed in

Table 3 Differently expressed proteins in SW1116csc and SW1116 cells

Protein index	Protein description	NCBI ID	Theoretical Mr	Theoretical pI	Protein coverage (%)	Summary score
7387	stratifin	gi 5454052	27774	4.68	3	41
6402	Raichu404X	gi 14595132	85016	6.45	10	102
6963	Ubiquitin fusion-degradation 1 like protein	gi 1654346	39170	6.04	7	58
7013	eukaryotic translation elongation factor 1 delta isoform 2	gi 25453472	31121	4.9	8	119
6840	tubulin beta	gi 223429	50223	4.67	6	79
7335	nuclear chloride channel protein	gi 4588526	27249	5.02	10	54
7049	glyceraldehyde-3-phosphate dehydrogenase	gi 31645	36202	8.26	8	143
6946	F-actin capping protein alpha-1 subunit	gi 5453597	32923	5.45	9	84
6852	hypothetical protein	gi 51476996	40696	6.43	7	68
7137	guanine nucleotide binding protein beta polypeptide 2-like 1	gi 21619296	35077	7.6	6	125

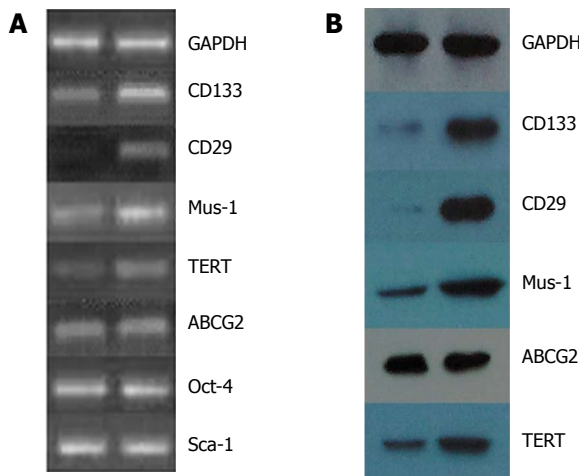


Figure 4 Expressions of CD133, CD29, Musashi-1, TERT, ABCG2, Oct-4 and Sca-1 genes (A) and CD133, CD29, Musashi-1, ABCG2 and TERT proteins (B) in SW1116csc and SW1116 cells (left: SW1116 cells, right: SW1116csc). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Mus-1: Musashi-1.

SW1116csc and SW1116 cells were presented. The protein spots detected in gels of total protein were 2115 ± 137 and 2133 ± 153 , respectively. One of the gels was selected as a reference gel. Student's t-test showed that the volume of 26 protein spots was significantly changed in gels (Figure 5A, $P < 0.05$). The expression levels were increased and decreased, respectively, in 15 and 11 out of the 26 protein spots of SW1116 cells (Figure 5B).

Identification of differentially expressed proteins in SW1116 cells

Ten out of the differentially expressed protein spots were chosen and identified as ubiquitin fusion-degradation 1 like protein, nuclear chloride channel protein, tubulin β , Raichu404X, stratifin, F-actin capping protein α -1 subunit, eukaryotic translation elongation factor 1 delta isoform 2, hypothetical protein, glyceraldehyde-3-phosphate dehydrogenase and guanine nucleotide binding protein β polypeptide 2-like 1, respectively, as detected by Western blotting (Table 3).

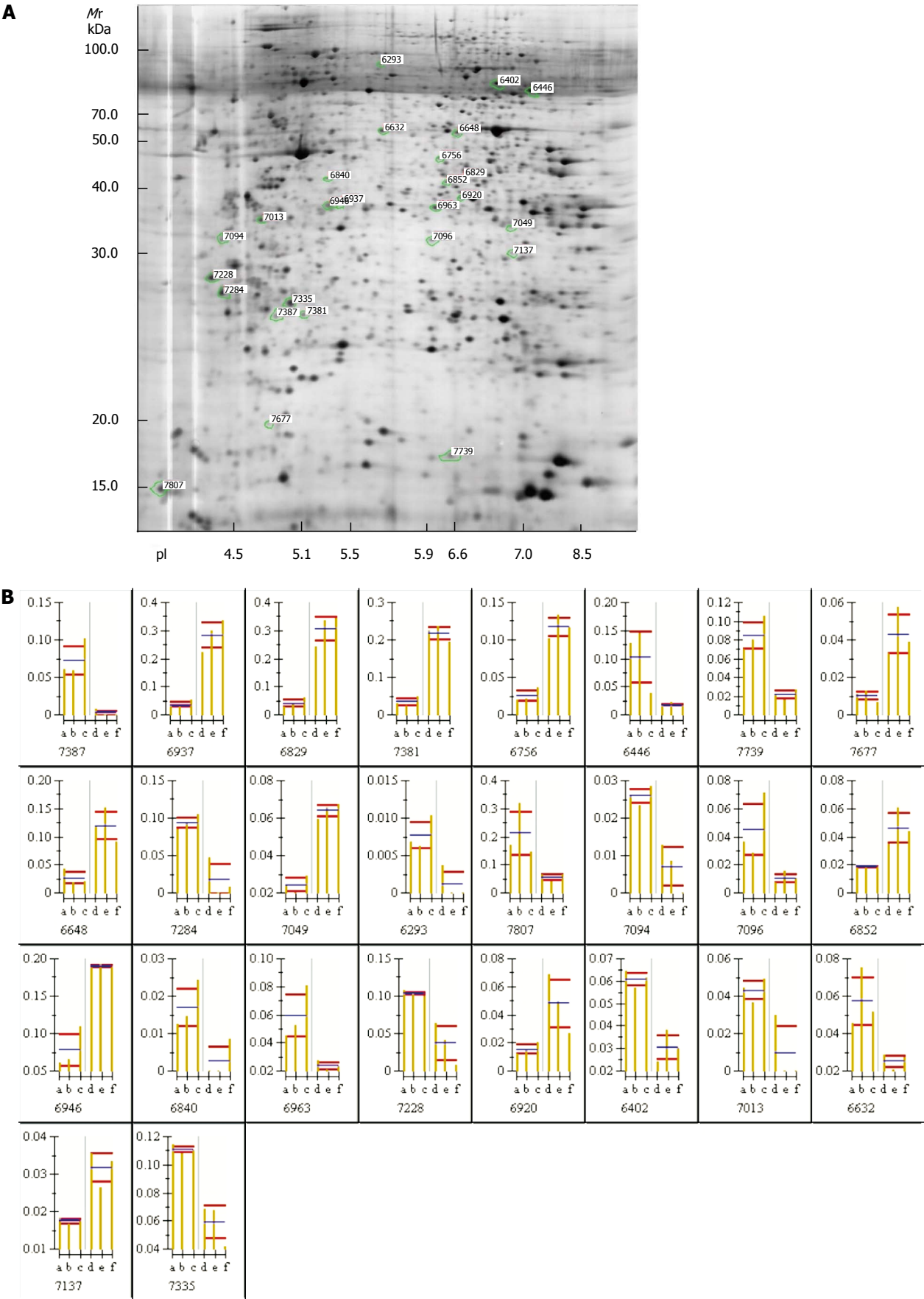
DISCUSSION

Cancer is composed of heterogeneous cells. A cancer

stem cell concept means that cancer cells exhibit a hierarchy and a small number of cancer cells are maintained as cancer stem cells able to renew and differentiate. Therefore, presumably all cancers come from stem cells and these cancer stem cells may be associated with metastasis, treatment resistance, and recurrence. Biologically distinct and relatively rare populations of tumor-initiating cells have been detected by several methods and markers in a variety of cancers. Putative breast cancer tumorigenic cells and $CD44^+ CD24^-$ / low ESA^+ cells are able to drive tumor formation when a few hundred cells are injected into the mammary fat pad of NOD/SCID mice^[13]. In addition, different populations of cancer cells derived from primary head and neck squamous cell carcinomas display a tumorigenic potential. A minority number of $CD44^+$ cells give rise to new tumors *in vivo*^[14]. Pancreatic cancer cells with the $CD44^+ CD24^+ ESA^+$ phenotype have stem cell properties and exhibit a 100-fold higher tumorigenic potential than nontumorigenic cancer cells^[15]. In this study, colon CSC were found in the $CD133^+ CD29^+$ fraction and colon cancer stem cells with this phenotype were biologically characterized by self-renewal, proliferation and differentiation.

CD133 (prominin-1, PROM1) is a 5-transmembrane glycoprotein of 865 amino acids with a total molecular weight of 120 kDa. It has been shown that CD133 antigen is expressed in undifferentiated endothelial progenitor cells^[16], hematopoietic stem cells^[17], fetal brain stem cells^[18], embryonic epithelium^[19], prostatic epithelial stem cells^[20], and myogenic cells^[21]. CD133 has also been found on cancer stem or tumor-initiating cells in cancers such as retinoblastoma^[22], teratocarcinoma^[23], leukemia^[24], brain tumor^[25], hepatocellular carcinoma^[26], and colon cancer^[27]. It was reported that a small number of $CD133^+$ cells from primary colon cancer tissue have a tumorigenic potential in immunodeficient mice^[4], indicating that $CD133^+$ cells in colon cancer tissue have a high tumorigenic ability.

Integrin is a non-covalently-bound heterodimeric cell adhesion molecule that links ECM to cytoskeleton. $\beta 1$ integrin family ($\beta 1$ integrins, CD29), the largest group of integrins, is composed of a $\beta 1$ and one of the 12 α subunits and functions predominantly as cell-ECM adhesion. Both subunits have single hydrophobic transmembrane domains and transmit information from ECM context surrounding cells into outside-in signaling cells, while



the extracellular binding activity of integrin is regulated from the inside of cells (inside-out signaling). It was reported that integrins are involved in many biological processes such as cell growth, differentiation, migration, and death^[28], suggesting that CD29 ($\beta 1$ integrin) is a new stem cell marker for colon cancer.

In mouse small intestine and human colon, Musashi-1 (Mus-1), a mammalian RNA-binding protein associated with the maintenance of neural stem cell state and its differentiation, has been found only in the lower third crypt, with a distribution that is compared in terms of cell position with the theoretical distribution of potential stem cells in the intestinal epithelium^[29]. It has been shown that Mus-1 can activate the Notch signaling pathway by suppressing the translation of the Notch inhibitor m-Numb^[30]. The Numb protein is asymmetrically distributed in neural progenitor cells in *Drosophila* and a similar asymmetrical distribution can maintain the intestinal epithelium stem cell compartment. However, interaction of Mus-1 with Notch, Delta and Tcf-4 (which appears to be intimately involved in intestinal stem cell maintenance) together with the wide variety of stem cells that express Mus-1^[31,32], suggest that this protein plays a general role in regulation of stem cell maintenance and differentiation, thus representing distinct progenitor cells.

In this study, the expression of stem cell genes (Musashi-1, TERT, ABCG2, Oct-4, Sca-1) was detected by RT-PCR, which showed that the expression levels of Musashi-1, TERT, ABCG2 genes were significantly increased in SW1116 cells, indicating that the expression of these genes in SW1116 cells is up-regulated at transcriptional level and that SW1116 cells can express stem cell genes and proteins biologically characterized by self-renewal, proliferation and differentiation.

Telomerase is a ribonucleoprotein that extends the telomeric ends of chromosomes to counterbalance their natural shortening due to incomplete DNA replication in eukaryotic cells. It has been demonstrated that telomerase is activated in 90% of malignant tumors, but is stringently repressed in normal somatic cells^[33,34], displaying that telomerase reactivation is a critical step in carcinogenesis. In this study, the activity of telomerase was increased in SW1116 cells, which is essential for the stabilization of telomere length in attaining cellular immortality.

In this study, proteomics of SW1116 cells was used to identify more specific phenotypic markers of colon CSC and elucidate the mechanism underlying their self-renewal and differentiation^[26]. Differential protein spots were found and 10 proteins were identified. Among the differentially expressed proteins, some may be essential for isolation and identification of colon CSC.

Ubiquitin fusion degradation 1 L (UFD1L) is a human homologue of the yeast ubiquitin fusion degradation 1 (Ufd1) gene. In yeast, Ufd1 protein is involved in a degradation pathway for ubiquitin fused products (UFD pathway). The biochemical role of UFD1L protein in human cells is unknown. Velazquez-Fernandez D^[35] used microarray to study the expression profiling of adrenocortical

neoplasms, and found that UFD1L is a molecular signature of malignancy. In this study, the UFD1L protein was intensively expressed in SW1116csc and hardly expressed in SW1116 cells, indicating that the degradation pathway for ubiquitin fused products is active in colon CSC.

Stratifin is a member of 14-3-3 protein family, a highly conserved group of proteins consisting 7 isoforms involved in numerous crucial intracellular functions such as cell cycle and apoptosis, regulation of signal transduction pathways, cellular trafficking, cell proliferation and differentiation, cell survival, protein folding and processing. In eukaryotes, peptide chain elongation is mediated by elongation factors, EF-1 and EF-2. EF-1 is composed of a nucleotide-binding protein EF-1 α , and a nucleotide exchange protein complex EF-1 β gamma. Elongation factors are highly conserved among different species and may be involved in functions other than protein synthesis, such as organization of the mitotic apparatus, signal transduction, developmental regulation, ageing and transformation. Increased expression levels of stratifin and EF-1 delta in SW1116 cells may be related to cell proliferation and differentiation.

In this study, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was down-regulated in SW1116 cells. GAPDH, a multifunctional protein with defined functions in numerous subcellular processes, plays an integral role in glycolysis. New investigations are needed to establish the primary role of GAPDH in a variety of critical nuclear pathways apart from its already recognized role in apoptosis. The new roles of GAPDH include its requirement for transcriptional control of histone gene expression^[36], its essential function in nuclear membrane fusion, its necessity for recognition of fraudulently incorporated nucleotides in DNA, and its mandatory participation in maintenance of telomere structure. To undertake these new functions, GAPDH is recruited to the nuclei in S phase or its intracellular distribution is regulated as a function of drug exposure. Further study is needed to explore the functions of GAPDH in SW1116 cells.

In conclusion, SW1116 cells and CD133⁺/CD29⁺ fraction in human colon cancer cells are biologically characterized by self-renewal, proliferation and differentiation. SW1116csc is also more chemoresistant than SW1116 cells. CD133, CD29 and Mus-1 may be used in isolation and identification of colon cancer stem cells.

COMMENTS

Background

Cancer stem cell hypothesis is currently at the centre of a rapidly evolving field, involving a change of perspective in development and treatment of cancers. However, research has been hampered by the lack of distinct molecular markers of cancer stem cells.

Research frontiers

Since the identification and characterization of cancer stem cells (CSC) in hematological malignancies, an increasing number of studies have described CSC in solid tumors such as ovarian tumor, colon tumor, lung tumor, breast tumor, liver tumor, melanoma and pancreatic tumor, raising that the cancer stem cell hypothesis can be applied to all neoplastic systems.

Innovations and breakthroughs

In our study, human CSC were isolated, which presented as spheroid and suspension growths in serum-free medium, with a strong ability of self-renewal, proliferation, differentiation and drug-resistance. Proteomic analysis showed that 26 differentially expressed protein spots were detected and 10 protein spots were chosen and identified.

Applications

The results of this study have important implications for future cancer treatment. The CSC hypothesis infers that if the CSC were eliminated, the tumor would simply regress due to cell differentiation and death. It is possible to treat patients with aggressive, non-resectable tumors and prevent their metastasis by selectively targeting CSC.

Terminology

Cancer stem cells are a sub-population of cancer cells that possess characteristics associated with normal stem cells, such as self renewal and differentiation into multiple cell types. CSC are tumorigenic while the bulk of cancer cells are non-tumorigenic. CSC persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors.

Peer review

The authors identified the biological characteristics and proteome of human colon cancer stem cells. The results are interesting and may be essential for CSC isolation and characterization.

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Treatment of pediatric refractory Crohn's disease with thalidomide

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Abstract

AIM: To assess the efficacy and tolerability of thalidomide in pediatric Crohn's disease (CD).

METHODS: Six patients with refractory CD received thalidomide at an initial dose of 2 mg/kg per day for one month, then increased to 3 mg/kg per day or decreased to 1 mg/kg per day, and again further reduced to 0.5 mg/kg per day, according to the individual patient's response to the drug.

RESULTS: Remission was achieved within three months. Dramatic clinical improvement was demonstrated after thalidomide treatment. Endoscopic and pathological improvements were also observed after thalidomide treatment, which was well tolerated by all patients.

CONCLUSION: Thalidomide is a useful drug for pediatric refractory CD.

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Key words: Inflammatory bowel disease; Thalidomide; Tumor necrosis factor- α ; Children

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INTRODUCTION

Crohn's disease (CD) is a chronic transmural inflammatory bowel disease (IBD) that may involve any part of the alimentary tract from mouth to anus, especially the distal ileum and colon. It may also accompany various intra- and extra-intestinal complications during its clinical course^[1,2]. In addition to causing serious clinical symptoms and complications, its chronic nature can adversely affect the quality of life of patients^[3,4]. Despite an increasing number of treatment options, many patients with CD continue to pose a therapeutic challenge because they do not adequately respond to therapy, or because they experience serious side effects of standard medical interventions. This is particularly true for those patients with refractory CD or concomitant tuberculosis. There is an urgent need to develop new treatment modalities for refractory CD.

Thalidomide, originally used to treat morning sickness in the early 1960s, has been withdrawn from the market because it leads to serious congenital birth defects. In 1991, it was discovered that thalidomide can inhibit the synthesis of cytokine, a tumor necrosis factor- α (TNF- α), by accelerating the degradation of its mRNA^[5]. Interest in thalidomide has intensified in recent years since its immunomodulatory^[6,7] and anti-angiogenic^[8,9] properties were identified and clarified. A series of clinical trials have demonstrated the efficacy of thalidomide in treatment of several clinical conditions such as human immunodeficiency virus (HIV)-associated wasting syndrome^[10], hereditary hemorrhagic telangiectasia^[11], refractory cutaneous lesions of lupus erythematosus^[12], multiple myeloma^[13], and Behcet's disease^[14],

for which there are few or no alternative treatment options. Based on these observed clinical responses and the apparent anti-TNF- α properties of thalidomide, we used it in treatment of a 12-year-old boy with concomitant refractory CD and tuberculosis infection, and achieved a dramatic success. With this experience, we started to treat refractory CD with thalidomide. Following is a report of 6 children with a complete remission after treated with thalidomide.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Ethics Committee of Children's Hospital of Fudan University and informed consent was obtained from all patients and their parents.

Clinical data

This is a retrospective study of 6 patients with refractory CD who visited the Children's Hospital of Fudan University in 2006-2010 (Table 1). Conventional therapy failed in these patients, and/or they could not receive steroid or immunosuppressive treatment because of concomitant tuberculosis infection.

Study design

Patients administered thalidomide in the evening at a starting dose of 2.0 mg/kg per day, which was increased to 2.5-3.0 mg/kg per day or decreased to 1.5 mg/kg per day, according to the individual patient's response to the drug. Patients were assessed at baseline, at weeks 2, 8 and 12, and then every three months for the following parameters, including physical examination, laboratory analyses and pediatric CD activity index (PCDAI) scoring. Endoscopies were repeated at six months after administration of thalidomide. Its side effects were intensely monitored during follow-up. CD was defined as refractory when standard induction therapy with high-dose intravenous steroids failed to induce remission either at diagnosis or during subsequent relapse. Efficacy was defined as thalidomide induced and maintained remission and mean time to achieve clinical remission (PCDAI^[15] < 7.5).

Statistical analysis

Results are expressed as mean \pm SD. Changes in interval PCDAI scores were evaluated by multivariate analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Patient A

Patient A was a 22-year-old boy with a 10-year history of CD. Steroids and 5-aminosalicylic acid (5-ASA) were administered for 4 years. However, abdominal pain, diarrhea and fever were still recurrent, and the patient remained seriously undergrown: 42.5 kg, below 10th percentile (< P10) weight-for-age and 162 cm (< P10 height-for-age). In 2006, an immunosuppressant, azathioprine, was started but could not be tolerated because of severe thrombocy-

topenia. He was then given thalidomide (2 mg/kg per day) in addition to prednisone (10 mg/d) and 5-ASA for four weeks, after which the dosage of steroid was reduced gradually while that of thalidomide was increased. After eight weeks of thalidomide treatment, abdominal pain and fever improved significantly, and the dosage of thalidomide was increased to 3 mg/kg per day while the dose of prednisone was reduced to 5 mg/d. After six months of treatment, this patient achieved complete clinical remission. The dose of thalidomide was reduced to 1.5 mg/kg per day and prednisone was withdrawn. Enteroscopic examination at this time showed that the mucosal ulceration and hyperplasia improved significantly. During thalidomide therapy, this patient experienced transient hepatic function abnormality which became normal after withdrawal of thalidomide. In August 2010, both thalidomide and prednisone were withdrawn and he was on 5-ASA for maintenance of remission without any clinical evidence for recurrence of the disease. His body weight was 67 kg (P75-P90, weight-for-age) and height was 168 cm (P10-P25, height-for-age).

Patient B

Patient B, a 13-year-old boy with a 5-year history of splenic tuberculosis, was diagnosed as CD 3 years ago. This patient also had a history of chronic diarrhea accompanying hematochezia, abdominal pain, weight loss, oral and perianal ulcers, anemia, fatigue, malnutrition and spiking fever up to 40°C. Symptoms did not improve upon anti-tuberculosis (TB) treatment with rifampin, isoniazid and pyrazinamide, and splenectomy was performed 3 years ago after which the symptoms persisted. In June 2009, double-balloon enteroscopy demonstrated segmental distribution of ulcers with different sizes and characteristics in the colon and ileum. Histological examination revealed acute and chronic mucosal inflammation and hyperplasia, with infiltration of a large number of neutrophils, plasma cells, macrophages and scanty eosinophils. Bone marrow culture identified *Mycobacterium*. Steroids, immunosuppressive agents and biologics such as infliximab could not be used due to concurrent tuberculosis infection, thalidomide was therefore chosen as the second-line therapy for his CD.

After he received thalidomide at a dose of 2 mg/kg per day for two weeks, his body temperature returned to normal, his abdominal pain and diarrhea were significantly alleviated, and his oral ulcers became more superficial and then completely disappeared. After one month of treatment, steroids were withdrawn. Six months later, thalidomide was reduced to 0.5 mg/kg per day. His body weight increased from 33 kg (< P10, weight-for-age) to 54 kg (P50-P75, weight-for-age). Endoscopic examination revealed that the intestine appeared almost normal, and laboratory data improved significantly with a normal erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). In August 2010, he was on thalidomide, 1.0 mg/kg per day, to maintain remission. No adverse effect was found throughout the treatment.

Patient C

Patient C was an 11-year-old boy who underwent two sur-

Table 1 Parameters of patients, doses of thalidomide and pediatric Crohn's disease activity index scores

	Patients					
	A	B	C	D	E	F
Gender	M	M	M	F	M	F
Current age	20	13	12	16	9	13
Age at onset (yr)	12	10	11	11	5	11
Duration of disease (yr)	8	3	1	5	4	2
Thalidomide start age (yr)	16	12	12	16	9	13
Duration of thalidomide treatment (mo)	12	14	10	10	7	7
Start dose (mg/kg per day)	2	2	2	2	2	2
Dose at sixth months (mg/kg per day)	1	0.2	0.5	0.3	1	1
PCDAI score before thalidomide treatment	55	67.5	70	65	72.5	65
PCDAI score after six months of thalidomide treatment	5	5	7.5	5	5	12.5

PCDAI: pediatric Crohn's disease activity index.

gical procedures for perianal abscess in September 2008 and February 2009 and had a positive history of close contact with a TB patient. TB-PPD test was strongly positive, but culture of intestinal mucosa for tuberculosis was negative. He had no response to 5-ASA and a limited response to immunosuppressive agents. In November 2009, colonoscopy identified several polyps and ulcers in the transverse and ascending colon. Because of suspected *Mycobacterium tuberculosis* infection, steroid was not used in this patient. Treatment with thalidomide at a dose of 2 mg/kg per day was started in November 2009. After two weeks of thalidomide treatment, his symptoms including fever, abdominal pain and diarrhea were significantly relieved. CRP and ESR became normal within two months after thalidomide treatment, and his body weight increased from 27 kg (< P10, weight-for-age) to 37.5 kg (P20-P50, weight-for-age). After six months of thalidomide treatment, the PCDAI was reduced to 7.5 from 70 before thalidomide treatment.

Patient D

Patient D, a 16-year-old girl with a 5-year history of CD, suffered from severe abdominal pain, chronic diarrhea and severe malnutrition. Growth failure was very marked in this patient. Her body weight was only 21 kg (< P10, weight-for-age) while her height was 130 cm (< P10, height-for-age) when she was 15 years old, and showed no secondary sexual characteristics. During the five years of treatment with steroids, immunosuppressive agents and 5-ASA, her clinical symptoms were relieved significantly, but her height or weight did not increase after the diagnosis of CD. In April 2010, thalidomide therapy was initiated. Three months after treatment, her appetite was significantly improved, her body weight increased from 21 to 33 kg (< P10, weight-for-age) and her height increased 1 cm.

Patient E

Patient E was a 9-year-old boy with a four-year history of CD accompanying recurrent fever, abdominal pain, diarrhea and poor weight gain. PPD test was regarded as positive when the induration was 18 mm × 18 mm. He was

diagnosed with intestinal tuberculosis and received anti-TB therapy for one year without any improvement in his symptoms. Because he was allergic to a variety of foods including egg, milk, soybean, peanut and wheat, his allergic colitis was treated by failed. During the last two years, he developed edema in lower limbs, ascites and pleural effusion attributable to serious malnutrition. In 2009, colonoscopy showed chronic granulomatous inflammation and segmental distribution of ulcers in the colon and terminal ileum, and CD was diagnosed. Infliximab was contraindicated in this patient because of the positive tuberculin test, thalidomide was therefore given to control his CD. After three months of treatment, the both CRP and ESR decreased significantly. His symptoms also improved quite remarkably.

Patient F

Patient F was a 13-year-old girl with a 2-year history of CD accompanying abdominal pain, chronic diarrhea and hematochezia. The patient received steroid treatment during the last two years and clinical symptoms were relieved significantly. However, when the dose of steroids was reduced gradually, her clinical symptoms appeared again. She became steroid-dependent with obvious cushingoid features developed last year. Based on the strong desire of the patient and her parents, thalidomide treatment was started at a dose of 2 mg/kg per day in April 2010. After three months of thalidomide treatment, steroids were withdrawn and her clinical symptoms improved significantly.

In conclusion, thalidomide is an effective treatment modality for refractory CD. However, its long-term efficacy and safety need to be further evaluated in a large-scale randomized controlled trial (Figures 1 and 2).

DISCUSSION

TNF- α , one of the most important immunologic mediators generated by cells of the monocyte-macrophage lineage, has a broad spectrum of biological functions and plays a crucial role in amplification of responses to infection, injury, and immune-mediated damage. As a cytokine, TNF- α can transmit signals between immune and other

cells, and is involved in apoptosis, metabolism, inflammation, thrombosis, and fibrinolysis^[16]. The importance of TNF- α and TNF- α signaling in response of the immune system to disease has become clearer as a result of a number of seminal studies^[17-19]. It was reported that mice lacking of TNF- α show a high susceptibility to infectious agents^[17] and also an impaired clearance of adenoviral vectors^[18]. In addition, gene knockout experiments (TNF- $\alpha^{-/-}$ mice) showed that TNF- α is necessary for adhesion molecule expression and recruitment of leukocytes to inflammatory sites^[19].

Although TNF- α has critical physiological functions, its overproduction plays a key role in physiopathology of a variety of diseases. Of relevance to IBD are the abilities of TNF- α to recruit circulating inflammatory cells to local tissue sites of inflammation, to induce edema, to activate coagulation cascade, and to initiate formation of granuloma^[20]. Clinical trials demonstrating symptomatic improvement and remission of CD by suppressing TNF- α have provided additional evidence of the role of TNF- α in pathogenesis of CD^[21-24].

Thalidomide was developed in the 1960s as a sedative but was subsequently withdrawn from widespread use because of teratogenicity. The drug has been banned for more than two decades when it was shown to inhibit TNF- α production^[5], thus leading to its revival in clinical settings. This property of thalidomide was first described in treatment of patients with systemic erythema nodosum leprosum^[25]. Such patients demonstrated an extremely high TNF- α level in their blood, which decreased significantly during thalidomide treatment, indicating that thalidomide therapy can reduce not only serum TNF- α level, but also clinical symptoms as well. Dermal infiltration of polymorphonuclear leukocytes and T cells are also diminished after treatment with thalidomide^[25]. Studies also demonstrated that thalidomide improves clinical symptoms in patients with HIV-associated wasting syndrome^[10], multiple myeloma^[13], Behcet's disease^[14] and CD^[26-30]. Suppression of TNF- α may be the mechanism underlying CD. It was reported that thalidomide treatment can restore the normal levels myeloperoxidase and TNF- α in a rat model of IBD^[27].

Tuberculosis is still quite common in China. Treatment of CD patients with simultaneous TB infection may be difficult. In this situation, administration of steroids or immunosuppressive agents may lead to reactivation of latent tuberculosis or its flare-up. In this study, thalidomide was a good choice for such patients, which not only improved the symptoms of CD, but also enhanced the response of the patients to anti-TB drugs.

Although thalidomide can more uniquely improve the symptoms of many diseases than other drugs, it may lead to significant problems due to its side-effects. The most serious side-effect is teratogenicity. Moreover, thalidomide often has other side-effects, including peripheral neuropathy. For these reasons, synthetic thalidomide analogues (IMiDs) with an increased TNF- α inhibitory activity and diminished side effects are desirable. Many analogues

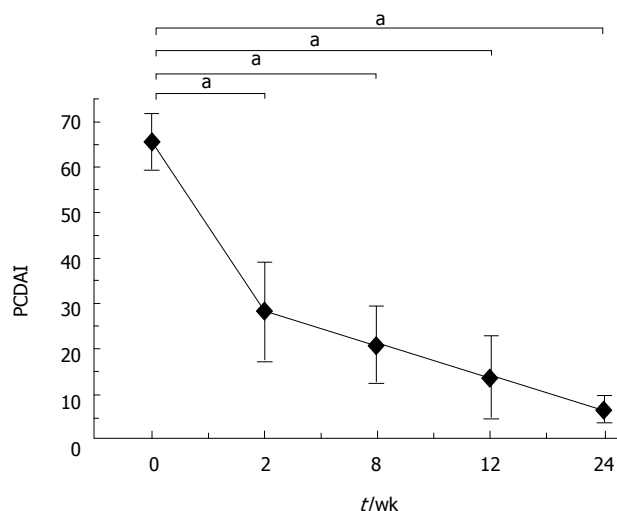


Figure 1 Mean pediatric Crohn's disease activity index during thalidomide treatment. ^a $P < 0.0005$. PCDAI: Pediatric Crohn's disease activity index.

exhibit a higher inhibition of TNF- α expression than thalidomide. In particular, the IMiDs, lenalidomide and pomalidomide (CC-4047), are respectively 2000- and 20000-fold more potent than thalidomide in inhibiting TNF- α and able to induce transcription and secretion of TGF- β and IL-10^[31]. The 4-amino analogues (in which an amino group is added to the fourth carbon of the phthaloyl ring of thalidomide) have been found to be up to 50000-fold more potent in inhibiting TNF- α than thalidomide *in vitro*^[32]. Several of these new compounds *in vivo* are able to reduce lipopolysaccharide-induced TNF- α levels in mice^[33] and inhibit development of adjuvant-induced arthritis in rats^[34]. Whether IMiDs have a greater therapeutic efficacy with fewer side-effects than thalidomide needs to be determined. It was reported that neuropathy may occur only after high cumulative doses of thalidomide are used^[26,29], indicating that thalidomide can be used as a maintenance modality.

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COMMENTS

Background

Despite an increasing number of treatment options, treatment of Crohn's disease (CD) is still a challenge of physicians due to its poor response to therapy or serious side effects of standard medical interventions. This is particularly true for those with refractory Crohn's disease or concomitant tuberculosis infection. Thalidomide, a synthetic glutamic acid derivative, has anti-inflammatory, anti-angiogenic and immunomodulatory properties. Studies suggest that thalidomide can significantly inhibit the synthesis of a tumor necrosis factor- α (TNF- α) and plays a role in up-regulation of Th2-type immunity. Thalidomide may be a good choice for Crohn's disease patients.

Research frontiers

Treatment of CD patients with tuberculosis infection may be difficult. In this situation, administration of steroids or immunosuppressive agents may lead to

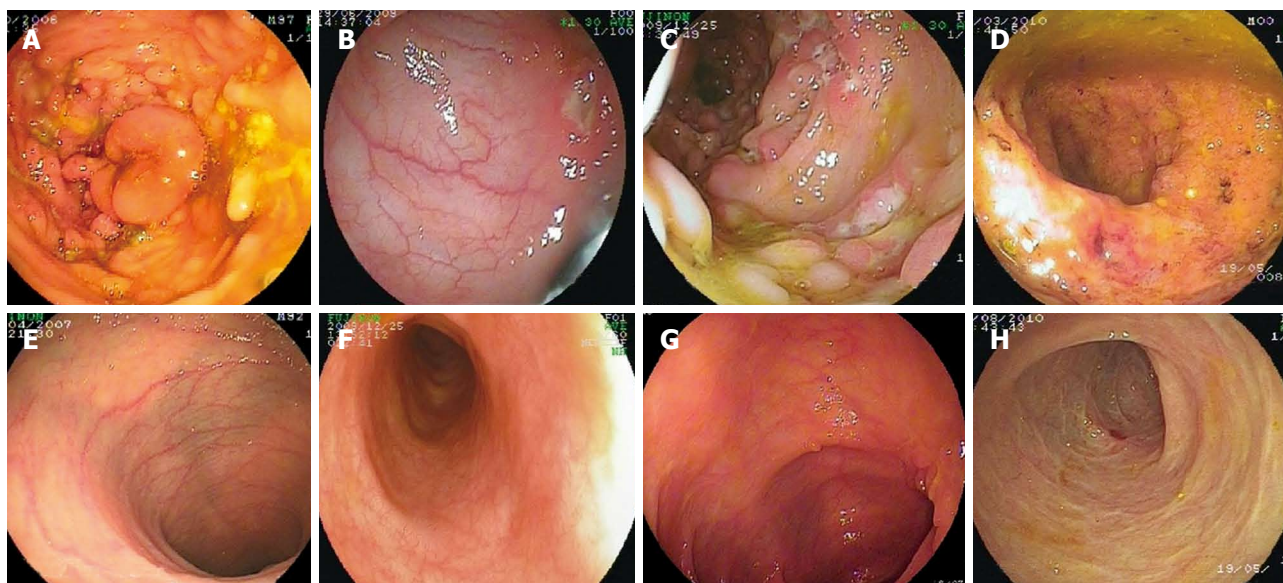


Figure 2 Endoscopy views of patients A, B, C, F before thalidomide treatment (A-D) and six months after thalidomide treatment (E-H).

reactivation of latent tuberculosis or tuberculosis infection flare-up, but tuberculosis infection is still quite common in some countries including China. It is therefore urgent to develop new treatment modalities for such patients.

Innovations and breakthroughs

Therapy for refractory Crohn's disease or concomitant tuberculosis infection is always a challenge. In the present study, thalidomide was a good choice for such patients, which can not only improve the symptoms of CD, but also enhance the response of the patients to anti-tuberculosis drugs. CD patients tolerate it quite well.

Applications

This study may offer a future strategy for therapeutic intervention of CD patients with a poor response to therapy or serious side effects of standard medical interventions.

Terminology

Refractory Crohn's disease refers to a condition in which standard induction therapy with high-dose intravenous steroids has failed to induce remission either at diagnosis or during subsequent relapse. Pediatric CD activity index is a rating scale used to assess the severity of pediatric Crohn's disease and correlates well with disease activity.

Peer review

Although the number of patients investigated was small, the study was interesting, and well written. This manuscript is acceptable as a short article in *WJG*.

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Hepatitis B virus infection: A favorable prognostic factor for intrahepatic cholangiocarcinoma after resection

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Abstract

AIM: To study the prognostic factors for intrahepatic cholangiocarcinoma (ICC) and evaluate the impact of chronic hepatitis B virus (HBV) infection on survival rate of ICC patients.

METHODS: A total of 155 ICC patients who underwent macroscopic curative resections (R0 and R1) were enrolled in this retrospective study and divided into group A with HBV infection and group B without HBV infection according to their chronic HBV infection, represented by positive hepatitis B surface antigen (HBsAg) in serum or in liver tissue. Clinicopathological characteristics and survival rate of the patients were evaluated.

RESULTS: All patients underwent anatomical resection. Their 1- and 3-year survival rates were 60.6% and 32.1%, respectively. Multivariate analyses revealed that HBV infection, hepatolithiasis, microscopic satellite

lesion, and lymphatic metastasis were the independent prognostic factors for the survival rate of ICC patients. The median disease-free survival time of the patients was 5.0 mo. The number of tumors, microscopic satellite lesion, and vascular invasion were the independent prognostic factors for the disease-free survival rate of the patients. The prognostic factors affecting the survival rate of ICC patients with HBV infection and those without HBV infection were not completely consistent. Alkaline phosphatase > 119 U/L, microscopic satellite lesion, vascular invasion, and lymphatic metastasis were the independent factors for the patients with HBV infection, while γ -glutamyltransferase > 64 U/L, microscopic satellite lesion, and poor tumor differentiation were the independent factors for the patients without HBV infection.

CONCLUSION: HBV infection is a valuable clinical factor for predicting tumor invasiveness and clinical outcome of ICC patients. ICC patients with HBV infection should be distinguished from those without HBV infection because they have different clinicopathological characteristics, prognostic factors and outcomes after surgical resection.

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Key words: Intrahepatic cholangiocarcinoma; Hepatitis B virus; Survival; Prognosis

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Zhou HB, Wang H, Li YQ, Li SX, Wang H, Zhou DX, Tu QQ, Wang Q, Zou SS, Wu MC, Hu HP. Hepatitis B virus infection: A favorable prognostic factor for intrahepatic cholangiocarcinoma after resection. *World J Gastroenterol* 2011; 17(10): 1292-1303 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i10/1292.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i10.1292>

INTRODUCTION

Cholangiocarcinoma originates from the extrahepatic bile duct, hilar bifurcation, and intrahepatic duct. Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic tumor after hepatocellular carcinoma (HCC). Primary sclerosing cholangitis (PSC)^[1,2], liver fluke infestation (particularly endemic *Opisthorcis viverrini*)^[3], and hepatolithiasis are the known risk factors for ICC^[4,5]. Recent evidence suggests that hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is also an important risk factor for ICC^[5-10]. Our previous study also demonstrated that the incidence of HBV infection is significantly higher in ICC patients than in those without cancer (48.6% *vs* 6.6%) and chronic HBV infection is the most important independent risk factor for ICC in Chinese^[11].

The prognosis of ICC patients is poorer than that of HCC patients, mainly due to frequent lymphatic involvement, periductal invasion, poor encapsulation, or difficulty of early diagnosis. These characteristics are more prominent in ICC patients with seronegative hepatitis B surface antigen (HBsAg) than in those with seropositive HBsAg^[11], indicating that ICC patients with HBV infection have a more favorable prognosis than those without HBV infection. Given a higher incidence of microvascular invasion, poor tumor differentiation, and liver function in seropositive-HBsAg ICC patients compared with seronegative-HBsAg ICC patients, the real difference in prognosis is still unclear.

In the present study, the prognostic factors for ICC and the impact of chronic HBV infection on the survival rate of ICC patients were studied.

MATERIALS AND METHODS

Patients

A total of 209 patients underwent surgical dissection for ICC (the diagnosis of ICC was confirmed by pathology) at three departments of hepatobiliary surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China) in January 2005 - December 2007. Of the 209 patients, 195 underwent macroscopic curative resection (R0 and R1), 14 underwent only laparotomy and biopsy because of advanced lesions, such as peritoneal seeding. Of the 195 patients, there were 155 patients with information on survival and 40 lost their follow-up due to death, loss of contact, or other unknown reasons. Finally, 155 patients were included in the study. The prognostic factors influencing their survival rate and tumor recurrence were analyzed. The study was approved by the local ethics committee.

Clinicopathological investigations

Demographic and clinicopathological information was obtained from medical records of the patients including age, gender, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), γ -glutamyltransferase (γ -GT), alkaline phosphatase (ALP), α -feto-

protein (AFP), carbohydrate antigen 19-9 (CA19-9), HBV infection, number of tumors, and tumor location, size, capsule formation, histologic type, differentiation and recurrence, as well as vascular invasion, microscopic satellite lesion, lymphatic and extrahepatic metastasis, surgical procedures, postoperative complications.

Statistical analysis

Patients were screened for carcinoembryonic antigen (CEA), AFP, CA19-9 and CT scan every 3-6 mo after operation. When recurrence was suspected, magnetic resonance imaging (MRI) or PET images were taken for confirmation. Disease-free survival was measured from the date of surgery to the date of recurrence. Survival was measured from the date of surgery. Follow-up of patients was continued until death or April 10, 2010.

Statistical analysis was performed using the SPSS, version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Overall and disease-free survival rates were calculated using the Kaplan-Meier method. Prognostic factors for the patients were evaluated using the univariate Kaplan-Meier method and compared with the log-rank test. Multivariate regression analysis was performed using the Cox proportional hazards model to identify the independent prognostic factors for the survival rate of patients and tumor recurrence. Variables to be entered into the multivariate analysis were selected on the basis of the results of univariate analysis ($P < 0.1$). χ^2 test was used for the comparison of categorical variables and t -test was employed for the comparison of discrete variables between the patients with HBV infection and those without HBV infection. $P < 0.05$ was considered statistically significant.

RESULTS

General characteristics of the patients and surgical procedures

One hundred and fifty-five ICC patients (102 men and 53 women with a male/female ratio of 1.92/1) were enrolled in this study. Their mean age was 54.97 ± 10.65 years (range, 27-76 years). Of the 87 patients with chronic HBV infection, 14 were positive for HBsAg in liver tissue, 29 were positive for HBsAg in serum, and 44 were positive for HBsAg both in serum and liver tissue. Of the 155 patients with anatomical *en bloc* resections, 111 (71.6%) underwent segmentectomy or bisegmentectomy or trisegmentectomy, 30 (19.4%) left hepatectomy, 2 (1.3%) left extended hemihepatectomy, and 14 (9.0%) right hemihepatectomy, 5 (3.2%) concomitant caudate segmentectomy, 6 (3.9%) common bile duct exploration for cholelithiasis or thrombus resection, and 3 (1.9%) Roux-en-Y cholangiojejunostomy. If the tumor invaded its adjacent organs grossly in the operative field, combined resection was performed to achieve complete removal of the tumor. An additional 21 combined resections of other organs were performed in 16 patients (10.3%) as shown in Table 1. Surgical complications occurred in 6 patients, including

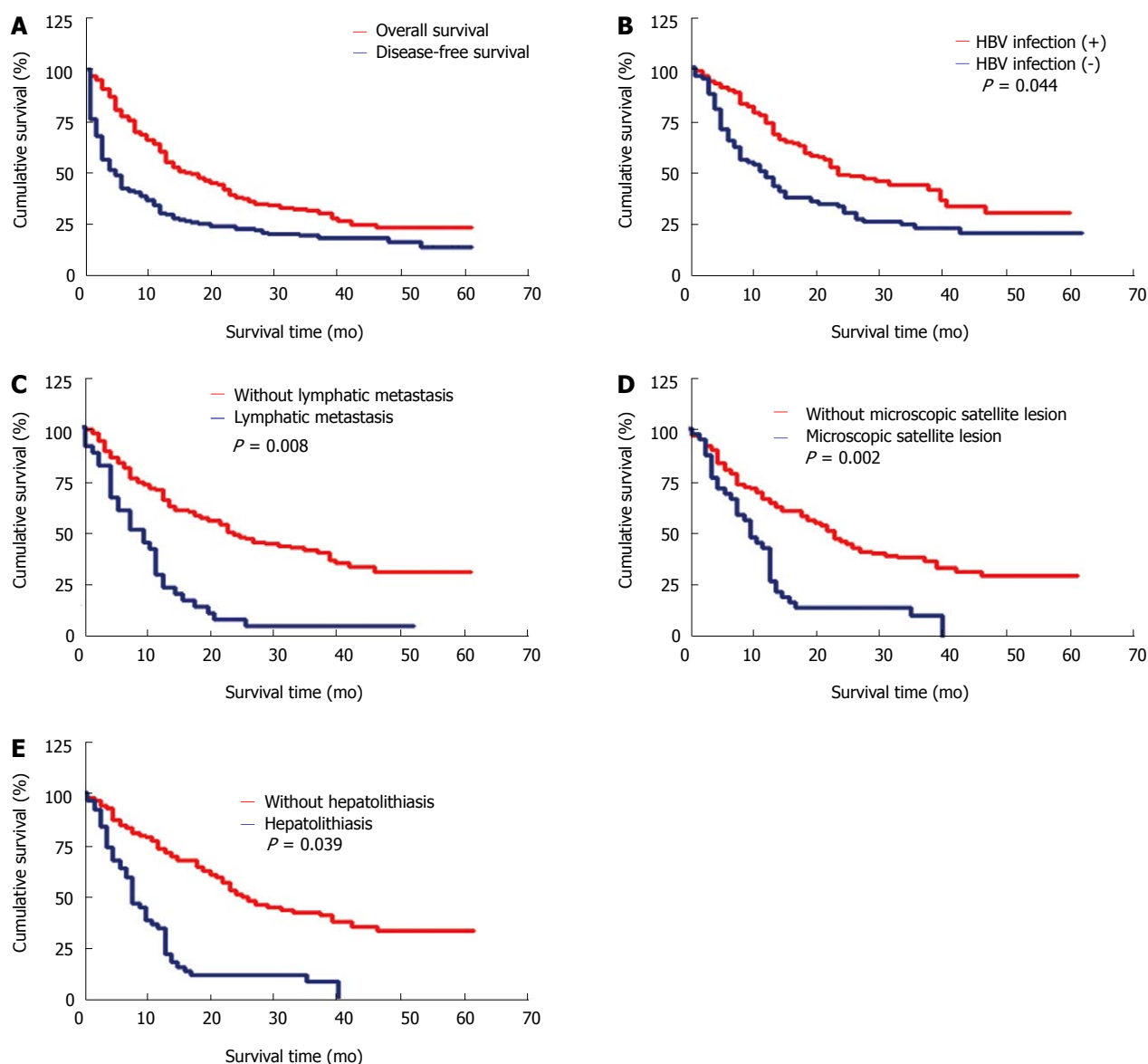


Figure 1 Overall and disease-free survival rates of patients with intrahepatic cholangiocarcinoma after surgical resection (A), higher survival rate of intrahepatic cholangiocarcinoma patients with hepatitis B virus infection than that of those without hepatitis B virus infection (B), significantly poorer survival rate of intrahepatic cholangiocarcinoma patients with lymphatic metastasis than that of those without lymphatic metastasis (C), significantly poorer survival rate of intrahepatic cholangiocarcinoma patients with microscopic satellite lesion than that of those without microscopic satellite lesion (D), and significantly poorer survival rate of intrahepatic cholangiocarcinoma patients with hepatolithiasis than that of those without hepatolithiasis (E).

biliary leakage in 2, subphrenic infection in 1, liver abscess in 2, and bleeding in 1, respectively.

Survival and recurrence

The cumulative 1- and 3-year survival rates were 60.6% and 32.1%, respectively, for the ICC patients (Figure 1). The median survival time was 17.0 mo and 35 patients survived more than 3 years. Univariate analysis demonstrated that absence of HBV infection, hepatolithiasis, $\text{r-GT} > 64 \text{ U/L}$, $\text{ALP} > 119 \text{ U/L}$, $\text{CA19-9} > 37 \text{ U/mL}$, multiple tumors, tumor size $\geq 5 \text{ cm}$ and location, microscopic satellite lesion, lymphatic metastasis, and extrahepatic metastasis were the significant prognostic factors for the poor survival rates of ICC patients. Cox regression analyses revealed that HBV infection (hazard ratio: 4.075), hepatolithiasis (hazard ratio: 4.254), microscopic satellite

Table 1 Surgical procedures performed for patients

Operation	<i>n</i>	Combination resection	<i>n</i>
Partial hepatectomy	111	Abdominal wall focus resection	1
Left hemihepatectomy	30	Diaphragm wedge resection	8
Right hemihepatectomy	14	Right adrenalectomy	2
Left extended hemihepatectomy	2	Omentumectomy	7
Caudate segmentectomy	5	Gallbladder removal	3

lesion (hazard ratio: 9.418), and lymphatic metastasis (hazard ratio: 7.078) were the significant factors for overall survival rates of ICC patients. Sex, age, AST, ALT, TBIL, AFP, cirrhosis, liver schistosomiasis, capsule formation,

Table 2 Univariate and multivariate analyses of prognostic factors for overall survival rate of intrahepatic cholangiocarcinoma patients included in this study

Factor	n	Survival rate (%)		P value		Hazard ratio	95% CI
		1-yr	3-yr	Univariate analysis	Multivariate analyses		
Age (yr)							
> 65	28	71.4	33.7				
≤ 65	127	58.3	31.8	0.590	NA	NA	NA
Sex							
Female	53	64.2	29.4				
Male	102	58.8	33.7	0.973	NA	NA	NA
HBV infection							
No	68	45.6	20.5				
Yes	87	72.4	41.8	0.003	0.044	4.075	0.418-0.987
Cirrhosis							
Yes	45	62.6	32.7				
No	110	60	31.9	0.964	NA	NA	NA
Hepatolithiasis							
Yes	12	16.7	< 0.01				
No	143	64.3	34.4	0.001	0.039	4.254	1.040-4.614
Liver schistosomiasis							
Yes	7	57.1	14.3				
No	148	60.8	33	0.063	0.612	0.257	0.532-2.920
ALT							
≤ 42 U/L	113	60.2	32.9				
> 42 U/L	42	61.9	29.8	0.956	NA	NA	NA
AST							
≤ 37 U/L	102	58.8	32.7				
> 37 U/L	53	64.2	30.5	0.949	NA	NA	NA
TBIL							
≤ 20 μmol/L	119	60.5	30.7				
> 20 μmol/L	36	61.1	37.5	0.355	NA	NA	NA
r-GT							
≤ 64 U/L	68	67.6	44.7				
> 64 U/L	87	55.2	22.4	0.003	0.102	2.667	0.918-2.547
ALP							
≤ 119 U/L	89	71.9	44.5				
> 119 U/L	66	45.5	15.8	< 0.001	0.386	0.75	0.758-2.047
AFP							
≤ 20 μg/L	125	58.4	32.8				
> 20 μg/L	30	70	29.3	0.788	NA	NA	NA
CA19-9							
≤ 37 U/mL	66	66.7	41.7				
> 37 U/mL	89	56.2	24.7	0.035	0.534	0.388	0.555-1.356
Tumor number							
Single	137	63.5	36				
Multiple	18	38.9	11.1	0.007	0.188	1.734	0.811-2.913
Tumor size							
< 5 cm	58	74.1	42.1				
≥ 5 cm	97	52.6	26.1	0.014	0.563	0.335	0.737-1.754
Tumor location							
Left lobe	55	52.7	32.2				
Right lobe	90	70	35.8				
Both lobes	10	20	< 0.01	0.008	0.314	1.015	0.962-1.128
Microscopic satellite lesion							
Yes	39	43.6	11.5				
No	116	66.4	38.9	< 0.001	0.002	9.418	1.287-3.140
Capsule formation							
Yes	17	64.7	45.8				
No	138	60.1	30.5	0.251	NA	NA	NA
Tumor differentiation							
Well to moderately	118	61.9	33.3				
Poorly	37	56.8	29.5	0.785	NA	NA	NA
Vascular invasion							
Yes	35	51.4	22.2				
No	120	63.3	36.3	0.211	NA	NA	NA
Lymphatic metastasis							
Yes	32	28.1	3.1				

No	123	69.1	40.8	< 0.001	0.008	7.078	1.193-3.203
Extrahepatic metastasis							
Yes	10	20	< 0.01				
No	145	63.4	33.7	0.001	0.225	1.474	0.743-3.541
CK19 (<i>n</i> = 153) ¹							
Yes	139	59.7	30.5				
No	14	71.4	45.9	0.178	NA	NA	NA

¹Number of tumors. HBV: Hepatitis B virus; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: α -fetoprotein; ALP: Alkaline phosphatase; r-GT: R-glutamyltransferase; CA 19-9: Carbohydrate antigen 19-9; CK19: Cytokeratin 19; NA: Not available.

Table 3 Univariate and multivariate analyses of prognostic factors for disease-free survival rate of intrahepatic cholangiocarcinoma patients included in this study

Factor	<i>n</i>	Survival rate (%)		<i>P</i> value		Hazard ratio	95% CI
		1-yr	3-yr	Univariate analysis	Multivariate analysis		
Age (yr)							
> 65	20	45	25				
≤ 65	112	28.6	19.4	0.243	NA	NA	NA
Sex							
Female	43	34.9	20.7				
Male	89	29.2	20.1	0.679	NA	NA	NA
HBV infection							
Yes	71	39.4	23.5				
No	61	21.3	16.4	0.087	0.351	0.869	0.524-1.258
Cirrhosis							
Yes	39	25.6	25.6				
No	93	33.3	18	0.606	NA	NA	NA
Hepatolithiasis							
Yes	7	14.3	< 0.01				
No	135	32	21.4	0.041	0.130	2.291	0.807-5.299
Liver schistosomiasis							
Yes	6	< 0.01	< 0.01				
No	126	32.5	21.2	0.307	NA	NA	NA
r-GT							
≤ 64 U/L	59	44.1	28.3				
> 64 U/L	73	20.5	13.7	0.005	0.289	1.124	0.797-2.140
ALP							
≤ 119 U/L	79	41.8	27.7				
> 119 U/L	53	15.1	9.4	0.001	0.589	0.293	0.713-1.813
AFP							
≤ 20 μg/L	104	34.6	21.8				
> 20 μg/L	28	17.9	14.3	0.212	NA	NA	NA
CA19-9							
≤ 37 U/mL	55	40	27.3				
> 37 U/mL	77	24.7	15.3	0.048	0.720	0.129	0.707-1.652
Tumor number							
single	115	35.7	23.2				
multiple	17	< 0.01	< 0.01	< 0.001	0.011	6.515	1.194-3.863
Tumor size							
< 5 cm	48	39.6	24.4				
≥ 5 cm	84	26.2	17.7	0.135	NA	NA	NA
Tumor location							
Left lobe	43	37.2	20.9				
Right lobe	81	30.9	21.9				
Both lobes	8	< 0.01	< 0.01	0.105	NA	NA	NA
Microscopic satellite lesion							
Yes	34	11.8	8.8				
No	98	37.8	24.2	0.002	0.017	5.736	1.106-2.745
Histological inflammation							
Yes	34	47.3	20.6				
No	98	25.5	20.3	0.332	NA	NA	NA
Capsule formation							
Yes	14	42.9	21.4				
No	118	29.7	20.1	0.683	NA	NA	NA
Tumor differentiation							
Well to moderately	98	33.7	21.1				

Poorly	34	23.5	17.6	0.647	NA	NA	NA
Vascular invasion							
Yes	31	16.1	9.7				
No	101	35.6	23.5	0.005	0.025	5.030	1.072-2.812
Lymphatic metastasis							
Yes	109	4.3	< 0.01				
No	23	36.7	24.5	< 0.001	0.182	1.781	0.834-2.597
Extrahepatic metastasis							
Yes	8	< 0.01	< 0.01				
No	124	33.1	21.6	0.059	0.609	0.261	0.551-2.764
CK19 staining (<i>n</i> = 130) ¹							
Positive	13	27.4	17.9				
Negative	117	61.5	34.6	0.038	0.522	0.410	0.597-2.762

¹Number of available data. AFP: α -fetoprotein; ALP: Alkaline phosphatase; r-GT: R-glutamyltransferase; CA 19-9: Carbohydrate antigen 19-9; CK19: Cytokeratin19; NA: Not available.

tumor differentiation, vascular invasion, and CK19 were not significantly correlated with the overall survival rate of ICC patients after hepatic resection (Table 2).

Tumor recurrence occurred in 108 patients. The disease-free survival rate was 31.1% and 20.3%, respectively, for the ICC patients 1 and 3 years after operation with a median disease-free survival time of 5.0 mo (Figure 1). Univariate analysis showed that hepatolithiasis, r-GT > 64 U/L, ALP > 119 U/L, CA19-9 > 37 U/mL, multiple tumors, microscopic satellite lesion, vascular invasion, lymph node metastasis, and positive CK19 were the significant risk factors for tumor recurrence in ICC patients. Multivariate analysis demonstrated that the number of tumors (95.0% CI = 1.194-3.863), microscopic satellite lesion (95.0% CI = 1.106-2.745), and vascular invasion (95.0% CI = 1.072-2.812) were the independent prognostic factors for disease-free survival rate of ICC patients (Table 3). The most common tumor recurrence sites were the remnant liver and regional lymph nodes. The treatment modalities for recurrent tumors included repeated operation (*n* = 9), transplantation (*n* = 2), radiation therapy (*n* = 7), radiofrequency ablation (*n* = 9), microwave coagulation (*n* = 3), percutaneous ethanol injection therapy (*n* = 23), and transarterial chemoembolization (*n* = 93).

Prognostic factors for ICC patients according to their HBV infection

The clinicopathological characteristics of ICC patients with HBV infection and those without HBV infection were compared to further interpret the influence of chronic HBV infection on their survival rate. Univariate analysis showed that the following variables were significantly different between the patients with HBV infection and those without HBV infection, including gender, AST, AFP, CA19-9, inflammation of liver tissue, cirrhosis, hepatolithiasis, tumor capsule formation, tumor differentiation, lymphatic metastasis, and positive immunohistochemical staining of CK19. Although perineural infiltration was not significantly different between them, it occurred more frequently in ICC patients without HBV infection than in those with HBV infection (Table 4).

The potential prognostic factors affecting the survival rate of the patients with HBV infection and those without HBV infection were compared to further clarify the difference in prognostic factors affecting their survival rate. The

Table 4 Clinicopathological features of intrahepatic cholangiocarcinoma patients according to their hepatitis B virus infection

	HBV infection		<i>P</i> value
	Yes (<i>n</i> = 87)	No (<i>n</i> = 68)	
Gender (M/F)	64/23	38/30	0.021
Age (> 65 yr) (%)	17 (19.54)	13 (14.94)	0.763
Hepatolithiasis (%)	2 (2.30)	10 (14.71)	0.004
Hepatic schistosomiasis (%)	2 (2.30)	5 (7.35)	0.133
ALT (> 42 U/L) (%)	27 (34.03)	15 (22.06)	0.212
AST (> 37 U/L) (%)	38 (43.68)	15 (22.06)	0.005
TBIL (> 20 μ mol/L) (%)	20 (22.99)	16 (23.53)	0.937
r-GT (> 64 U/L) (%)	49 (56.32)	38 (55.88)	0.956
ALP (> 119 U/L) (%)	33 (37.93)	33 (48.53)	0.185
AFP (> 20 μ g/L) (%)	25 (28.74)	5 (7.35)	0.001
CA19-9 (> 37 U/mL) (%)	44 (50.57)	45 (66.18)	0.051
CA19-9 (> 200 U/mL) (%)	20 (22.99)	32 (47.06)	0.002
Tumor location (%)			0.520
Left lobe	28 (32.18)	27 (39.71)	
Right lobe	54 (62.07)	36 (52.94)	
Both lobes	5 (5.75)	5 (7.35)	
Tumor size			0.069
< 5 cm	38 (43.68)	20 (29.41)	
\geq 5 cm	49 (56.32)	48 (70.59)	
Tumor number (%)			0.958
Single	77 (88.51)	60 (88.24)	
Multiple	10 (11.49)	8 (11.76)	
Histological inflammation (%)	35 (40.23)	4 (11.76)	< 0.001
Cirrhosis (%)	40 (45.98)	5 (7.35)	< 0.001
Capsule formation (%)	15 (17.24)	2 (2.41)	0.005
Tumor differentiation (%)			0.036
Well	0 (< 0.01)	5 (7.35)	
Moderately	66 (75.86)	47 (69.12)	
Poorly	21 (24.14)	16 (23.53)	
Vascular invasion (%)	22 (25.29)	13 (19.12)	0.362
Perineural infiltration (%)	0 (< 0.01)	3 (4.41)	0.082
Microscopic satellite lesion (%)	23 (26.44)	16 (23.53)	0.679
Lymphatic metastasis (%)	12 (13.79)	20 (29.41)	0.017
Extrahepatic metastasis (%)	5 (5.75)	5 (7.35)	0.749
Immunohistochemical examinations			
CK18 positive staining (%) (<i>n</i> = 135) ¹	78 (89.66)	57 (86.36)	0.531
CK19 positive staining (%) (<i>n</i> = 139) ¹	75 (86.21)	64 (96.97)	0.022

¹Number of available data. HBV: Hepatitis B virus; M: Male; F: Female; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: α -fetoprotein; ALP: Alkaline phosphatase; r-GT: R-glutamyltransferase; CA19-9: Carbohydrate antigen 19-9; CK: Cytokeratin.

prognostic factors for the patients with HBV infection and

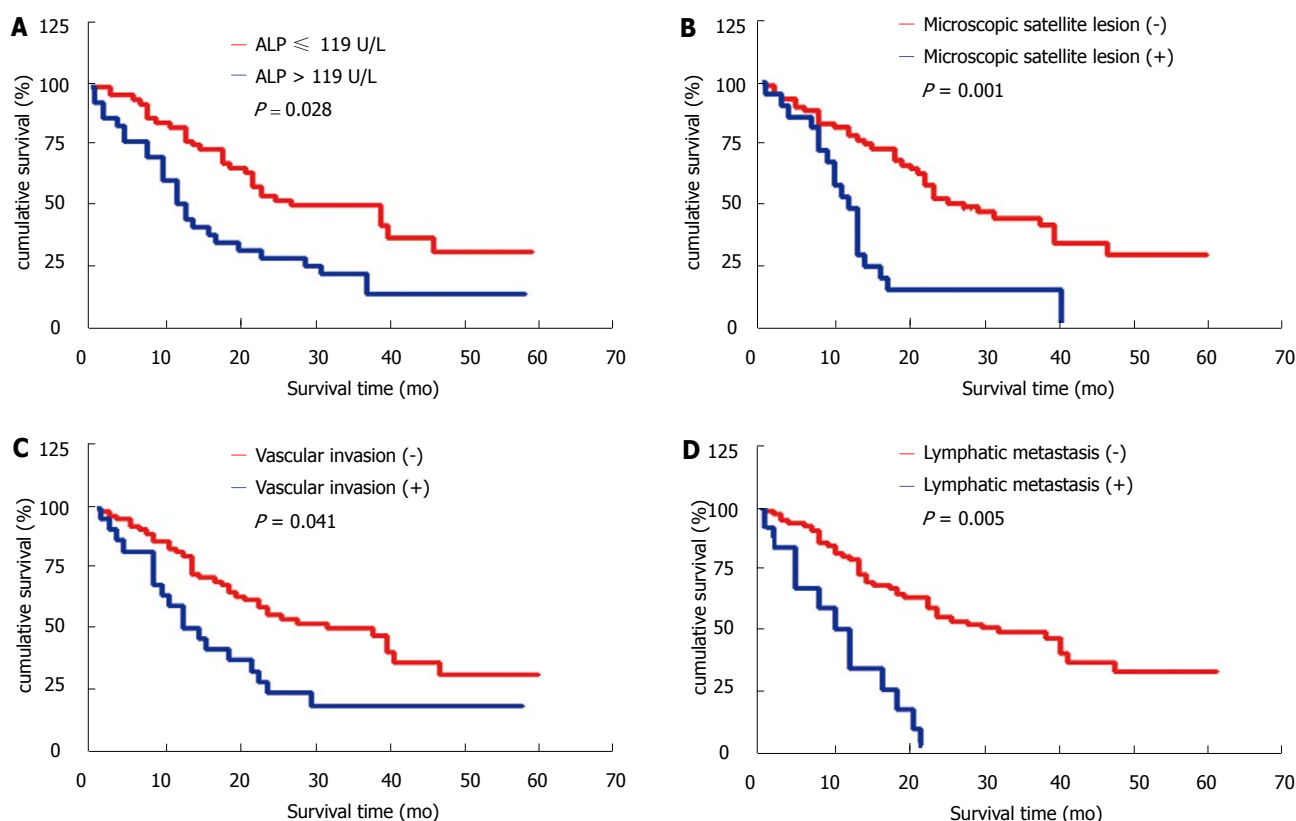


Figure 2 Adjusted survival curves according to the independent prognostic factors by multivariate analysis (Cox model) for intrahepatic cholangiocarcinoma with hepatitis B virus infection after resection. A: Alkaline phosphatase (ALP); B: Microscopic satellite lesion; C: Vascular invasion; D: Lymphatic metastasis.

those without HBV infection were not completely consistent (Tables 5 and Table 6). Univariate analysis demonstrated that ALP > 119 U/L, microscopic satellite lesion, tumor size ≥ 5 cm, lymphatic metastasis, and vascular invasion were the significant poor prognostic factors for the survival rate of patients with HBV infection (Table 5), while r-GT > 64 U/L, CA19-9 > 37 U/mL, hepatolithiasis, microscopic satellite lesion, lymphatic and extrahepatic metastasis were the significant poor prognostic factors for the survival rate of those without HBV infection (Table 6). Cox regression analysis demonstrated that ALP > 119 U/L (hazard ratio: 4.800), microscopic satellite lesion (hazard ratio: 12.066), lymphatic metastasis (hazard ratio: 7.887), and vascular invasion (hazard ratio: 4.167) were the independent poor prognostic factors for patients with HBV infection (Table 5, Figure 2), while r-GT > 64 U/L (hazard ratio: 4.157), microscopic satellite lesion (hazard ratio: 5.965), and poor tumor differentiation (hazard ratio: 5.844) were the independent poor prognostic factors for those without HBV infection (Table 6, Figure 3).

DISCUSSION

Although a number studies are available on the correlation between chronic HBV infection and ICC^[5-11], the impact of HBV infection on the survival rate of ICC patients remains unclear. In the present study, HBV infection was found to be a favorable prognostic factor for the patients with ICC after resection, thus ICC patients with HBV in-

fection should be distinguished from those without HBV infection. First, ICC patients with HBV infection and those without HBV infection are different in their clinicopathological characteristics. It was reported that the number of male ICC patients with HBV infection is more, with a younger age, a higher abnormal liver function and a higher serum AFP level, a worse histological inflammation and cirrhosis, a poorer tumor differentiation and encapsulation, a lower serum CA19-9 level, and a lower frequency of lymphatic metastasis and positive CK19 than those of ICC patients without HBV infection^[11]. Second, ICC patients with HBV infection have a more favorable outcome after surgical resection than those without HBV infection. Third, the prognostic factors for the survival rate of ICC patients with and those without HBV infection are different.

Surgical resection remains the curable procedure for ICC. However, the prognosis of ICC after surgery is poor because of its high recurrence rate. The median survival time of ICC patients after operation is 11.0-37.4 mo^[12-18]. The overall 1- and 3-year survival rates of ICC patients after operation are 46.3%-73.3% and 23.0%-55.0%, respectively^[14,15,19-23], which are consistent with the findings in our study. It was reported that the preoperative CA19-9 level, vascular invasion, perineural invasion, lymph node metastasis, intrahepatic metastasis, the number and differentiation of tumors are the significant prognostic factors for the overall survival rate of ICC patients^[13,15-19]. In the present study, several clinicopathological factors that significantly influence the survival rate of ICC patients and

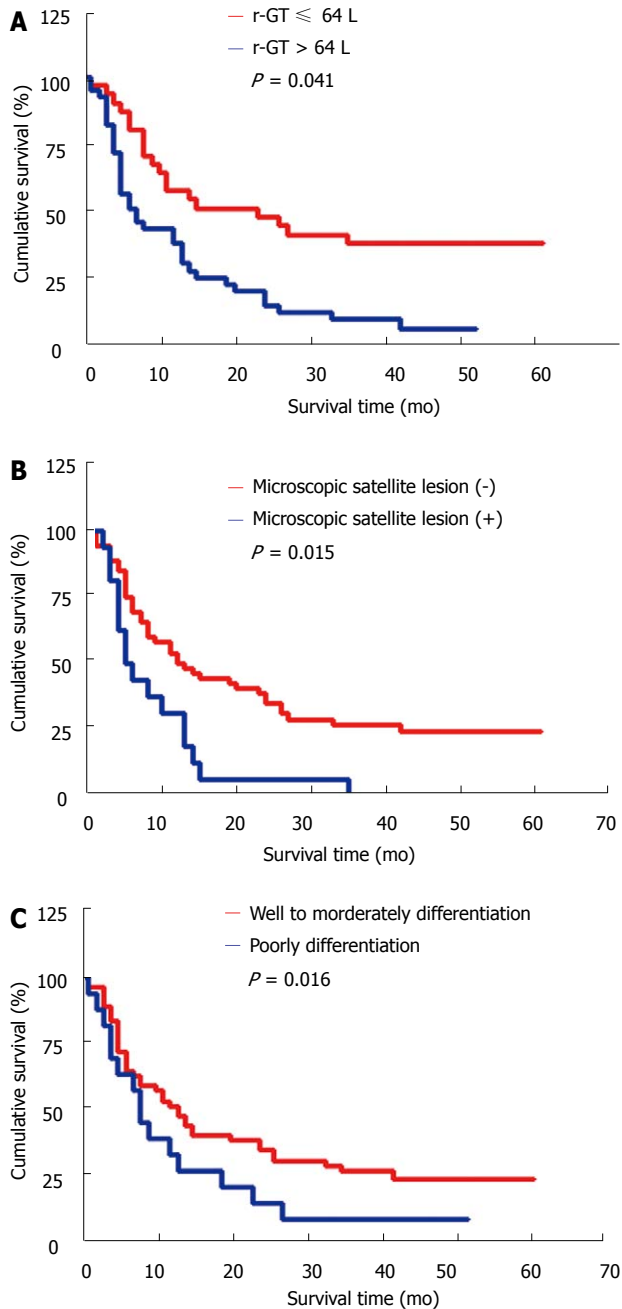


Figure 3 Adjusted survival curves according to the independent prognostic factors by multivariate analysis (Cox model) for intrahepatic cholangiocarcinoma without hepatitis B virus infection after resection. A: R-glutamyl-transferase (r-GT); B: Microscopic satellite lesion; C: Tumor differentiation.

tumor recurrence were investigated, and univariate analysis showed that absence of HBV infection, hepatolithiasis, high CA19-9 or ALP or r-GT level before operation, multiple tumors, tumor location, microscopic satellite lesion, lymphatic and extrahepatic metastasis, tumor size greater than 5 cm in diameter, were the significantly poor prognostic factors for the survival rate of ICC patients. Multivariate analysis revealed that the presence of HBV infection, hepatolithiasis, lymph node metastasis, microscopic satellite lesion were independent prognostic factor on survival. However, tumor differentiation, vascular invasion, tumor capsule formation were not found to be the

significant prognostic factors for the survival rate of ICC patients.

In Asia, intrahepatic duct stone (IHDS) is one of the factors highly related with ICC^[24]. Since Sanes and MacCallum^[25] reported two cases of hepatolithiasis-related cholangiocarcinoma discovered incidentally at autopsy for the first time in 1942, the correlation between IHDS with ICC has been reported in case series from all over the world^[4,5]. Our previous study also demonstrated that the incidence of IHDS is significantly higher in ICC patients than in non cancer patients (7.8% *vs* 1.1%), and IHDS is an independent risk factor for the development of ICC in Chinese (OR = 11.020, 95%CI = 4.238-28.657)^[11], which are consistent with the findings in the current study. IHDS was also found to be a negative prognostic factor affecting the survival rate of ICC patients in this study. Our explanation is that it is more difficult to diagnose early ICC with IHDS than to diagnose ICC without IHDS, and the recurrence of early ICC with IHDS is higher than that of ICC without IHDS.

It was reported that a high preoperative CA19-9 level (> 37 U/mL) greatly influences the overall survival rate of ICC patients after hepatic resection^[26]. It has been demonstrated that the preoperative CA19-9 level is an indication of ICC in patients without primary sclerosing cholangitis, and the serum CA19-9 level is related to tumor burden^[27]. In our study, the median survival time of ICC patients with their preoperative CA19-9 level ≤ 37 U/mL was significantly longer than that of those with their preoperative CA19-9 level > 37 U/mL (23 mo *vs* 13 mo).

It has been shown that lymph node metastasis is a significant factor for the poor prognosis of ICC patients^[12,14,15,17,21]. The presence of lymph node metastasis is correlated with other poor prognosis factors, such as gross type of tumor, poorly or undifferentiated tumor, vascular invasion, and perineural invasion. In the current study, multivariate analysis showed that lymph node metastasis was correlated with both the overall and disease-free survival rates of ICC patients. The median survival time of ICC patients without lymph node metastasis was significantly longer than that of those with lymph metastasis (23 mo *vs* 8 mo).

CK19 belongs to type 1 cytokeratin with a molecular weight of 40-56 kDa^[28] and is normally expressed in ductal epithelium (bile ducts, pancreas, and renal collecting tubules) and in mucosa of the gastrointestinal (GI) tract^[29]. Most adenocarcinomas of the GI tract including cholangiocarcinoma are CK19 positive^[30]. It was reported that CK19, as a prognostic marker, plays a role in the pathogenesis of papillary thyroid carcinoma^[31], hepatocellular carcinoma^[32-33] and colorectal adenocarcinoma^[34]. For example, CK19 expressing HCCs had a higher rate of recurrence (hazard ratio 12.5) after transplantation^[35]. It has been shown that the expression level of CK19 in HCC patients increases with a worse prognosis of HCC patients and a faster recurrence of it after surgical treatment^[36-39], indicating that CK19 is a useful prognostic marker for HCC. However, the role of CK19 as a prognostic marker in ICC has not been explored. In the current study, CK19 was expressed more frequently in ICC patients in the

Table 5 Univariate and multivariate analyses of prognostic factors for survival rate of intrahepatic cholangiocarcinoma patients with hepatitis B virus infection

Factor	n	HBV infection		P value		Hazard ratio	95% CI
		1-yr (%)	3-yr (%)	Univariate analysis	Multivariate analyses		
r-GT							
≤ 64 U/L	38	76.3	52.1				
> 64 U/L	49	69.4	34.1	0.223	NA	NA	NA
ALP							
≤ 119 U/L	54	85.2	53.2				
> 119 U/L	33	60.6	24.2	0.001	0.028	4.800	1.075-3.662
CA19-9							
≤ 37 U/mL	43	69.8	41.0				
> 37 U/mL	44	75.0	42.5	0.575	NA	NA	NA
Tumor number							
single	77	75.3	44.9				
multiple	10	50.0	20.0	0.066	0.058	3.599	0.972-5.587
Tumor size							
< 5 cm	38	86.8	52.0				
≥ 5 cm	49	61.2	33.7	0.049	0.655	0.199	0.436-1.686
Tumor location							
Left lobe	28	64.3	42.9				
Right lobe	54	79.6	45.5				
Both lobes	5	40.0	< 0.01	0.065	0.933	0.007	0.899-1.122
Microscopic satellite lesion							
Yes	23	52.2	21.7				
No	64	79.7	48.8	0.001	0.001	12.066	1.648-6.014
Capsule formation							
Yes	15	60.0	45.7				
No	72	75.0	41.0	0.979	NA	NA	NA
Tumor differentiation							
Well to moderately	66	71.2	40.3				
Poorly	21	76.2	47.1	0.354	NA	NA	NA
Vascular invasion							
Yes	22	50.0	17.0				
No	65	80.0	50.1	0.007	0.041	4.167	1.030-4.388
Lymphatic metastasis							
Yes	12	33.3	< 0.01				
No	75	78.7	48.5	< 0.001	0.005	7.887	1.408-6.848
Extrahepatic metastasis							
Yes	5	40.0	< 0.01				
No	82	74.4	43.1	0.099	0.857	0.032	0.251-3.154

NA: Not applicable; ALP: Alkaline phosphatase; r-GT: R-glutamyltransferase; CA 19-9: Carbohydrate antigen 19-9.

absence of HBV infection and the tumor disease-free survival rate of patients with CK19 expressing ICC was also lower after curative resection.

Recent studies showed that HBV infection is an important risk factor for the development of ICC^[5-11]. In the current study, 73 ICC patients (47.1%) were positive for serum HBsAg. Interestingly, 14 out of the 155 ICC patients were positive for HBsAg only in liver tissue, indicating that occult HBV infection is also a risk factor for the development of ICC as for HCC. In the current study, HBV infection was significantly correlated with some important clinicopathological factors, such as hepatolithiasis, high preoperative CA19-9 level, capsule formation, lymph node metastasis, perineural invasion, and positive CK19 (Table 4), which is consistent with the findings in our previous study^[11], indicating that the absence of HBV infection may be a predictor for the invasiveness of ICC and the poor survival rate of ICC patients. In the current study, the outcome of ICC patients with HBV infection was better than

that of those without HBV infection after curative resection. The median survival time of patients without HBV infection was significantly shorter than that of those with HBV infection (11 mo *vs* 23 mo), the prognostic factors affecting the survival rates of ICC patients with HBV infection and those without HBV infection were not completely consistent. Univariate analysis demonstrated that high preoperative ALP level, microscopic satellite lesion, tumor size greater than 5cm in diameter, lymph node metastasis, and vascular invasion were the poor prognostic factors for the survival rate of ICC patients with HBV infection (Table 5), while high preoperative r-GT or CA19-9 level, hepatolithiasis, microscopic satellite lesion, lymph node and extrahepatic metastasis were the poor prognostic factors for the survival rate of those without HBV infection (Table 6). Cox regression analysis demonstrated that high preoperative ALP level (hazard ratio: 4.800), microscopic satellite lesion (hazard ratio: 12.066), lymphatic metastasis (hazard ratio: 7.887), and vascular invasion (hazard ratio: 4.167)

Table 6 Univariate and multivariate analyses of prognostic factors for survival of intrahepatic cholangiocarcinoma patients without hepatitis B virus infection.

Factor	n	No HBV infection		P value		Hazard ratio	95% CI
		1-yr (%)	3-yr (%)	Univariate analysis	Multivariate analyses		
Hepatolithiasis							
Yes	10	10.0	< 0.01				
No	58	51.7	24.1	0.013	0.084	2.979	0.900-5.223
r-GT							
≤ 64 U/L	30	56.7	36.4				
> 64 U/L	38	36.8	7.9	0.001	0.041	4.157	1.035-5.614
ALP							
≤ 119 U/L	35	51.4	31.4				
> 119 U/L	33	39.4	9.1	0.017	0.407	0.688	0.316-1.595
CA19-9							
≤ 37 U/mL	23	60.9	43.5				
> 37 U/mL	45	37.8	8.9	0.001	0.173	1.858	0.806-3.311
Tumor number							
Single	60	48.3	23.2				
Multiple	8	25.0	< 0.01	0.050	0.392	0.734	0.609-3.540
Tumor size							
< 5 cm	20	50.0	24.0				
≥ 5 cm	48	43.8	18.8	0.308	NA	NA	NA
Tumor location							
Left lobe	27	40.7	21.6				
Right lobe	36	55.6	22.2				
Both lobes	5	< 0.01	< 0.01	0.137	NA	NA	NA
Microscopic satellite lesion							
Yes	16	31.2	< 0.01				
No	52	50.0	26.9	0.003	0.015	5.956	1.183-4.657
Tumor differentiation							
Well to moderately	52	50.0	24.9				
Poorly	16	31.2	6.2	0.076	0.016	5.844	1.172-4.569
Vascular invasion							
Yes	13	53.8	30.8				
No	55	43.6	18.0	0.459	NA	NA	NA
Lymphatic metastasis							
Yes	20	25.0	5.0				
No	48	54.2	26.9	0.015	0.430	0.624	0.683-2.452
Extrahepatic metastasis							
Yes	5	< 0.01	< 0.01				
No	63	49.2	22.1	< 0.001	0.065	3.403	0.933-9.940

NA: Not applicable; ALP: Alkaline phosphatase; r-GT: R-glutamyltransferase; CA 19-9: Carbohydrate antigen 19-9.

were the independent prognostic factors for ICC patients with HBV infection (Table 5), while high preoperative r-GT level (hazard ratio: 4.157), microscopic satellite lesion (hazard ratio: 5.965), and poor tumor differentiation (hazard ratio: 5.844) were the independent prognostic factors for those without HBV infection (Table 6), indicating that ICC patients with HBV infection should be distinguished from those without HBV infection.

It has been shown that vascular invasion or poor differentiation of tumor is a negative prognosis factor for ICC patients^[40], in this study, however, vascular invasion or differentiation of tumor was not a significant predictor for the overall survival rate of ICC patients. We hypothesize that compared ICC without HBV infection, ICC with HBV infection are associated with more vascular invasion and poor differentiation. While HBV infection is a favorable prognostic factor for survival, this may decrease the influence of vascular invasion or tumor differentiation on overall survival. Vascular invasion and poor differentiation were independent negative prognostic factors in ICC with HBV

infection and in ICC without HBV infection, respectively (Tables 5 and 6). The result may indirectly provide support for our hypothesis.

In conclusion, absence of HBV infection, hepatolithiasis, microscopic satellite lesion, and lymphatic metastasis are the independent predictors for a dismal prognosis of ICC patients. HBV infection is a valuable clinical factor for the invasiveness of tumor and the clinical outcome of ICC patients. ICC patients with HBV infection should be distinguished from those without HBV infection.

COMMENTS

Background

Although the correlation between chronic Hepatitis B virus (HBV) infection and intrahepatic cholangiocarcinoma (ICC) has been documented, the impact of HBV infection on the survival rate of ICC patients remains unclear.

Research frontiers

One hundred and fifty-five ICC patients who underwent macroscopic curative resections (R0 and R1) were classified according to their chronic HBV infection represented by positive hepatitis B surface antigen (HBsAg) in serum or in liver

tissue. The clinicopathological characteristics and survival rate of these patients were evaluated.

Innovations and breakthroughs

Multivariate analyses revealed that HBV infection, hepatolithiasis, microscopic satellite lesion, and lymphatic metastasis were the independent prognostic factors for the survival rate of ICC patients. The prognostic factors affecting the survival rate of ICC patients with HBV infection and those without HBV infection were not completely consistent. Alkaline phosphatase (ALP) > 119 U/L, microscopic satellite lesion, vascular invasion, and lymphatic metastasis were the poorer prognoses of ICC patients with HBV infection, and α -glutamyltransferase (α -GT) > 64 U/L, microscopic satellite lesion, and poor tumor differentiation were the poorer prognoses of ICC patients without HBV infection.

Applications

HBV infection in ICC patients is a valuable clinical factor for predicting the invasiveness of tumor and the clinical outcome of ICC patients. ICC patients with HBV infection should be distinguished from those without HBV infection because they have different clinicopathological characteristics, prognostic factors and favorable outcomes after surgical resection.

Terminology

ICC is a fatal cancer of the biliary epithelium, arising from the intrahepatic bile ducts. Globally, ICC is the most common primary hepatic malignancy, after hepatocellular carcinoma (HCC). The incidence of ICC varies greatly in different areas of the world, and is related to the distribution of risk factors. HBV or hepatitis C virus, primary sclerosing cholangitis, liver fluke infestation particularly the endemic *Opisthorcis viverrini*, and hepatolithiasis are the known risk factors for ICC.

Peer review

This study showed that HBV infection, hepatolithiasis, microscopic satellite lesion, and lymphatic metastasis were the independent prognostic factors for the survival rate of ICC patients. HBV infection was a valuable clinical factor for the invasiveness of tumor and the clinical outcome of ICC patients and ICC patients with HBV infection should be distinguished from those without HBV infection, thus providing certain accurate data for the diagnosis of ICC.

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Does deep sedation impact the results of 48 hours catheterless pH testing?

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Abstract

AIM: To study a cohort of patients undergoing 48 h Bravo pH testing receiving deep sedation with propofol.

METHODS: We retrospectively reviewed the charts of 197 patients (81 male, 116 female) who underwent Bravo esophageal pH monitoring from July 2003 to January 2008. All patients underwent Bravo pH probe placement *via* esophagogastroduodenoscopy (EGD) and received propofol for sedation. Patients on a proton pump inhibitor (89 patients) were excluded. Acid reflux variables measured included the total, upright, and supine fractions of time at pH < 4 and DeMeester score, and were compared between day 1 and day 2.

RESULTS: Of the 108 patients that were included in the study, the most common indication for Bravo pH monitoring was heartburn, with chest pain being the second most common. A signed rank test revealed no

statistically significant difference between day 1 and day 2 reflux episodes.

CONCLUSION: Patients who received propofol for sedation for EGD with Bravo pH capsule placement did not experience any significant difference in reflux episodes from day 1 to day 2.

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Key words: Esophagus; Bravo study; Propofol; pH capsule; Gastroesophageal reflux disease

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INTRODUCTION

Esophageal pH monitoring is widely used for the diagnosis of gastroesophageal reflux disease (GERD). Some indications for monitoring, as defined by expert panels, include refractory symptoms or continuation of atypical symptoms despite proton pump inhibitor (PPI) treatment, evaluation for anti-reflux surgery and recurrence of symptoms following anti-reflux surgery^[1-3].

Traditionally, the transnasal placement of a pH catheter probe was used to monitor esophageal pH. This technique often produced discomfort, inconvenience and often restricted activity and diet, thereby underestimating the amount of reflux^[4]. The limitations of the conventional catheter system (CCS) led to the development of a

catheter-less system (CLS) which utilizes a radiotelemetry pH-sensing capsule clipped to the esophageal wall. Compared to CCS, CLS is better tolerated by patients and permits increased duration of pH recording^[5].

Despite the wide use of the CLS, there have not been any standardized guidelines for the placement of the catheter. For example, the Bravo capsule may be clipped to the esophagus on the same day as esophagogastroduodenoscopy (EGD) with sedation or on a different day, with or without sedation.

Studies in the past on the CLS, have examined the differences in day 1 (d1) and day 2 (d2) with limited sample sizes and varied results. A few have suggested that a probable cause of increased reflux episodes on d1 may be the effect of sedation. Until now, there has been limited data examining the effect of deep sedation on d1 and d2 results.

MATERIALS AND METHODS

We retrospectively reviewed the charts of 197 patients who underwent 48-h Bravo pH monitoring (Medtronic) for suspected GERD over a 5-year period at Winthrop University Hospital. This study was approved by the Institutional Review Board of our institution. Eighty nine patients who were on anti-reflux medications at the time of the study were excluded.

All patients that were included in the study underwent EGD under deep sedation (propofol) with placement of the capsule at the time of the procedure. All patients underwent EGD with Bravo placement by the same endoscopist (KRK). The capsule was placed at 6 cm above the gastroesophageal junction. Following deployment of the capsule, accurate placement was confirmed with direct visualization. Following recovery from sedation, patients were instructed to keep the data recorder around their waist and were encouraged to resume their normal daily activities and usual diets. Patients were also instructed to keep a diary of daily activities (e.g. when they ate, periods of sleep), and occurrence of symptoms.

Patients returned 48 h later and turned in their receivers and diaries. The data was downloaded and analyzed by Medtronic software, which then generated a summary report, which was reviewed by one gastroenterologist (KRK). The summary report included percent of total time at pH < 4, percent of upright time at pH < 4, percent of supine time at pH < 4, total number of reflux episodes and the DeMeester score.

Statistical methods

All continuous variables were summarized using mean \pm SD. We calculated delta scores of our parameters, representing the difference from d1 to d2, i.e. to determine the effects of deep sedation. Since these difference scores were not normally distributed, we utilized the signed-rank test (instead of the paired *t*-test for normally distributed difference scores) to assess the statistical significance of these differences. All calculations were performed using SAS 9.1 for Windows (SAS Institute, Cary,

Table 1 Indication for Bravo monitoring

Indication	Frequency	Percent (of <i>n</i> = 108)
Heartburn	63	58.30%
Chest pain	32	29.60%
Chronic cough	9	8.30%
Laryngitis	23	21.30%
Bloating	38	35.20%

Total percentages exceed 100% since about half of the patients had two distinct indications.

NC, USA); results were considered significant when $P < 0.05$.

RESULTS

Our analysis included 108 patients (47 male, 61 female; mean age \pm SD, 54.74 \pm 14.67) after excluding 89 patients on anti-reflux medications. Table 1 shows the indications for Bravo pH monitoring. The most frequent indication was heartburn (58% of the patients) followed by chest pain. The total percentages in Table 1 exceed 100% since about half of the patients presented with two indications for the study.

Tables 2-5 indicate descriptive statistics for each of our parameters for d1, d2, and delta = d2-d1. None of the difference scores were significantly different from zero by the signed rank test, although one parameter (upright) showed a trend toward significance ($P = 0.0576$).

DISCUSSION

GERD is defined as symptoms or mucosal damage produced by the abnormal reflux of gastric contents into the esophagus^[3]. The typical clinical manifestations include heartburn and regurgitation; however an atypical presentation should be considered when chronic cough, non-cardiac chest pain, and otolaryngological (ENT) symptoms are present. It is appropriate to offer empiric therapy with a PPI to patients with symptoms consistent with GERD. However symptoms that are refractory to high-dose PPI would usually prompt the clinician to perform an endoscopy. The majority of symptomatic patients will have a normal endoscopy which does not necessarily indicate either less severe symptoms or a more easy to control form of GERD^[6].

Studies demonstrate that those GERD patients without esophagitis have symptoms that are just as difficult and at times more difficult to control^[7]. The diagnosis of GERD in the symptomatic patient without endoscopic findings requires prolonged ambulatory pH monitoring. This technique assesses the magnitude of esophageal acid exposure, and also allows one to assess whether a correlation exists between symptoms reported by the patient and acid reflux events^[8]. The only way to perform ambulatory pH monitoring, up until recently, has been by the transnasal placement of a pH catheter probe left in

Table 2 Time pH < 4 - upright (%)

	Mean	Median	SD	Minimum	Maximum
Day 1 (d1)	7.31	5.70	7.55	0	41.4
Day 2 (d2)	6.52	4.85	7.89	0	59.6
Delta = d2-d1	-0.79	-0.50	5.81	-19.9	24.8

Mean Delta (-0.79) not significantly different from zero ($P = 0.0576$) by signed rank test.

Table 4 Time pH < 4 - total (%)

	Mean	Median	SD	Minimum	Maximum
Day 1 (d1)	6.36	4.85	7.60	0.0	48.1
Day 2 (d2)	6.02	4.25	8.02	0.0	67.1
Delta = d2-d1	-0.34	0.00	6.13	-22.5	29.6

Mean Delta (-0.34) not significantly different from zero ($P = 0.73$) by signed rank test.

place for 24 h. Patients often alter their daily physical and dietary activities, or do not complete the study, as they find a transnasally placed pH electrode conspicuous and uncomfortable. These changes result in “false negative” results if the activity and/or dietary limitations reduce esophageal acid exposure to within the normal limits^[4].

The Bravo pH monitoring system is a newer modality in which a pH-sensing capsule is endoscopically clipped to the esophageal wall, and transmits pH data to a recorder worn by the patient. Bravo esophageal pH monitoring is better tolerated by patients and permits increased duration of pH recording compared to the traditional catheter-based pH system^[9].

Many studies have been performed to investigate if a difference exists for gastroesophageal reflux as measured by the Bravo pH system between d1 and d2 of the study. The results have been somewhat conflicting. In a study performed by Bhat *et al*^[10], 217 patients underwent endoscopy and capsule placement. Of these, 56% were abnormal with 32.2% being abnormal on both days, and showed increased acid exposure on d1 compared to d2. The higher likelihood of abnormal results for d1 was associated with a significantly increased esophageal acid exposure during the first 6 h after capsule insertion on d1 compared with the corresponding time on d2 without differences in esophageal acidification during the remaining time or differences in recorded activity. The significantly higher likelihood of abnormal findings during the initial period of pH monitoring suggests an influence of endoscopy and associated sedation, typically performed prior to capsule insertion, which needs to be considered when pH data are analyzed^[10]. Bechtold *et al*^[11] performed a retrospective review on 27 consecutive adult patients who underwent Bravo pH monitoring with intravenous doses of midazolam and fentanyl. Their results demonstrated that people with moderate conscious sedation experience significantly more acid reflux on d1 than d2.

Other studies performed have reported no consistent differences. Pandolfino *et al*^[12] found no differences

Table 3 Time pH < 4 - supine (%)

	Mean	Median	SD	Minimum	Maximum
Day 1 (d1)	4.01	0.25	8.94	0.0	60.2
Day 2 (d2)	4.66	0.00	12.20	0.0	87.4
Delta = d2-d1	0.65	0.00	11.80	-33.1	63.3

Mean Delta (0.65) not significantly different from zero ($P = 0.56$) by signed rank test.

Table 5 DeMeester score

	Mean	Median	SD	Minimum	Maximum
Day 1 (d1)	22.0	16.3	25.7	0.0	163.7
Day 2 (d2)	21.1	15.2	25.6	0.0	213.0
Delta = d2-d1	-0.9	0.0	21.0	-100.5	82.2

Mean Delta (-0.9) not significantly different from zero ($P = 0.90$) by signed rank test.

between d1 and d2 in their patients who underwent same day EGD with 50 to 75 mg of meperidine and 1 to 5 mg of midazolam. Prakash *et al*^[13] evaluated patients who underwent endoscopic placement of a wireless capsule with conscious sedation, employing a variety of different medications at the time of Bravo placement, and found no differences in reflux variables between d1 and d2.

The limitations of our study include the fact that it was retrospective and that a variety of different anesthesiologists administered the anesthesia. However, all Bravo placements and interpretations were performed by the same gastroenterologist.

In conclusion, there is great variability in different studies between the number of reflux episodes from d1 to d2. However, this was not demonstrated in our study when looking at patients undergoing deep sedation with wireless ambulatory esophageal pH monitoring, thereby supporting deep sedation as the optimal method for patient comfort and accuracy of data. Future prospective studies looking at larger sample sizes are needed to substantiate our findings.

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COMMENTS

Background

The prevalence of esophageal pH monitoring for the diagnosis of gastroesophageal reflux disease (GERD) has increased substantially. Despite its widespread use, there are no standard guidelines for the placement of the catheter.

Research frontiers

Some studies in the past have suggested that the effects of sedation used during placement of a 48 h catheter-less pH monitoring device may account for the difference in day 1 (d1) to day 2 (d2) data. In this study, the authors demonstrate that using deep sedation in the placement of a wireless pH monitoring device reduces the difference in d1 and d2 data.

Innovations and breakthroughs

Many studies have been performed to investigate if a difference exists for gastroesophageal reflux as measured by the Bravo pH system between d1 and d2 of the study. The results are conflicting. This study suggests that deep sedation may eliminate the variability in data from d1 to d2.

Applications

By using deep sedation during placement of a catheter-less pH monitoring device, the authors could limit the number of false positive results (i.e. acid reflux episodes) during d1.

Terminology

Bravo is a catheter-less pH monitoring device that is endoscopically clipped to the esophageal wall, and the data is transmitted to a recorder worn by the patient.

Peer review

This is an interesting study, delving into how to best use the Bravo capsule. Disadvantages include (as the authors state) that it is a retrospective study. Since there is no comparison group in the study either, it is difficult to generalize this study to other settings.

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Impact of remote ischemic preconditioning on wound healing in small bowel anastomoses

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Abstract

AIM: To investigate the influence of remote ischemic preconditioning (RIPC) on anastomotic integrity.

METHODS: Sixty male Wistar rats were randomized to six groups. The control group ($n = 10$) had an end-to-end ileal anastomosis without RIPC. The preconditioned groups ($n = 34$) varied in time of ischemia and time of reperfusion. One group received the amino acid L-arginine before constructing the anastomosis ($n = 9$). On postoperative day 4, the rats were re-laparotomized, and bursting pressure, hydroxyproline concentration, intra-abdominal adhesions, and a histological score concerning the mucosal ischemic injury were collected. The data are given as median (range).

RESULTS: On postoperative day 4, median bursting pressure was 124 mmHg (60-146 mmHg) in the control group. The experimental groups did not show a statistically significant difference ($P > 0.05$). Regarding the hydroxyproline concentration, we did not find any significant variation in the experimental groups. We detected significantly less mucosal injury in the RIPC groups. Furthermore, we assessed more extensive intra-abdominal adhesions in the preconditioned groups than in the control group.

CONCLUSION: RIPC directly before performing small bowel anastomosis does not affect anastomotic stability in the early period, as seen in ischemic preconditioning.

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Key words: Anastomotic healing; Hydroxyproline; Bursting pressure; Mucosal injury index; Wound healing; Remote ischemic preconditioning

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INTRODUCTION

Wound healing in intestinal anastomoses is dependent on operative technique, the underlying medical condition, medical treatment, and individual, often unknown

factors^[1,2]. Primarily, the stability of an intestinal anastomosis is regulated by the suture holding capacity, due to a high activity of collagenases that peaks in the first 48-72 h. Over time, the suture holding capacity decreases, whereas the tissue strength increases because of upregulated collagen synthesis and remodeling^[3]. Despite optimal treatment, anastomotic insufficiency occurs, which causes significantly higher morbidity and mortality^[4-6]. The underlying medical condition and individual genetic factors are different in each case. Accordingly, investigational interest is focused on fundamentals in gastrointestinal wound healing^[1,2] and on innovative perioperative management to improve anastomotic healing, especially in patients at risk.

Ischemic preconditioning (IPC) in general was first described by Murry in 1986, when a delay in cell death in the myocardium after preconditioning was described^[7]. Concerning IPC in the intestine, Hotter *et al.*^[8] have found an increased NO level, which was the first report of such a feature. Several local and systemic effects of IPC have been described, including decreased bacterial translocation^[9], mucosal injury^[9], epithelial apoptosis^[10], and on the other hand, an improvement in microvascular perfusion and oxygenation^[11,12] after ischemia-reperfusion injury (IRI). Our group previously has shown that IPC of the superior mesenteric artery improves stability of intestinal anastomoses^[13].

Contrary to IPC, remote ischemic preconditioning (RIPC) is induced by temporary occlusion of different arteries, which are not responsible for direct blood supply of the organ of interest. Less is known about the influence of RIPC on the gastrointestinal tract, and especially on its impact on anastomotic healing in the small intestine. RIPC was first reported in the literature in 1993 when Przyklenk *et al.*^[14] discussed the effect of temporary coronary occlusion on virgin myocardium not affected by the artificial ischemia. Subsequent studies have all suggested an increased tolerance to IRI in different tissue types^[15-18]. To the best of our knowledge, Colak *et al.*^[19] are the only group that has investigated the effect of RIPC on intestinal anastomosis. They have performed an anastomosis in the descending colon after branching off the superior mesenteric artery in an IR setting. No beneficial effect was found concerning IRI-induced delay of anastomotic healing. Neither in IPC nor RIPC are the optimal IR intervals defined.

The aim of the present study was to investigate the effect of RIPC, directly before anastomotic construction, in the early period (postoperative day 4) when suture holding capacity has already decreased and tissue strength has gained more importance. We intentionally did not perform an IRI interval because we claim that an anastomosis itself is a temporary ischemic injury. Moreover, we were interested in the implication of L-arginine, as an NO progenitor and semi-essential proteinogenic amino acid, on intestinal wound healing because of its reported importance in this process^[20,21].

MATERIALS AND METHODS

Subjects

The local Ethics Committee at the University of Freiburg

Table 1 Observations during the planned re-laparotomy on postoperative day 4

Group	n	Dehiscence	Abscess	Hemorrhage	Ileus	Expelled
Control	10	1	0	1	1	3
RIPC 5/20	9	1	1	0	0	1
RIPC 5/30	11	1	1	0	0	1
RIPC 10/20	14	0	0	0	0	0
RIPC 10/30	10	0	0	0	0	0
Arginine	9	0	1	0	0	1

RIPC: Remote ischemic preconditioning

approved all animal experiments. Male Wistar rats (Charles River, Sulzfeld) weighing 219-350 g were used for all experiments. The animals were housed two per cage, fed standard chow and given water *ad libitum*. Twelve hours before anesthesia, rats were fasting but had free access to water. Postoperatively, rats also had free access to water but were fed stepwise following a specified increasing amount of chow to prevent postoperative ileus.

Experimental design

Randomization (closed envelopes) took place after a pre-operative acclimatization under laboratory conditions of 5-7 d. Rats were assigned to one of the six groups. Each group consisted of at least nine (9-14) Wistar rats (Table 1): the RIPC 5/20 ($n = 9$) group was characterized by 5 min of intraoperative ischemia followed by 20 min of reperfusion, before constructing an anastomosis. This was compared with the RIPC 5/30 ($n = 11$), RIPC 10/20 ($n = 14$) and RIPC 10/30 ($n = 10$) groups. In the control group (CO) and the L-arginine group, the same anastomosis was created without preconditioning.

Operative procedure

Rats were operated upon by the same investigator under sterile laboratory conditions. After induction of anesthesia with isoflurane (4% isoflurane in 3 L/min oxygen) in an acrylic glass box, narcosis was maintained, after transferring the rats to an operating table, through a mask (1.5% isoflurane in 3 L/min oxygen). A 26 G silicon venous catheter was placed into the tail vein. A catheter was used for continuous infusion (9 mL/h per kg body weight) of an iso-osmolar electrolyte solution (Jonosteril; Fresenius, Bad Homburg, Germany: 137 mmol/L Na⁺, 4 mmol/L K⁺, 1.65 mmol/L Ca²⁺, 1.25 mmol/L Mg²⁺, 110 mmol/L Cl⁻, 18 mmol/L CHCOO⁻, pH 5.0-7.0; osmolality 291 mosm/L). After anesthesia was established, the abdominal coat was shaved and disinfected with polyvidone (Beta-isadonna; Mundipharma, Limburg, Germany). A 4-5-cm midline incision in the lower half of the abdomen was performed to accomplish optimal exposition.

In animals assigned to one of the four RIPC groups, the infrarenal aorta was prepared. Clamping off the infrarenal aorta directly above the bifurcation was achieved with an atraumatic microsurgical clamp (Medicon, Germany), following the different intervals of IR, as mentioned above. Intervals were similar to our previously published

paper on IPC. In the control group, the infrarenal aorta was prepared but not branched off. The arginine group received 200 µg/kg L-arginine (L-Arginin-Hydrochlorid; Fresenius) intraoperatively without preparing the abdominal aorta.

Afterwards, an approximately 1-cm segment, about 15 cm oral to the ileocecal valve was resected. Ileal continuity was restored by eight inverting interrupted sutures (Prolene 8/0; Ethicon, Germany). We used a silicon catheter (Heidelberger Verlängerung; Braun, Melsungen, Germany; diameter 5 mm) to standardize and simplify the suture technique. The silicon catheter was inserted into both lumina of the small intestine. At first, front-wall sutures were made, then we turned around the anastomosis to perform the back-wall sutures, removing the catheter just before the last two sutures. The distance between the single sutures and the stitches to the resection margin was 1-2 mm. The abdominal cavity was closed using a two-layer technique: musculo-peritoneal layer (Monocryl 4/0 SHplus; Ethicon), and fasciocutaneous layer (Vicryl 4/0 SHplus; Ethicon).

On postoperative day 4, the rats were re-laparotomized and sacrificed by cardiac puncture with injection of a lethal dose of potassium. The abdomen was opened by a complete midline incision combined with a transverse incision to gain maximum exposure. Careful exploration was carried out to look for signs of inflammation, adhesions, anastomotic insufficiency, and abscesses. Without dissecting the directly adjacent tissue around the anastomosis, an approximately 4-6-cm segment bearing the anastomosis was harvested for further analysis.

Bursting pressure

The bowel segment was water-tight and connected to an infusion pump (Perfusor fm; Braun) filled with iso-osmolar saline solution (0.9% NaCl; Braun) *via* a 14 G silicon catheter (Vasofix Safety; Braun) and to a digital pressure transducer (Codman ICP Express; Ethicon). Intraluminal pressure was increased by an infusion rate of 60 mL/h. Monitoring included the bursting pressure recorded just before sudden loss of tension and the site of rupture (mesenterial *vs* anti-mesenterial). Subsequently, the bowel wall was released from all adhering tissue and the complete suture line was excised within a total length of the bowel of 1 cm. The anastomosis was opened at the mesenterial site and gently washed using a saline solution (0.9% NaCl, Braun). The anastomosis was divided at the anti-mesenterial site into two parts of the same length for paraffin embedding and measurement of hydroxyproline concentration. The former was achieved by immediately fixing the specimen in 4% phosphate-buffered formaldehyde (pH 7.3), and the latter by preservation of the anastomotic strip in an Eppendorf tube at -80°C until spectrophotometric measurement.

Hydroxyproline concentration

The specimens used for hydroxyproline concentration measurement were desiccated in an oven (Heraeus Elec-

Table 2 Mucosal injury scale from Chiu *et al*^[23]

Grade	Definition
0	Normal mucosal villi
1	Development of subepithelial Gruenhagen's space at the apex of the villus, often with capillary congestion
2	Extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria
3	Massive epithelial lifting down the sides of villi, possibly with few denuded tips
4	Denuded villi with lamina propria and dilated capillaries exposed, possibly with increased cellularity of lamina propria
5	Digestion and disintegration of the lamina propria, hemorrhage and ulceration

tronic UT5042EK, Germany) until a constant dry weight was achieved. Hydroxyproline concentration was determined by using the Chloramine-T spectrophotometric method as previously described by Reddy *et al*^[22]. The practice is based on alkaline hydrolysis of the tissue homogenate and the consecutive measurement of free hydroxyproline. Before measurement, Chloramine-T was used to oxidize the free hydroxyproline in a pyrrole. Adding Ehrlich's reagent resulted in a chromophore that could be recorded at 550 nm. Data finally were calculated to express the results as micrograms of hydroxyproline per gram dry weight of tissue.

Histological evaluation

After fixation in formalin and embedding in paraffin, histological sections were stained with hematoxylin-eosin. Mucosal injury, inflammation and hyperemia/hemorrhage were assessed and graded in a blinded manner by two pathologists. Pathologists used the injury scale (Table 2) as described by Chiu *et al*^[23].

Statistical analysis

All data are expressed as median and range. Overall significance was proved using the Kruskal-Wallis test. Subsequent comparison of subgroups was done using the Mann-Whitney *U* test; *P* < 0.05 was assumed to be significant. SPSS for Windows version 14.0.2 (Chicago, IL, USA) was used.

RESULTS

General observations

One rat died intraoperatively in the RIPC 10/20 group because of acute mesenterial bleeding. One anastomosis in the RIPC 10/20 group had to be redone because of a technical issue. Two rats in the RIPC group died on postoperative day 3: one had advanced peritonitis caused by bacterial translocation in ileus without anastomotic dehiscence; and the other had ileus without macroscopic signs of peritonitis.

The findings during planned re-laparotomy on postoperative day 4 are summarized in Table 1. There were three

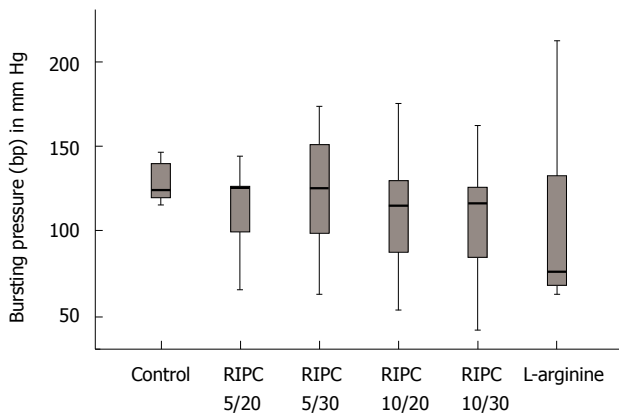


Figure 1 Bursting pressure after different remote ischemic preconditioning settings and after arginine application expressed as box plot. There was no significant overall difference between the groups ($P > 0.05$). RIPC: Remote ischemic preconditioning.

insufficient anastomoses. Further observations were ileus, mesenteric hemorrhage, and three abscesses. Two of the three abscesses were adjacent to the anastomosis without macroscopic signs of insufficiency, whereas one was distant from the anastomosis. These nine rats were excluded from further examination (hydroxyproline concentration, bursting pressure and histology). We observed that intra-abdominal adhesions, especially near the anastomosis, were more pronounced in the RIPC groups than in the control or the arginine group.

Bursting pressure

Control vs RIPC groups: During bursting pressure measurement, the site of rupture was equally allocated to both the mesenteric and anti-mesenteric site along the suture line, and never occurred distant to the anastomosis. Median bursting pressure in the control group was 124 mm Hg (60-146 mmHg). Similar bursting pressure ($P > 0.05$) was documented in all of the RIPC groups: 125 mmHg (65-144 mmHg) in the RIPC 5/20 group; 125 mmHg (65-144 mmHg) in the RIPC 5/30 group; 130 mmHg (52-175 mmHg) in the RIPC 10/20; and 117 mmHg (41-162 mmHg) in the RIPC 10/30 group (Figure 1).

Control/RIPC vs arginine: The arginine group showed a median bursting pressure of 90 mmHg (65-212 mmHg) and was not different to the control and RIPC groups (Figure 1).

Hydroxyproline levels

Hydroxyproline was measured to establish a relationship with the collagen content in the anastomotic region. Median hydroxyproline concentration in the control group was 86 $\mu\text{g/g}$ dry weight (36-160 $\mu\text{g/g}$). Hydroxyproline concentration in the RIPC groups was not significantly different from the control group ($P > 0.05$): 58 $\mu\text{g/g}$ (30-158 $\mu\text{g/g}$) in the RIPC 5/20 group; 78 $\mu\text{g/g}$ (19-227 $\mu\text{g/g}$) in the RIPC 5/30 group; 99 $\mu\text{g/g}$ (40-250 $\mu\text{g/g}$) in the RIPC 10/20 group; and 108 $\mu\text{g/g}$ (57-198 $\mu\text{g/g}$) in

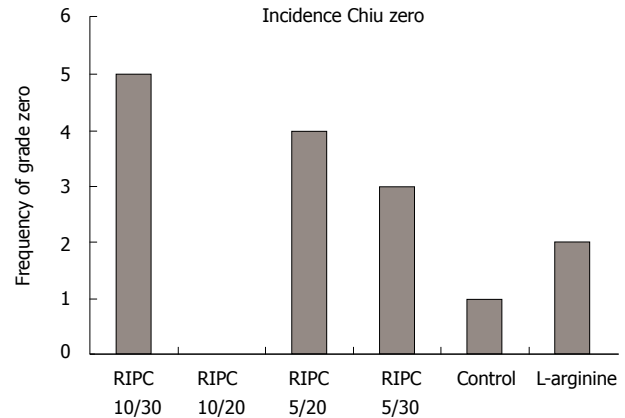


Figure 2 The histological mucosal damage score by Chiu^[23] showed significantly more low-grade alterations in the remote ischemic preconditioning groups (Remote ischemic preconditioning, 27% vs control, 10%). RIPC: Remote ischemic preconditioning.

the RIPC 10/30 group. The arginine group also showed no significant disparity: 78 $\mu\text{g/g}$ (21-130 $\mu\text{g/g}$).

Histological examination

The above described histological score for mucosal damage according to Chiu *et al.*^[23] was applied to evaluate the structural damage due to the creation of the anastomosis. Different grades of damage (0-3) were seen in all groups at the suture line. Maximum damage was seen up to grade 3, which means massive epithelial lifting down the sides of the villi, with possibly a few denuded tips. Mucosal damage was observed in the first few villi on both sides of the anastomotic line. Twelve rats (27%) in the RIPC groups did not show any mucosal damage (grade 0); and five in the RIPC 10/30 group, four in the RIPC 5/20 group, and three in the RIPC 5/30 group. The control group had only one rat (10%) with grade 0 damage (Figure 2).

DISCUSSION

Our research group has focused on fundamental aspects of gastrointestinal wound healing^[13,24,25], because anastomotic insufficiency still occurs and results in high morbidity and mortality^[4-6,26,27]. We previously have presented data on the influence of IPC on the stability of small intestine anastomoses. IPC has a beneficial effect on wound healing in the gastrointestinal tract^[13]. In the present study, we are the first to present experimental data on the effect of RIPC of the infrarenal aorta on anastomotic healing in the small bowel, with RIPC being performed directly before anastomotic construction.

RIPC has been shown to have consistently favorable effects in several other organs, such as heart, lungs, brain and kidney. Mostly, and contrary to our data, the effect has been measured by a reduction in IRI^[15-18,28-31]. However, in most publications, the best practice intervals for IR in IPC and RIPC are not clear. It may be that there are differences according to which organ is being studied. Colak *et al.* have performed left colonic anastomoses after temporary clo-

sure of the superior mesenteric artery (SMA). Their experimental setting consisted of four groups: a control group without preconditioning and without IRI, a remote IRI group in which the anastomosis was executed after a temporary closure of the SMA for 40 min; a preconditioned IRI group with two cycles of preconditioning (5 min closure of SMA) before IRI; and a preconditioned group without IRI. IRI significantly impaired anastomotic healing in terms of a decreased bursting pressure in the non-preconditioned and preconditioned groups. Furthermore, they observed the lowest bursting pressure in the preconditioned group without IRI on postoperative day 7^[19]. The data are contrary to our findings because we worked out a non-inferiority of RIPC *vs* the control group. It could be that re-laparotomy on postoperative day 7, and the fact that they performed colonic anastomoses, are responsible for their findings, but on the other hand, there also could have been a delayed negative effect of remote preconditioning in general. Colonic anastomoses lose 70% of their initial strength and approach 75% of normal strength at 4 mo, whereas small intestine anastomoses primarily lose less strength and reach their original state at 4 wk^[32].

In our current experimental model, we could not demonstrate a superiority of RIPC over non-preconditioned rats, as expressed by bursting pressure and hydroxyproline concentration. Also, in our non-remote preconditioned model, hydroxyproline levels were not significantly higher than in the control group, whereas the bursting pressure was. In the literature, a positive but also a negative correlation between anastomotic stability and hydroxyproline concentration has been reported^[33,34]. Two of the studies have demonstrated the influence of pentoxifylline^[35] and doxycycline^[33] on intestinal anastomoses, thus indicating further factors that are involved in early anastomotic healing (e.g. other extracellular matrix molecules). Ahrendt *et al* have subdivided the total protein content of a tissue sample bearing an anastomosis into collagenase-digestible protein (CDP) and non-collagenous protein^[36]. Although they have primarily focused on the impact of sepsis on collagen synthesis, an alteration of non-collagenous protein synthesis after construction of an anastomosis is ascertainable^[36]. On the other hand, hydroxyproline concentration measurements do not distinguish between collagen subtypes (especially types I and III), which are known to influence wound stability in subject to their ratio^[36-41]. IPC may result in a higher collagen type I / III ratio or in a higher degree of crosslinking. Moreover, hydroxyproline measurement just reflects the total amount of collagen, but does not discern between pre-existing structural collagen and newly synthesized collagen^[36]. We suggest that anastomotic stability is not strictly correlated to the amount of collagen, but rather to the quality of collagen and other extracellular proteins that have not yet been identified to have an influence on anastomotic healing.

The physiological process of anastomotic healing is potentially disturbed by hypoperfusion, tension, hypovolemia, infection, drugs, malnutrition and immunodeficiency^[42-48].

Tension resulting in hypoperfusion, or hypoperfusion itself at the region of the anastomosis, primarily has to be avoided by the surgeon *via* adequate mobilization of the oral and aboral segment, and foresighted transection to maintain sufficient perfusion. Jönsson *et al*^[3] have shown that collagen synthesis is dependent on tissue oxygenation, thus indicating disturbed anastomotic healing in cases of insufficient blood supply at the site of transection. Moreover, collagenase activity simultaneously is elevated in hypoxia^[43]. Against the background of the above-mentioned facts, any improvements in (micro)circulation could be beneficial to gastrointestinal wound healing in general. Although we did not find an impact on anastomotic stability in our RIPC model, the trend towards a lower grade of mucosal injury could be shown.

Even if significant improvements in bursting pressure and/or hydroxyproline concentration are not visible on postoperative day 4, a beneficial effect in the later phase cannot be excluded. The effects of preconditioning in general are subdivided into an early and a delayed response. Furthermore, subdivision into a humoral, neural and systemic pathway has been described previously^[15,49]. The early response leads to a release of trigger substances such as adenosine, bradykinin, norepinephrine, endocannabinoids, calcitonin gene-related peptide (CGRP), and opioids at the site of ischemia. Via membrane bound receptors and subsequent intracellular signaling, rapid protection in terms of avoiding necrosis and apoptosis is induced locally. Early protection lasts for 2-3 h but reappears approximately 24 h after preconditioning, and is dependent on *de novo* synthesis of molecules such as inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and heat shock proteins (HSPs)^[15]. In RIPC, the early local protection is lacking because the site of preconditioning is distant to the region of interest. After the washout (reperfusion), mediators in an RIPC setup also reach the site of the anastomosis/IRI, but this secondary local effect seems to be attenuated compared with an IPC setting^[50]. The systemic effect of RIPC resulting in transcription of anti-apoptotic and anti-inflammatory proteins seems to be similar to IPC^[29,51]. Considering the attenuated local effect, it may be speculated that a positive effect of RIPC on intestinal wound healing becomes evident in a later phase due to newly synthesized molecules.

In our study, one group received 200 µg/kg L-arginine to test whether increased availability of an NO progenitor (L-arginine) and/or proteinogenic amino acid influenced the anastomotic stability. Compared to promising results after oral application^[20], we did not find any positive results compared to the control group. Whether a dose-dependent effect of arginine on anastomotic healing plays an important role remains to be proven. Thornton and colleagues have pointed out a possible dose-dependent effect of NO on collagen synthesis in sepsis^[52]. Stechmiller *et al*^[20] have summed up that NO is of paramount importance in the early phase (inflammation stage) of wound healing, but disturbs it in the pro-

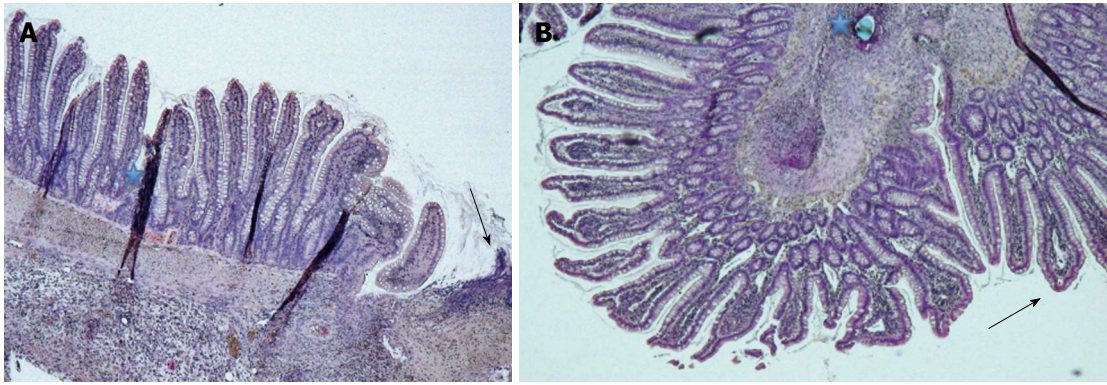


Figure 3 Histological view. A: Chiu grade 0 with intact epithelium and a few villi along one side of the anastomosis (HE staining; magnification 5 ×), Region of the anastomosis (arrow) and staining artefacts (*); B: Chiu grade 2 with a pronounced subepithelial space in the villi next to the inverted anastomosis (arrow) and a suture hole (*) (HE staining; magnification 5 ×).

liferation phase^[53]. Infusion of a substrate regarding the applied dose therefore is not as productive as mimicking preconditioning *via* direct mediators (pharmacological preconditioning)^[54]. In general, mimicking the effect of (remote) ischemic preconditioning could be promising because it could easily be implicated in clinical routine, and could avoid any harm to the site of preconditioning (e.g. vascular dissection in preconditioning)^[54].

The partly massive adhesions around the anastomosis, but also the whole abdominal cavity in the RIPC groups, are highly relevant for surgeons. Preparation on postoperative day 4 was significantly more difficult and technically demanding in the RIPC groups. There were two rats in the RIPC 10/20 group that died due to ileus. Although adhesions are not constant, re-laparotomy on postoperative day 4 could be problematic. Concerning these adhesions, re-laparotomy in the later phase (e.g. postoperative day 7, as mentioned above) in our experimental setting could shed light on the dynamics of these adhesions. Otherwise, it is known that the primary sealing of the anastomosis emanates from the serosal layer, therefore, the adhesions could imply better primary sealing of the anastomosis.

The most interesting issue is the tendency to a lower degree of mucosal injury in the RIPC groups. This could in part be a mark of improved microcirculation, at least at the mucosal level. Additionally, the mucosal injury index of Chiu^[23] (Table 2) is definitely affected by the stitching technique during surgery. Only strictly extramucosal stitches avoid partial mucosal necrosis; transmural stitches would have resulted in a higher degree of mucosal injury. In our highly standardized anastomotic technique, we paid attention to a strictly correct suture technique in a single surgical design. Figure 3 shows a sample of two different Chiu grades. Early or improved mucosal repair is important for the formation of an antimicrobial barrier, and furthermore, for untroubled anastomotic healing at a submucosal level. Mucosal integrity itself is definitely not responsible for anastomotic stability, but aseptic milieu at the submucosa accelerates wound healing and avoids disturbance caused by inflammation due to infection. This

could be useful for patients at risk (e.g. genetic alteration of collagen synthesis) in whom collagen synthesis and/or remodeling takes longer, or for patients suffering from an immunodeficiency who are susceptible to infection.

Given the fact that oxygenation at the site of anastomosis is of paramount importance, angiogenesis and especially drugs that boost or restrict the expression of pro-angiogenic proteins [e.g. vascular endothelial growth factor; (VEGF)] are of note. Ishii *et al.*^[55] have demonstrated an increased bursting pressure and hydroxyproline content on postoperative day 4 in colonic anastomosis. Another interesting approach is VEGF gene therapy, as reported by Enestvedt *et al.*^[56]. After esophagogastrectomy and gastric tube formation, a VEGF plasmid vector is injected at the site of anastomosis. Subjects treated with VEGF transfection resulted in an increased bursting pressure and neovascularization^[56]. They assumed a strong correlation between the number of microvessels and bursting pressure, which supports the enormous importance of oxygenation in anastomotic healing. In contrast, anti-angiogenic agents impair anastomotic integrity if applied shortly before surgery. It may be that (R)IPC can prevent the negative effect of agents such as bevacizumab^[57]. Pro-angiogenic agents could improve gastrointestinal wound healing in the absence of anti-angiogenic drugs, especially in patients with life-time steroid and/or combined immunosuppressive therapy.

The informative value of the current study could be influenced by the small groups and the wide data range, but the data are equally distributed within the range of each group. Therefore, it remains to be proven if larger groups would have led to distinctive findings. Moreover, the group size calculation was based on a presumed high difference in primary outcome measure between the groups indicating a potentially high clinical relevance. As mentioned above, we sacrificed the rats on postoperative day 4 to investigate early anastomotic healing and to match the results with our IPC study. Before postoperative day 4, anastomotic stability is more influenced by suture holding capacity than by tissue regeneration due to wound healing. Postoperative day 4 is a vulnerable phase in anastomotic healing because

suture holding capacity has already decreased and collagen synthesis increases and just overcomes collagenolysis. In fact, increasing the stability of anastomoses on postoperative day 4 would be of great clinical relevance. Given the fact that RIPC results in a commensurate effect, we cannot exclude a positive impact in a later phase of anastomotic healing. Optimum intervals of ischemia and reperfusion are not exactly established, therefore, we adopted the intervals from our previous study. It is not clear whether these are suitable or whether ideal intervals in RIPC differ from those in IPC, and moreover in different effector organs. Our current experiment is a highly mechanistic model, and it is not known whether it can be transferred into clinical routine, but it is easy to repeat and it is one small piece of experimental research on the fundamentals of gastrointestinal wound healing.

In conclusion, RIPC of the infrarenal aorta does not seem to have an influence on anastomotic stability, as was shown for IPC of the SMA. However, according to our previous results with IPC, mucosal injury seems to be less in the RIPC groups, thus indicating improved mucosal microcirculation at the anastomotic region. The observed relevant intra-abdominal adhesions around the anastomosis support the presumption of a gaugeable effect of RIPC on intestinal anastomoses. Further studies, especially in respect to the mucosal and submucosal microcirculation at the anastomotic region, will be of interest to verify the potential implementation of RIPC in intestinal wound healing.

ACKNOWLEDGMENTS

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COMMENTS

Background

Wound healing is a widely researched topic. Many experimental studies of wound healing, especially in the skin, have been published. The healing of an intestinal anastomosis in phases is similar to wound healing in the skin. The dehiscence of an anastomosis results in higher morbidity and mortality. To avoid anastomotic dehiscence, the best surgical technique is a prerequisite. Even though an optimal surgical technique for dehiscence is used, it is necessary to research the fundamentals of gastrointestinal wound healing to improve and innovate perioperative management for lowering the rate of anastomotic dehiscence.

Research frontiers

To study the fundamentals of gastrointestinal wound healing, ischemic preconditioning (IPC) and remote ischemic preconditioning (RIPC) were used. Other studies have investigated the effect of different volume regimens perioperatively, and the application of additive medication before or after installation of an anastomosis.

Innovations and breakthroughs

The Surgical Metabolic and Anastomotic Research Team has shown that IPC improves the stability of small-intestinal anastomoses. Other researchers who have used RIPC have not found an advantage in colonic anastomoses. Examination of mucosal injury in anastomoses is an innovation but its consequences are not clear.

Applications

Preconditioning in general could probably be transferred to clinical routine although there are risks, but RIPC has shown no benefit in terms of increasing anastomotic stability. The reduction of mucosal injury in this setting should be

confirmed by *in vivo* microscopy.

Terminology

In preconditioning, ischemia is induced by temporarily branching off an artery, followed by an interval of reperfusion in order to minimize the IR injury of subsequent prolonged ischemia. The ischemia following the preconditioning in our setting is the creation of an anastomosis. In (direct) IPC, the branched off artery supplies the region of interest, and in that case, it would be the later constructed anastomosis. In RIPC, the clamped artery does not supply the region of interest but other organs/regions.

Peer review

In this experimental work, the authors examined the effect of RIPC on small-intestinal anastomosis healing and the paper is interesting.

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Vigorous, but differential mononuclear cell response of cirrhotic patients to bacterial ligands

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Peripheral blood mononuclear cells were isolated and exposed to lipopolysaccharide or lipoteichoic acid. CD14, Toll-like receptor 2 and 4 expression was determined by flow cytometry, and tumor necrosis factor (TNF) α , interleukin (IL)-1 β , IL-6, IL-12 and IL-10 secretion in supernatants was determined by ELISA.

RESULTS: Higher CD14, Toll-like receptor 2 and 4 expression was observed in peripheral blood mononuclear cells from cirrhotic patients, ($P < 0.01$, $P < 0.006$, $P < 0.111$) respectively. Lipopolysaccharide and lipoteichoic acid induced a further increase in CD14 expression ($P < 0.111$ lipopolysaccharide, $P < 0.013$ lipoteichoic acid), and a decrease in Toll-like receptor 2 ($P < 0.008$ lipopolysaccharide, $P < 0.008$ lipoteichoic acid) and Toll-like receptor 4 ($P < 0.008$ lipopolysaccharide, $P < 0.028$ lipoteichoic acid) expression. With the exception of TNF α , absolute cytokine secretion of peripheral blood mononuclear cells was lower in cirrhotic patients under non-exposure conditions ($P < 0.070$ IL-6, $P < 0.009$ IL-1 β , $P < 0.022$ IL-12). Once exposed to lipopolysaccharide or lipoteichoic acid, absolute cytokine secretion of peripheral blood mononuclear cells was similar in cirrhotic and non-cirrhotic patients, determining a more vigorous response in the former ($P < 0.005$ TNF α , IL-1 β , IL-6, IL-2 and IL-10 lipopolysaccharide; $P < 0.037$ TNF α ; $P < 0.006$ IL-1 β ; $P < 0.005$ IL-6; $P < 0.007$ IL-12; $P < 0.014$ IL-10 lipoteichoic acid). Response of peripheral blood mononuclear cells was more intense after lipopolysaccharide than after lipoteichoic acid exposure.

CONCLUSION: Peripheral blood mononuclear cells of cirrhotic patients are able to respond to a sudden bacterial ligand exposure, particularly lipopolysaccharide, suggesting that immune regulation mechanisms are still present.

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Abstract

AIM: To study the role of gram-positive and gram-negative bacteria in the pathogenesis of liver injury, specifically the activation of inflammatory mediators.

METHODS: Peripheral blood mononuclear cells of 20 out-patients were studied, 10 of them with cirrhosis.

Key words: Liver cirrhosis; Toll-like receptors; Cytokines; Lipopolysaccharide; Lipoteichoic acid

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INTRODUCTION

Patients with cirrhosis frequently present with intestinal bacterial overgrowth of both gram-negative and gram-positive bacteria. Coexisting increased intestinal permeability facilitates bacterial translocation into the portal vein^[1]. The resulting bacteremia and endotoxemia can not be efficiently cleared by the injured liver^[2], leading to a rise of systemic proinflammatory cytokines^[3]. This is thought to aggravate the underlying liver damage. The role of gram-negative bacteria in the pathogenesis of liver injury has been extensively studied. As to gram-positive bacteria, a similar deleterious role has been proposed^[4], but still remains to be proven.

It is known that bacterial cell wall products, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan (PGN) fragments, trigger monocyte expression of many inflammatory cytokines. LPS, also known as endotoxin, a major constituent of the outer membrane of gram-negative bacteria, elicits an immune reaction which is responsible for many of the harmful effects seen in septic shock patients. LPS binds to the LPS-binding protein (LBP), a member of a binding and transport protein family. It requires either mCD14 or sCD14 receptors to be transferred to the toll-like receptor 4 (TLR4), a transmembrane signaling receptor, and translocated into the hydrophobic pocket of myeloid differentiation factor-2 (MD-2)^[5]. This signaling pathway activates a variety of transcription factors such as nuclear factor (NF)- κ B (p50/p65) and AP-1 (c-Fos/c-Jun), which induce the production of many inflammatory mediators^[6].

Nowadays, it has become clear that LPS can not reproduce all clinical features of sepsis. This emphasizes the participation of other contributing factors. Gram-positive bacteria, which lack LPS, are responsible today for a substantial part of sepsis incidence. The rapid transmission and acquisition of antibiotic-resistance genes among gram-positive bacteria, and their propensity to adhere and persist on vascular catheter surfaces and other implantable medical devices have contributed to an increasing incidence of gram-positive pathogens as a cause of sepsis^[7]. The major

Table 1 Biochemical characteristics of non-cirrhotic and cirrhotic patients

	Non-Cirrhotic (n = 10)	Cirrhotic (n = 10)
Bilirubin (mg/dL)	0.8 (0.6-1.2)	1.2 (0.5-28.5)
Albumin (g/dL)	4.0 (1.8-4.2)	3.3 (1.2-4.1)
PT (sec/ctl)	11.8 (9.7-15.3)	11.6 (10.2-18.4)
ALT (IU/L)	23 (15-61)	32.5 (19-57)
AST (IU/L)	23 (17-48)	43 (28-180)
Alkaline phosphatase (IU/L)	78 (56-204)	145.5 (56-479)

Data are expressed as median (minimum -maximum) values. PT: Prothrombin time; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

wall components of gram-positive bacteria, LTA and PGN, are thought to contribute to the development of sepsis, septic shock^[8] and multiple organ dysfunction syndrome (MODS)^[9]. Like LPS, LTA can interact with CD14 to initiate signal transduction pathways that lead to NF- κ B activation^[10]. It has been observed recently, that LTA is recognized by TLR2, which heterodimerises with either TLR1 or TLR6^[11,12]. Activation of the TLR2/6 heterodimer is greatly facilitated by CD36 in a similar way as TLR4 by CD14^[13].

This study compares, in cirrhotic and non-cirrhotic patients, the ability to activate inflammatory pathways of both gram-negative and gram-positive bacteria ligands. We therefore assessed the response of peripheral blood mononuclear cells (PBMC) of cirrhotic and non-cirrhotic patients to LPS and LTA exposure in terms of receptor expression (CD14, TLR2 and TLR4) and cytokine secretion [tumor necrosis factor (TNF) α , interleukin (IL)-1 β , IL-6, IL-12 and IL-10].

MATERIALS AND METHODS

Patients

Twenty out-patients were studied, ten of them with cirrhosis. Diagnosis of cirrhosis was supported clinically, by laboratory tests and ultrasound. Cirrhosis was due to alcohol in 4 patients, cryptogenic in 5, and due to portal thrombosis in 1. Child-Pugh classification was A in 5 patients, B in 3, and C in 2. Male:female ratio was 1:1 and the median age was 56.5 (36-79) years. Coexisting disorders were diabetes in 2 patients, hypertension in 1 and systemic sclerosis in 1. Laboratory tests are summarized in Table 1. Non-cirrhotic controls were patients with dyslipidemia (4), peptic ulcer disease (3), hypothyroidism (2), major depression (1), diabetes (1), hypertension (1), and achalasia (1). Their male:female ratio was 1:4 and median age 54.5 (41-75) years. At the time of inclusion, subjects neither had a concurrent infectious disorder, nor were receiving antibiotic or immune-modulating therapy. They all signed an informed consent before entry. The protocol of the study was approved by the Human Biomedical Research Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Isolation and stimulation of PBMC

Peripheral blood mononuclear cells were used as experimental units, given that they represent well-suited low-cost proxy-measures of monocytic response^[14]. Peripheral venous blood was collected with heparinized sterile pyrogen-free disposable syringes (Becton Dickinson). PBMC were isolated from blood samples on a lymphoprep gradient (Axis Shield). After washing, PBMC were adjusted to 10^6 cells/mL in RPMI 1640 (Life Technologies, Invitrogen), and supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen) and 1% penicillin-streptomycin 500 U/mL-500 µg/mL (GIBCO, Invitrogen). Then, 3×10^6 cells were plated on 2 mL media in 6-well round bottom tissue culture plates (NUNC). After stabilization at 37°C and 5% CO₂, cells were stimulated (duplicate experiments) with either 0.1 µg/mL ultra-purified *Escherichia coli* endotoxin (Sigma Chemical Co.) or 0.1 µg/mL *Streptococcus faecalis* lipoteichoic acid (Sigma Chemical Co.). In order to establish the optimal concentration of activation, PBMC from blood donors were cultured with LPS or LTA at different concentrations such as 0.01, 0.1, 1 and 10 µg/mL and 0.1, 1.0, 10 and 20 pg/mL, respectively. Cultures were incubated for 24 h before supernatant harvest and TNFα concentration measurement. TNFα levels were found highest with a concentration of 0.1 pg/mL. Also, to establish the optimal time of activation, normal PBMC were cultured with 0.1 pg/mL of LPS or LTA, and supernatants harvested after 6, 24 and 48 h. TNFα levels were highest after 24 h (data not shown). We therefore used 0.1 pg/mL of LPS or LTA for a 24-h exposure. Supernatants were harvested after 24 h and stored at -70°C until analysis.

CD14, TLR2 and TLR4 expression

5×10^5 freshly isolated or cultured PBMC were kept unexposed (NE), or were treated with LPS or LTA for 24 h. The expression of CD14, TLR2 or TLR4 was determined by flow cytometry. Briefly, treated PBMC were resuspended at 5×10^5 cells/mL in blocking buffer (PBS containing 2% FBS, 2% rabbit serum, 5 mM EDTA and 0.1% sodium azide) and incubated on ice for 30 min. Cell suspension was centrifuged and stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 (Santa Cruz Biotechnology), phycoerythrin (PE)-conjugated anti-human TLR2 (Santa Cruz Biotechnology), and PE-conjugated anti-human TLR4 (Santa Cruz Biotechnology). Isotype-matched nonbinding control goat anti-mouse IgG_{2a} (Santa Cruz Biotechnology) was used. The cells were incubated for 15 min in the dark, washed twice with FACS buffer (PBS containing 2% FBS, 5 mmol/L EDTA and 0.1% sodium azide) and fixed with 4% paraformaldehyde in PBS (pH 7.2) for 30 min and analyzed on an EPICS-ALTRA (Beckman-Coulter). A total of 20 000 events was obtained for each sample. Data were analyzed with WinMDI 2.8 software. CD14, TLR2 and TLR4 values were expressed as % fluorescence.

Cytokine assays

After activation, cell-free culture supernatants were harvested and concentrations of TNFα, IL-1β, IL-6, IL-12 and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) (OptEIA™, BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Detection limits for each assay were 4 pg/mL for TNFα, IL-1β, IL-6, and IL-10, and 15 pg/mL for IL-12. In each patient, every test was run in duplicate.

Data are summarized as median (minimum and maximum) values. Taking the NE condition as reference, absolute and relative (%) differences were determined for LPS or LTA exposed PBMC of cirrhotic and non-cirrhotic patients. The Mann-Whitney test was used to analyze differences between cirrhotic and non-cirrhotic groups, and the Wilcoxon sign-rank test to analyze differences between exposure and non-exposure to LPS or LTA. A *P* value < 0.05 was considered as statistically significant, and a *P* < 0.10 as tendency towards significance. The Stata v7 statistical package was used.

RESULTS

CD14, TLR2 and TLR4 expression

Expression of CD14, TLR2 and TLR4 by NE PBMC was higher in cirrhotic than non-cirrhotic patients. Median CD14 expression was 13.3% (8.9-34.6) *vs* 6.7% (3.5-27.5) (*P* < 0.01), median TLR2 expression was 9% (4.8-19.5) *vs* 4.8% (1.7-7.9) (*P* < 0.006), and median TLR4 expression was 26.9% (5.9-36.4) *vs* 8.5% (1.2-30) (*P* < 0.111), respectively. (Figure 1A-C) Non-exposure (NE), LPS or LTA exposure, bars represent median values.

After exposure to LPS, CD14 expression by PBMC of non-cirrhotic patients [5.6% (2-28.2)] was not significantly different from corresponding NE values [6.7% (3.5-27.5), NS], but TLR2 and TLR4 expressions were significantly lower [2% (1-6.5) *vs* 4.8% (1.7-7.9), *P* < 0.047, and 3.5% (0.9-26.1) *vs* 8.5% (1.2-30), *P* < 0.028]. PBMC of cirrhotic patients showed, after the same exposure, an increased CD14 expression [23.2% (3.2-48.5) *vs* 13.3% (8.9-34.6), *P* < 0.111], and significantly decreased TLR2 [4.9% (1.1-10.8) *vs* 9% (4.8-19.5), *P* < 0.008] and TLR4 [14.8% (1.2-32) *vs* 26.9% (5.9-36.4), *P* < 0.008] expression (Figure 1A-C). Taking the NE condition as 100% reference, the median relative difference in CD14 expression tended to be higher in cirrhotic than non-cirrhotic patients after LPS exposure (*P* < 0.096). As to TLR2 and TLR4 expression, LPS exposure induced a non-significant trend towards larger median relative differences in cirrhotic than non-cirrhotic patients (Table 2).

LTA exposure did not affect significantly CD14 expression in non-cirrhotic patients [6.5% (3.6-26.9)] when compared to NE conditions [6.7% (3.5-27.5), NS], neither did it affect TLR2 [2.1% (1-8.2) *vs* 4.8% (1.7-7.9), NS] expression. TLR4 expression was, however, significantly decreased [2.7% (0.7-28.8) *vs* 8.5% (1.2-30), *P* < 0.013]. LTA challenged PBMC of cirrhotic patients showed sig-

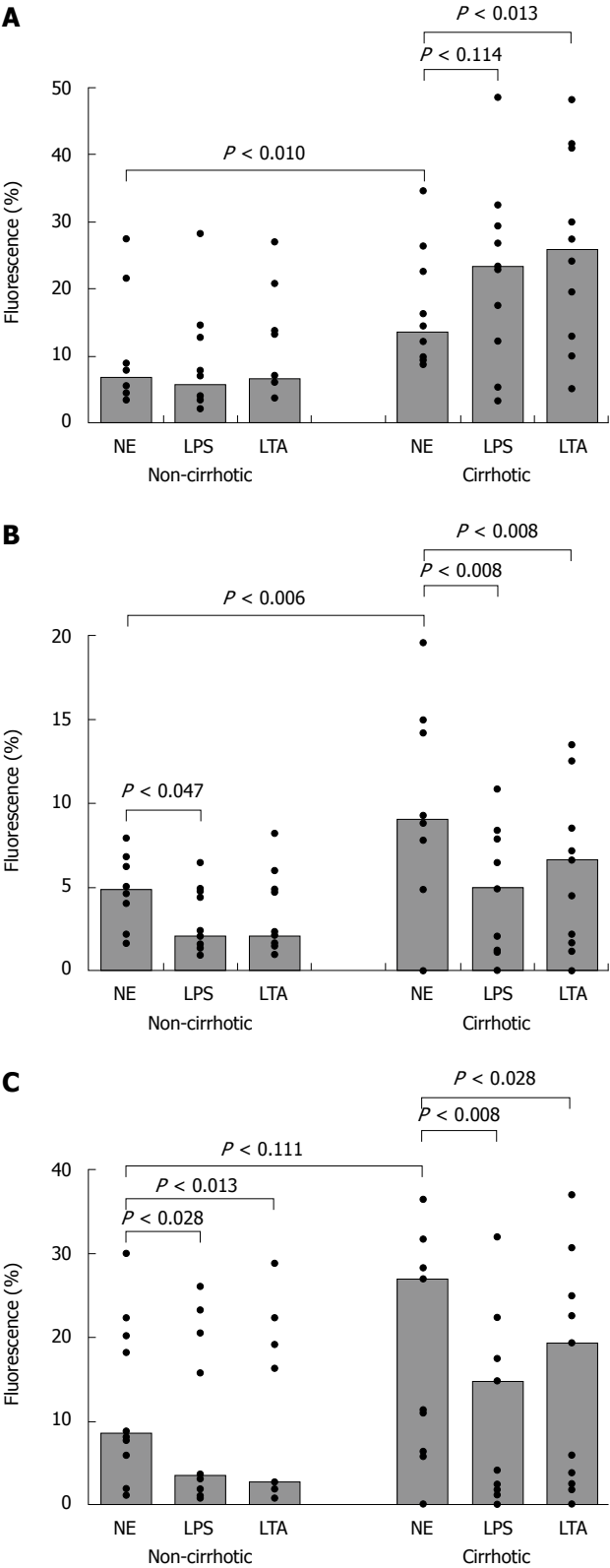


Figure 1 Receptor expression from peripheral blood mononuclear cells of non-cirrhotic and cirrhotic patients under conditions of non-exposure, lipopolysaccharide or lipoteichoic acid exposure for CD14, toll-like receptor 2 and toll-like receptor 4 expression. Bars represent median values. $P < 0.05$ denotes statistical significance and $P < 0.10$ denotes tendency to statistical significance. A: CD14 expression; B: TLR2 expression; C: TLR4 expression. NE: Non-exposure; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid.

Table 2 Median relative difference ¹ in receptor expression and cytokine secretion by peripheral blood mononuclear cells of non-cirrhotic and cirrhotic patients after exposure to lipopolysaccharide and lipoteichoic acid				
		Non-cirrhotic (%) <i>n</i> = 10	Cirrhotic (%) <i>n</i> = 10	<i>P</i> value
Expression	CD14			
	LPS	-9	+39	< 0.096 ^b
	LTA	-3.50	+55	< 0.028 ^a
	TLR2			
TLR2	LPS	-10	-60	< 0.121
	LTA	0	-42	< 0.289
TLR4	LPS	30	-53	< 0.221
	LTA	-19.50	-29	< 0.935
Secretion				
TNF α	LPS	+7400	+8770	< 0.940
	LTA	+190	+360	< 0.970
IL-1 β	LPS	+70.50	+1164	< 0.019 ^a
	LTA	-6	+71	< 0.049 ^a
IL-6	LPS	+91	+319	< 0.174
	LTA	+125	+246	< 0.326
IL-12	LPS	+3324	+6219	< 0.151
	LTA	+503	+1786	< 0.227
IL-10	LPS	+1768	+5844	< 0.364
	LTA	+50	+415	< 0.571

¹Difference with the non-exposure value (considered as the reference or 100%). A negative value reflects a decrease, whereas a positive value reflects an increase. ^aDenotes statistically significant ($P < 0.05$) differences between non-cirrhotic and cirrhotic patients. ^bDenotes tendency towards statistically significant ($P < 0.10$) differences between non-cirrhotic and cirrhotic patients. LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; TLR: Toll-like receptor; IL: Interleukin; TNF: Tumor necrosis factor.

13.3% (8.9-34.6), $P < 0.013$], and decreased TLR2 [6.6% (1.2-13.4) *vs* 9% (4.8-19.5), $P < 0.008$] and TLR4 [19.4% (1.9-37) *vs* 26.9% (5.9-36.4), $P < 0.028$] expression (Figure 1A-C). LTA induced median relative differences in CD14, TLR2 and TLR4 expression were similar to those induced by LPS (Table 2).

TNF α , IL-1 β , IL-6, IL-12 and IL-10 secretion

NE PBMC of non-cirrhotic *vs* cirrhotic patients secreted similar amounts of TNF α [≤ 4 pg/mL (≤ 4 -143) *vs* ≤ 4 pg/mL (≤ 4 -42), NS] and IL-10 [26 pg/mL (≤ 4 -275) *vs* 6 pg/mL (≤ 4 -72), NS]. Secretion of IL-6 tended to be higher in non-cirrhotic [401 pg/mL (12-1530)] than cirrhotic [168 pg/mL (5-459)], patients ($P < 0.070$). Secretion of IL-1 β and IL-12 was significantly higher in non-cirrhotic [26 pg/mL (≤ 4 -159) and 19 pg/mL (≤ 15 -959)] than cirrhotic [≤ 4 pg/mL (≤ 4 -10) and ≤ 15 pg/mL (≤ 15 -38)] patients ($P < 0.009$ and < 0.022) (Figure 2A-E).

Taking NE values as reference [≤ 4 pg/mL (≤ 4 -143) and ≤ 4 pg/mL (≤ 4 -42)], LPS exposure triggered significant increases in TNF α secretion by both non-cirrhotic [443 pg/mL (52-658), $P < 0.005$] and cirrhotic [355 pg/mL (52-713), $P < 0.005$] PBMC. Similar increases were observed for IL-1 β , IL-6, IL-12 and IL-10 secretion. Specifically, IL-1 β PBMC secretion increased from NE values of 26 pg/mL (≤ 4 -159) in non-cirrhotic and ≤ 4 pg/mL (≤ 4 -10) in cirrhotic patients, to 61 pg/mL (8-192) and 51 pg/mL (17-286) after LPS exposure, respectively ($P < 0.028$ and < 0.005). As for IL-6, secre-

nificantly increased CD14 expression [25.7% (5-48.2) *vs*

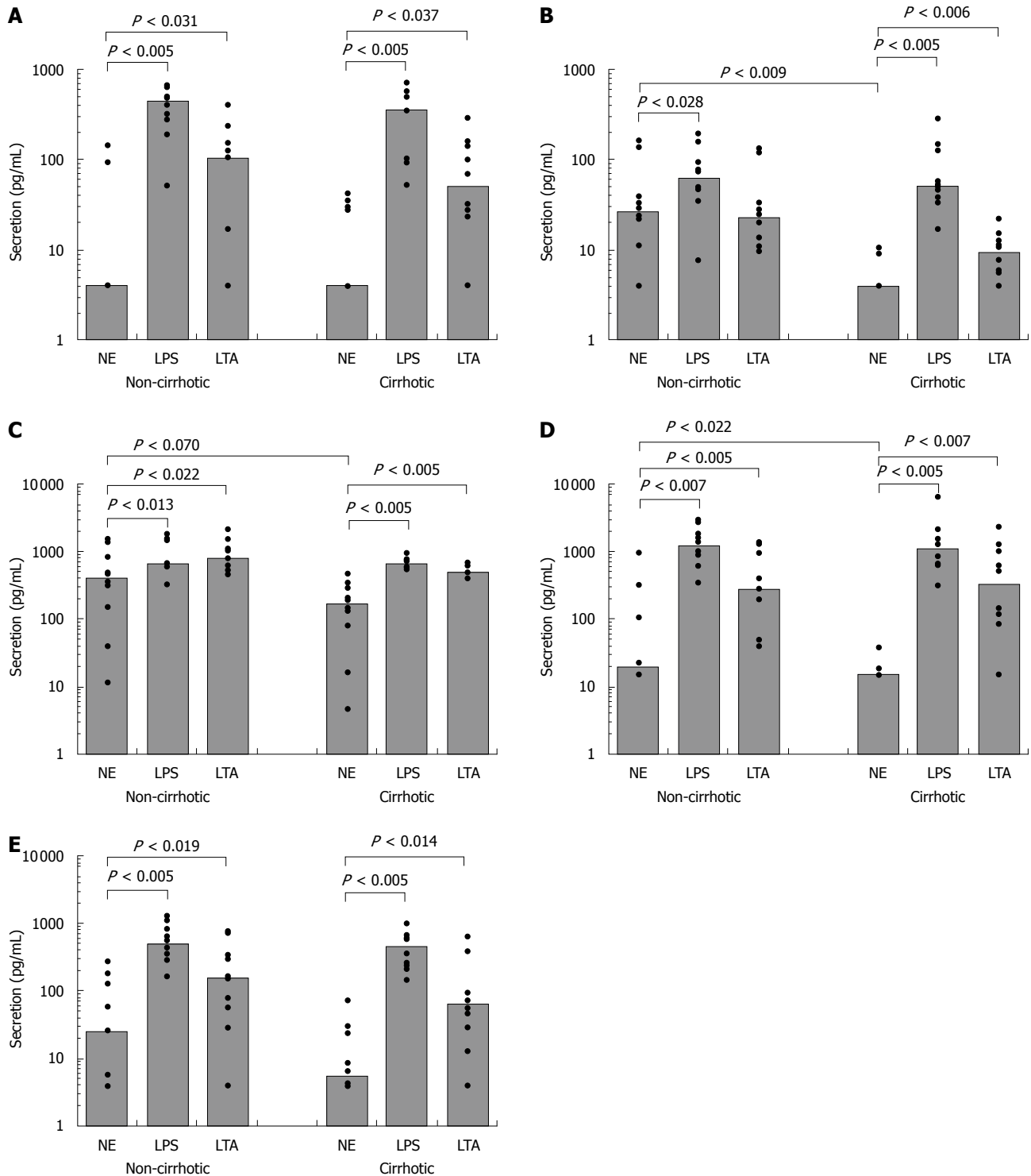


Figure 2 Cytokine secretion from peripheral blood mononuclear cells of non-cirrhotic and cirrhotic patients under conditions of non-exposure, lipopolysaccharide or lipoteichoic acid exposure for tumor necrosis factor α , interleukin-1 β , interleukin-6, interleukin-12 and interleukin-10 secretion. Bars represent median values. $P < 0.05$ denotes statistical significance and $P < 0.10$ denotes tendency to statistical significance. A: Tumor necrosis factor α secretion; B: Interleukin (IL)-1 β secretion; C: IL-6 secretion; D: IL-12 secretion; E: IL-10 secretion. NE: Non-exposure; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid.

tion increased from NE values of 401 pg/mL (12-1530) and 168 pg/mL (5-459), to 645 pg/mL (325-1793) and 660 pg/mL (540-946), $P < 0.013$ and < 0.005 . IL-2 secretion showed an increase from 19 pg/mL (≤ 15 -959) and ≤ 15 pg/mL (≤ 15 -38), to 1201 pg/mL (15-2850) and 1074 pg/mL (317-6397), $P < 0.007$ and < 0.005 . IL-10

secretion was 26 pg/mL (≤ 4 -275) and 6 pg/mL (≤ 4 -72) under NE conditions, and 498 pg/mL (163-1292) and 464 pg/mL (146-1010) after LPS exposure, $P < 0.005$ and < 0.005 . Median relative difference in cytokine secretion between LPS exposure and NE tended to be higher in cirrhotic than non-cirrhotic patients, reaching statistical

significance in IL-1 β only ($P < 0.019$) (Table 2).

To a lesser degree than LPS, LTA exposure also induced increases in cytokine secretion. TNF α secreted by PBMC of non-cirrhotic and cirrhotic patients increased from NE values of ≤ 4 pg/mL ($\leq 4-143$) and ≤ 4 pg/mL ($\leq 4-42$), to 105 pg/mL (4-409) and 51 pg/mL (4-288), $P < 0.031$ and < 0.037 . IL-1 β secretion was 26 pg/mL ($\leq 4-159$) and ≤ 4 pg/mL ($\leq 4-10$) under NE conditions, and 23 pg/mL (10-133) and 9 pg/mL (4-22) after LTA exposure, NS and $P < 0.006$. IL-6 secretion increased from 401 pg/mL (12-1530) and 168 pg/mL (5-459), to 802 pg/mL (454-2155) and 509 pg/mL (397-705), $P < 0.022$ and < 0.005 . IL-12 secretion increased from 19 pg/mL ($\leq 15-959$) and ≤ 15 pg/mL ($\leq 15-38$), to 275 pg/mL (40-1385) and 334 pg/mL ($\leq 15-2339$), $P < 0.005$ and < 0.007 . IL-10 secretion increased from 26 pg/mL ($\leq 4-275$) and 6 pg/mL ($\leq 4-72$), to 157 pg/mL ($\leq 4-756$) and 64 pg/mL ($\leq 4-638$), $P < 0.019$ and < 0.014 (Figure 2A-E). Median LTA-induced relative differences in cytokine secretion tended to be more vigorous in cirrhotic than in non-cirrhotic patients, reaching statistical significance in IL-1 β only ($P < 0.049$) (Table 2).

DISCUSSION

In this study, higher PBMC CD14, TLR2 and TLR4 expression was observed in cirrhotic patients under NE and LPS/LTA exposure conditions. LPS and LTA exposure induced an increase in CD14 expression in cirrhotic patients, and a decrease in TLR2 and TLR4 expression in both non-cirrhotic and cirrhotic patients. With the exception of TNF α , PBMC absolute cytokine secretion was lower in cirrhotic patients under NE conditions. However, once exposed to LPS or LTA, cytokine secretion was similar in both non-cirrhotic and cirrhotic patients, determining a more vigorous response in the latter, as shown by the corresponding relative differences. As to LPS, and with the exception of IL-6 secretion, this bacterial ligand triggers a more vigorous cytokine response than LTA.

CD14, TLR2 and TLR4 expression

Higher PBMC CD14 expression in cirrhotic patients under NE conditions reflects a state of hyperactivation, conditioned probably by a long-standing exposure to intestinal microorganisms and their products. This hyperactivation leads to vigorous reactions with any further bacterial stimuli^[15]. It should be kept in mind that PBMC expression in our study is summarized as percentage of control baseline fluorescence conditions. In terms of the mean fluorescence intensity (MFI)^[16-18], no significant differences in CD14, TLR2 or TLR4 expression among cirrhotic and non-cirrhotic PBMC before and after exposure to LPS and LTA were observed (data not shown). This means that the herein reported differences in PBMC expression reflect differences in the number of activated cells, not in the amount of antibody bound per cell.

Chronic increase in circulating LPS, and the resulting state of PBMC hyperactivation has been associated

to low levels of high-density lipoprotein (HDL), a well-known complication of cirrhosis. HDL is able to bind LPS and neutralize its bioactivity. HDL can also down-regulate monocyte CD14 expression, and has other anti-inflammatory properties^[19]. Low HDL levels could explain the increased CD14 expression observed in PBMC of our cirrhotic patients under both NE and exposed conditions.

As to LTA, this ligand relies, at least in part, on CD14 to initiate signal transduction pathways^[10,20]. It has been shown recently, that CD14 expression enhances markedly LTA binding to plasma cell membranes^[21]. It seems, therefore, that increased CD14 expression in cirrhosis is due to high circulating levels of both LPS and LTA. Increased circulating levels of LPS and proinflammatory cytokines have been documented in patients with chronic liver disease, even in the absence of infection. However, no significant correlation between LPS and these inflammatory mediators has been shown, raising the possibility that other agents, besides LPS, may play a role. Recent studies on TLR expression in cirrhotic patients show that this might be in fact true. TLR4, in the presence of LPS, triggers the signal transduction that leads to TNF α production. When PGN and LTA are present, TLR2 is required for signaling and activation of the inflammatory cascade. Recently, PBMC expression of TLR2, but not TLR4 was shown to correlate significantly with circulating levels of both TNF α and anti-inflammatory soluble TNF receptors. These findings suggest that gram-positive microbial stimuli might be important in the proinflammatory state of chronic liver disease. If proven true, this would contraindicate the use of probiotic agents, such as gram-positive lactobacilli, in cirrhotic patients. Current evidence, however, shows that probiotic use is associated with a significant increase of fecal lactobacilli and a decrease of potentially pathogenic gram-positive and gram-negative bacterial species. Probiotics reverse bacterial overgrowth and improve minimal hepatic encephalopathy. They improve the Child-Pugh class at the expense of serum bilirubin, albumin and prothrombin. Also, serial ALT levels show a significantly reduced hepatic necro-inflammatory activity, suggesting that probiotics can protect against hepatocellular damage^[22].

In our study, PBMC of cirrhotic patients expressed more TLR2 and TLR4 under NE conditions than PBMC of non-cirrhotic patients. Exposure to LPS and LTA decreased expression of both receptors in all patients. (Figure 1B and C) A similar decrease in TLR2 expression was observed by Riordan *et al.* after exposing PBMC of cirrhotic patients to gram-positive bacteria products *in vitro*. However, *in vivo*, they observed an increased PBMC expression of TLR2, but not TLR4, in cirrhotic subjects^[4]. It has been shown recently, that monocyte expression of TLR4 is down-regulated in cirrhotic patients with Child-Pugh class C, whereas TLR2 expression is equivalent to controls. In our study, we included patients with Child-Pugh class A or B mainly, or patients with a reasonably preserved liver function and immune competence. TLR4 down-regulation in advanced cirrhosis is associated with

LPS tolerance, enhanced bacterial translocation and portal venous endotoxemia^[23]. In this context, endotoxin tolerance is viewed as a regulation mechanism that protects the cell from “over expression” or sustained activation. It is regarded as a protection mechanism that aims to limit tissue damage due to excessive immune response. Another explanatory mechanism of TLR down-regulation is receptor internalization, which has been shown for TLR2 and TLR4^[24].

After exposure to LPS, PBMC of both cirrhotic and non-cirrhotic patients showed a lower TLR2 and TLR4 expression. A similar but smaller decrease was observed after LTA exposure, suggesting that these two TLRs might not be completely specific. It is well documented that TLR2 recognizes LPS as well as LTA, while TLR4 recognizes LPS mainly^[5,25]. From our results, we can not exclude a cross-recognition of LPS and LTA that could lead to an “additive activation” of signaling pathways.

Differences and changes in CD14, TLR2 and TLR4 expression observed in our study support the so called hyperactivation state in cirrhotic patients which, compared to the non-cirrhotic patients, does not appear to be an uncontrolled response, but a process of cellular reprogramming or adaptation to bacteria or their products^[26]. We should point out that our non-cirrhotic controls had dyslipidemia, peptic ulcer disease, hypothyroidism, major depression, diabetes, hypertension, and/or achalasia. It is known that some of these entities compromise, up to certain degree, the immune response. In spite of this, PBMC response to bacterial stimuli among cirrhotic patients was significantly different to their non-cirrhotic counterpart.

TNF α , IL-1 β , IL-6, IL-12 and IL-10 secretion

Cytokines, chemokines, and growth factors such as TNF α , IL-1 β , IL-6, interferon- γ , IL-8, macrophage inflammatory protein-1, macrophage chemoattractant factor-1, and transforming growth factor, are all upregulated in patients with cirrhosis^[1]. This upregulation varies according to the degree of liver damage, or Child-Pugh score^[4,19]. *In vitro*, PBMC exposure to bacterial and viral ligands results in an elevated production of inflammatory cytokines, particularly IL-1 β , IL-6, IL-8, and TNF α , β ^[16]. In our study, PBMC exposure to LPS or LTA triggered a significant TNF α , IL-1 β , IL-6, IL-12 and IL-10 secretion in both cirrhotic and non-cirrhotic patients. Due to sample size restrictions, no correlation with the Child-Pugh score was observed.

LPS elicited a more vigorous cytokine secretion than LTA, irrespective of the presence or absence of cirrhosis. This “attenuated” response to LTA has been observed by other investigators and attributed, *in vivo*, to serum components such as lipoproteins and LBP^[19,27]. *In vitro*, to get a proinflammatory response in monocytes and hepatic stellate cells, the minimal active concentration of PGN or LTA needs to be 100 times higher than that of LPS^[1]. We used 0.1 μ g/mL of LTA and LPS based on dose-response experiments. With this exposure dosage, the highest TNF α secretion was obtained, which was quantitatively

lower for LTA than LPS.

As to IL-6, a higher secretion was observed after LTA than after LPS exposure. This cytokine plays a pivotal role in the acute response to bacterial products. Wang *et al.* reported that whole human blood is a potent source of IL-6 production after stimulation with *S. aureus* LTA^[28]. However, other investigators failed to induce IL-6 release from monocyte cultures^[29]. This discrepant IL-6 secretion has been attributed to non-monocytic cells present in the whole blood, not well characterized paracrine factors absent in monocyte cultures^[28], variable LTA exposure dosage^[29], and inter- and intra-species LTA variations^[29].

We should consider that, in cirrhosis, the innate immunity hyper-responsiveness observed in this and other studies do not occur in isolation to alterations in adaptive immunity. It is known that cirrhotic patients are prone to get frequent bacterial infections due to an immunosuppressed state. Contrary to the expected, their T lymphocytes are activated. The proportion of CD4+ T cells expressing CD25 and CD122 antigens is increased significantly, and so is the proportion of memory CD4+ and CD8+ T cells with characteristics of senescent cells. It is thought that repeated cycles of inflammation and damage lead to a continuous recruitment of effector leucocytes within the liver and amplify effector responses exerted by T cells, macrophages, natural killer cells or neutrophils^[30]. The contribution of these immune derangements, separately and as a whole, to chronic liver injury remains to be documented.

PBMC of cirrhotic patients show a hyperactivation state in terms of CD14, TLR2 and TLR4 expression. Exposure to LPS or LTA decreases this expression in both cirrhotic and non-cirrhotic PBMC, suggesting that control mechanisms are still present in chronic liver disease. Given that PBMC receptor expression changed after exposure to both LPS and LTA, our data suggest a non-specific cross-activation. Decreased CD14, TLR2 and TLR4 expression is accompanied by an increased TNF α , IL-1 β , IL-6, IL-12 and IL-10 secretion. This secretion is relatively higher in cirrhotic than non-cirrhotic patients. How this systemic hyperactivation relates to the progression of liver injury is still speculative. Both LPS and LTA elicit a PBMC response, but to a different degree. The impact of this differential response needs to be evaluated, particularly when potentially beneficial gram-positive bacteria (probiotics) are involved.

COMMENTS

Background

Liver diseases figure as the fifth cause of death in Mexico. They are the third cause of death in subjects between 35-44 years, and the fourth cause of death in subjects aged 45-64 years. Patients with advanced chronic liver disease or cirrhosis frequently present with intestinal bacterial overgrowth of both gram-negative and gram-positive bacteria. This leads to infectious complications such as spontaneous bacterial peritonitis or sepsis, and to a chronic proinflammatory state.

Research frontiers

The role of gram-negative bacteria in the pathogenesis of liver injury has been extensively studied. It involves intestinal bacterial translocation and decreased

liver clearance, leading to inflammation, tissue injury and, eventually, cirrhosis. As to gram-positive bacteria, a similar damaging role has been proposed, but still remains to be proven.

Innovations and breakthroughs

It became clear that lipopolysaccharide, a gram-negative bacterial cell wall product, cannot reproduce all the clinical features observed in sepsis. This emphasizes the participation of other contributing factors. Gram-positive bacteria, which lack lipopolysaccharide, are responsible today for a substantial part of sepsis incidence. Peripheral blood mononuclear cells of cirrhotic patients are able to respond to a sudden bacterial ligand exposure, particularly lipopolysaccharide, in terms of a decreased expression of CD14, Toll-like receptor 2 and 4, and an increased tumor necrosis factor α , interleukin (IL)-1 β , IL-6, IL-12 and IL-10 secretion. The authors suggest that immune regulation mechanisms persist in chronic liver disease, at least in Child-Pugh A and B stages.

Applications

Both lipopolysaccharide and lipoteichoic acid elicit a peripheral blood mononuclear cells response, but to a different degree, suggesting that gram-positive microbial stimuli might be important in the proinflammatory state of chronic liver disease. The impact of this differential response needs to be evaluated, particularly when potentially beneficial gram-positive bacteria (probiotics) are involved. Current evidence shows that probiotic use is associated with a significant increase of fecal lactobacilli and a decrease of potentially pathogenic gram-positive and gram-negative bacterial species.

Terminology

Intestinal bacterial overgrowth is a major promoting factor of bacterial translocation in cirrhosis. It is defined as bacterial migration from the intestinal lumen to the mesenteric lymph nodes or other extra-intestinal sites. Sepsis is a common cause of death in cirrhotic patients. Toll-like receptors are transmembrane receptor proteins that play a critical role in the induction of innate immunity to microbial pathogens via recognition of conserved molecular patterns.

Peer review

The paper is very scientific, has copious data and is well written.

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Lower esophageal sphincter relaxation is impaired in older patients with dysphagia

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Abstract

AIM: To characterize the effects of age on the mechanisms underlying the common condition of esophageal dysphagia in older patients, using detailed manometric analysis.

METHODS: A retrospective case-control audit was performed on 19 patients aged ≥ 80 years (mean age 85 ± 0.7 year) who underwent a manometric study for dysphagia (2004-2009). Data were compared with 19 younger dysphagic patients (32 ± 1.7 years). Detailed manometric analysis performed prospectively included

basal lower esophageal sphincter pressure (BLESP), pre-swallow and nadir LESP, esophageal body pressures and peristaltic duration, during water swallows (5 mL) in right lateral (RL) and upright (UR) postures and with solids. Data are mean \pm SE; a P -value < 0.05 was considered significant.

RESULTS: Elderly dysphagic patients had higher BLESP than younger patients (23.4 ± 3.8 vs 14.9 ± 1.2 mmHg; $P < 0.05$). Pre-swallow LESP was elevated in the elderly in both postures (RL: 1 and 4 s $P = 0.019$ and $P = 0.05$; UR: $P < 0.05$ and $P = 0.05$) and solids ($P < 0.01$). In older patients, LES nadir pressure was higher with liquids (RL: 2.3 ± 0.6 mmHg vs 0.7 ± 0.6 mmHg, $P < 0.05$; UR: 3.5 ± 0.9 mmHg vs 1.6 ± 0.5 mmHg, $P = 0.01$) with shorter relaxation after solids (7.9 ± 1.5 s vs 9.7 ± 0.4 s, $P = 0.05$). No age-related differences were seen in esophageal body pressures or peristalsis duration.

CONCLUSION: Basal LES pressure is elevated and swallow-induced relaxation impaired in elderly dysphagic patients. Its contribution to dysphagia and the effects of healthy ageing require further investigation.

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Key words: Dysphagia; Elderly; Esophageal Motility; Lower Esophageal Sphincter; Aging

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INTRODUCTION

Dysphagia is common in older individuals, with mealtime difficulties being reported in 87% of residential care clients who are predominantly elderly^[1]. This condition is associated with significant morbidity (anxiety and depression) and, in some cases, increased mortality from malnutrition and aspiration pneumonia^[2]. People aged over 65 years constitute more than 15% of the South Australian population and this proportion is predicted to increase substantially over the next 10 years^[3]. Moreover, this demographic trend is consistently noted across the developed world. There is thus the need for a greater understanding of the pathophysiology of dysphagia in older individuals.

An increase in esophageal motor abnormalities has been reported with advancing age^[4]. Studies in patients with gastroesophageal reflux disease suggest that only 62% of swallows are normal in subjects aged over 64 years, compared with 95% of younger patients^[5]. Despite the magnitude of this clinical problem in the elderly, no studies have detected significant differences in the mechanism of esophageal dysphagia between age groups, although older patients tend to find dysphagia more clinically troublesome^[6].

In the absence of a structural esophageal lesion, more than 50% of all patients with dysphagia have abnormal motility on manometry^[7]. However, the most common diagnosis made after investigation is non-specific esophageal motility disorder (NSMD). This is a “default position” when esophageal motor function is abnormal, but does not satisfy the diagnostic features of classic motility disorders, such as diffuse esophageal spasm or achalasia. Advances in high resolution manometry have improved the ability to identify the specific motility changes underlying such conditions^[8], which has significantly improved treatment and patient outcomes^[8,9]. However, the absence of a clearly understood pathogenic mechanism for dysphagia in older adults currently limits the provision of specific therapeutic recommendations for these patients.

Much of our current understanding of the effects of aging on esophageal physiology is based on data from healthy older individuals without dysphagia. These studies have shown age-related esophageal motility changes that include decreased upper esophageal sphincter pressure with reduced duration of relaxation^[10], low amplitude peristalsis and increased stiffness of the esophageal body^[11]. Changes in lower esophageal sphincter function, however, have been harder to identify in healthy ageing^[12] and studies are rarely performed in adults above the age of 80 years. Alterations that may occur with extreme age are increasingly relevant as the proportion of the population surviving into their ninth decade increases.

The few studies looking specifically at esophageal motor function in symptomatic older patients have yielded conflicting results, but do not appear to support an earlier concept of “presbyesophagus”. This refers to a cluster of dysfunctions, including decreased contractile activity, polyphasic waves in the esophageal body and incomplete

relaxation with esophageal dilatation^[13]. Although no differences in broad diagnostic category have been demonstrated between older and younger dysphagic patients^[6,14], we hypothesize that older individuals may comprise a distinct subgroup of NSMD. The pathophysiologic abnormalities in these patients may involve more subtle motor sequencing problems that are not readily detected using standard manometry. Recognition of such abnormal motor patterns would improve understanding of the effects of ageing on the esophagus, as well as potentially provide a more targeted approach to treatment for the increasing number of older individuals with dysphagia.

The aim of the current study, therefore, was to compare esophageal motility between older (> 80 years) and younger adults with dysphagia, using analysis of high resolution manometric data.

MATERIALS AND METHODS

Subject selection

Subjects were identified from a prospectively collected database at the Repatriation General Hospital, Daw Park, a tertiary referral centre providing an esophageal manometry service to an ageing predominantly veteran population along with younger public hospital patients. Esophageal manometric studies were performed on patients presenting with a variety of symptoms suggestive of disease, including dysphagia, atypical chest pain, heartburn and cough. Of these, the database was audited to identify all patients aged ≥ 80 years investigated for dysphagia (as their primary symptom) between January 2004 and November 2009. These were gender-matched to a group of younger dysphagic patients. Exclusion criteria included (1) incomplete manometric data; (2) diagnosis of achalasia; (3) previous fundoplication; and (4) diabetes mellitus.

The standard manometric diagnoses as provided to the referring physician, and symptoms of older and younger dysphagic patients from this database have previously been reported in a clinical audit^[6]. The current study, however, provides a more specific evaluation of the possible motor mechanisms underlying dysphagia, with a detailed re-analysis of the previous manometric data.

The manometry database of this laboratory was approved by the Human Research Ethics Committee of the Repatriation General Hospital. All subjects provided written informed consent prior to the inclusion of their results in the database.

While all patients included in the study described dysphagia, each patient was asked to report whether it was present with solids, liquids or both and whether it was at the level of upper, mid or lower esophagus. These data were recorded prior to performing their esophageal manometry investigation.

Manometric technique

Esophageal pressures were measured using high resolution manometry (HRM). This technique allows visualization of esophageal contractility as a continuum of pressure and

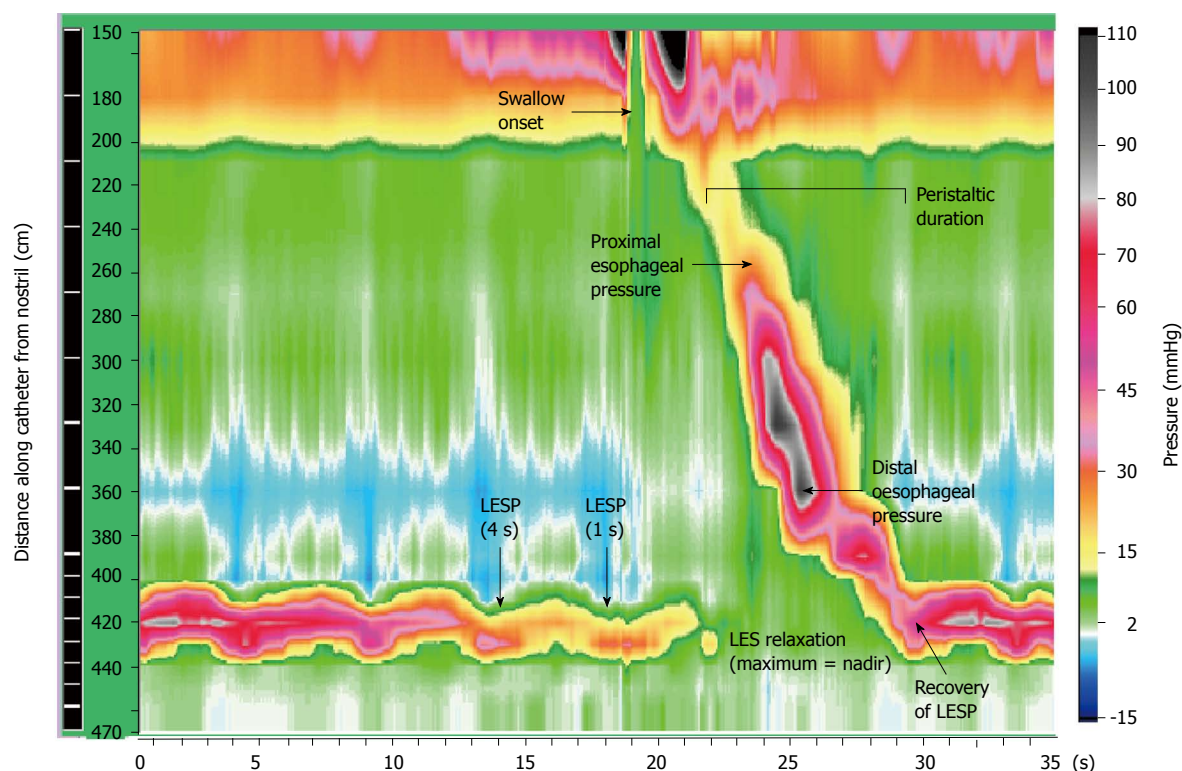


Figure 1 Example of swallow pressure topography spanning from the pharynx (15–20 cm) to stomach (44–47 cm), in a young patient with normal peristalsis and lower esophageal sphincter relaxation. Pressure data (amplitudes shown by colour gradient) are displayed with time on the x-axis and location of sensors on the y-axis. Points of measurement for motility parameters are indicated with arrows. Pressure sensors located in the region of the lower esophageal sphincter (LES) are spaced 1 cm apart, spanning a 6 cm segment. LES: Lower esophageal sphincter pressure.

time (Figure 1) using a colour display. To achieve this, pressure sensors were spaced at close intervals (1–3 cm apart) and at multiple recording sites, enabling extrapolation of pressure values between sensors without loss of accuracy. Compared to conventional manometry, HRM has several technical advantages that include greater efficiency, higher quality recordings and more objective interpretation. Intraluminal pressures were measured using a 16 channel silicone-rubber manometric assembly (Dentsleeve International, Toronto, Canada). The 9 most proximal side-holes (channels 1–9) were spaced 3 cm apart and spanned the pharynx and esophageal body (24 cm). The remaining 7 side-holes (channels 10–16) were spaced at 1 cm intervals and positioned astride the LES, with the most distal channel in the proximal stomach. All lumina were perfused with degassed distilled water at a rate of 0.15 mL/min using a low compliance perfusion pump (Dentsleeve International, Ontario Canada). Data were recorded at 25Hz and analyzed using specialized software (Trace Version 1.2v, Hebbard, Melbourne, Australia). All pressures were referenced to the intra-gastric pressure and could be displayed as a spatio-temporal color plot or conventional line-plot.

Study protocol

All studies were performed after a minimum 4-h fast. The manometric assembly was passed transnasally into the stomach, *via* an anaesthetized nostril. Subjects were placed in the right lateral (RL) posture and allowed time to adapt to the assembly. A basal lower esophageal sphincter

pressure (BLESP) was recorded for 30 s. Ten 5 mL liquid (water) swallows were performed in the RL posture, and repeated seated upright (UR). Manometry was also recorded during 5 standardized solid boluses (1/8 sliced white bread with crust removed) in the UR position. Subjects were asked to chew the bread and indicate when they were ready to swallow. The presence or absence of dysphagia was recorded for each swallow.

Data analysis

The original clinical manometry recordings were re-analyzed manually by 2 observers (LB and CB), neither of whom was responsible for the initial report. Both observers were blinded to patient age, gender and initial manometric diagnosis. Detailed motility analysis was performed, comprising (1) BLESP at end-expiration; (2) LES at 1 and 4 s before the onset of each swallow; (3) number of successful LES relaxations (defined as > 75% reduction in LES); (4) nadir LES (point of most complete LES relaxation) and time to nadir; (5) time to recovery of LES tone after swallow-induced relaxation; (6) amplitude of proximal and distal esophageal pressures (measured 6 cm below the upper esophageal sphincter and 4 cm above the LES, respectively); and (7) duration of peristalsis (time between peak amplitudes of channels 1–9). The sites at which esophageal pressure measurements were taken are shown in Figure 1. Subjects were excluded if more than 30% of their manometric data were missing, or more than 30% double swallows were encountered.

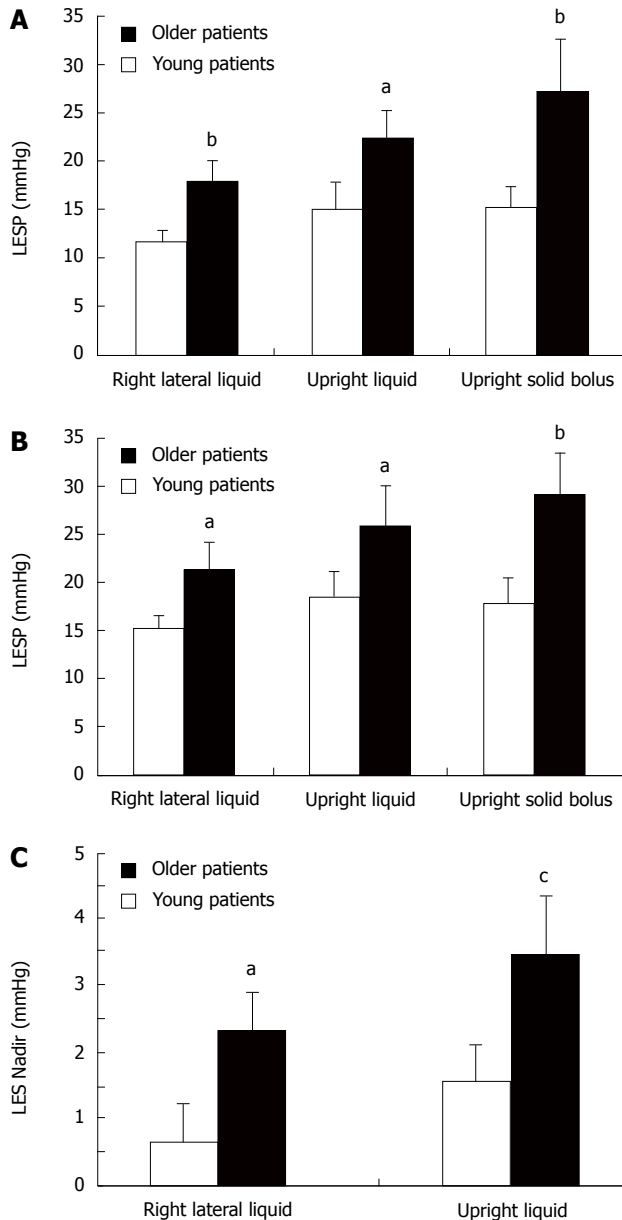


Figure 2 Lower esophageal sphincter pressure at 1 s (A) and 4 s (B) prior to swallow, and lower esophageal sphincter nadir pressure (C), in right lateral and upright postures with liquids and solids, in young ($n = 19$; 32 ± 1.7 years) and older ($n = 19$; 85 ± 0.7 year) patients with dysphagia. ^a $P < 0.05$; ^b $P < 0.01$; ^c $P = 0.01$ vs young. LES: Lower esophageal sphincter; LESP: Lower esophageal sphincter pressure.

Statistical analysis

All data are expressed as a mean \pm SE. Manometric parameters were compared between groups using Student unpaired *t*-test (two-tailed). A *P*-value < 0.05 was considered significant.

RESULTS

Nineteen patients aged older than 80 years (9 male, 10 female; mean age 85 ± 0.7 year) were included. The pattern of dysphagia was characterised as upper (21%), mid (42%) or lower (37%) esophagus, present with solids (47%), liquids (6%) or both (47%). Data were compared

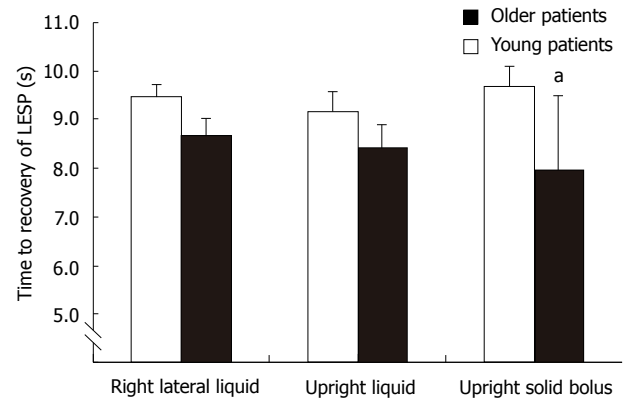


Figure 3 Time to recovery of lower esophageal sphincter tone after relaxation in right lateral and upright postures and with solids, in young and older patients with dysphagia. ^a $P = 0.05$ vs young. LESP: Lower esophageal sphincter pressure.

Table 1 Proximal and distal esophageal peristaltic amplitudes (mmHg) in older and young patients with dysphagia (mean \pm SE)

	Right Lateral water	Upright water	Upright solids
Older			
Proximal	39.1 \pm 7.3	41.4 \pm 5.7	45.2 \pm 7.5
Distal	53.8 \pm 7.8	67.3 \pm 16.4	61.7 \pm 14.8
Young			
Proximal	27.9 \pm 3.0	29.6 \pm 3.9	35.7 \pm 3.1
Distal	62.3 \pm 6.0	55.5 \pm 6.8	57.9 \pm 8.1

to 19 younger patients (9 male, 10 female; mean age 32 ± 1.7 years) with esophageal dysphagia in the upper (48%), mid (26%) or lower (26%) esophagus, with solids (79%), liquids (10%) or both (11%).

Mean basal LESP was higher in the older patients (23.4 ± 3.8 mmHg) compared to younger dysphagic patients (14.9 ± 1.2 mmHg) ($P < 0.05$).

Pre-swallow LESP was higher in older dysphagic patients in both RL (elderly *vs* young; 1 s: 17.8 ± 2.2 mmHg *vs* 11.6 ± 1.2 mmHg, $P = 0.019$; and 4 s: 21.3 ± 2.8 mmHg *vs* 15.3 ± 1.3 mmHg, $P = 0.05$) and UR (1 s: 22.3 ± 2.9 mmHg *vs* 14.9 ± 2.9 mmHg, $P < 0.05$; and 4 s: 25.9 ± 4.2 mmHg *vs* 18.3 ± 2.7 mmHg, $P = 0.05$) postures, and with solid boluses (1 s: 27.3 ± 5.5 mmHg *vs* 15.2 ± 2.2 mmHg, and 4 s: 29.1 ± 4.2 mmHg *vs* 17.7 ± 2.8 mmHg, $P < 0.01$ for both) (Figure 2A and B).

Mean nadir LESP was higher in older patients with liquid swallows in both RL (2.3 ± 0.6 mmHg *vs* 0.7 ± 0.6 mmHg, $P < 0.05$) and upright (3.5 ± 0.9 mmHg *vs* 1.6 ± 0.5 mmHg, $P = 0.01$) postures, when compared to younger patients (Figure 2C). There were no differences in nadir pressure with solids, time to nadir, or number of complete LES relaxations, between age groups.

Time to recovery of LES tone after swallow-induced relaxation was shorter in the older group after a solid bolus ($P = 0.05$), with a similar trend with liquid swallows in the RL posture ($P = 0.07$) (Figure 3).

There were no significant differences between age groups in mean amplitude of proximal or distal esophageal body contractions with liquids or solids (Table 1).

The duration of esophageal peristalsis was similar between age groups with liquid swallows in both postures (older *vs* young patients; RL 8.5 ± 0.5 s *vs* 8.6 ± 0.3 s; UR 8.9 ± 0.8 s *vs* 8.0 ± 0.3 s), and with solid boluses (9.4 ± 1.7 s *vs* 8.3 ± 0.5 s).

DISCUSSION

This study is the first to evaluate motility changes specific to dysphagia in the elderly. It demonstrates subtle differences in LES function between older and younger patients with dysphagia. In particular, increased age was associated with a higher resting pressure, incomplete relaxation and a shorter time to recovery of tone after swallowing. There were, however, no differences in esophageal body pressures or peristaltic duration between age groups. These findings of LES dysfunction provide new insights into the possible mechanisms underlying dysphagia in elderly patients.

Using standard clinical manometric analysis, previous studies reported no differences in the frequency of diagnostic categories between older and younger subjects with dysphagia^[6,14]. This suggests consistency amongst regular reporters of these clinical studies. However, the current study demonstrates specific motility differences between age groups using a more detailed approach to analysis. In agreement with Pandolfino *et al.*^[15], these data suggest that standard manometric assessment may not be sufficiently sensitive to detect potentially relevant abnormalities.

Adequate LES relaxation is pivotal in allowing bolus transit across the gastroesophageal junction (GEJ); this is both intuitive and demonstrated in physiologic and mechanical studies of esophageal emptying^[16]. Studies in patients with achalasia^[17-19] or post-fundoplication^[20,21] confirm the functional consequence of inadequate LES relaxation. Therapeutic modalities aimed at reducing sphincter pressure and thus enhancing bolus clearance are known to reduce dysphagia in these patients^[22,23]. These findings support the concept that high LES nadir pressure and incomplete relaxation observed in elderly patients could impede bolus clearance, and may be directly relevant to the production of their dysphagia.

Increased basal LES pressure observed in our elderly patients did not meet current criteria used to define Hypertensive LES (HLES) syndrome (mean LESP > 45 mmHg)^[24]. However, we postulate that subtle increases in LESP, in combination with failure to relax adequately, may contribute to their dysphagia. Gockel *et al.* demonstrated an association between elevated resting LESP and dysphagia in 100 patients with HLES (defined as BLESP > 26 mmHg). Of these, 71% of subjects reported moderate dysphagia in the absence of any other motility disorder^[25]. There is, however, inconsistency in dysphagia rates between studies, and the pathophysiology and clinical significance of this are uncertain^[24,26,27]. It has also been reported in HLES patients that LES nadir pressure is higher than that seen in healthy subjects, with a shorter relaxation duration^[25,26]. Similar dynamics of the LES were observed in the elderly patients of the current study.

Disturbances in the activation of vagal pathways to the LES (in particular nitric oxide release) underlie impaired sphincter relaxation in motility disorders such as achalasia^[28]. A loss of vagal motor control of the LES has not yet been studied in older patients with dysphagia, however it is known that ageing is associated with degeneration of inhibitory ganglion cells in the intramural esophageal plexus^[29,30]. A significant reduction in the number of neurons innervating the LES leads to increased basal pressure and poor relaxation^[31]. If this age-associated neuronal loss occurs to a pathologic extent, it may produce dysphagia in a subset of older people. Another possibility may be decreased LES compliance, reflecting either changes in the characteristics of the circular muscle or adjacent anatomic structures. Specifically, this may include the crural fibres of the diaphragm and, in some patients, compression due to ectasia of the descending aorta; however further investigation is required.

Adequate propagation and strength of distal esophageal pressures are pivotal in efficient bolus clearance. Studies using impedance and fluoroscopic techniques have both confirmed that a minimum contractile amplitude of 30 mmHg in the distal esophagus is required for sufficient flow across the GEJ^[32,33]. In patients with hypertensive LES, Gockel *et al.*^[25] showed that a quarter of subjects generated pressures in the distal esophagus above 180 mmHg, which is consistent with “nutcracker esophagus”. Previous data regarding the physiologic effect of ageing on esophageal body function, however, suggests an expected decrease in peristaltic amplitude in healthy older people. In the current study, distal esophageal amplitudes in elderly patients with dysphagia were similar to those of younger patients, despite a higher basal LES pressure and impaired LES relaxation. This failure to compensate for increased resistance at the GEJ may further contribute to their symptoms.

In conclusion, these data indicate that subtle impairment of LES relaxation, and thus increased resistance at the GEJ, is a potential contributor to dysphagia in elderly patients. This may reflect the normal ageing process or the pathogenesis of dysphagia, and warrants further study. As there are limited data on LES function in healthy asymptomatic elderly subjects, detailed examination of this area may be of benefit.

COMMENTS

Background

Esophageal dysphagia is common in older individuals and adversely affects both morbidity and mortality. Previous studies have shown changes in esophageal function with advancing age. However, data on the specific mechanisms underlying dysphagia in older patients have yielded conflicting results. A better understanding of the pathophysiology in this group would enable a more targeted approach to treatment.

Research frontiers

Much of our current understanding on esophageal motor abnormalities reported with advancing age is based on data from asymptomatic older individuals. In over 50% of patients with dysphagia, abnormal esophageal motility is seen on manometry. However, no specific differences in the mechanism underlying dysphagia in older patients have been identified. In this study, the authors demonstrate subtle differences in lower esophageal sphincter (LES) function in elderly dysphagic patients using a detailed manometric approach. These findings provide new insights into the possible mechanisms underlying dysphagia in this patient group.

Innovations and breakthroughs

The few studies examining esophageal motor function in older patients with dysphagia provide conflicting data. Recent studies do not support an earlier concept that age-related changes (presbyesophagus) are responsible for symptoms. Broad manometric diagnoses are similar between age-groups, although it is possible that elderly patients comprise a distinct sub-group of non-specific motility disorders. In the current study, LES basal pressure was elevated and relaxation impaired in elderly patients. A possible etiology may be degeneration of neurons innervating the LES, which has been described with ageing.

Applications

There is a need for a greater understanding of esophageal motor changes that are specific to older patients with dysphagia. The absence of a clearly understood pathogenic mechanism in this group currently limits the provision of specific therapeutic recommendations.

Terminology

High resolution manometry is a methodology that improves visualization of esophageal contractility. Intraluminal pressures are displayed as a colour plot over time, using multiple closely spaced pressure sensors (time on x-axis and location of sensors on y-axis). Pressure values between sensors are extrapolated and amplitudes displayed as different colours in the plot.

Peer review

In this manuscript, Besanko *et al* compared esophageal motility between older and younger adults with dysphagia using high resolution manometric data analysis. The trial is carefully performed and clinically important.

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Gastroesophageal reflux disease symptoms: Prevalence, sociodemographics and treatment patterns in the adult Israeli population

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traditional remedies (e.g. soda, milk, almonds, *etc.*). Antacids were used by 35.1%, H₂ blockers by 13.2% and PPIs by 17.5%.

CONCLUSION: We found that 12.5% of the adult Israeli population experience weekly GERD symptoms. GERD prevalence and sociodemographics are similar to those described in other Western countries, and treatment is still suboptimal.

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Key words: Gastroesophageal reflux disease; Prevalence; Sociodemographics

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Abstract

AIM: To evaluate the prevalence and sociodemographics of gastroesophageal reflux disease (GERD) symptoms and to identify treatment patterns among GERD patients.

METHODS: A telephone survey of a representative sample of the adult Israeli population was conducted. The questionnaire included detailed sociodemographics, history of GERD symptoms and the various treatments used.

RESULTS: The survey included 2027 subjects. Twice weekly, once weekly and monthly GERD symptoms were reported by 8.4%, 12.5% and 21.5% of subjects, respectively. There was no difference in prevalence between men and woman; however, GERD symptoms were significantly more prevalent within the older age group and lower socioeconomic status. Among those reporting weekly symptoms, a quarter did not use any kind of therapy and another quarter used various

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a highly prevalent gastrointestinal (GI) disorder and is one of the most common GI illnesses encountered in clinical practice^[1,2]. Heartburn and acid regurgitation are the most common symptoms reported by patients with GERD^[3,4]. The prevalence of GERD has been studied in many parts of the world and has been shown to be high in the Western population^[5]. In the United States, 44% of the adult population have reported experiencing heartburn at least once

a month, 14% have reported experiencing it weekly, and 7% have reported experiencing it daily^[6]. Previous studies have shown an association between GERD and several demographic and behavioral factors such as gender, age, educational level and socioeconomic status^[1,7]. However, inconsistent results have been reported for both GERD prevalence rates and possible associated risk factors in different countries. These inconsistencies may be the result of geographical variation, different lifestyle habits and methodological differences concerning the definition and evaluation of GERD symptoms^[8].

The aim of the present study was to define the prevalence of GERD in Israel, to assess the effect of sociodemographic factors on the occurrence of GERD and to evaluate treatment patterns and use of medications among GERD patients.

MATERIALS AND METHODS

Subjects

The study was conducted in late 2006. A total of 2033 individuals aged 18 years or more were included in the study.

Questionnaire

A telephone questionnaire designed for use in large, population-based studies to assess the prevalence of GERD was administered. The interview was performed by non-health related professional reviewers (Geocartography Group Information Strategy & Solutions, Israel). The interviewers were totally familiar with the questionnaire and were instructed to better explain any eventual question that might not have been fully understood by the interviewed individuals.

After disclosing their age and giving voluntary consent to participate in the study, the participants were asked if they had ever experienced heartburn or gastric content regurgitation. Heartburn was defined as burning feeling starting in the stomach and radiating towards the throat. The questionnaire included questions related to the following information: presence and characteristics of heartburn or regurgitation, frequency of symptoms and type of treatments that were used. The questionnaire also included demographic variables such as: age, gender, ethnic origin, education, monthly income and living conditions.

Statistical analysis

Categorical variables were summarized with number and percentage of patients. The χ^2 and Fisher exact tests were used to compare categorical variables whereas Kruskal-Wallis one-way analysis of variance was used to analyze the demographic data. A $P < 0.05$ was considered statistically significant. Data were analyzed using SPSS version 15.0 (SPSS Inc. Chicago, IL).

RESULTS

The study population consisted of 2027 subjects. There

Table 1 Frequency of gastroesophageal reflux disease symptoms among the studied population

Frequency of heartburn events	No. of patients (%)
Daily	81 (4)
4-6 times/wk	32 (1.6)
2-3 times/wk	57 (2.8)
Once weekly	82 (4.1)
Once/2 wk	65 (3.2)
Once/mo	117 (5.8)
< Once/mo	145 (7.2)
Never	1448 (71.3)
Total	2027 (100)

Table 2 Frequency of gastroesophageal reflux disease symptoms according to various demographic parameters

Parameter	No.	> 1/wk (%)	< 1/wk (%)	None (%)	P value
Gender					
Male	967	12.4	16.1	71.5	0.1
Female	1060	12.5	16.4	71.1	
Age (yr)					
18-34	744	8.7 ¹	17.6	73.7	0.0002
35-54	677	12.2	16	71.8	
> 55	606	17.2 ¹	15.2	67.6	
Education					
Basic	774	15.4 ¹	16.8	67.8	0.03
High school	417	12.2	18.9	68.9	
Academic	836	9.5 ¹	16.7	73.8	
Income (IS)					
< 7000	855	17.8 ¹	19.4	62.9	0.008
7000-10000	571	7.5 ¹	20	72.5	
> 10000	601	9.9 ¹	12.9	77.1	
Ethnic origin					
Oriental	585	16.8 ¹	15.2	68	0.003
Western	539	10	15.1	74.8	
Israeli born	516	9.6 ¹	17.2	73.2	
New immigrant	387	12.8	18.5	68.8	
Number of family Members					
1-2	702	14	16.7	69.3	0.56
3-4	730	12.1	16.5	71.4	
≥ 5	595	11	18.4	70.6	

¹Statistically significant.

were 1060 (52.3%) females and 967 (47.7%) males. The mean age was 44 ± 14 years. 572 individuals (29.6%) reported GERD symptoms at any frequency over the previous year, and 252 individuals (12.5%) reported reflux symptoms occurring at least once a week (Table 1).

Table 2 shows the frequency and severity of GERD symptoms in relation to sociodemographic parameters. There was no difference between males and females regarding GERD symptoms, but GERD symptoms were more frequent among older subjects ($P = 0.0002$). GERD symptoms were significantly more common among subjects with lower education level ($P = 0.03$) and in those with lower income ($P = 0.008$). GERD symptoms were also more frequent among subjects of Oriental ethnic origin, and lower among Israeli-born subjects ($P = 0.003$). All the differences were significant only among the sub-

Table 3 Type of treatment according to heartburn severity (More than one type may be used)

Type of treatment	High heartburn frequency > 1/wk n = 253 (%)	Low heartburn frequency < 1/wk n = 330 (%)	P value
Proton pump inhibitors	50 (19.8)	22 (6.7)	< 0.001
H2 Blockers	33 (13)	24 (7.2)	0.02
Commercial antacids	89 (35.2)	71 (21.5)	< 0.001
Natural and traditional remedies (milk, almonds, ice cubes, etc.)	57 (22.5)	83 (25.2)	0.46
Analgesics	1 (0.4)	10 (3)	0.04
Prescription drugs (does not remember the name)	8 (3.2)	2 (0.6)	0.04
OTC (does not remember the name)	2 (0.8)	10 (3)	0.11
Other	27 (10.7)	31 (9.4)	0.6
Does not remember	14 (5.5)	13 (3.9)	0.36
Does not take any treatment	65 (25.7)	127 (38.5)	0.001

jects who had significant GERD symptoms defined as having symptoms at least once a week.

Table 3 shows the pattern of anti-reflux medications that were used by the study population. Twenty-five percent of subjects with significant GERD (at least once a week GERD episode), and 38.5% of those with less frequent GERD, did not take any treatment. Natural and traditional remedies were used by 22%-25% of subjects and only 20% of subjects with significant GERD used proton pump inhibitors (PPI).

DISCUSSION

The results of the present study show that the prevalence of typical GERD defined by heartburn and/or acid regurgitation in the general adult population in Israel is close to 30%, and 12.5% of this population has suffered from at least one of these symptoms once weekly over the past year. These rates are similar to those reported in prior studies among Western populations, and recently in Israel^[1,3-6,9].

In our study, GERD symptom prevalence was strongly associated with increasing age (17.2% in the > 55 years age group and 12.2% in the 18-34 years age group). However, higher prevalence was observed only in the high frequency patient group (\geq once/wk) but not in the lower frequency group. The effect of increasing age on the prevalence of GERD symptoms has been reported by two European studies^[3,10], but was not seen in a study from Minnesota, USA^[6]. In the UK GP database study, the trend of increased GERD incidence with age was reversed at the age of 69 years^[11], while in the Georgia Medicaid study, the trend reversed earlier, at 55 years^[12]. In all of these studies, GERD was defined on a symptomatic basis which lacks the ability to evaluate whether objective aspects of GERD, such as reflux esophagitis, are more prevalent or severe in older individuals.

A recent study from Israel reported that GERD symptoms are more common and severe among men^[9]. However, our study, similar to several previous cross-sectional and longitudinal studies, did not find a significant association between gender and GERD^[3,6,7,10,12].

An interesting observation of the current study is the effect of low socioeconomic status on the prevalence of

GERD symptoms. Increased prevalence of GERD symptoms was reported in our study by subjects with lower income, lower level of education and by those of Oriental ethnic origin. Previous studies have found an increased GERD prevalence among subjects with lower educational level^[13-14]. Recently, Nocon *et al*^[15] from Germany reported similar results. A reasonable explanation for this observation is that people from a lower social class are more likely to have lifestyle-related risk factors, such as smoking or overweight. However, Nokon *et al*^[15] have found that even after adjusting for smoking and BMI, being in middle or lower social class nearly doubled the risk of severe GERD symptoms. The association of GERD and *Helicobacter pylori* (*H. pylori*) is controversial. While some argue for a preventive role of *H. pylori* in the pathogenesis of GERD by its ability to alter the nature of gastric refluxate, others find no link between the infection and GERD^[16]. The prevalence of *H. pylori* in Israel has been found to be similar to that of other Western countries.

Another important finding of the current study is that a third of the subjects suffering from GERD symptoms do not use any of the accepted medical treatments, such as antacids or antisecretory drugs. Self-medication is still very common, even in those who have reported frequent symptoms, and popular and traditional remedies (as well as antacids) are still widely used.

There are several limitations to this study, and interpretation of the results should be cautious. The questionnaire included only the major and most common GERD symptoms: heartburn and acid regurgitation, but not other symptoms. Extra-esophageal manifestations of GERD were not included. Moreover, symptoms were assessed only by using patient questionnaires and not by a physician. Indeed, in the absence of a gold standard for diagnosing GERD, patient questionnaires remain the common outcome in clinical or epidemiological studies. Finally, we also were unable to obtain data on body mass index, diet, smoking status, and alcohol intake, which may have been confounding factors. However, despite these limitations, our findings are in accordance with population-based studies from other Western countries and with a recent report from Israel.

In conclusion, we found that the prevalence of typical GERD symptoms in the general adult population in Israel

is close to 30%, and 12.5% of this population has suffered from at least one of these symptoms once weekly over the past year. These figures are similar to those found in other Western populations. There is an association between the frequency and severity of GERD symptoms and increased age, low income and low educational level. Antacids and traditional remedies are still widely used, and PPI treatment has not reached its full potential.

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COMMENTS

Background

Gastroesophageal reflux disease, commonly referred to as Gastroesophageal reflux disease (GERD), is defined as chronic symptoms or mucosal damage induced by abnormal regurgitation of fluids from the stomach into the esophagus. The most typical symptoms are heartburn and regurgitation. In most cases, this is a result of inappropriate lower esophageal sphincter (LES) function. Gastroesophageal refluxate contains a variety of noxious agents, mainly acid but also pepsin, bile salts and pancreatic enzymes, and the exposure of the esophageal mucosa to these agents may result in significant injuries to the esophageal mucosa. These injuries include: reflux esophagitis, esophageal stricture, Barrett's esophagus and esophageal adenocarcinoma. Over the past three decades, the incidence of GERD as well as esophageal adenocarcinoma has risen rapidly, especially in Western countries. Lifestyle modifications combined with proton pump inhibitors (PPI) are currently the first-line treatment for subjects with GERD. It is important, therefore, to identify populations at risk for GERD and to recognize those who are inappropriately or inadequately treated. The aim of the current study was to assess the prevalence and risk factors of GERD, presenting as heartburn or regurgitation, among the Israeli adult population and to identify treatment patterns exhibited by this population.

Research frontiers

In the current study the authors placed our emphasis on the importance of sociodemographic factors as risk factors for GERD. In addition, the authors examined to what extent subjects who suffer from GERD are treated appropriately according to current recommendations in the literature.

Innovations and breakthroughs

The authors have found an increased prevalence of GERD symptoms among subjects from lower socioeconomic status, defined as those with lower income, lower level of education and those of Oriental ethnic origin. The authors have also found that a significant proportion of GERD patients are not treated adequately.

Applications

More efforts are needed to identify subjects who are at risk for GERD, to promote lifestyle improving programs and to encourage adequate treatment for GERD in order to prevent complications, especially esophageal adenocarcinoma.

Peer review

In this manuscript, the authors described the prevalence of GERD and its related factors in an adult Israeli population. The study was uniquely performed and interesting.

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Non-sequential narrow band imaging for targeted biopsy and monitoring of gastric intestinal metaplasia

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tive value (PPV), negative predictive value (NPV), and likelihood ratio of positive test (LR+). The number of early gastric cancer cases detected was also reported.

RESULTS: NBI targeted biopsy was performed in 38 and 26 patients during the first and second surveillance endoscopies, respectively. There were 2 early gastric cancers detected in the first endoscopy. No cancer was detected from the second study. Surgical and endoscopic resections were successfully performed in each patient. Sensitivity, specificity, PPV, NPV, and LR+ of all 3 endoscopic criteria during the first/second surveillances were 78.8%/91.3%, 82.5%/89.1%, 72.8%/77.8%, 86.8%/96.1, and 4.51/8.4, respectively. LBC provided the highest LR+ over VP and LLC.

CONCLUSION: Non-sequential NBI is useful for GIM targeted biopsy. LBC provides the most sensitive reading. However, the optimal duration between two surveillances requires further study.

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Key words: Gastric intestinal metaplasia; Gastric cancer; Non-sequential narrowband imaging

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Abstract

AIM: To evaluate the efficacy of non-sequential narrow band imaging (NBI) for a better recognition of gastric intestinal metaplasia (GIM).

METHODS: Previously diagnosed GIM patients underwent targeted biopsy from areas with and without GIM, as indicated by NBI, twice at an interval of 1 year. The authors compared the endoscopic criteria such as light blue crest (LBC), villous pattern (VP), and large long crest (LLC) with standard histology. The results from two surveillance endoscopies were compared with histology results for sensitivity, specificity, positive predic-

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INTRODUCTION

In Correa's gastric cancer cascade, gastric intestinal metaplasia (GIM) is an important precancerous lesion for the development of intestinal type gastric cancer^[1,2]. Although diffuse type gastric cancer can arise independently of GIM, in many cases GIM may be found nearby^[3]. The benefit of annual endoscopic surveillance in these high risk patients has been shown to improve survival by enhancing the detection of gastric cancer at the early stage^[4]. However, conventional white light endoscopy has a limitation in selection of the area for surveillance biopsy^[5]. Multiple random biopsies under white light endoscopy to detect GIM are quite cumbersome as they are time-consuming and have the possibility of missing a small lesion. Methylene blue chromoendoscopy is the only technique that provides a good validity score for GIM targeted biopsy^[6,7]. However, this technique still has a limitation because of the need for dye spraying. Moreover, it has been shown that DNA damage can occur in columnar cell-lined mucosa after chromoendoscopy with methylene blue, thus DNA damage to the GIM epithelium may also occur as it is also an intestinal type columnar cell^[8]. Magnifying narrow band imaging (NBI) has been introduced which negates the need for dye spray. This technique provides better details of the mucosa and vascular patterns of minute lesions, including early gastric adenocarcinoma and GIM^[9,10]. Uedo *et al.*^[11] reported on the excellent accuracy (91%) of the light blue crest (LBC) pattern detected by NBI in predicting the likelihood of GIM. Using a similar method but without magnification, a group from Missouri demonstrated that the ridge/villous pattern of the gastric mucosa detected by NBI was also useful for the diagnosis of GIM, with a sensitivity and specificity of 80% and 100%, respectively^[12]. However, these pioneer investigators used the sequential NBI system that is mainly available in Japan and South Korea. This system is not widely available for other commercial users. The slight differences in color spectrum of sequential and non-sequential NBI processors provide some differences in color images^[13]. Therefore the excellent results from the sequential system may not be replicable in the non-sequential system. To date there has been no study on the role of non-sequential NBI for the detection of precancerous gastric lesions. We therefore conducted a prospective endoscopic study to validate the feasibility of non-sequential NBI by using a combination of endoscopic criteria to determine the leading area for GIM biopsy.

MATERIALS AND METHODS

Non-sequential NBI

In the non-sequential NBI system, there is a rotating interference narrow band filter (R/G/B). The specific colors are transferred directly without alteration in R/G/B color pattern from the color charge-couple device (CCD) as a picture containing different mucosal depth patterns. This

incomplete image is further transformed with a matrix element, coefficient K, by a computer to a more complete image that provides a better contrast^[14]. The scope that we used in this study was a magnifying gastroscope (Olympus GIF Q160Z, Olympus, Tokyo, Japan) which is compatible with the non-sequential light source and processor EVIS Exera II (Olympus, Tokyo, Japan). This scope had a zoom lens placed just distal to the CCD. The CCD was located at the tip of the endoscope, and the optical power of magnification was 115 times. To maintain the optimal distance of the magnifying focus, a transparent plastic cap was attached at the scope tip.

Patients

From November 2007 to May 2009, at the King Chulalongkorn Memorial Hospital Endoscopy Unit, we recruited all patients with GIM previously diagnosed by routine endoscopic biopsy. Generally, the initial endoscopy was performed for dyspepsia, and a random biopsy was performed at that time. Patients with abnormal coagulopathy, patients who refused to give informed consent, and patients who had previous subtotal gastrectomy were excluded from the study. There were 38 eligible patients enrolled who had a prior history of GIM. All patients underwent, 1 year apart, two upper endoscopies with NBI targeted biopsy. The last procedure was performed in May 2009. The study protocol was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University.

Endoscopic criteria for GIM diagnosis

All of the endoscopic readings were performed at 115 x magnification. Based on the previous studies results^[11,12], we selected three criteria as positive readings for GIM: (1) Normal gastric mucosa was defined as a uniform pattern of round pit gastric mucosa (Figure 1A); (2) Light blue crest (LBC) was defined as a fine, blue-white line on the crests of the epithelial surface, which looked like light reflection from the mirror (Figure 1B)^[11]; (3) Villous pattern (VP) was defined as a raised area of villi above the gastric mucosal surface (Figure 1C)^[12]; and (4) Large long crest (LLC) was defined as a combination of linear dark and light areas that differed from the normal gastric epithelium (Figure 1D)^[12].

Procedures and biopsy protocol

All endoscopies were performed by one gastroenterology fellow (BI) under supervision of one senior attending endoscopist (RR). Both endoscopists had experience in using NBI for other lesions, including colonic adenoma and minimal erosive reflux esophagitis, in more than 200 cases. After obtaining consent, 5 mg intravenous midazolam was injected to sedate each patient. In addition, 10 mg hyoscine was also given intravenously to decrease bowel movement for easier endoscopic visualization. Simethicone solution was used to reduce mucus and gas bubbles in the stomach. We elected to do biopsies from the an-

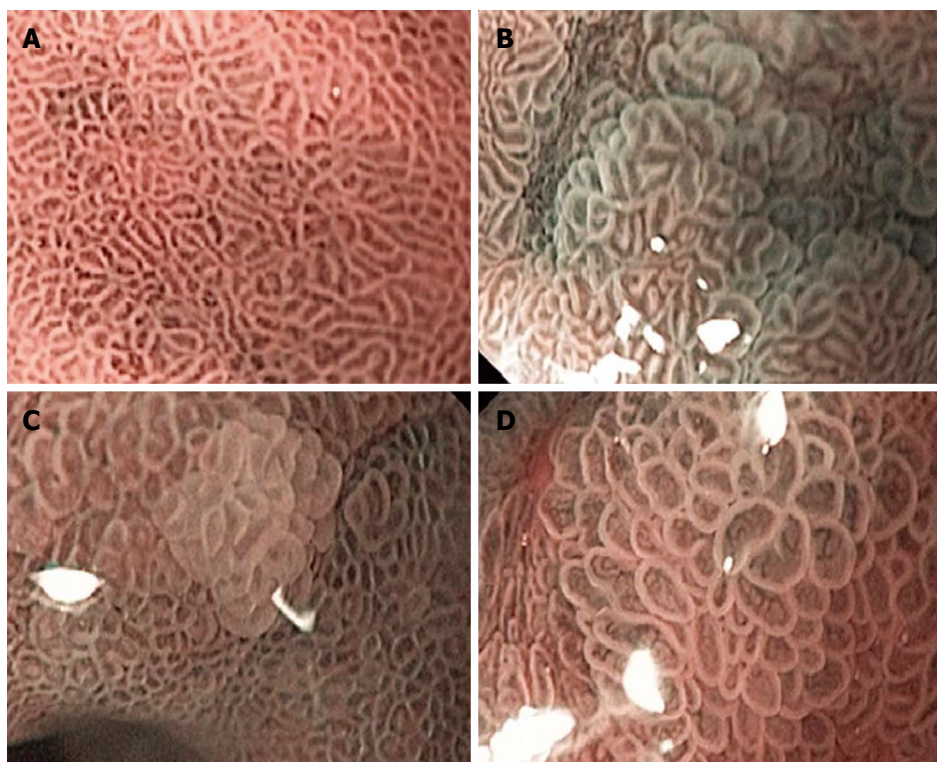


Figure 1 Endoscopic findings with narrow band imaging mode. A: Normal gastric mucosa under magnifying narrow band imaging (NBI); B: Light blue crest (LBC) under 115 × magnification of NBI; C: A raised area of gastric villi compatible with villous pattern (VP); D: Large long crest (LLC) is a combination of linear dark and light areas that differ from the normal gastric epithelium.

Table 1 Baseline characteristics of 38 patients

Average age (yr), mean ± SD (range)	59.9 ± 11.5 (27-80)
Gender	
Male:Female	20:18
Median interval duration after initial diagnosis (yr)	1
Type of GIM	All complete GIM
Positive rapid urease test in 1st endoscopy	12/38
Positive rapid urease test in 2nd endoscopy	8/26
Dyspeptic symptoms	5/38
Smoking	None
Family history of gastric cancer	None
Significant underlying disease	5/38

GIM: Gastric intestinal metaplasia

trum and incisura (area with the possible highest probability of GIM). In detail, a screening non-magnifying white light endoscopy was performed first. Then NBI was performed to target GIM. Six NBI snapshot images were obtained from four quadrants of the antrum and two areas of the gastric incisura. If there was any positive finding by one of the three criteria mentioned above (LBC or VP or LLC), that area was counted as positive for GIM and a targeted biopsy was taken from the positive lesion(s). If there was no positive finding from that area, one random biopsy was taken from that quadrant as a GIM negative specimen. Therefore, six magnifying images and their

targeted biopsies were derived from each patient. Pilot cohorts ($n = 10$) were used for training in biopsy techniques and endoscopic readings. *Helicobacter pylori* infection was examined by a rapid urease test. Eradication of *H. pylori* was carried out if the urease test or histology was positive for infection. A standard triple therapy in this study consisted of amoxicillin, clarithromycin, and omeprazole given orally for 14 d. One year after the first endoscopy, eligible patients underwent the second surveillance gastrointestinal (GI) endoscopy, using a similar protocol to the first surveillance.

Histological assessment and monitoring of GIM

All gastric specimens were immersed in formalin and processed by embedding in a paraffin block. A 4 µm section of the specimen was later stained with hematoxylin-eosin, Alcian blue and Giemsa stain. A clinically blinded pathologist (NK) reviewed all the specimens. The updated Sydney classification^[15] was referred to as the gold standard for gastric histological classification. Regarding GIM monitoring on each patient, a comparison was made between the first and second gastric biopsy results. Patients were classified as having persistence, regression, or progression of GIM based on the existence or absence of GIM on the first and second histologies.

Diagnostic accuracy and statistical analysis

The diagnostic accuracies of all three criteria (LBC or VP or LLC) were judged after the index endoscopy by

Table 2 Validity scores with confidence intervals of the first endoscopic targeting biopsy for gastric intestinal metaplasia, mean (range)

Criteria	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR + (%)
LBC	70.59 (59.57-79.72)	83.22 (75.75-88.75)	71.43 (60.37-80.49)	82.64 (75.24-88.25)	4.21 (2.85-6.21)
VP	29.41 (20.28-40.43)	97.90 (93.51-99.45)	89.28 (70.63-97.19)	70.00 (63.06-76.16)	14.02 (4.36-45.03)
LLC	17.65 (10.53-27.75)	95.10 (89.79-97.84)	68.18 (45.12-85.26)	66.02 (59.06-72.36)	3.60 (1.53-8.48)
All criteria	78.82 (68.34-86.64)	82.51 (75.08-88.16)	72.83 (62.38-81.33)	86.76 (79.63-98.73)	4.51 (3.11-6.55)

PPV: Positive predictive value; NPV: Negative predictive value; LR +: Likelihood ratio of positive test; LBC: Light blue crest; VP: Villous pattern; LLC: Large long crest.

comparing the endoscopic diagnosis with histology results from that site. A descriptive comparison between endoscopic and pathological readings was performed. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio of positive test (LR+) for the diagnosis of GIM were calculated. The calculations were performed with SPSS statistical software (SPSS version 15, Inc., Chicago, IL, USA).

RESULTS

During the initial surveillance endoscopy, there were 38 patients (20 male, 18 female) with a previous diagnosis of GIM (Table 1). The mean age \pm SD was 59.9 ± 11.5 years (range, 27-80 years). The median time from diagnosis of GIM to the first surveillance endoscopy was 2.2 years. The median interval between the two surveillance endoscopies was 1 year. Twenty-six patients returned for a second surveillance study. The reasons for those 12 patients not attending the second study were: being diagnosed as having another type of cancer and requiring further treatment ($n = 4$), withdrawal from the second study ($n = 4$), loss to follow-up ($n = 2$), and diagnosis with early gastric cancer from the first surveillance endoscopy ($n = 2$).

From the first surveillance endoscopy, most of the background endoscopic findings indicated atrophic gastritis. Thirty one of 38 patients (81.6%) were found to have GIM. The overall number of positive specimens was 85/228 (37.3%). Each GIM positive patient had two specimens positive for GIM on average. All GIM cases were complete GIM (type I). Of these, 12 patients had a positive rapid urease test (31.6%) and all received standard triple therapy for eradication. Under standard white light endoscopy, five patients were suspected to have GIM because of a whitish color change with plaques or patches. With NBI, the sensitivity, specificity, PPV, NPV, and LR+ of the three endoscopic criteria were 78.8%, 82.5%, 72.8%, 86.8%, and 4.5, respectively, while LBC possessed the highest LR+ compared with VP and LLC (Table 2).

The first surveillance study was able to detect early gastric cancer in two patients. The first patient was diagnosed with GIM a year before recruitment. NBI was able to demonstrate a focal area of depressed mucosa with loss of the normal vascular pattern. At that time, the technique of endoscopic submucosal dissection (ESD) was

not available in Thailand, hence the patient was sent for a subtotal gastrectomy. The gastric pathology confirmed a minute area of signet ring carcinoma involving only the submucosal area. The patient has been doing well without evidence of recurrence after a 2-year follow-up. The second patient had been treated as having a *Helicobacter*-associated antral ulcer, and found to have diffuse GIM. Six months later, at the first surveillance endoscopy, *H. pylori* eradication and healing of the previous ulcer were confirmed but a new elevated lesion with abnormal subepithelial vascular network in the lesser curvature was discovered. Pathology confirmed it as an intestinal type gastric adenocarcinoma. This patient underwent a successful ESD. The resected specimen showed malignant cells confined to the submucosal level without any vascular invasion.

During the second surveillance endoscopy, two patients still had persistent *H. pylori* infection confirmed by a rapid urease test. Six other patients became positive for *H. pylori* infection in the second rapid urease test. All these patients, including those with a persistent positive rapid urease result, received standard triple therapy for eradication. Clarithromycin was replaced with metronidazole for the second round of triple therapy in the two patients with persistent infection. During the second surveillance endoscopy, 17/26 patients were persistently positive for GIM, 5/26 had disappearance of GIM, and 4/26 were persistently negative for GIM. No new GIM was detected in those who were previously negative for GIM. No gastric cancer was found in this second study. The sensitivity, specificity, PPV, NPV, and LR+ from the second endoscopy were 91.3%, 89.1%, 77.8%, 96.1%, and 8.4, respectively (Table 3).

DISCUSSION

Sequential NBI has been well recognized as an excellent tool to detect abnormal mucosal GI lesions in certain Asian countries such as Japan, Korea, and Hong Kong, and in some US/Europe research centers^[16-18]. However, most GI endoscopists worldwide have no access to this system and instead, they are more familiar with the non-sequential system. Many endoscopists are impressed with the difference in the contrast and the brightness of the non-sequential image compared with those images published in the literature using sequential NBI^[13,19].

Table 3 Validity scores with confidence intervals of the second endoscopic targeting biopsy for gastric intestinal metaplasia, mean (range)

Criteria	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR + (%)
LBC	82.61 (68.05-91.68)	92.73 (85.74-96.58)	82.61 (68.05-91.68)	92.73 (85.74-96.58)	11.36 (5.75-22.42)
VP	21.74 (11.45-36.76)	98.15 (92.81-99.67)	83.33 (50.88-97.06)	74.65 (66.53-81.40)	11.74 (2.67-51.48)
LLC	43.48 (29.25-58.79)	99.07 (94.20-99.95)	95.24 (74.13-99.75)	80.45 (72.49-86.61)	46.95 (6.49-339.58)
ALL	91.30 (78.31-97.17)	89.09 (81.36-93.98)	77.78 (64.05-87.52)	96.07 (89.69-98.73)	8.37 (4.87-14.38)

PPV: Positive predictive value; NPV: Negative predictive value; LR +: Likelihood ratio of positive test; LBC: Light blue crest; VP: Villous pattern; LLC: Large long crest.

Studies of the feasibility of NBI to detect a minute abnormality in the GI tract mucosa have been widely published^[10-12,16-18]. Certain patterns of abnormal gastric mucosa have been described^[16-18]. LBC is an important endoscopic marker for GIM described by distinctive sharp blue and white on the gastric epithelial crest. This lesion can be best assessed by at least 80 × magnification with NBI. When sequential NBI was applied in 107 consecutive GIM patients, Uedo *et al*^[11] reported that LBC criteria had a sensitivity of 89% [95% confidence interval (CI): 83-96], a specificity of 93% (95% CI: 88-97), a PPV of 91% (95% CI: 85-96), a NPV of 92% (95% CI: 87-97), and an accuracy of 91% (95%CI: 88-95).

VP and LLC represent the thickness of the abnormal GIM epithelium on top of the regular gastric surface. In one non-magnifying sequential NBI study, Bansal *et al*^[12] reported that the sensitivity, specificity, and PPV of the VP and LLC criteria (the original series naming villous and ridge patterns) to diagnose GIM were 80%, 100%, and 100%, respectively.

We found that LBC was the most sensitive recognized endoscopic pattern (71.2% and 82.6% during the first and second endoscopic surveillances, respectively). It seems that our LBC sensitivity was lower than the results reported by Uedo *et al*^[11]. We speculate that different types of NBI may have had a bearing on the difference in the results. In addition, from our observation that the background gastric mucosa in Thais with GIM contained some gastric inflammation, determining LBC from an unclear gastric mucosal background can be quite difficult. Despite magnifying the images, we found less impressive results using VP and LLC criteria when compared with the original series results (46.2% during the first endoscopy and 65.2% during the second endoscopy). When we combined all three criteria together (LBC, VP and LLC), the overall sensitivities went up to 78.8% and 91.3% during the first and second surveillances respectively. In addition, our VP and LLC criteria provided comparable results on the specificity (82.7%-99.1%), PPV (71.4%-88.2%), NPV (69.7%-96.1%), and LR+ (69.2%-89.7%) with those of LBC.

When compared with the first surveillance results, the second surveillance in our study provided higher levels of all validity scores (sensitivity, specificity, PPV, NPV, and LR+). However, these did not reach statistical difference.

During the present study, we were able to detect two

early gastric cancers. Both were detected during the first surveillance and underwent successful resection. After resection both patients have been doing well.

Although a gastric cancer screening and surveillance program is only available in certain countries such as Japan and Korea, many countries have seen an increase in the prevalence of this cancer^[20]. In Thailand, gastric cancer screening has not yet become a national health policy due to an insufficient number of the endoscopists, and a lower prevalence of gastric cancer in Thailand^[21].

H. pylori infection, smoking, chronic atrophic gastritis, heavy alcohol use, and several dietary factors have been recognized as risk factors for gastric cancer^[22]. The potential factors for gastric cancer in our population are chronic/atrophic gastritis and *H. pylori* infection. The majority of our patients had chronic or atrophic gastritis as their background. *H. pylori* infection rates were found in one third. No other significant risk factors could be determined in our cohorts. None of our patients were smokers and none had a family history of gastric cancer. They were in middle age and the youngest patient was only 32 years old. Despite few risk factors being identified, we were able to detect two early gastric cancers from these cohorts.

To put this into a more practical surveillance protocol, we may have to look for patients with atrophic gastritis by standard white light endoscopy first and later do a targeted GIM biopsy by NBI. However, cost effectiveness may be an issue for a country with low gastric cancer prevalence, such as Thailand.

GIM can either progress or regress depending on its nature and environmental stimuli^[23]. *H. pylori* eradication may help the regression of GIM. All patients infected with *H. pylori* (12/38; 31.6%) received an eradication regimen after the first surveillance. During the second surveillance, we found disappearance of GIM in only 5/26 patients (19.2%). Interestingly, those were patients with positive for *H. pylori* infection from the first study and GIM disappeared after *H. pylori* eradication (data not shown).

Furthermore, we identified two patients who had persistent infection with *H. pylori* during the follow-up endoscopy. These two received different triple therapy. In addition, six more patients had newly discovered *H. pylori* infections during the second endoscopy. All were patients persistently positive for GIM. The explanation for the finding of new infection was uncertain, but we speculate that these were false negative cases in the first urease tests.

It had been our routine to discontinue proton pump inhibitor, H2 blocker, and antibiotics at least 2 wk prior to the test for *H. pylori*. Thus prior medication use was not a cause for our false negative. Gastric mucosa has dynamic regeneration. This regenerated mucosa may be a factor in the re-growth of the occult *H. pylori* infection.

The limitations of the present study are mainly the small number of patients and the short 1-year duration of follow-up. Some patients dropped out from the second surveillance endoscopy for a variety of reasons. Despite these limitations, we were able to identify two early gastric cancers (5.3%) from this high risk cohort, and this was the first time in Thailand that we were able to select a group that may gain benefit from this intensive endoscopic surveillance.

In conclusion, non-sequential NBI is an interesting technique that can facilitate GIM detection by targeted biopsy. It helps the detection of early gastric cancer in patients with atrophic gastritis and GIM. However, the precise protocol regarding the frequency and duration of follow-up, and how to identify this target group still requires further study.

COMMENTS

Background

Gastric intestinal metaplasia (GIM) is considered as a precancerous lesion. The benefit of annual endoscopic surveillance in patients with GIM has been shown to improve survival by enhancing the detection of gastric cancer at the early stage. However, conventional white light endoscopy has a limitation in its sensitivity. In addition, this technique is quite cumbersome because it is time consuming and may miss small lesions.

Research frontiers

Previously, chromoendoscopy has been proven to provide a good validity score for GIM targeted biopsy. Without a need of dye spray and only switching on a button, magnifying NBI has become a good alternative for the detection of GI precancerous lesions including colonic polyps, Barrett esophagus, and GIM. However, these pioneer results were based on the sequential NBI system and there may be slight differences in color spectrum between sequential and non-sequential NBI.

Innovations and breakthroughs

The present study supports the majority of practices that use the non-sequential system. It showed a higher accuracy for targeted biopsy in GIM suspicious lesions over previously reports with conventional white light endoscopy.

Applications

Instead of using a dye spray, the non-sequential NBI system can be more practical in routine practice. With comparable accuracy to the sequential system, it provides high diagnostic yields for GIM detection.

Terminology

Light blue crest (LBC) is defined as a fine, blue-white line on the crests of the epithelial surface, which looks like light reflection from a mirror. Villous pattern (VP) is defined as a raised area of villi above the gastric mucosal surface. Large long crest (LLC) is defined as a combination of linear dark and light areas that are different from normal gastric epithelium.

Peer review

The present study described the usefulness of non-sequential NBI, that is much more available worldwide, for targeted biopsy of GIM and surveillance for early gastric cancer. This system showed high sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio of positive test for GIM prediction. Among the three criteria purposed by the authors, LBC seems to provide the best diagnostic value. Although the number of patients was limited, these patients with GIM provided a good set for this observation.

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Biochemically curative surgery for gastrinoma in multiple endocrine neoplasia type 1 patients

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Abstract

AIM: To search for the optimal surgery for gastrinoma and duodenopancreatic neuroendocrine tumors in patients with multiple endocrine neoplasia type 1.

METHODS: Sixteen patients with genetically confirmed multiple endocrine neoplasia type 1 (MEN 1) and Zollinger-Ellison syndrome (ZES) underwent resection of both gastrinomas and duodenopancreatic neuroendocrine tumors (NETs) between 1991 and 2009. For localization of gastrinoma, selective arterial secretagogue injection test (SASI test) with secretin

or calcium solution was performed as well as somatostatin receptor scintigraphy (SRS) and other imaging methods such as computed tomography (CT) or magnetic resonance imaging (MRI). The modus of surgery for gastrinoma has been changed over time, searching for the optimal surgery: pancreaticoduodenectomy (PD) was first performed guided by localization with the SASI test, then local resection of duodenal gastrinomas with dissection of regional lymph nodes (LR), and recently pancreas-preserving total duodenectomy (PPTD) has been performed for multiple duodenal gastrinomas.

RESULTS: Among various types of preoperative localizing methods for gastrinoma, the SASI test was the most useful method. Imaging methods such as SRS or CT made it essentially impossible to differentiate functioning gastrinoma among various kinds of NETs. However, recent imaging methods including SRS or CT were useful for detecting both distant metastases and ectopic NETs; therefore they are indispensable for staging of NETs. Biochemical cure of gastrinoma was achieved in 14 of 16 patients (87.5%); that is, 100% in 3 patients who underwent PD, 100% in 6 patients who underwent LR (although in 2 patients (33.3%) second LR was performed for recurrence of duodenal gastrinoma), and 71.4% in 7 patients who underwent PPTD. Pancreatic NETs more than 1 cm in diameter were resected either by distal pancreatectomy or enucleations, and no hepatic metastases have developed postoperatively. Pathological study of the resected specimens revealed co-existence of pancreatic gastrinoma with duodenal gastrinoma in 2 of 16 patients (13%), and G cell hyperplasia and/or microgastrinoma in the duodenal Brunner's gland was revealed in all of 7 duodenal specimens after PPTD.

CONCLUSION: Aggressive resection surgery based on accurate localization with the SASI test was useful for biochemical cure of gastrinoma in patients with MEN 1.

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Key words: Gastrinoma; Duodenopancreatic neuroendocrine tumors; Multiple endocrine neoplasia type 1; Selective arterial secretagogue injection test; Somatostatin receptor scintigraphy; Pancreas-preserving total duodenectomy; Pancreaticoduodenectomy

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INTRODUCTION

Controversy has surrounded the treatment strategy for gastrinoma and neuroendocrine tumors (NETs) in patients with multiple endocrine neoplasia type 1 (MEN 1) and Zollinger-Ellison syndrome (ZES)^[1-14]. It has been confirmed that ZES in patients with MEN 1 is caused mostly by duodenal gastrinomas^[15,16]. Some surgeons have not recommended surgery for duodenopancreatic gastrinoma, because of both low biochemical cure rate of gastrinoma and early recurrence of gastrinoma after surgery^[8,9]. In contrast, surgeons who have performed aggressive duodenopancreatic resection have reported a higher biochemical cure rate of gastrinoma after surgery, although these studies included relatively small numbers of patients^[4-7,11-14].

We have performed curative resection surgery for gastrinoma in 41 patients with ZES guided by localization using the selective arterial secretagogue injection test (SASI test)^[17,18]. Guided by localization with the SASI test, pancreaticoduodenectomy (PD) was performed for 10 patients, of whom 3 patients were classified as MEN 1, and all of them have been cured of gastrinoma postoperatively. Pathological examination of the duodenopancreatic specimens resected from the MEN 1 patients revealed single or multiple gastrinomas < 10 mm only in the duodenum, but not in the pancreas head. Thus, we have changed the modus of resection surgery for gastrinomas in patients with MEN 1 from PD to transduodenal excisions of the duodenal gastrinomas or partial duodenectomy (LR) with dissection of the regional lymph nodes, while seeking for less invasive and optimal surgical resection for gastrinomas in MEN 1 patients. Recently, we have performed pancreas-preserving total duodenectomy (PPTD) for MEN 1 patients with multiple gastrinomas and/or numerous microgastrinomas in the duodenum^[19,20]. Here, we report the results of our surgical strategy for both gastrinoma and pancreatic NETs in MEN 1 patients, and discuss the optimal surgery for

patients with MEN 1 and gastrinomas from a viewpoint of the staging of both gastrinoma and pancreatic NET in these patients.

MATERIALS AND METHODS

Patients

Sixteen patients with genetically confirmed MEN 1 and gastrinoma underwent resection surgery for gastrinomas and pancreatic NETs by a team comprising a chief surgeon (senior author) and co-surgeons (co-authors) at the Departments of Surgery of Graduate School of Medicine, Kyoto University, Osaka Saiseikai Noe Hospital and Kansai Electric Production Company Hospital between March 1991 and March 2010.

All patients were examined for MEN 1 gene mutations by a co-author (MK) at the Medical Gene Research Center, Kyoto University. A diagnosis of ZES was established by confirming the co-existence of gastric hyperacidity and hypergastrinemia. Levels of gastrin were > 80 pg/mL in patients who had undergone distal or total gastrectomy and > 200 pg/mL in patients who had not undergone distal gastrectomy^[21]. Gastric hyperacidity was confirmed using 24 h pH monitoring, and was diagnosed when the percentage of the time that the gastric pH was 0-4 was > 70%^[21]. Either the secretin test or the calcium test was performed for all patients^[22-24]. The secretin test was performed by bolus intravenous injection of secretin (3 U/kg body weight). Blood samples were collected from a cubital vein before and 2, 4, and 6 min after secretin injection. An increase in serum immunoreactive gastrin concentration (IRG) both of > 20% of the basal serum IRG and > 80 pg/mL, 4 min after secretin injection was considered positive. The calcium test was performed by injecting 1.17 mEq calcium solution (1 mL of 0.39 mEq calcium gluconate hydrate) diluted with 2 mL physiological saline over 30 s into a cubital vein^[22-24]. The intraoperative secretin test was performed using the same method as the pre-operative secretin test, and results were obtained within 60 min using rapid radioimmunoassay of serum gastrin levels^[25].

Localization of gastrinoma

For localization of gastrinoma, the SASI test with secretin (Secrepan[®] 30 units) or calcium solution (0.39 mEq calcium gluconate diluted with 2 mL physiological saline) was performed for all patients as described previously^[17,18,26]. The principle of the SASI test is to identify the feeding artery of gastrinoma by stimulating gastrinoma to release gastrin using a secretagogue^[17]. We used secretin until 2004, since then we have used calcium gluconate hydrate solution, because production of secretin in Japan ended in 2004^[10]. CT, MRI or US have been used primarily for detection of distant metastases, such as hepatic metastases or large lymph nodes^[11,10,11].

PPTD

PPTD was performed using a new technique described

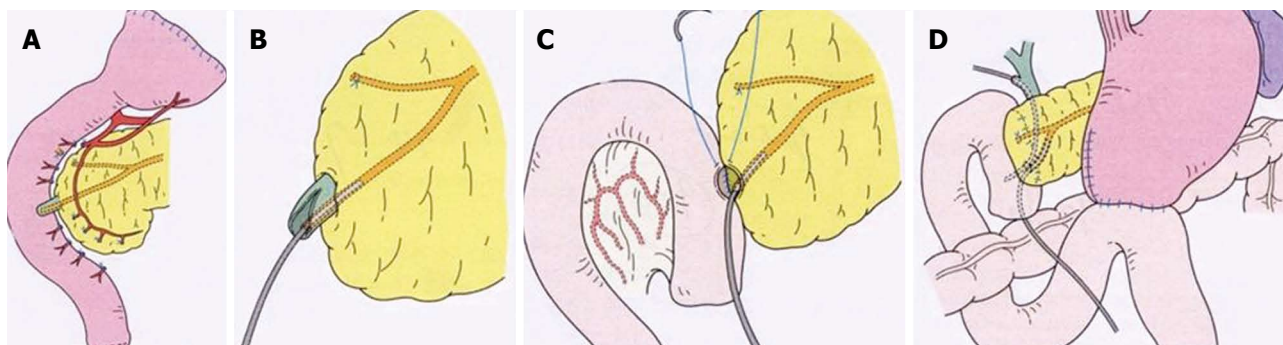


Figure 1 Pancreas-preserving total duodenectomy technique. A: The duodenum is separated from the head of the pancreas by cutting the branches of the pancreaticoduodenal arcade vessels. The choledochal trunk is saved and only the membrane of the major papilla is shaved sharply. The accessory pancreatic duct is ligated and cut; B: Papillotomy is performed on the major papilla at 0, and then a catheter is inserted into the main pancreatic duct for stenting; C: Bilio-jejunal reconstruction. The edge of the common bile duct is sewn to a small opening on the jejunum with 4-0 absorbable knotted sutures; D: The final reconstruction schema of the alimentary tract.

elsewhere^[20]; briefly, when resecting the entire duodenum, only a mucosectomy is performed on the duodenal major papilla portion, retaining the structure of the major papilla, and after an 8 mm long sphincterotomy, the opened papilla is anastomosed to the incisional opening of the jejunum^[20]. Details of the procedure are shown in Figure 1.

Pathological examination of the resected specimens

Resected duodenopancreatic tissues including any suspected NETs or lymph nodes were fixed in a 10% formalin solution and embedded in paraffin. Paraffin-embedded sections were stained with Masson-Fontana, Grimelius, and Hellerstrom-Hellman silver stains. Immunohistochemical staining was performed with Simple Stain MAX-PI (Multi) (mouse and rabbit/horseradish peroxidase) reagent (Nichirei, Tokyo, Japan) using polyclonal rabbit anti-human gastrin serum (Dako, Glostrup, Denmark).

Criteria of biochemical cure of gastrinoma

Cure of gastrinoma was defined as a normal fasting serum IRG < 150 pg/mL in patients without a history of gastrectomy and < 80 pg/mL in patients with a history of gastrectomy, and/or a negative secretin test or a negative calcium test during the 6 mo follow-up surveillance period. Survival curve analysis was performed using the Kaplan-Meier method.

RESULTS

PD

Between 1991 and 1997, PD was performed for 3 patients with ZES based on localization guided only by the SASI test, because imaging methods (CT, MRI, US) did not visualize any tumor in the abdomen (Table 1). In 3 patients, the SASI test localized the gastrinoma in the upper part of the duodenum and/or the head of the pancreas, thus PD was performed for all patients. Preoperative localization by the SASI test was correct, and gastrinomas were proved in the duodenum; that is, 7 duodenal gastrinomas in 1 patient (No. 2) and only 1 duodenal gastrinoma in 2 patients

(Nos. 1 and 3). Metastatic lymph nodes associated with the duodenal gastrinoma were identified in 2 patients. Two patients (Nos. 1 and 2) had multiple nonfunctioning NETs in the head of the pancreas (Table 1). The preoperative serum IRG of these patients ranged between 310 and 800 pg/mL, and the postoperative serum IRG decreased in all patients to < 33 pg/mL. The postoperative secretin test was negative in all patients. One patient died of other causes 4 year after undergoing the PD. Two patients are alive and well, and biochemical cure of gastrinoma has continued for 18 year 5 mo and 12 year, postoperatively.

LR

Since 1996, 5 patients have successively undergone local resection of duodenal gastrinoma through duodenectomy after a 7 year intermission. 1 patient (No. 9) underwent DX in 2009 based on localization by the SASI test, duodenoscopy revealed duodenal submucosal NETs in 3 patients, and CT visualized a few metastatic lymph nodes more than 2 cm with a pancreatic NET more than 1 cm in 2 patients (Table 2). Localization by the SASI test was correct in all of them. In case No. 9, gastrinoma was located not only in the duodenum, but also in the head of the pancreas. Size of duodenal gastrinoma was between 1-15 mm in diameter. Any pancreatic NETs > 1 cm were treated by enucleation and/or distal pancreatectomy. In 3 patients, metastatic lymph nodes were associated with duodenal gastrinoma.

Most of these patients were biochemically cured of gastrinoma after the first LR, but ZES recurred in 2 patients (Nos. 5 and 6). In patient No. 5, the serum IRG increased from 140 to 170 pg/mL 8 year postoperatively, and in patient No. 6, the serum IRG increased from 68 to 400 pg/mL 6 year postoperatively. Based on localization by the SASI test, a second LR was performed for these patients, and their serum IRG levels decreased to within normal ranges postoperatively. They have been biochemically cured of gastrinoma since the second LR for 5 year, 8 mo and 2 year, 7 mo, respectively, postoperatively.

Patient No. 9 had undergone a distal pancreatectomy

Table 1 Results of pancreaticoduodenectomy for patients with multiple endocrine neoplasia type 1

No.	Age	Gender	ZES	Ulcer diseases	Ulcer related operation	MEN 1 related diseases	Pre-PD IRG (pg/mL)	Localization of gastrinoma			Operation	Post-PD IRG (pg/mL)	Gastrinoma		Metastases		Pancreas NET	Prognosis present status (post-op yr)	Postoperative secretin or calcium test		
								SASI	GIF	CT			Location	Number	Size (mm)	N				L	
1	44	M	+	DU 1984	-	Pit NET 1987	310	GDA ND ND	IPDA	ND	PitX T PitX PD	1989 Mar 1990 Mar 1991 Mar	26	D	1	5	1	0	5	Alive well with Pit NET, PNET (18 yr 5 mo)	Negative
2	39	F	+	GU perf 1982 Ileus 1983 JU 1990	GX 1982 JX 1983	HPT 1991 Nov	800	GDA ND ND	IPDA	ND	PD, PX St ParX	1991 Mar 1991 Nov	33	D	7	1-7	1	0	6	DOD (4 yr) no recur	Negative
3	21	M	+	DU, GU 1997	-	Pit NET 1997	583	GDA ND ND	ND	ND	PD PitX	1997 Aug 1997 Oct	25	D	1	10	0	0	0	Alive will (12 yr) no recur	Negative

ZES: Zollinger-Ellison syndrome; PD: Pancreaticoduodenectomy; SASI: Selective arterial secretagogue injection test; GIF: Gastrointestinal fibroscopy; CT: Computed tomography; IRG: Serum immunoreactive gastrin concentration; Ni: Lymph node metastasis; L: Liver metastasis; NET: Neuroendocrine tumor; postop: Postoperative; F: Female; M: Male; DU: Duodenal ulcer; GU: Gastric ulcer; JU: Jejunal ulcer; perf: Perforation; GX: Partial gastrectomy; JX: Partial jejunectomy; Pit: Pituitary; PitX: Extirpation of pituitary tumor; T PitX: Total resection of pituitary gland; HPT: Hyperparathyroidism; St ParX: Subtotal parathyroidectomy; GDA: Gastroduodenal artery; IPDA: Inferior pancreaticoduodenal artery; ND: Not detected; PX: Partial resection of the pancreas; D: Duodenum; P: Pancreatic; DOD: Died of other disease; Dsmt: Duodenal submucosal tumor; diff: Diffuse; D-EUS: Duodenal endoscopic ultrasonography; no recur: No recurrence.

for multiple insulinoma 31 year before visiting our clinic. She also had a history of a total parathyroidectomy with a forearm subcutaneous parathyroid transplantation and gamma knife therapy for a pituitary NET. Her serum IRG was 49 500 pg/mL. Multiple submucosal gastric NETs and multiple duodenal submucosal NETs were identified by gastroduodenoscopic examination. A few large metastatic lymph nodes around the head of the pancreas were visualized using CT; therefore, advanced stage of gastrinoma was suspected. The SASI test localized the gastrinoma in the duodenum and/or the head of the pancreas. We performed LR and an enucleation NET in the head of the pancreas with dissection of the peripancreatic lymph nodes. A partial resection of the middle part of the stomach for multiple gastric tumors was also performed. Her serum IRG decreased to < 150 pg/mL and plasma chromogranin A concentration was normalized. Pathological examination revealed 3 duodenal gastrinomas and 1 pancreatic gastrinoma with 3 metastatic lymph nodes from duodenopancreatic gastrinoma. The gastric NET was a type II NET.

PPTD

PPTD was first performed for case No. 10, in whom a substantial numbers of NETs were palpated intraoperatively and a few large metastatic lymph nodes were detected, without any pancreatic head tumors. Pathological study revealed numerous submucosal microgastrinomas throughout the duodenum. Her serum IRG did not decrease to within normal range and she developed hepatic metastases 3 year after the PPTD. In order to save the head of the pancreas, PPTD was performed for the following 6 patients in whom the SASI test diagnosed gastrinoma in the pancreatic head and/or the duodenum and considerable numbers of duodenal NETs were suspected during surgery (Table 3). In one patient (case 16) the SASI test localized gastrinoma not only in the head of the pancreas and/or the duodenum, but also in the tail of the pancreas, so PPTD and a distal pancreatectomy were performed curatively, and the patient has since been free of gastrinoma. Any serious postoperative morbidity was experienced in this series of patients.

Hyperplasia of G cells or microgastrinomas in the duodenal Brunner's gland

In 7 PPTD patients, duodenal gastrinomas were numerous in only 2 patients, and there were 4 or more in 2 additional patients. In 3 other patients, only 1 tumor was diagnosed as gastrinoma, and the other submucosal tumors were mostly diagnosed as hyperplasia of the duodenal Brunner's gland. Not expecting these results, we carefully re-examined the duodenal mucosal membrane with anti-gastrin antibody and identified clusters of gastrin-producing cells in or adjacent to the Brunner's gland, some of which were diagnosed as microgastrinoma. The clusters of gastrin-producing cells were found in all 7 duodenal specimens after PPTD (Figure 2).

Five patients post-PPTD have been cured of gastrinoma for lengths of time ranging from 2 year to 6 year 8 mo. However, in 2 patients in whom their preoperative serum IRG levels were as high as 18 200 pg/mL or



Table 3 Results of pancreas-preserving total duodenectomy for duodenal gastrinomas in patients with multiple endocrine neoplasia

No.	Age	Gender	ZES	Ulcer diseases	Ulcer related operation	MEN 1 related diseases	Pre-PPTD IRG (pg/mL)	Localization of gastrinoma			Operation	Post-PPTD IRG (pg/mL)	Gastrinoma		Metastases		Pancreas NETs	Prognosis (post-op yr)	Post-PPTD secretin or calcium test	
								SASI	GIF	CT			Location	Number	Size (mm)	N				L
10	51	F	+	DU 1997 Dec	-	HPT	54800 ↓ 18200 (post ParX)	GDA, IPDA	ND	#6 LN #13 LN	St ParX 2003 Apr PPTD, DP 2003 Nov	216	D	num	1-4	2	0	9 (1; gluc)	Alive well with L Mets (IRG 900) (6 yr 8 mo)	Positive
11	30	M	-	-	-	HPT Pit NET	820 ↓ 206 (post ParX)	GDA, IPDA	ND	PNET (uncus tail)	T ParX, TX 2004 Apr PPTD, DP 2004 Jul	110	D	1	5	0	0	1	Alive well (6 yr) no recur	Negative
12	33	M	+	DU 2004 Mar	-	HPT	3050 ↓ 710 (post ParX)	GDA, IPDA	Dsmt	ND	T ParX, TX 2003 Aug PPTD, DP 2004 Aug	57	D	1	5	0	0	multi	Alive well (6 yr) no recur	Negative
13	48	F	-	-	-	HPT	687 (post ParX)	IPDA, DPA	Dsmt diff PNET (D-EUS)	#13 LN PNET (< 3 mm)	PPTD 2007 Apr T ParX, TX 2007 Sep	59	D	num	1-5	1	0	multi diff	Alive well (2 yr 11 mo) no recur	Negative
14	33	M	+	JU perf 2007 Jan	Patch	HPT	13900 (post ParX)	GDA	Dsmt diff PNET (D-EUS)	#17 LN Dsmt	T ParX, TX 2001 Dec PPTD 2007 Nov	255	D	1	8	2	0	multi diff	Alive well with N Mets (IRG 371) (2 yr 8 mo)	Positive
15	57	F	+	DU perf 2006 Jan JU perf 2006 Jul Ileus 2007 May JU perf 2008 Jul	GX JX	HPT	720 ↓ 646 (post ParX)	GDA, IPDA	ND	ND	T ParX, TX 2007 Mar PPTD, TG 2008 Aug	42	D	7	2	1	0	0	Alive well (2 yr) no recur	Negative
16	32	M	+	Es bleeding 2002 Oct DU JU perf 2006 Jan JU perf 2008 Nov	JX GX, JX	HPT	1630	DGA, SPA	ND	PNET (17 mm)	ParX, PPTD 2008 P(T)X Aug	450	D, P(T)	3, 1	3, 10, 11, 10	3	0	3 (> 5 mm)	Alive well (2 mo) no recur	Negative

ZES: Zollinger-Ellison syndrome; PPTD: Pancreas preserving total duodenectomy; SASI: Selective arterial secretagogue injection test; op: Operation; GIF: Gastrointestinal fibroscopy; CT: Computed tomography; IRG: Serum immunoreactive gastrin concentration; N: Lymph node metastasis; L: Liver metastasis; NET: Neuroendocrine tumor; F: Female; M: Male; postop: Postoperative; DU: Duodenal ulcer; JU: Jejunal ulcer; perf: Perforation; Es: Esophagus; Patch: Omental patch; GX: Partial gastrectomy; JX: Partial jejunectomy; HPT: Hyperparathyroidism; Pit NET: Pituitary neuroendocrine tumor; ParX: Parathyroidectomy; GDA: Gastroduodenal artery; IPDA: Inferior pancreaticoduodenal artery; DPA: Dorsal pancreatic artery; SPA: Splenic artery; ND: Not detected; Dsm: Duodenal submucosal tumor; diff: Diffuse; D-EUS: Duodenal endoscopic ultrasonography; LN: lymph node; PNET: Pancreatic neuroendocrine tumor; St ParX: Subtotal parathyroidectomy; T ParX: Total parathyroidectomy; TX: Transplantation of the parathyroid; DP: Distal pancreatectomy; PX: Partial resection of the pancreas; D: Duodenum; P(T): Pancreas tail; num: Numerous; gluc: Glucagonoma; mult: Multiple; NP: Not performed; diff: Diffuse; Met: Metastasis; no recur: No recurrence.

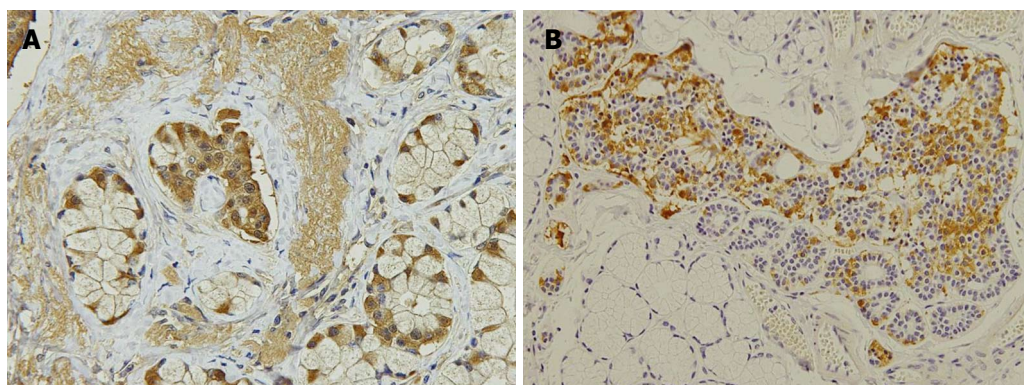


Figure 2 Hyperplasia (A) and a cluster of gastrin-producing cells (B) in the duodenal Brunner's glands (in patient No. 12, who underwent pancreas-preserving total duodenectomy for numerous duodenal microgastrinomas) were detected by immunohistochemical gastrin staining.

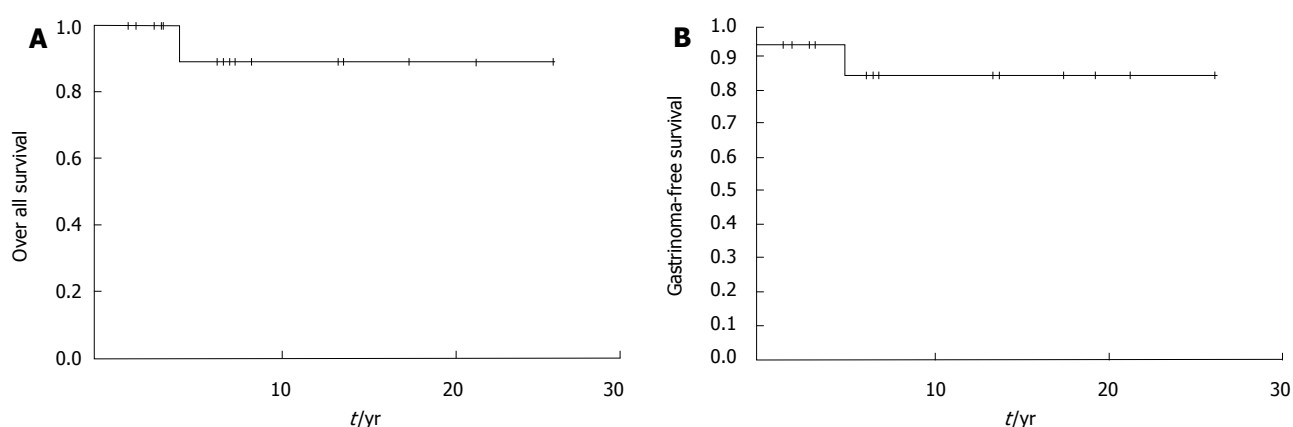


Figure 3 Survival curve of 16 patients with multiple endocrine neoplasia type 1 and neuroendocrine tumors. A: Overall survival after initial resection surgery. The survival rate at 10 years was 90.9%; B: Gastrinoma-free survival after initial resection surgery. The survival rate at 10 years was 82.0%.

13 900 pg/mL after parathyroidectomy, and advanced stages of gastrinoma were suspected, PPTD was non-curative (Table 3). In one of them, hepatic metastases have become apparent on CT film within 3 year postoperatively, and in the other patient, distant lymph nodes metastases have developed.

Results of surgery and survival curves

Of the 16 patients in this series, 7 patients had single duodenal gastrinoma and 9 patients had multiple gastrinomas. More specifically, 2, 4, 5, 6, and 9 duodenal gastrinomas were detected in 1 patient each; 7 duodenal gastrinomas in 2 patients; and numerous duodenal gastrinomas in 2 patients. In 2 patients (13%), pancreatic gastrinoma co-existed with duodenal gastrinoma which were localized by the SASI test.

To date, 14 patients have been cured of gastrinoma and 2 patients have been noncurative, postoperatively. The overall patient survival curve is shown in Figure 3A, with a survival rate of 90.9% at 10 years. The gastrinoma-free survival curve is shown in Figure 3B, with a survival rate of 82.0% at 10 years.

DISCUSSION

Controversy has surrounded the treatment strategy for

gastrinoma and pancreatic NET in patients with MEN 1 and ZES^[1-14]. It is difficult to determine whether aggressive surgical resection of both gastrinoma and pancreatic NETs improves survival rates and the long term biochemical cure of gastrinoma in MEN 1 patients, because of the rarity of the disease^[1-4,10-14]. Many recently published articles support aggressive surgery, such as PD or multiple LR of a few duodenal gastrinomas and distal pancreatectomy for pancreatic NETs, for both biochemical cure of gastrinoma and prolongation of survival^[1,4,10-14]. On the other hand, Gibril *et al*^[27] reported the results of an important prospective study on the natural history of gastrinoma in patients with MEN 1, in which 57 patients with MEN 1 and ZES were followed for 8 year without performing surgical resection for duodenopancreatic NETs until the tumors grew to > 2.5 cm. In this study, 13 patients (23%) developed hepatic metastases and 3 patients died of duodenopancreatic NETs. They suggested that biochemical cure of gastrinoma might be impossible in patients with MEN 1 and that prolongation of survival of MEN 1 patients with an aggressive type of NETs would not be realized until the development of a tool to differentiate an aggressive type of NET from another slow growing type of NET. Their results themselves, we think, support the idea that early resection should be necessary for decreasing the rate of hepatic metastases from duodenopancreatic NETs

in MEN 1 patients.

The present study shows that aggressive surgical resection for gastrinoma in MEN 1 patients using PD or aggressive LR, or PPTD guided by localization with the SASI test, was useful for long term biochemical cure of duodenopancreatic gastrinoma, and that aggressive resection of pancreatic NETs was also useful for prevention of hepatic metastases. So, we would like to recommend early aggressive surgical resection of duodenopancreatic NETs for MEN 1 patients.

Goudet *et al.*^[28] performed a cohort study of 758 patients with MEN 1 and found that gastrinoma was a statistically significant high-risk factor for death of patients with MEN 1 secondary to the nonfunctioning pancreatic NETs, and suggested earlier resection surgery for both gastrinoma and nonfunctioning pancreatic NETs in patients with MEN 1. Gauger and Thompson reported a 94% 15 year survival rate of patients with functioning NETs (gastrinoma or insulinoma) in MEN 1 patients after local resections of duodenal gastrinomas and a distal pancreatectomy with enucleations of NETs in the head of the pancreas without any operative morbidity^[2]. These results suggest that early resection of gastrinoma in MEN 1 patients is useful for normalization of serum gastrin levels and prevention of distant metastases.

Identification of gastrinoma among multiple NETs in the duodenopancreatic region of patients with MEN 1 is essentially impossible by imaging techniques alone^[17,18]. The SASI test localizes gastrinomas or metastatic lymph nodes by judging whether or not gastrin is secreted from NETs in the area of interest by stimulation with a secretagogue, so it can differentiate functioning gastrinoma among multiple NETs in MEN 1 patients.

On the other hand, SRS and other imaging methods [CT or MRI or ultrasonography (US)] are useful for identification of hepatic metastases, although it is difficult to tell the absence of gastrinoma in the area of interest. We have used secretin for stimulating gastrinoma to release gastrin during the SASI test for a long time, but now we use calcium gluconate hydrate solution (Calciol[®]), because secretin has not been produced in Japan since 2004. We compared the results with both secretagogues and found the results were identical^[21].

In 1991, imaging methods were not sensitive for visualizing < 1 cm gastrinoma; thus we performed resection surgery of both gastrinoma and microgastrinoma based on localization with the SASI test. When the SASI test localized gastrinomas in the feeding area of the gastroduodenal artery, we performed PD. In the first 3 patients with MEN 1, the SASI test localized < 1 cm gastrinomas in the head of the pancreas and/or the duodenum, so we performed PD for them and all of them were cured of gastrinoma; 2 patients have been alive and healthy for more than 12 year, although a patient died of other causes 4 year postoperatively (Table 1). In the resected specimens of the first 3 patients, < 1 cm gastrinomas were located only in the duodenum and not in the pancreas. In those days, endocrine surgeons working in the USA or EU gradually found that the gastrinomas in patients with MEN 1

were localized mostly in the duodenum and rarely in the pancreas^[15,16]. Thompson *et al* have started to perform LR for duodenal gastrinoma and distal pancreatectomy with enucleation of NET in the head of the pancreas in MEN 1 patients^[1]. According to our results and theirs, we also started to perform local excisions of duodenal gastrinomas and enucleation or a distal pancreatectomy for pancreatic NETs, which are less invasive compared to PD. Since then, 6 patients have undergone LR for duodenal gastrinomas, which has been successful in all patients, although in 2 patients duodenal gastrinoma recurred and second LR was performed 8 year 8 mo and 6 year after the first LR.

We performed PPTD for 7 patients in whom duodenal gastrinomas were thought to number more than 5 during surgery. The duodenal gastrinomas were numerous in only 2 of 7 patients and the duodenal tumors in the other 5 patients were mostly diagnosed as hyperplasia of the duodenal Brunner's glands postoperatively (Table 3). Not expecting these results, we immunohistochemically stained the duodenal wall with anti-gastrin antibody and found a cluster of gastrin-producing cells or microgastrinomas in or adjacent to the Brunner's gland. The clusters of gastrin-producing cells in the Brunner's gland were found in all of the duodenal specimens after PPTD.

Klöppel *et al.*^[29] have reported that in patients with MEN 1, mutations in the *menin* gene can cause hyperplasia of gastrin-producing cells in the duodenal Brunner's glands, which are the precursor lesion of gastrinoma. Our results are consistent with their report. Thus, in the duodenum of MEN 1 patients with substantial numbers of duodenal gastrinomas and/or microgastrinomas, *de novo* gastrinoma might develop during the patient's lifetime.

Of the 16 patients in the present study, 7 patients (43.8%) had 1 duodenal gastrinoma and 9 patients (56.2%) had multiple duodenal gastrinomas. Gastrinoma did not recur in patients belonging to the former group, but recurred in 2 patients (22.2%) belonging to the latter group who had 3 and 5 duodenal gastrinomas, respectively. PPTD may be useful for preventing both residual microgastrinoma and recurrence due to development of *de novo* duodenal gastrinoma in MEN 1 patients with substantial numbers of gastrinomas and microgastrinomas.

In 7 patients who underwent PPTD, no postoperative complications, such as pancreatic leakage, acute pancreatitis, abscess or surgical site infections, have been experienced. Thus, PPTD is less invasive surgery compared to PD. On the other hand, dissection of the regional lymph nodes may be incomplete by PPTD compared to PD. As duodenal gastrinoma metastasizes to the regional lymph nodes independent of size, any regional lymph nodes around both the pancreas head and the common hepatic artery have to be dissected. Lymph nodes along the superior mesenteric artery have to be resected when they are palpated hard^[20].

When considering the optimal surgery for patients with MEN 1 and gastrinoma, we must first seriously consider the risk of hepatic metastases from pancreatic NETs^[1,7-9,14,28,30]. Hepatic metastases from pancreatic

NETs are more serious than those from duodenal gastrinoma, and the rate of hepatic metastases from pancreatic NETs is at least several times more frequent than those from duodenal NETs^[6,7,16,28,30]. Thus, we recommend distal pancreatectomy for pancreatic NETs with enucleations of NETs in the pancreatic head more than 1 cm, as recommended by Thompson^[1].

As for optimal surgical resection for sporadic duodenal NET, recently several articles have dealt with the subject relating to the staging of duodenal nonfunctioning NETs. Evans's group have performed a retrospective analysis of patients with duodenal NETs operated at their institute and they proposed a standard strategy for duodenal NETs using a staging based on the depth of tumor invasion and the grading of the development of the distant metastases^[31]. Sarr's group also published a similar study^[32]. Both groups recommended endoscopic excisions for duodenal NET smaller than 1 cm, and open transduodenal resection with dissection of the regional lymph nodes for duodenal NET between 1 cm and 2 cm, because rate of lymph node metastases cannot be ignored in duodenal NET between 1 and 2 cm in diameter. Both groups recommended PD for duodenal NET more than 2 cm with lymph node metastases^[31,32]. However, both groups intentionally excluded duodenal gastrinoma from their retrospective analytical studies, because the natural history of duodenal gastrinoma seemed quite different from other duodenal NETs, which suggested a more aggressive progression of duodenal gastrinoma^[31,32].

In our study, 7 of 16 patients had only 1 duodenal gastrinoma, but 3 of the 7 patients had metastatic lymph nodes, and 1 of them (No. 14) had distant metastases resulting in noncurative resection of gastrinoma (Table 3). So, instead of endoscopic excision, local resection with dissection of lymph nodes may be recommended for a few < 1 cm duodenal gastrinomas in MEN 1 patients^[1,2]. For substantial numbers of < 1 cm duodenal gastrinomas with multiple pancreatic NETs in MEN 1 patients, we would like to recommend PPTD with distal pancreatectomy and enucleation of > 1 cm NETs in the head of the pancreas, because cure of duodenal gastrinoma is not likely to be achieved for a long time due to both possible residual microgastrinoma and development of de novo gastrinomas in the duodenum^[20,29]. PD might be indicated for MEN 1 patients with a substantial number of both duodenal gastrinomas and metastatic regional lymph nodes with a few > 1 cm pancreatic NETs. Of course, curative resection has to be indicated before development of hepatic micrometastases.

In this series, only one patient has died of other diseases and the other patients have been alive and well to date. Overall survival curve of the patients is shown in Figure 3A. Evaluating together with the gastrinoma-free survival curve of these patients (Figure 3B), we would like to conclude that resection surgery was useful for cure of gastrinoma and prolongation of survival of the patients with MEN 1 and gastrinoma.

Given that pancreatic gastrinoma co-existed with duo-

denal gastrinoma in 12.5% of our patients, caution is advised, because many surgeons and pathologists have believed that pancreatic gastrinoma is rare in MEN 1 patients^[33]. To date, total pancreatectomy has rarely been performed for MEN 1 patients, but we think that total pancreatectomy may be indicated for a few MEN 1 patients according to decisions based on the clinicopathological genetic analysis of pancreatic NET in such patients^[34].

In conclusion, aggressive resection surgery based on accurate localization was useful for biochemical cure of gastrinoma in patients with MEN 1 and gastrinoma. Given that pancreatic gastrinoma co-existed with duodenal gastrinomas in 2 of 16 patients (13%), we would like to recommend the SASI test for preoperative localization of gastrinoma in MEN 1 patients.

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COMMENTS

Background

Treatment strategy for gastrinoma and pancreatic neuroendocrine tumors (NETs) in patients with multiple endocrine neoplasia type 1 (MEN 1) has been controversial. Most doctors have thought that gastrinomas in MEN 1 cannot be cured because curative resection is rare and recurrence rate is high, and pancreatectomy for pancreatic NETs in MEN 1 does not make sense, since NETs and micro-NETs exist diffusely in the pancreas. On the other hand, recent reports by a few aggressive surgeons show that a high cure rate of gastrinomas and long term prolongation of survival have been achieved by aggressive surgery. For achieving curative resection of gastrinomas in MEN 1, correct localization of gastrinomas is essential for guiding curative surgery, and in order to prolong the life of patients with MEN 1 and duodenopancreatic NETs, surgical resection of these NETs before development of hepatic metastases is essential, because hepatic metastases is the most significant prognostic factor.

Research frontiers

The authors should select an optimal modus of surgery for curing gastrinoma and pancreatic NETs in MEN 1 patients, otherwise surgery may end non-curatively or may become too invasive to ensure quality of life for patients. For the best balance between curability of surgery and postoperative good quality of life, the best modus of surgery should be applied for patients with MEN 1 and gastrinoma by estimating the stage of duodenopancreatic NETs.

Innovations and breakthroughs

The present study shows that cure of gastrinomas in MEN 1 patients can be obtained when you resect gastrinomas guided by localization with the SASI test, and prevention of hepatic metastases can be obtained by resection of > 1 cm pancreatic NETs by pancreatectomy of enucleations. As for the modus of surgery, we are the first to propose pancreas-preserving total duodenectomy (PPTD) for multiple or numerous duodenal gastrinomas in MEN 1 as the optimal extent of aggressive surgery. The authors have also proved that pancreatic gastrinoma co-exists with duodenal gastrinoma in 13% of patients with MEN 1, although recently most surgeons and some pathologists have reported that gastrinomas exist only in the duodenum in MEN 1 patients.

Applications

By understanding the fact that curative surgical resection is possible by correct localization, and by further development of clinicopathological genetic analysis

of the disease, the optimal surgical strategy corresponding to the stage of the disease will be established for gastrinomas and duodenopancreatic NETs in MEN 1 patients in the near future.

Terminology

PPTD is the modus of surgery by which total duodenum is resected without resecting pancreas tissue. Traditionally, for resecting malignant tumors in the duodenum, pancreatoduodenectomy has been used by which one third of the pancreas is resected with the duodenum.

Peer review

The study evaluates the standard surgery for patients with gastrinoma in MEN 1 guided by accurate preoperative localization.

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Hemi-hepatectomy in pediatric patients using two-surgeon technique and a liver hanging maneuver

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Mochizuki K, Eguchi S, Hirose R, Kosaka T, Takatsuki M, Kanematsu T. Hemi-hepatectomy in pediatric patients using two-surgeon technique and a liver hanging maneuver. *World J Gastroenterol* 2011; 17(10): 1354-1357 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i10/1354.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i10.1354>

Abstract

AIM: To evaluate the efficacy of the two-surgeon technique with the liver hanging maneuver (LHM) for hepatectomies in pediatric patients with hepatoblastoma.

METHODS: Three pediatric patients with hepatoblastoma were enrolled in this study. Two underwent right hemi-hepatectomies and one underwent a left hemi-hepatectomy using the two-surgeon technique by means of saline-linked electric cautery (SLC) and the Cavitron Ultrasonic Surgical Aspirator (CUSA; Valleylab, Boulder, CO) and the LHM.

RESULTS: The mean operative time during the parenchymal transections was 50 min and the mean blood loss was 235 g. There was no bile leakage from the cut surface after surgery. No macroscopic or microscopic-positive margins were observed in the hepatic transections.

CONCLUSION: The two-surgeon technique using SLC and CUSA with the LHM is applicable to even pediatric patients with hepatoblastoma.

INTRODUCTION

The safety and efficacy of saline-linked electric cautery (SLC) in hepatectomies has been described^[1,2]. A two-surgeon technique utilizing SLC and the Cavitron Ultrasonic Surgical Aspirator (CUSA) is used in the hepatic resections performed in our department, especially in living donor liver transplantations (LDLT), to reduce intraoperative hemorrhage and to prevent bile leakage from the cut surface^[3].

The liver hanging maneuver (LHM) was reported to be a safe approach for hepatectomies during parenchymal transections in many investigations including ours^[4].

We herein describe the hepatectomy procedure using this two-surgeon technique with the LHM in pediatric patients with hepatoblastoma.

MATERIALS AND METHODS

From January 2007 to December 2009, 3 hepatectomies for hepatoblastoma were performed in pediatric patients. Two underwent right hemi-hepatectomies, while one underwent a left hemi-hepatectomy.

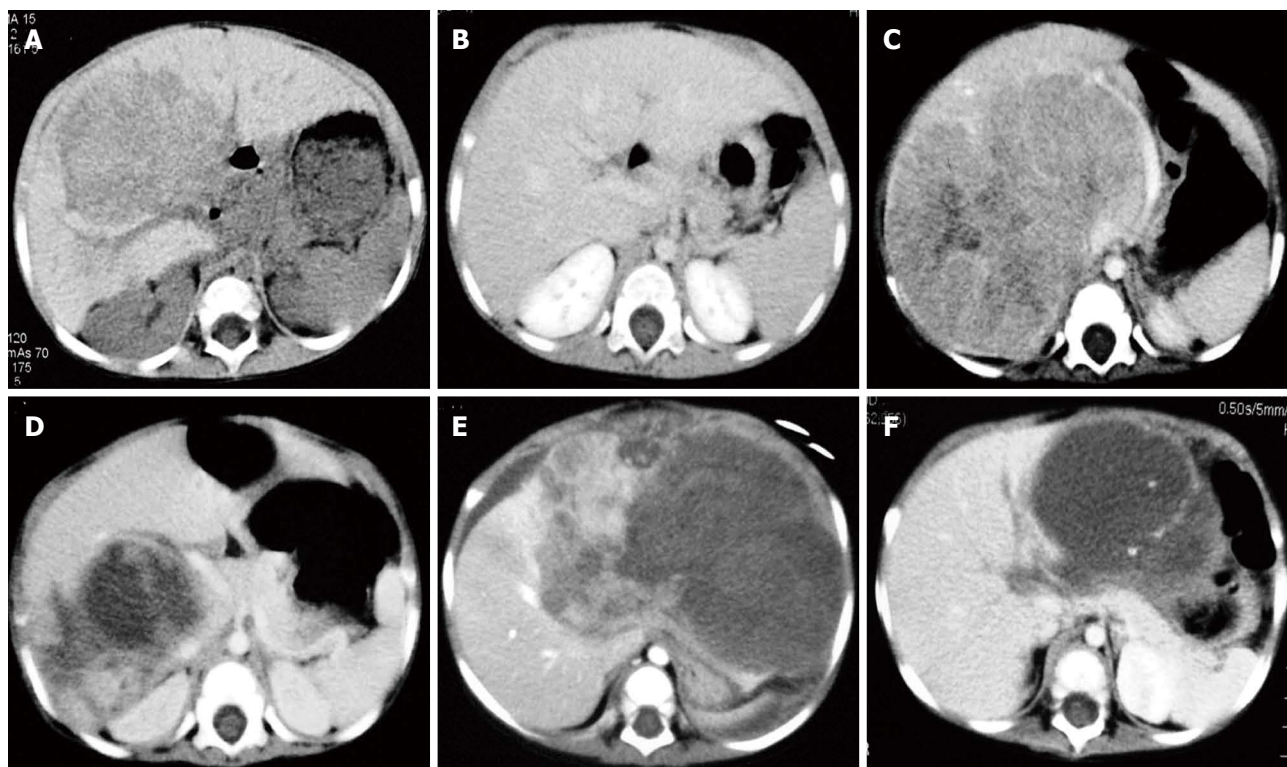


Figure 1 Each tumor location on contrast-enhanced computed tomography. A: Prechemotherapeutic computed tomography (CT) of case 1; B: Preoperative CT of case 1; C: Prechemotherapeutic CT of case 2; D: Preoperative CT of case 2; E: Prechemotherapeutic CT of case 3; F: Preoperative CT of case 3.

Following laparotomy, the upper surface of the liver was exposed up to the anterior surface of the suprahepatic inferior vena cava (IVC). The space between the right and middle hepatic veins was dissected and the tape was pulled upward and to the left to allow the exposure of the anterior surface of the infrahepatic portion of the IVC^[5]. In LHM, the mobilization of liver was not needed in adult patients^[5]. However, for right hemi-hepatectomies, we mobilized the right liver lobe prior to the dissection procedure since blind dissection between the liver and the IVC is challenging with a very narrow space, especially in pediatric cases. For the left hemi-hepatectomies, the middle hepatic vein was carefully encircled. Subsequently, tape was entered between the middle hepatic vein and the left hepatic vein and then placed along Arantius' ligament^[4].

The 3-0 polypropylene stay sutures were placed at the antero-caudal edge of the liver along the plane of intended transection. The chief surgeon dissected the hepatic parenchyma from the patient's right side using the CUSA System, while the assistant surgeon used the SLC device (Dissecting Sealer DS 3.5; TissueLink Medical, Inc, Dover, NH) from the patient's left side. The occlusion of the hepatic arterial and portal inflow was not used in any case. The liver parenchyma was dissected with CUSA, and the intraparenchymal vascular anatomy was defined so that a decision on the hemostatic technique could be made based on the vessel size. The SLC device was used to coagulate and divide the dissected vessels that were 3 mm or smaller in diameter. Vessels larger than 3 mm in diameter were ligated with 3-0 or 4-0 silk ties and were sharply di-

vided. The few larger vessels were ultrasonically dissected and controlled with 4-0 absorbable monofilament transfixing sutures, and were then sharply divided. The traction on the stay sutures was used to separate and to expose the deepening transection plane^[3].

During the parenchymal dissection, the upward traction on the tape (hanging maneuver) allowed the surgeon to follow a direct plane and facilitated the exposure and hemostasis of the deeper parenchymal plane in front of the IVC^[5].

A closed suction drain was inserted at the conclusion of each procedure.

RESULTS

Patient data were shown in Table 1. All patients were 1 year old and male. The mean height was 74 cm, and mean weight was 8.53 kg. Each tumor location was shown in Figure 1.

The results were shown in Table 1. The mean operative time was 292 min (range: 178-368 min). The mean operative time during the parenchymal transections was 50 min (range: 30-84 min). The number of vessel ligations during the parenchymal transections was 23 in the first patient, but only 9 in the 2 subsequent patients. The mean blood loss was 235 g (excluding irrigation saline). In case 2, the blood loss was increased in separation/adhesion between tumor and right adrenal gland. There was no bile leakage from the cut surface after surgery. There were no intraoperative or postoperative complica-

Table 1 Patient data and results

Case	Age (mo)	Gender	Height (cm)	Weight (kg)	Tumor location	Hepatectomy	Operative time (min)	Blood loss (g)	Parenchymal transection		Complication	Outcome	Follow up (mo)
									Operative time (min)	Number of ligation			
1	18	M	75	9.2	Right lobe	Right	368	125	84	23	None	CR	43
2	12	M	70	8.4	Right lobe	Right	178	420	38	9	None	CR	34
3	17	M	77	8	Left lobe	Left	330	160	30	9	None	CR	29

tions and no macroscopic or microscopic-positive margins were seen in any of the patients.

All 3 patients could restart projected postoperative chemotherapy treatments from postoperative day 7, and all had complete remissions and no recurrences during the writing of this manuscript.

DISCUSSION

Reducing blood loss is one of the goals in liver surgery, and several technical inventions have been introduced to achieve it including the Pringle maneuver^[6] and selective vascular occlusion^[7], among other techniques. CUSA has contributed to safe hepatectomies by making it easy to identify the vessels during parenchymal transections^[8]. However, because CUSA has no function in the sealing of tissues, meticulous ligation is required to avoid bleeding or bile leakage from the cut surface of the liver. SLC is another novel device that contributes to the ligation reduction during liver parenchymal transections with its effect on tissue sealing^[1]. To guard against these possible disadvantages, Aloia *et al.*^[2] introduced a two-surgeon technique in hepatectomies for neoplasms in adults with promising results. Palavecino *et al.*^[9] demonstrated the mean intra-operative blood loss was significantly decreased after introduction of the two-surgeon technique compared with other techniques (stapling alone, ultrasonic dissection alone, saline-linked cautery alone, and clamp-crush technique). There were no differences in mean operative time between our data (292 min) and those data reported by Tannuri *et al.*^[10] (290.4 min) using the CUSA or LigaSure electrocautery by the chief surgeon and bipolar electrocautery by the assistant surgeon. There were no differences in mean intraoperative blood loss between our data (235 g) and those reported by Liu *et al.*^[11] (221 cm³) using total hepatic occlusion.

We previously demonstrated that SLC could be safely adapted to living liver donor surgery without injuring either the graft or the remnant liver^[3]. In pediatric patients, SLC is more effective because the vessels are smaller than those in adult patients. On the other hand, the LHM is more difficult because the space between the right and middle hepatic veins is narrower. In our department, a safer and more comfortable technique is performed to encircle the liver using a surgical probe with smaller tape^[4]. In these pediatric patients, the reason why the number of vessel ligations and the operative time in parenchymal transection decreased with the latter two patients without increased blood loss was because we could make progress

developing the technique.

The two-surgeon technique using SLC and CUSA with the LHM is therefore considered to be a feasible and safe surgical modality for hepatectomies in pediatric patients.

COMMENTS

Background

A two-surgeon technique utilizing saline-linked electric cautery (SLC) and the Cavitron Ultrasonic Surgical Aspirator (CUSA), and a liver hanging maneuver (LHM) is reported to be a safe approach for hepatectomies.

Research frontiers

CUSA is an easy way to identify the vessels and SLC contributes to the ligation reduction with its effect on tissue sealing during liver parenchymal transections.

Innovations and breakthroughs

Recent reports have highlighted the safety and efficacy of a two-surgeon technique and a liver hanging maneuver for hepatectomies in adults. This study reports the feasibility and safety of the two-surgeon technique and the LHM for hepatectomies in pediatric patients.

Applications

This study shows that the two-surgeon technique and the LHM could become common therapeutic modalities in the hepatectomies of pediatric patients in the future.

Terminology

The SLC device is used to coagulate and divide the dissected vessels that are 3 mm or smaller in diameter. In the two surgeon technique, the chief surgeon dissects the hepatic parenchyma using the CUSA system, while the assistant surgeon uses the SLC device. In the liver hanging maneuver, the upward traction on the tape allowed the surgeon to follow a direct plane and facilitated the exposure and hemostasis of the deeper parenchymal plane in front of the IVC.

Peer review

The authors described their technique and outcomes of performing hepatectomy using two-surgeon technique and liver hanging maneuver (LHM) in children. Data regarding such technique in children is quite limited in literature.

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Congenital bronchoesophageal fistula in adults

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Abstract

AIM: To study the clinical characteristics, diagnosis and surgical treatment of congenital bronchoesophageal fistulae in adults.

METHODS: Eleven adult cases of congenital bronchoesophageal fistula diagnosed and treated in our hospital between May 1990 and August 2010 were reviewed. Its clinical presentations, diagnostic methods, anatomic type, treatment, and follow-up were recorded.

RESULTS: Of the chief clinical presentations, non-specific cough and sputum were found in 10 (90.9%), recurrent bouts of cough after drinking liquid food in 6 (54.6%), hemoptysis in 6 (54.6%), low fever in 4 (36.4%), and chest pain in 3 (27.3%) of the 11 cases, respectively. The duration of symptoms before diagnosis ranged 5-36.5 years. The diagnosis of congenital bronchoesophageal fistulae was established in 9 patients by barium esophagography, in 1 patient by esophagoscopy and in 1 patient by bronchoscopy, respectively. The congenital bronchoesophageal fistulae communicated with a segmental bronchus, a main bronchus, and an intermediate bronchus in 8, 2 and 1 patients, respectively.

The treatment of congenital bronchoesophageal fistulae involved excision of the fistula in 10 patients or division and suturing in 1 patient. The associated lung lesion was removed in all patients. No long-term sequelae were found during the postoperative follow-up except in 1 patient with bronchial fistula who accepted reoperation before recovery.

CONCLUSION: Congenital bronchoesophageal fistula is rare in adults. Its most useful diagnostic method is esophagography. It must be treated surgically as soon as the diagnosis is established.

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Key words: Congenital bronchoesophageal fistula; Adult; Esophagography; Surgical treatment

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Zhang BS, Zhou NK, Yu CH. Congenital bronchoesophageal fistula in adults. *World J Gastroenterol* 2011; 17(10): 1358-1361 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i10/1358.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i10.1358>

INTRODUCTION

Congenital bronchoesophageal fistula is usually associated with esophageal atresia and readily diagnosed in infancy. However, it may persist until adulthood if not associated with esophageal atresia. Its pathogenesis is not absolutely clear. A persistent attachment between tracheobronchial tree and esophagus, produced by abnormal growth of the trachea during its severance from the esophagus, has been accused for it. The chief symptoms are bouts of cough after drinking and recurrent respiratory infections. Eleven cases of congenital bronchoesophageal fistula in adults were diagnosed and oper-

ated in our hospital between May 1990 and August 2010. Its etiology, pathophysiology, clinical presentations, diagnosis, complications, and treatment were discussed for its early diagnosis.

MATERIALS AND METHODS

Eleven adult cases (7 males and 4 females) of congenital bronchoesophageal fistula admitted to our hospital between 1990 and 2010 were reviewed. Their average age was 36 years (range 20-66 years) at diagnosis. Its clinical presentations, diagnostic methods, anatomic type, treatment, and follow-up were recorded.

RESULTS

Clinical presentations

The chief symptoms included nonspecific cough in 10 patients, recurrent bouts of cough after taking liquid food in 6 patients, hemoptysis in 6 patients, and bouts of chest pain in 3 patients, respectively. The duration of symptoms before diagnosis was 5-36.5 years and exceeded 15 years in 9 patients (Table 1).

Of the 11 patients, 3 had a history of heavy smoking, 1 had a history of tuberculosis, and 7 had no history of malignant disease, heavy smoking, tuberculosis, chest trauma, or occupational exposure to toxic agents.

Diagnosis

The diagnosis of congenital bronchoesophageal fistula was established by barium esophagography in 9 patients (Figure 1), by esophagoscopy in 1 patient, and by instillation of methylene blue into the esophagus during bronchoscopy in 1 patient (Table 1). Esophagoscopy showed esophageal orifice of the fistula in 7 out of 10 patients and bronchoscopy showed bronchial orifice of the fistula in 4 out of 9 patients, respectively. Preoperative chest computed tomography (CT) showed the extent of associated lung lesions (Figure 2).

Anatomic types

The exact levels of connection between esophagus and tracheobronchial tree are listed in Table 2. The fistula was communicated with the bronchial tree on the right side in 7 patients and on the left side in 4 patients, with a segmental bronchus in 8 patients and a main or intermediate bronchus in 3 patients. The fistula communicated with the lower third of the esophagus in 9 patients and with the middle third in 2 patients.

Treatment

Thoracotomy was performed from the right side in 7 patients and from the left side in 4 patients. The fistula was completely removed in 10 patients and the tract was simply divided with the end sutured in 1 patient. A pleural flap was inserted between esophagus and bronchial tree in all patients (Table 1). The associated lung lesion was removed due to sequestration, multiple epithelialized

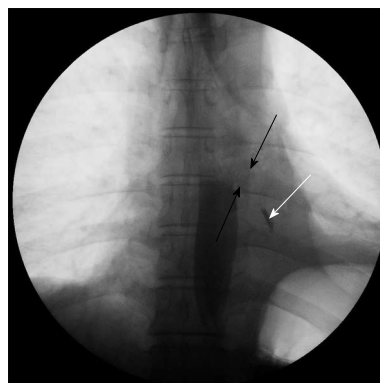


Figure 1 Barium esophagography showing the esophageal orifice of fistula (black arrows) and some barium in the left lower lobe (white arrow) demonstrating the downward direction of the fistula from esophagus to the left lower lobe bronchus.



Figure 2 Preoperative chest computed tomography scan demonstrating abnormal communication between the lower third of esophagus and the left segmental bronchus (arrow) and a mass measuring 3.0 cm in diameter with irregular borders encircling the basal segmental bronchi of the left lower lobe caused by bronchoesophageal fistula.

pulmonary cysts, and bronchiectasis, respectively, in 1, 6, and 4 patients (3 underwent lobectomy and 1 pneumonectomy). Extensive conglutination was found in the thorax of all patients. Large-volume bleeding (200-1000 mL) occurred during the operation, with a blood loss of over 400mL in 8 patients (Table 3).

Outcome

The postoperative course was uneventful in all patients but one who developed a bronchial fistula which was settled with conservative management and reoperation before recovery (Table 3). One patient died of chronic respiratory failure 10 years after operation.

DISCUSSION

Congenital bronchoesophageal fistula or tracheoesophageal fistula was first reported by Negus in 1929^[1]. Congenital bronchoesophageal or tracheoesophageal fistula is rare in adults^[2-5]. Its diagnosis is difficult due to the nonspecific nature of its symptoms. Benign bronchoesophageal fistula can remain undiagnosed for years.

Table 1 Clinical features, diagnosis, and treatment of congenital bronchoesophageal fistula

Symptoms	Patients (n)	Diagnosis	Patients (n)	Treatment	Patients (n)
Cough (nonspecific)	10	Esophagography	9	Right thoracotomy	7
Cough (after drinking)	6	Esophagoscopy	1	Left thoracotomy	4
Hemoptysis	6	Bronchoscopy	1	Resection of fistula	10
Chest pain	3			Division and suture	1
Low fever	4			Pleural flap insertion	11
				Associated lobectomy	10
				Associated pneumonectomy	1

Table 2 Anatomic types of congenital bronchoesophageal fistulae

Anatomic types	Patients (n)
Lower third of esophagus	9
Main bronchus	1
Intermediate bronchus	1
Segmental bronchus	7
Middle third of esophagus	2
Main bronchus	1
Segmental bronchus	1

Table 3 Blood loss during operation, hospital stay time, and outcome of patients with congenital bronchoesophageal fistula

Blood loss (mL)	Patients (n)	Hospital stay time	Patients (n)	Postoperative complication	Patients (n)
200-400	3	≤ 2 wk	8	Bronchial fistula	1
400-800	6	2 wk-1 mo	2	Esophageal fistula	0
800-1000	2	>1 mo	1	Respiratory failure	1
				Circulation failure	0

Table 4 Braimbridge and Keith's classification of bronchoesophageal fistula

Type I	Congenital bronchoesophageal fistula associated with congenital oesophageal diverticulum
Type II	Simple bronchoesophageal fistula
Type III	Bronchoesophageal fistula with an intralobar cyst
Type IV	Bronchoesophageal fistula communicating with a pulmonary sequestration

It was reported that bouts of cough when swallowing liquids (Ohno's sign) are pathognomonic for this condition and present in 65% of all cases^[3,4]. The duration of symptoms varies from 6 mo to 50 years before diagnosis^[2,4,6,7]. The duration of symptoms in our 11 patients before diagnosis was 5-36.5 years and over 15 years in 9 patients. Of the 11 patients, 6 were misdiagnosed as multiple pulmonary cysts, 4 as bronchiectasis, and 1 as empyema, with a misdiagnosis rate of 100%. Smith^[8] has given an embryologic explanation for the development of these fistulae. He stated that they are the result of persistent attachment between tracheobronchial tree and esophagus due to rapid elongation of the trachea and its separation from the esophagus.

From the pathological point of view, three points should be considered for congenital bronchoesophageal fistulae, including the long standing respiratory signs and symptoms even from infancy, the presence of bronchial or oesophageal epithelium lining the interior of the fistulous tract, and the absence of relevant inflammatory lesions in periphery of the fistula. Our 11 patients fulfilled these criteria and were therefore considered to have a congenital bronchoesophageal fistula. The fact that this type of lesions causes symptoms later in life can be explained by the occasional presence of membranes that can become permeable with time.

Conventional barium esophagography is the most sensitive means for the diagnosis of bronchoesophageal fistula^[4-7,9,10]. However, it is hard to detect tiny fistulae in a single scan. Therefore, a repetitive multi-positional esophagography is helpful for improving the detection rate of bronchoesophageal fistula. In this study, congenital bronchoesophageal fistulae were found in 6 cases during the first scan, and tiny fistulae were found in 3 cases during the multi-positional esophagography, indicating that both bronchoscopy and esophagoscopy should be performed

in these cases because they cannot always demonstrate the fistulous orifice. However, they help us to choose the *modus operandi*^[2,4-7]. CT scanning can rule out the presence of neoplasm and adenopathy, and define the extent of coexisting pulmonary disease, which may need resection^[5,7,11].

Braimbridge *et al.*^[12] have classified congenital bronchoesophageal fistula into 4 types (Table 4). Type II is the most prevalent and comprises almost 90% of all cases in some series^[2]. In our series, types I - IV were found in 2, 7, 2, and 1 cases, respectively, with an associated lung sequestration.

Despite the benign nature of this disease, it may lead to fatal complications if untreated^[5,7,13]. In order to avoid distal pulmonary lesions, early diagnosis and treatment are important. A high index of suspicion should be borne in mind for patients with repeated pulmonary infections or with coughing spells associated with ingestion.

Thoracotomy is the traditional treatment procedure for most cases of fistula^[2,4,6,7]. The two main treatment procedures are division and suturing of the ends of fistula and complete resection. The insertion of a muscular or pleural flap can prevent any refistulization^[4,7,9,13]. Pulmonary resection is often needed in patients with coex-

istent pulmonary diseases such as bronchiectasis and recurrent pneumonitis. In our series, fistula and associated lung lesions were completely removed in 10 patients, and the ends of fistula were sutured in 1 patient. The prognosis of the patients was excellent after the procedure. The postoperative course was uneventful in all patients but one who was complicated by bronchial fistula due to intraoperative contamination of the pleural space by oesophageal materials. However, the patient recovered after treatment. The less effective treatment modality is occlusion of the esophageal opening with biologic glue or a Celestin tube^[14], or with sodium hydroxid and acetic acid solution *via* a bronchoscope or an esophagoscope^[15]. We believe these techniques should be considered only when the condition of patients does not allow a thoracotomy.

Currently, most studies concerning congenital bronchoesophageal fistula in adults are case reports, and few clinical data are available based on large samples. In this study, 11 cases of congenital bronchoesophageal fistula admitted to our hospital in recent 20 years were analyzed.

In conclusion, a high index of suspicion should be borne in mind for patients with repeated pulmonary infections, especially with symptoms of recurrent bouts of cough after taking liquid food. Conventional barium esophagography is presently the most sensitive means for the diagnosis of congenital bronchoesophageal fistula. However, multi-positional esophagography should be performed for tiny or horizontal fistulae. Due to the long duration before diagnosis and repeated pulmonary infections, patients often suffer from severe conglutination in the thorax. A large volume bleeding may occur during operation and adequate blood preparation is necessary. Surgical management is the vital point for the prognosis of patients with congenital bronchoesophageal fistula. Fistula and associated lung lesions shall be completely removed to avoid recrudescence.

COMMENTS

Background

Congenital bronchoesophageal or tracheoesophageal fistula is rare in adults. Its diagnosis is difficult due to the nonspecific nature of its symptoms.

Research frontiers

The clinical characteristics, diagnosis and surgical treatment of congenital bronchoesophageal fistulae in adults were investigated.

Innovations and breakthroughs

Currently, most studies concerning congenital bronchoesophageal fistula in adults are case reports, and few clinical data are available based on large samples. In this study, 11 cases of congenital bronchoesophageal fistula admitted to hospital in recent 20 years were analyzed.

Peer review

This is an interesting case series report on a difficult and often misunderstood clinical condition.

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Polymorphisms of interleukin-10 promoter are not associated with prognosis of advanced gastric cancer

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Abstract

AIM: To evaluate the association between of the interleukin-10 (IL-10) promoter polymorphisms and survival of advanced gastric cancer (GC) patients.

METHODS: The IL-10 (-1082, rs1800896; -819, rs1800871; and -592, rs1800896) genotypes in 234 patients with advanced gastric cancer and in 243 healthy controls were determined by polymerase chain reaction-restriction fragment length polymorphism assay. Odds ratios (OR) and 95% confidence intervals (CI) were calculated by unconditional logistic regression for the associations between IL-10 genotypes and the risk of GC. The Kaplan-Meier method with log-rank testing was used to evaluate the association between genotype and survival of the patients.

RESULTS: The IL-10 -1082 G allele and GCC (-1082, -819 and -592) haplotype were associated with increased gastric cancer risks (OR 1.2, 95% CI 0.6-3.2, $P = 0.007$, for -1082 G allele, OR = 2.3, 95% CI, 1.2-4.1, $P = 0.005$, for GCC haplotype, respectively). However, none of the three IL-10 gene polymorphisms (-1082, -819 and -592) was correlated with gastric cancer survival ($P > 0.05$), and none of the genotypes of the three IL-10 sites was found as independent prognostic risk factors in the multivariate test.

CONCLUSION: IL-10 gene promoter polymorphisms may not be associated with the prognosis of advanced gastric cancer.

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Key words: Interleukin-10; Cytokine; Genetic polymorphism; Gastric cancer; Prognosis

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INTRODUCTION

Deregulated components of the immune system, such as cytokines, may be linked to the incidence and clinical course of malignant diseases with development of acute or chronic inflammatory reactions at tumor sites. Deregulated expression of defined subsets of cytokines

was found to be associated with the transformation of lymphatic cells either as autocrine growth factors for the transformed cells or as factors in rebuilding the tumor microenvironment, likely affecting tumor progression and dissemination^[1].

The mechanisms underlying differences in immune response between individuals are complex, but include inherited genetic variation. Some reports suggest that functional polymorphisms in the genes regulating the immune and inflammatory response may contribute to the susceptibility to and clinical outcome of gastric cancer^[2-4]. Interleukin-10 (IL-10) is a pleiotropic cytokine produced by macrophages, T-helper 2 cells and B lymphocytes and can suppress and stimulate the immune response^[5-7]. IL-10 has been shown to inhibit various immune functions, such as antigen presentation, cytokine production, macrophage activation, and antigen-specific T-cell proliferation^[8,9]. By interfering with antigen-presenting cells, IL-10 reduces antigen-specific T-cell proliferation. It has been postulated that IL-10 plays a key role in the oncogenetic and metastatic ability of neoplasms^[10,11]. Increased levels of serum IL-10 were found in patients with solid and hematopoietic tumors^[12]. However, a large body of evidence in different animal tumor models showed that IL-10 can favor immune-mediated cancer rejection^[13,14].

The gene encoding IL-10 is located on chromosome 1 (1q31-1q32). Several SNPs have been identified in the IL-10 gene promoter region. These include IL-10-1082 A/G, -819T/C and -592A/C, which influence the transcription of IL-10 messenger RNA and the expression of IL-10 *in vitro*^[15,16]. Although several studies have shown the possible involvement of IL-10 in the pathogenesis of gastric cancer^[17-21], its association with prognosis of gastric cancer was not extensively studied. The only study by Deans *et al*^[22] found that GG for IL-10-1082 was associated with reduced survival of gastric cancer patients, but it was not an independent prognosis factor for gastric cancer. The aim of this study was, therefore, to explore the relationship between polymorphisms of IL-10 -1082, -819 and -592 and the prognosis of patients with advanced gastric cancer in the northern area of China.

MATERIALS AND METHODS

Study population

A total of 234 patients (162 men and 72 women) with histologically or cytologically confirmed advanced gastric cancer registered from July 2005 to July 2008 at the Department of Oncology, Shandong Cancer Hospital and Institute were included in this study. The patients should have a histological diagnosis of gastric carcinoma with an unresectable primary tumor and/or metastases that were measurable or assessable by means of clinical examination, X-ray, computed tomography (CT) or ultrasound. The median age of the patients was 61.2 years (range, 27-79 years). There were 147 cases of antrum gastric cancer, 35 gastric cardia cancer, and 52 cases of other types, including 150 cases at stage III B and 84 cases at stage IV. Pathologically, 10 patients had

well-differentiated adenocarcinoma, 45 had moderately differentiated adenocarcinoma, 161 poorly differentiated adenocarcinoma, and 18 signet ring cell carcinoma. Two hundred and six patients were treated with conventional and 5-fluorouracil (5-FU)-based chemotherapy. Among them, 96 cases were treated with capecitabine/fluorouracil + cisplatin/oxaliplatin regimen and 89 cases were treated with docetaxel + oxaliplatin + fluorouracil regimen. The median chemotherapy courses consisted of 5 (1-11) cycles. Follow-up time was calculated from the initiation of diagnosis to July 2009, with a median of 13.3 mo (range, 3.5-34.4 mo). Thirty-three patients were lost to follow-up and 188 patients died, including 3 patients who died from causes other than gastrointestinal carcinoma during the follow-up.

We also selected 243 control individuals (aged 26-79 years) who visited the Shandong Cancer Hospital and Institute between July 2005 and July 2008 for general physical exams. The control individuals were screened to ensure that none had ever been diagnosed with cancer or other serious diseases. The selected controls were age matched to the cases (± 5 years). All the subjects were unrelated ethnic Han Chinese, and written informed consent was obtained from each participant. The study was approved by the Review Board of Shandong Cancer Hospital and Institute. A 2-mL peripheral blood sample was collected from each study participant.

Genotyping

Genomic DNA was extracted from peripheral blood using a Genomic DNA Extraction Kit (Fastagen, Shanghai, China) according to the manufacturer's protocol. IL-10 promoter polymorphisms were identified by PCR amplification and restriction analysis (PCR-RFLP), (Table 1). Each PCR reaction was performed in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, Foster, CA) at a final volume of 25 μ L (containing 5 pmol for each primer, 50 ng genomic DNA, 1.5 mmol/L MgCl₂, 5 μ mol/L dNTPs and 1 U of Taq DNA polymerase in PCR buffer containing 10 mmol/L Tris). PCR cycles used were as follows: 95°C for 5 min, 35 cycles of denaturing at 95°C for 40 s, annealing at the indicated temperature for 1 min, extension at 72°C for 40 s, and a single final extension at 72°C for 10 min. The amplified products were digested with corresponding restriction endonucleases (New England Biolabs, MA, USA), and separated by electrophoresis on a 10% polyacrylamide gel stained with silver nitrate for visualization.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS, Florida, USA). Demographic data between the study groups were compared using the Chi-square test and Student's *t* test. Each polymorphism was tested for deviation from the Hardy-Weinberg equilibrium by comparing the observed and expected genotype frequencies using the χ^2 test. Genotype frequencies of IL-10 were compared between groups using the χ^2 test, and odds ratios (OR) and 95% confidence intervals (CIs) were calculated using unconditional logistic regression

Table 1 Interleukin-10 promoter polymorphism identified by PCR-RFLP analysis

Polymorphism	Primer sequence	Annealing temperature	Restriction enzyme	Allele size
IL-10-1082G/A	5'-CTCGCTGCA ACCCAACTGGC-3' 5'-TCTTACCTATCCCTACTTCC-3'	58°C	<i>Mnl</i> I	A: 139 bp G: 106, 33 bp
IL-10-819C/T	5'-TCATTCTATGTGCTGGAGATGG-3' 5'-TGGGGGAAGTGGTAAGAGT-3'	59°C	<i>Mae</i> III	C: 125, 84 bp T: 209 bp
IL-10-592C/A	5'-GTGAGCACTACCTGACTAGC-3' 5'-CCTAGGTCACAGTGACGTGG-3'	58°C	<i>Rsa</i> I	C: 412 bp A: 175, 237 bp

Table 2 Genotype and allele frequencies of interleukin-10 promoter SNP in gastric cancer patients and controls

Genotypes/allele	Cases <i>n</i> (%)	Controls <i>n</i> (%)	Adjusted OR (95% CI) ¹	<i>P</i>
IL-10-1082	234	243		
AA	189 (80.4)	217 (89.3)	1	
AG	39 (17.2)	23 (9.7)	1.9 (1.1-3.4)	0.019 ^a
GG	6 (2.4)	3 (1.0)	2.4 (0.6-9.4)	0.244
A	417 (89.1)	457 (94.0)	1	
G	51 (10.9)	29 (6.0)	1.2 (0.6-3.2)	0.007 ^a
IL-10-819				
TT	99 (42.3)	109 (44.9)	1	
TC	96 (41.0)	106 (43.6)	0.9 (0.7-1.6)	0.975
CC	39 (16.8)	28 (11.5)	1.6 (0.9-2.8)	0.145
T	294 (62.8)	324 (66.7)	1	
C	174 (37.2)	162 (33.3)	1.2 (0.9-1.6)	0.221
IL-10-592				
AA	99 (42.3)	109 (44.9)	1	
AC	96 (41.0)	106 (43.6)	0.9 (0.7-1.6)	0.975
CC	39 (16.8)	28 (11.5)	1.6 (0.9-2.8)	0.145
A	294 (62.8)	324 (66.7)	1	
C	174 (37.2)	162 (33.3)	1.2 (0.9-1.6)	0.221
Haplotype				
ATA	282 (60.3)	314 (64.6)	1	
ACC	135 (28.9)	143 (29.4)	1.1 (0.8-1.5)	0.731
GCC	39 (8.3)	19 (3.9)	2.3 (1.2-4.1)	0.005 ^a
GTA	12 (2.5)	10 (2.1)	1.3 (0.6-3.2)	0.504

¹Adjusted for age, gender, and smoking status; ^a*P* < 0.05. OR: Odds ratio.

with adjustment for age and sex. The haplotypes of IL-10 (-1082, -819 and -592) were analyzed using the SHEsis software (Bio-X Inc., Shanghai, China), which uses a full-precise-iteration (FPI) algorithm to reconstruct haplotypes. The Kaplan-Meier method with a log-rank test was used to evaluate the association between genotype and survival. Multivariate analysis was performed using Cox proportional hazards regression. Statistical significance was interpreted as *P* < 0.05.

RESULTS

IL-10 polymorphism and gastric cancer risks

The genotype and allele frequencies of the IL-10 SNP in 234 gastric cancer patients and 243 healthy controls are shown in Table 2. All genotype frequencies in both patient and control groups were in the Hardy-Weinberg equilibrium (*P* < 0.05). There were significant differences in the genotype and allele frequencies of the IL-10 promoter -1082 A/G polymorphism between gastric cancer and

control groups. The -1082 AG genotypes were associated with a significantly increased risk of gastric cancer as compared with the -1082 AA genotypes (OR 1.9, 95% CI 1.1-3.4, *P* = 0.019). The -1082 G allele was associated with a significantly increased risk of gastric cancer as compared with the -1082 A allele (OR 1.2, 95% CI 0.6-3.2, *P* = 0.007). However, genotype and allele frequencies of the IL-10 -819 T/C and -592 A/C polymorphisms in gastric cancer patients were not significantly different compared with those in healthy controls (*P* > 0.05). The estimated haplotype frequencies of IL-10 polymorphisms in gastric cancer patients and controls are also shown in Table 2. Complete linkage disequilibrium was observed between locus -819T/C and locus -592A/C. Four possible haplotypes were demonstrated in our population. The most frequent haplotype in both patients (60.3%) and controls (64.6%) was the ATA (-1082A, -819T and -592A) haplotype. By haplotype analyses, we found that the GCC (-1082G, -819C and -592C) haplotype was associated with a significantly increased risk of gastric cancer as compared with the ATA haplotype (OR = 2.3, 95% CI 1.2-4.1, *P* = 0.005).

IL-10 polymorphism and survival of advanced gastric cancer

Of the 234 patients, 33 were lost to follow-up. We compared the IL-10 genotype with survival time in 201 patients with advanced gastric cancer. The median follow-up time was 13.3 mo (range, 3.0-30.3 mo), and the median survival time was 11.2 mo. The median survival time was 11.4 mo for patients with the IL-10 -1082AA genotype, 10.8 mo for patients with the GA genotype, and 11.0 mo for patients with the GG genotype. The IL-10 -1082 genotypes were not associated with the prognosis of gastric cancer (*P* = 0.709, log-rank test). Similarly, the median survival time was 11.5 mo for patients with the IL-10 -819TT/-592AA genotype, 10.8 mo for patients with the -819TC/-592AC genotype, and 10.2 mo for patients with the -819CC/-592CC genotype (*P* = 0.090, log-rank test). When stratified by the clinical stage, the association between the IL-10 gene polymorphisms (-1082, -819 and -592) and the gastric cancer survival was not statistically significant either in stage IIIB or stage IV (Figure 1). Multivariate Cox regression was used to analyze the effect of different risk factors (IL-10-1082A/G, -819T/C and -592A/C genotype, chemotherapy regimen, age, gender, disease stage, and histology) on survival time. However, none of the genotypes of the three IL-10 sites

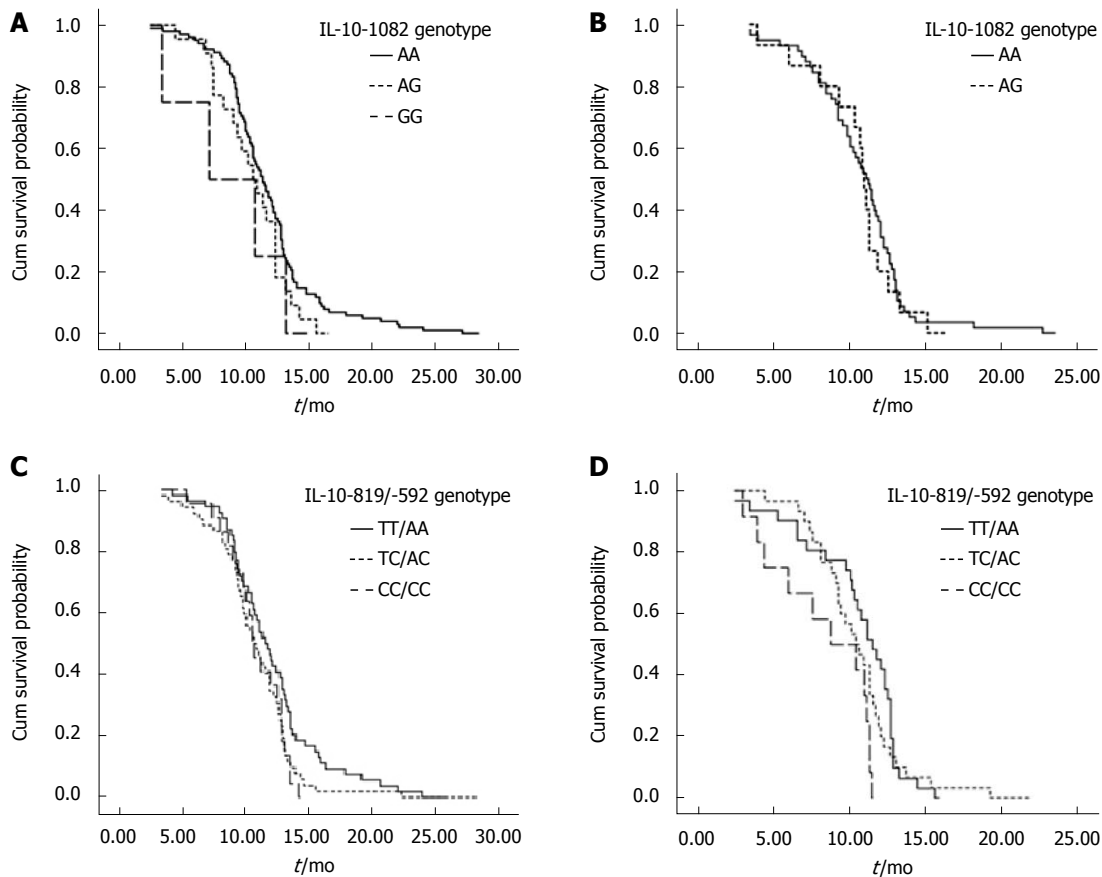


Figure 1 Kaplan-Meier survival plot for advanced gastric cancer patients by interleukin-10 genotype. A: Interleukin (IL)-10 -1082 genotype in stage III B; B: IL-10 -1082 genotype in stage IV; C: IL-10 -819/-592 genotype in stage III B; D: IL-10 -819/-592 genotype in stage IV.

Table 3 Cox regression analysis of variables affecting prognosis in advanced gastric cancer patients

Variable	P	Hazard ratio	95% CI for hazard ratio	
			Lower	Upper
IL-10-1082 genotype	0.815	1.2	0.8	1.7
IL-10-819/-592 genotype	0.671	1.1	0.5	1.9
Treatment method	0.426	1.4	0.9	2.3
Age	0.595	0.9	0.7	1.3
Stage	0.234	1.2	0.9	1.5
Histology	0.519	1.2	0.7	2.1

was recognized as an independent prognostic indicator (Table 3).

DISCUSSION

Cytokines play a crucial role in the regulation of key pathways of immunity, the balance between cell-mediated (Th1) and humoral (Th2) responsiveness. IL-10, which is produced mainly by macrophages and T lymphocytes, is an important anti-inflammatory and immunosuppressive cytokine, it inhibits the Th1-type pathway activation, prevents antigen-presenting cells (APC) from obtaining access to tumor antigens, and down-regulates surface expression of costimulatory molecules CD80 or CD86 on

tumor cells^[5-7]. Due to its immunosuppressive and anti-inflammatory properties, it has been hypothesized that IL-10 may contribute to the escape of tumor cells from immune surveillance and favor tumor growth. On the other hand, several studies showed that IL-10 may regulate angiogenesis in various cancers and is believed to play a protective and preventive role against tumors^[13,14].

Although the genetic control of IL-10 expression is not clearly understood yet, polymorphisms in promoter regions have been reported to determine inter-individual differences in IL-10 production. Previous studies indicated that three common-1082 A/G, -819 T/C and -592 A/C polymorphisms of the IL-10 promoter may influence production and expression of IL-10. IL-10 promoter-1082G allele or GCC haplotype (defined by three SNPs at positions of -1082, -819 and -592) is associated with increased IL-10 production and ATA haplotype is generally assumed to be a lower IL-10 responder^[15,16].

In this study we evaluated the association between the polymorphisms of the IL-10 promoter and advanced gastric cancer in a Chinese population. Our data showed significant differences in allele, genotype and haplotype frequencies between gastric cancer patients and healthy controls. In concordance with our study, Lee *et al.*^[20], Sugimoto *et al.*^[21] and several studies from China^[18,19] reported that the IL-10 gene promoter polymorphisms were associated with the risk of gastric cancer in Korean, Japanese and Chinese populations. However, these

results are not consistent with studies previously conducted in American and Spain, in which the IL-10 promoter genotype was not associated with gastric cancer risk^[23,24]. Although it is difficult to determine the reasons behind the contradictory results in these studies, the different genetic background of study populations may be one of the main factors.

Although the IL-10 promoter polymorphisms are associated with increased risk of advanced gastric, few studies have investigated the relationship between IL-10 promoter polymorphisms and gastric cancer prognosis. In this study, we investigated whether there are any associations between the three IL-10 promoter SNPs and survival time of advanced gastric cancer patients. Our results showed that the three IL-10 gene polymorphisms (-1082, -819 and -592) did not correlate with the advanced gastric cancer survival. Multivariate testing also found no genotype of the three IL-10 sites as independent prognostic risk factors. Several studies have observed that serum IL-10 is of independent prognostic utility in patients with advanced gastrointestinal carcinoma, and the reasonable interpretation is that the higher serum IL-10 level is secreted by the tumor itself rather than the inflammatory infiltration^[25,26]. However, only one study focused on the polymorphisms of cytokine genes and gastric cancer prognosis and found GG in IL-10-1082 to be associated with reduced survival of gastric cancer patients, but not an independent prognosis factor for gastric cancer^[22]. The reason why our study did not show the association between IL-10 genotype and gastric cancer prognosis may be the relatively small series of patients examined (the number of gastric cancer patients with GG genotype at position -1082 in our study population is small, only six cases). It remains to be confirmed whether the relationship is reproducible in larger Chinese samples.

In conclusion, our results suggested that IL-10 promoter polymorphisms were associated with an increased risk of gastric cancer, but these polymorphisms did not influence the prognosis of the Chinese patients with advanced gastric cancer. As this is the first report of the association between IL-10 promoter polymorphism and prognosis of the Chinese patients with gastric cancer, the results of the present study should be viewed cautiously. Since the dual biological effects of IL-10 and the functional significance of IL-10 promoter polymorphisms in determining IL-10 expression still need to be elucidated, further investigations are required to explore the relationship of the polymorphisms of IL-10 promoter and clinical outcome of gastric cancer.

COMMENTS

Background

The immune dysfunction may be linked to the incidence and clinical course of malignant cancers. Interleukin-10 (IL-10) is a pleiotropic immunoregulatory cytokine which is involved in inflammatory reaction and immune regulation and is considered to exert effects in malignant transformation. IL-10 promoter polymorphisms have been reported to determine inter-individual differences in IL-10 production and are associated with the risk and pathogenesis of several cancers.

Research frontiers

Several studies have shown the possible involvement of IL-10 polymorphisms in the pathogenesis of gastric cancer, although its association with prognosis of gastric cancer was not well explored. The aim of this study was to explore the association between polymorphisms of IL-10 promoter and the prognosis of patients with advanced gastric cancer.

Innovations and breakthroughs

This study indicated that IL-10 promoter polymorphisms were associated with an increased risk of gastric cancer, but these polymorphisms did not influence the prognosis of the patients with advanced gastric cancer.

Applications

This is the first report of the association between IL-10 promoter polymorphism and prognosis of the Chinese patients with gastric cancer. The results of this study will help understand the genetic background of the immune-related gene and gastric cancer incidence and prognosis.

Terminology

IL-10 is an important anti-inflammatory and immunosuppressive cytokine, which inhibits the Th1-type pathway activation, prevents antigen-presenting cells from obtaining access to tumor antigens, and down-regulates surface expression of costimulatory molecules CD80 or CD86 on tumor cells.

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Efficacy of endoscopic therapy for gastrointestinal bleeding from Dieulafoy's lesion

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Abstract

AIM: To investigate the endoscopic hemostasis for gastrointestinal bleeding due to Dieulafoy's lesion.

METHODS: One hundred and seven patients with gastrointestinal bleeding due to Dieulafoy's lesion were treated with three endoscopic hemostasis methods: aethoxysklerol injection (46 cases), endoscopic hemoclip hemostasis (31 cases), and a combination of hemoclip hemostasis with aethoxysklerol injection (30 cases).

RESULTS: The rates of successful hemostasis using the three methods were 71.7% (33/46), 77.4% (24/31) and 96.7% (29/30), respectively, with significant differences between the methods ($P < 0.05$). Among those who had unsuccessful treatment with aethoxysklerol injection, 13 were treated with hemoclip hemostasis and 4 underwent surgical operation; 9 cases were successful in the injection therapy. Among the cases with unsuccessful treatment with hemoclip hemostasis,

7 were treated with injection of aethoxysklerol and 3 cases underwent surgical operation; 4 cases were successful in the treatment with hemoclip hemostasis. Only 1 case had unsuccessful treatment with a combined therapy of hemoclip hemostasis and aethoxysklerol injection, and surgery was then performed. No serious complications of perforation occurred in the patients whose bleeding was treated with the endoscopic hemostasis, and no re-bleeding was found during a 1-year follow-up.

CONCLUSION: The combined therapy of hemoclip hemostasis with aethoxysklerol injection is the most effective method for gastrointestinal bleeding due to Dieulafoy's lesion.

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Key words: Dieulafoy's lesion; Gastrointestinal bleeding; Endoscopic therapy; Aethoxysklerol; Therapeutic efficacy

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INTRODUCTION

Dieulafoy's lesion was first reported by Gallad in 1884, and a systemic description was made by Dieulafoy in 1903. It is also called Dieulafoy's vascular malformation^[1]. Patients with gastrointestinal bleeding due to Dieulafoy's lesion in our hospital were treated with the endoscopic

hemostasis, and a satisfactory therapeutic efficacy was achieved.

MATERIALS AND METHODS

Patients

One hundred and seven patients with gastrointestinal bleeding due to Dieulafoy's lesion were enrolled in this study. There were 70 males and 37 females, with a mean age of 54 years. Clinical presentations were repeated hematemesis, a dark or bloody stool, the amount of blood loss of 800-2000 mL, shock occurring in some patients, and hemoglobin 46-80 g/L. No obvious epigastric discomfort and abdominal pain were noted, and there was no history of peptic ulcer or heredity disease.

Diagnostic standards

(1) The bottom of the ulcer had exposed vessels (most were dilated small arteries); (2) The ulcer was superficial and small, and the diameter was less than 0.5 cm; (3) Spurting blood from the ulcer was observed in the active phase, and if bleeding stopped, a black blood scab was observed with endoscopic examination and was easily misdiagnosed; (4) Dieulafoy's lesion occurred mostly in elderly people, possibly because of a small vascular malformation or arteriosclerosis; and (5) Dieulafoy's lesion was mostly located at the gastric body, or at the juncture of the gastric fundus and the gastric body^[2-4].

Endoscopic findings

Emergent endoscopic examination was performed for all the patients. Blood clotting was observed and they were flushed with saline. Fresh blood clotting covered the mucosa in 42 cases (39.3%), blood spurting from a small artery occurred in 32 cases (29.9%), ruptured vessels occurred in 33 cases (30.8%), and no obvious hyperemia was observed in the surrounding mucosa. Lesions were found in the following areas: the upper part of the gastric corpus (36 cases, 33.6%), surrounding the cardia (28 cases, 26.2%), the gastric fundus (18 cases, 16.8%), the middle of the gastric body (5 cases, 4.7%), the lower part of the gastric body (4 cases, 3.7%), the gastric antrum (3 cases, 2.8%), the duodenum (2 cases, 1.9%), the descending part of the duodenum (2 cases, 1.9%), the rectum and colon (6 cases, 5.6%), and the esophagus (3 cases, 2.8%).

Endoscopic therapy

Patients were randomly divided into 3 groups according to the three types of endoscopic therapy used.

Group one: patients received aethoxysklerol injection ($n = 46$ cases). A concentration of 1% aethoxysklerol was injected around the blood vessels beneath the mucosa and 0.5 mL was used for each injected site. Three to 6 sites were injected and the amount of aethoxysklerol used was 3-5 mL. Aethoxysklerol was injected into the ruptured site of the blood vessel (Figure 1).

Group two: patients were treated with endoscopic hemoclip hemostasis ($n = 31$ cases). We used an Olym-

pus HX-5LR-1 and Olympus clip HX-600-135 for bleeding arteries. Hemoclip hemostasis was performed until the vessel stopped bleeding (Figure 2).

Group three: patients received a combined therapy of hemoclip hemostasis and aethoxysklerol injection ($n = 30$ cases). For bleeding arteries, hemoclip hemostasis was used until the bleeding stopped. A concentration of 1% aethoxysklerol was injected around the blood vessel beneath the mucosa, and 0.5 mL was used for each injected site. Three to 6 sites were injected and the amount of aethoxysklerol used was 3-5 mL (Figure 3).

Criteria for determining successful endoscopic hemostasis

The criteria for determining successful endoscopic hemostasis were as follows. (1) Endoscopic demonstration: blood spurting or capillary hemorrhage stopped, and the endoscopic field of view became clear; (2) Clinical manifestations: there was no hematemesis or dark stool after treatment, blood pressure rose to a normal range and was stable, and pulse rate decreased and strengthened.

Criteria for determining unsuccessful endoscopic hemostasis

Criteria for determining unsuccessful endoscopic hemostasis were as follows: (1) hematemesis and/or dark stool occurred 48 h after endoscopic treatment; (2) hemoglobin was decreased by more than 20 g/L; (3) there was evidence of hypovolemic shock; and (4) there was manifestation of bleeding and blood transfusion was necessary. Re-bleeding was confirmed at the original site as demonstrated by endoscopic examination.

Statistical analysis

Rates of successful hemostasis and perforation among the groups were compared using the χ^2 test (SPSS 10.0). $P < 0.05$ was considered as a significant difference.

RESULTS

One hundred and seven patients received endoscopic hemostasis in this study. Eight cases had unsuccessful treatment with endoscopic hemostasis and underwent a surgical operation. No serious complications such as perforation occurred in the 99 cases whose bleeding was treated with endoscopic hemostasis, and no re-bleeding was found during a 1-year follow-up.

Success of hemostasis

The success rate of hemostasis by aethoxysklerol injection was 71.7% (33/46). Re-bleeding occurred in 13 cases in a short period after treatment (less than 48 h). For the re-bleeding cases, the hemoclip hemostasis was used; 9 cases were successfully treated and 4 cases whose bleeding was not controlled received a surgical operation.

The success rate of hemostasis by endoscopic hemoclip hemostasis was 77.4% (24/31). Re-bleeding occurred in 7 cases in a short period after treatment (less than 48 h)



Figure 1 Bleeding in Dieulafoy's lesion from the gastric fundus. A: Bleeding from the ruptured site of the vessel; B: Injection with aethoxysklerol; C: Bleeding stopped and the field of view became clear.



Figure 2 Bleeding in Dieulafoy's lesion from the greater curvature of the stomach. A: Spurting blood; B: After hemoclip hemostasis, the spurting of blood was stopped.

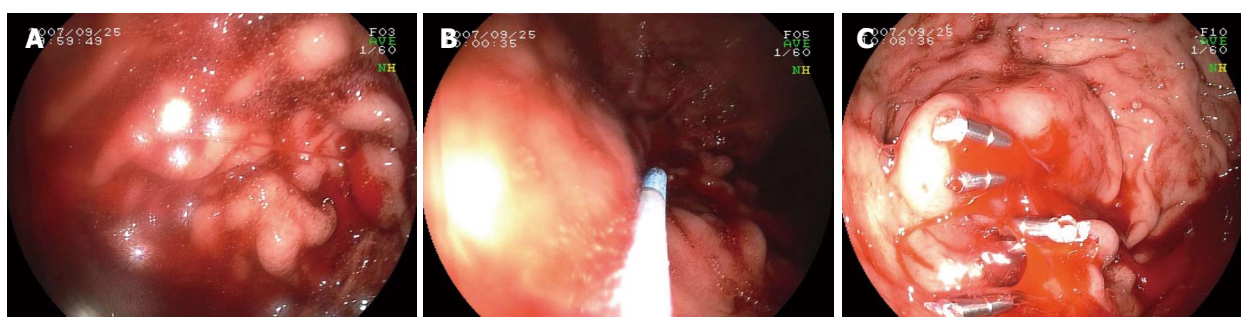


Figure 3 Bleeding in Dieulafoy's lesion from the gastric fundus. A: Spurting blood; B: Injection with aethoxysklerol; C: Bleeding stopped after hemoclip hemostasis and aethoxysklerol injection.

and was treated with aethoxysklerol injection. Four cases were successfully treated and 3 unsuccessful cases were treated surgically.

The successful rate of hemostasis by the combined therapy of hemoclip hemostasis with aethoxysklerol injection was 96.7% (29/30). Re-bleeding occurred in 1 case in a short period after treatment (less than 48 h), and he was treated by surgical operation.

DISCUSSION

Dieulafoy's lesion is a type of rare congenital vascular malformation, and it is also called "Dieulafoy's ulcer" or "constant diameter artery bleeding". The lesion can occur in any part of the gastrointestinal tract, such as the esophagus, colon, and small intestine. However, it often

occurs in the lesser curvature of the stomach 6 cm from the cardioesophageal junction^[5-8]. While Dieulafoy's lesion has no specific symptoms and is not easily diagnosed, the emergent endoscopic examination is an effective means for its diagnosis. The endoscopic presentations of Dieulafoy's lesion are as follows: (1) a superficial notch in the gastric mucosa, blood vessels in the mucosa, and coagulum on its surface; (2) a focal defect of lesser curvature of stomach mucosa complicated with active bleeding; (3) small arteries can protrude on the mucosa and active bleeding can occasionally be detected; and (4) occasionally, blood permeation can be detected from the mucosa, and is often detected when bleeding^[9-12].

The bleeding of Dieulafoy's lesion can be unexpected, with no obvious cause. Patients often do not have other bleeding diseases such as peptic ulcers and cirrhosis, since

the ruptured vessel is an artery with a constant diameter. If the bleeding is serious, patients often complain of hematemesis or hematemesis accompanied by a dark stool^[13-16]. The pathogenesis of Dieulafoy's lesion is considered as a congenital vascular malformation. Normally, the diameter of the gastrointestinal artery gradually decreases to 0.12-0.2 mm, branching to the mucosa. However, the diameter of the artery in a Dieulafoy's lesion does not decrease; it is abnormally dilated and its diameter is 0.4-4 mm. This constant arterial diameter leads to the pathology of Dieulafoy's lesion. The abnormally dilated artery travels under the mucosa. The mucosa in Dieulafoy's lesion is compressed and becomes ischemic, atrophied and thin. A small ulcer can form with the decay of digestive juice and friction from chyme, and if the small artery is exposed, it can rupture and bleed. The artery is often the branch of the left gastric artery; therefore, the bleeding lesion is often located at the lesser curvature of the stomach 6 cm from the cardioesophageal junction^[17-19]. When a small artery bleeds, blood pressure decreases, and vasoconstriction and thrombosis occur in the bleeding artery. Bleeding can temporarily stop and the artery is invisible when bleeding stops. It might not be detected by endoscopic examination, even with a surgical operation. If blood pressure rises to normal or the blood clot is shed, serious bleeding can reoccur.

Generally, it is considered that medical treatment plays a minor role in the treatment of Dieulafoy's lesion; endoscopic therapy and surgery are often performed. The use of pituitrin and somatostatin causes bowel vessels to contract and blood flow to decrease, and these drugs are used for endoscopic therapy and surgical operations. The Dieulafoy's lesion before the 1980's was mainly treated with surgical operations. Since Boron first successfully treated 3 cases of Dieulafoy's lesion in 1987 with the endoscopic therapy^[20], an increasing number of doctors have treated this lesion with endoscopic therapy. The death rate decreased from 60%-70% to 20%. Endoscopic treatment has the advantages of being simple in operation, which is easily replicated, is microtraumatic, safe and useful, and it can be performed during the examination. Methods used include injection beneath the mucosa, electric coagulation, laser, heat probe, microwaves, ligation and hemoclips. The efficacy was reported to be more than 80%^[21-28].

Three types of endoscopic therapy were used in this study for the treatment of bleeding due to Dieulafoy's lesion. Aethoxysklerol injection therapy can result in sclerosis and emphysema to prevent re-bleeding. Local tissue edema occurred and the surrounding pressure of the bleeding site increased when aethoxysklerol was injected. The artery was compressed and thrombosis occurred in the vessel. When aethoxysklerol was injected around the vessel, edema and inflammation rapidly occurred, with fibroblast proliferation. Forty-six cases received this type of therapy and the rate of successful hemostasis was 71.7% (33/46). We also treated patients with endoscopic hemoclip hemostasis. The mechanism of hemoclip hemostasis is similar to that of surgical vascular suturing. In our study, 31 cases received this type of therapy, and the rate of suc-

cessful hemostasis was 77.4% (24/31). The third method we used in this study was a combined therapy of hemoclip hemostasis with injection of aethoxysklerol. Thirty cases received this therapy, and the rate of successful hemostasis was 96.7% (29/30). We found that this combined therapy was the most effective method for stopping bleeding from a Dieulafoy's lesion, and it could effectively reduce the re-bleeding rate as well.

Hemoclip hemostasis is widely used for non-variceal active bleeding, and it is indicated for Dieulafoy's lesion^[29,30]. Our study used the following procedure. A clip was assembled on the delivery device. When a bleeding lesion was detected, the delivery device was inserted into the endoscopic working channel to push it to the anterior extremity of the endoscope. The clip was stretched out of the endoscope, and then the clip was stretched open to the largest width (1.2 cm). The direction of the clip could be adjusted with the rotating device on the delivery system. The stretched clip was collimated to the lesion, the gliding lug on the device was pushed back, and the clip was locked. The clip was then released and the delivery device was pulled out. One clip was used for ordinary lesions, and for larger lesions, 2-3 clips were needed. We believe that the key points for successful clipping are as follows: (1) the lesion needs to be directly observed; (2) the lesion and its surrounding tissue should be fully exposed, and the angle of the clip and bleeding site should be in a range of 45-90°; and (3) the depth of the clip should be considered. The optimal depth is where the exposed vessel and deep tissue are able to be clipped. The clip should not be superficial, and if it is superficial, the clip can come off in a short period, and then re-bleeding is inevitable. The clip often releases automatically in 1-3 wk. It is mixed with food debris and the stool, and it is then eliminated from the body. This method is considered as microtraumatic. If it is not successful at the first time, it can be replicated. Even if it fails, it can mark the bleeding site, thereby making the surgical operation easier, and avoiding blind resection. This method is effective for the treatment of Dieulafoy's lesion, and it is increasingly recognized by doctors. We found that if aethoxysklerol was injected after clipping, the re-bleeding rate in Dieulafoy's lesion was effectively decreased.

COMMENTS

Background

Generally, it is considered that medical treatment plays a minor role in the treatment of bleeding from Dieulafoy's lesion. Endoscopic therapy and surgical operations are the preferred treatments for this lesion. In recent years, an increasing number of doctors have treated bleeding from a Dieulafoy's lesion with endoscopic therapy, and the death rate has decreased. The purpose of this study was to explore a new endoscopic method for the treatment of bleeding due to Dieulafoy's lesion.

Research frontiers

Patients with gastrointestinal bleeding due to Dieulafoy's lesion were treated with the endoscopic hemostasis. Three methods were used: aethoxysklerol injection, endoscopic hemoclip hemostasis, and a combination of hemoclip hemostasis with aethoxysklerol injection.

Innovations and breakthroughs

The authors concluded that a combined therapy of hemoclip hemostasis with aethoxysklerol injection is the most effective method for gastrointestinal bleeding due to Dieulafoy's lesion.

Applications

A combined therapy of hemoclip hemostasis with injection of aethoxysklerol, can be applied successfully in the treatment of bleeding due to a Dieulafoy's lesion.

Terminology

Dieulafoy's lesion: Dieulafoy's lesion is a type of rare congenital vascular malformation, and it is also called "Dieulafoy's ulcer" or "constant diameter artery bleeding". It was first reported in 1898 by a French surgeon named Dieulafoy. Endoscopic hemostasis: A kind of endoscopic method to control bleeding.

Peer review

Since the nature of bleeding from Dieulafoy's lesion poses several technical difficulties in its identification and its nonsurgical treatment, several endoscopic modalities have been used, but the treatment of choice is still unclear. Therefore, comparison of the efficacy and safety among two single hemostatic methods and a combined method applied to more than 100 patients provided useful results. In addition, the technical details given in the text may be helpful for endoscopists for the diagnosis and treatment of bleeding from a Dieulafoy's lesion.

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Autoantibodies against MMP-7 as a novel diagnostic biomarker in esophageal squamous cell carcinoma

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Abstract

AIM: To evaluate the diagnostic values of serum autoantibodies against matrix metalloproteinase-7 (MMP-7) in patients with esophageal squamous cell carcinoma (ESCC).

METHODS: The MMP-7 cDNA was cloned from ESCC tissues, and MMP-7 was expressed and purified from a prokaryotic system. MMP-7 autoantibodies were then measured in sera from 50 patients with primary ESCC and 58 risk-matched controls, using a reverse capture enzyme-linked immunosorbent assay (ELISA) in which autoantibodies to MMP-7 bound to the purified MMP-7 proteins. In addition, MMP-7 autoantibody levels in sera from 38 gastric cancer patients and from control serum samples were also tested.

RESULTS: The optimum conditions for recombinant MMP-7 protein expression were determined as 0.04 mmol/L

Isopropyl-β-D-Thiogalactopyranoside (IPTG) induction at 37°C for four hours. The levels of serum autoantibodies against MMP-7 were significantly higher in patients with ESCC than in the matched-control samples ($OD_{450} = 1.69 \pm 0.08$ vs $OD_{450} = 1.55 \pm 0.10$, $P < 0.001$). The area under the receiver operating characteristic (ROC) curve was 0.87. The sensitivity and specificity for detection of ESCC were 78.0% and 81.0%, respectively, when the OD_{450} value was greater than 1.65. Although the levels of autoantibodies against MMP-7 were also significantly higher in patients with gastric cancer compared to control samples ($OD_{450} = 1.62 \pm 0.06$ vs $OD_{450} = 1.55 \pm 0.10$, $P < 0.001$), the diagnostic accuracy was less significant than in ESCC patients. The area of ROC curve was 0.75, whereas the sensitivity and specificity were 60.5% and 71.7%, respectively, when the cut-off value of OD_{450} was set at 1.60.

CONCLUSION: Serum autoantibody levels of MMP-7 may be a good diagnostic biomarker for esophageal squamous cell carcinoma.

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Key words: Matrix metalloproteinase-7; Serum autoantibody; Esophageal squamous cell carcinoma; Gastric cancer; Biomarker

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC), the major

histological form of esophageal cancer in East Asian countries, is one of the leading causes of cancer death worldwide^[1,2]. The high mortality of this disease is largely due to the lack of a screening strategy to detect early stage disease. ESCC survival is highly stage dependent. Patients with localized disease are 12 times more likely to survive five years than those with distant disease^[3]. Unfortunately, most cases are diagnosed with ESCC when they have already reached an advanced stage^[4]. Thus there is a growing need to identify useful biological markers for early, non-invasive diagnosis of ESCC^[5].

The traditional serological tumor markers for ESCC, which are mainly secreted tumor antigens, i.e. CEA, SCCA, CYFRA21-1, and DKK-1, have been found to be useful but not sufficiently sensitive for early detection of the disease^[6,7]. Autoantibodies against tumor-associated antigens were recently reported in sera from patients with ESCC and other cancers as a promising approach for early cancer detection^[8-10]. Changes in the level of gene expression and aberrant expression of tissue-restricted gene products are factors that lead to humoral immune response in cancer patients, usually at the early stage of cancer development^[11,12]. Therefore identifying novel autoantibody biomarkers may lead to early diagnosis or prediction of disease progression in patients with ESCC.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes capable of degrading the extracellular matrix (ECM). MMPs play a key role in the physiological degradation of the ECM in angiogenesis, tissue repair, and tissue morphogenesis^[13]. They also regulate cell growth and inflammation by cleaving non-matrix proteins like growth factors, cytokines and chemokines, and their respective receptors^[14]. MMP-7, as the smallest molecule of the MMPs, has been found overexpressed in a variety of epithelial and mesenchymal tumors, such as esophagus, colon, liver, renal, and pancreas. Its expression is correlated with unfavorable prognosis^[15]. Increased circulating levels of MMP-7 proteins were correlated with the presence of metastatic disease and poor patient survival in colorectal and renal cell cancer^[15-17]. However, as one of the early immune responses to cancer development, levels of autoantibodies against the MMP-7 protein have not been studied. This humoral immune response to MMP-7 might be a good early indicator of ESCC.

In this study, we evaluate the diagnostic values of serum autoantibodies against MMP-7 in ESCC and gastric cancer patients using purified MMP-7 proteins. We further discuss the possibility of the utility of these autoantibodies to MMP-7 as tumor markers in clinical diagnosis.

MATERIALS AND METHODS

Clinical samples

A total of 108 individuals' serum samples (50 ESCC patients and 58 risk-matched controls) were collected from Baoding Tumor Hospital, Hebei, China. About 10 g of ESCC tumor samples were also collected in parallel. In ad-

Table 1 Characteristics of normal and patient serum *n* (%)

	Control (<i>n</i> = 58)	Patients with esophageal squamous cell carcinoma (<i>n</i> = 50)	Patients with gastric cancer (<i>n</i> = 38)
Age			
≤ 45	5 (8.62)	3 (6.00)	4 (10.53)
45-55	7 (12.07)	4 (8.00)	6 (15.79)
55-65	24 (41.38)	23 (46.00)	13 (34.21)
65-75	17 (29.31)	19 (38.00)	12 (31.58)
≥ 75	5 (8.62)	1 (2.00)	1 (2.63)
Sex			
Female	11 (18.97)	9 (18.00)	13 (34.21)
Male	47 (81.03)	41 (82.00)	25 (65.79)

dition, 38 serum samples were collected from patients with gastric cancer from the same hospital. Detailed information on the serum samples is listed in Table 1. All the clinical samples were collected after informed consents were obtained.

Cloning the MMP-7 cDNA from ESCC tissues

About 10 g of each ESCC tissue sample was snap-frozen in liquid nitrogen. RNA extraction was performed according to procedures described in the TRIzol Reagent manual (Invitrogen, USA). Primers were designed according to the sequence of the MMP-7 mRNA (GenBank, NM_002423.3). The sequence of the forward primer was 5'-GGAATTC⁺CATATGTC⁺ACTATTTTC-CAAATAGCCC-3' and the sequence of reverse primer was 5'-CCCAAGC⁺TTTATCCATATAGTTTCTGAATGCC-3'. *Nde* I and *Hind* III (underlined) restriction sites were introduced into the sequences of forward and reverse primers, respectively.

RT-PCR reactions were carried out under the following conditions. After heating at 94°C for 4 min, the reactions were exposed to 30 cycles of 94°C for 30 s, 63°C for 40 s, and 72°C for 1 min; with a final extension at 72°C for 10 min. The final products were then subjected to electrophoresis on 1% agarose. The amplified inserts were purified using a DNA purification kit (QIAGEN, USA), digested with *Nde* I and *Hind* III, and then ligated to a prokaryotic expression vector pET28b(+) (Novagen, USA) that was also digested with the same restriction enzymes. The constructed plasmid was transformed into competent *E. coli* DH5α cells and grown in Luria-Bertani (LB) broth supplemented with kanamycin (30 μg/mL). The recombinant plasmid was confirmed by double endonuclease digestion and DNA sequencing.

MMP-7 protein expression and purification

The recombinant plasmid pET-28b/MMP-7 was transformed into expression strain *E. coli* Rosetta (DE3) cells by heat-shock. One colony was picked and grown in

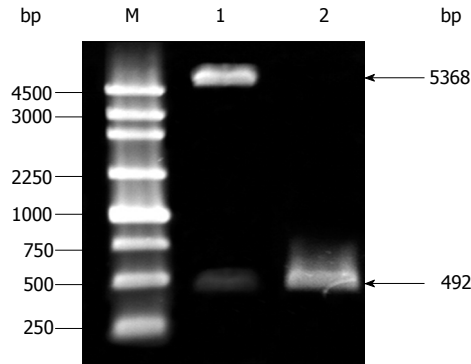


Figure 1 Double endonuclease digestion of the recombinant vector pET-28b/matrix metalloproteinase-7. M: DNA maker; Lane 1: Double digestion with NdeI and HindIII; Lane 2: Positive bacterial clone.

20 mL LB medium containing 30 µg/mL kanamycin at 37°C until an optical density (OD) at 600 nm of 0.6 was reached. Isopropyl-β-D-Thiogalactopyranoside (IPTG) was then added to induce protein expression at 28°C and 37°C. To determine the optimal condition for MMP-7 expression, DE3 cells were induced at different concentrations (0.4, 0.8, and 1.0 mmol/L) of IPTG for different lengths of time (1, 2, 4, 6 and 7 h). At the end of each condition, cells were harvested by centrifugation, resuspended in 1 mL PBS, and sonicated on ice until the suspension became transparent (5 min). The lysate was centrifuged for 30 min at 12000 *g*, and then both the supernatant and the pellets were tested for the MMP-7 protein expression by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To purify this His-tagged MMP-7 recombinant protein, a QIAexpressionist (QIAGEN, USA) kit was used. Briefly, 3 mL of cell culture at the most optimal expression conditions was pelleted and resuspended in 600 µL lysis buffer (8 mol/L Urea, 10 mmol/L NaH₂PO₄, 10 mmol/L Tris.Cl, pH8.0) at room temperature for 4 h. Cell debris was cleared by centrifugation, and the supernatants were transferred to a fresh tube and incubated with 60 µL of a 50% slurry of Ni-NTA resin (10 µL resin has a capacity for 50-100 µg His-tagged protein) for 60 min at 4°C with agitation. The resin was then pelleted by centrifugation and washed twice with 300 µL wash buffer (8 mol/L Urea, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris.Cl, pH6.3). The protein was then eluted three times with 30 µL elution buffer (8 mol/L Urea, 100 mmol/L NaH₂PO₄, 10 mmol/L Tris.Cl, pH4.5). The purification process was tested by 15% SDS-PAGE followed by Coomassie Brilliant Blue staining.

Measurement of serum autoantibodies against MMP-7

Ninety-six-well Costar ELISA plates (Jet Biofil, Beijing, China) were coated with 2 µg/mL of the purified MMP-7 protein and incubated overnight at 4°C. The plates were washed four times with PBST (PBS buffer containing 0.05% Tween 20), and then blocked with PBS containing 1% BSA at 37°C for 1 h, followed by four washes in PBST. Serum samples (ESCC or gastric cancer patients

or control) were diluted 1/150 in 1% BSA and incubated in the MMP-7-coated ELISA plates at 37°C for 1 h. After washing four times with PBST, 100 µL of goat anti-human IgG-HRP (1:1000 dilutions) was added to each well for 1 h at 37°C. After washing four times with PBST, the color was developed with 3,4,5-trimethoxy benzaldehyde (TMB) for exactly 15 min, and then stopped with 0.5 mol/L H₂SO₄. The absorbance of each well was read at 450 nm by a plate microplate reader (Beijing's New Air Electrical Technology, Beijing, China). Each serum sample was tested in triplicate.

Statistical analysis

To analyze the difference of autoantibodies reaction to MMP-7 proteins between cancer and matched control sera, the absorbance of each serum sample in the ELISA plate was averaged from triplicate experiments. Student's *t*-test was performed between cancer and matched-control samples. Nonparametric receiver-operating curves (ROCs), in which the value for sensitivity was plotted against false-positive rate (1-specificity), were generated. In addition, an area under the ROC curve (AUC) with 95% confidence intervals (CI) was calculated for each marker. In all tests, a *P*-value of ≤ 0.05 was considered to be statistically significant. All statistical analysis was done with the SPSS software package version 16.0 (SPSS, Chicago, IL, USA).

RESULTS

MMP-7 expression and purification

The cDNA of MMP-7 was cloned from ESCC tissues and inserted into a prokaryotic expression plasmid, pET-28b. After double endonuclease digestion and PCR confirmation, a single band of 492 bp was obtained (Figure 1) at the expected location. The insert DNA was further purified and sequenced. The sequence was found to be a perfect match to the sequence of MMP-7 deposited in NCBI GenBank.

This pET-28b/MMP-7 plasmid was transformed into DE3 cells for MMP-7 expression. Optimal IPTG concentration and time course determinations were performed for the kinetics of protein expression in the bacterial culture. The results showed that DE3 cells had the highest MMP-7 protein expression level after 4 h of 0.4 mmol/L IPTG induction at 37°C (Figure 2A and B). The pET-28b/MMP-7 recombinant proteins were mainly observed in the precipitate of the DE3 lysate, which indicated that the expressed protein was mainly in sequestered to inclusion bodies. The expressed proteins were further purified to approximately 95% purity by Ni-NTA resin (Figure 3).

Comparison of MMP-7 autoantibodies between cancer and control samples

To test serum autoantibodies against MMP-7 proteins, the purified MMP-7 proteins were coated onto 96-well ELISA plates. Serum samples from 50 patients with ESCC or 38 with gastric cancer were tested, along with 58 control serum samples, using indirect ELISA. The mean OD450

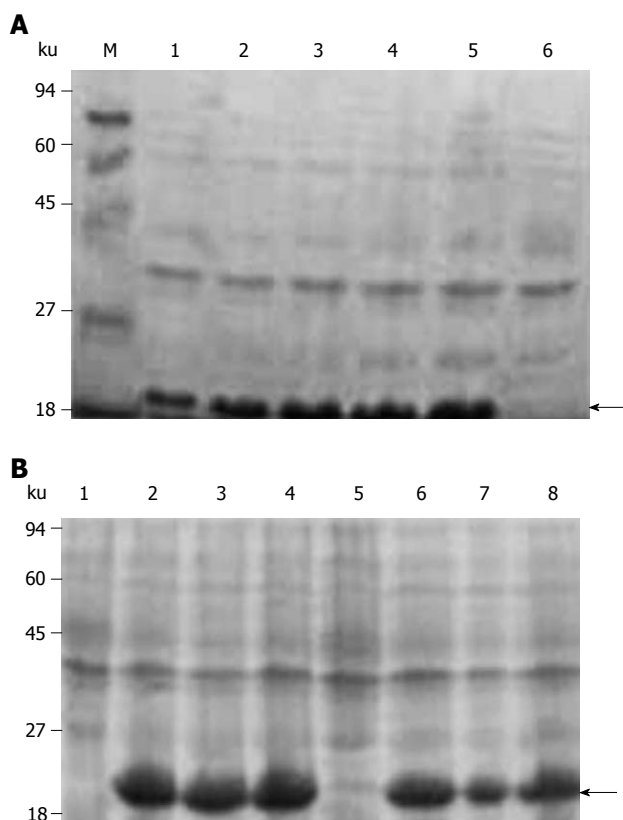


Figure 2 pET-28b/matrix metalloproteinase-7 protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Panel A: Time course analysis of pET-28b/matrix metalloproteinase-7 (MMP-7) protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). M: Protein marker; 1-5: Samples for different time points (1, 2, 4, 6, and 7 h) at 37°C after 0.4 mmol/L IPTG induced; 6: Uninduced bacterial lysate. Panel B: SDS-PAGE showing pET-28b/MMP-7 protein expression induced by different amounts of IPTG. 1, 5: Uninduced bacterial lysate; 2-4: Samples induced at different concentrations of IPTG (0.4, 0.8, 1 mmol/L) at 37°C for 4 h; 6-8: Samples induced at different concentrations of IPTG (0.4, 0.8, 1 mmol/L) at 28°C for 4 h.

(\pm SD) of serum autoantibodies against MMP-7 in 50 ESCC patients was 1.69 ± 0.08 , and was 1.55 ± 0.10 in 58 control individuals. The mean OD450 (\pm SD) of serum autoantibodies against MMP-7 in 38 gastric cancer patients was 1.62 ± 0.06 . The serum levels of autoantibodies against MMP-7 were significantly higher in ESCC than in healthy donors ($P < 0.001$, Figure 4A). The difference between healthy individuals and gastric cancer patients was also significant ($P < 0.001$, Figure 4B).

ROC curves were plotted to identify a cut-off value that would distinguish case from control samples. According to the ROC curve, the optimal cutoff value for ESCC was 1.65, providing a sensitivity of 78.0% and a specificity of 81.0%. The AUC for MMP-7 was 0.87 (95% CI: 0.80-0.93, Figure 4C) in ESCC patients. For gastric cancer, according to the ROC curve, the optimal cutoff value was 1.60, providing a sensitivity of 60.5% and a specificity of 71.7%. The AUC for MMP-7 was 0.75 (95% CI: 0.64-0.84, Figure 4D).

DISCUSSION

Immune response with antibody production can be elic-

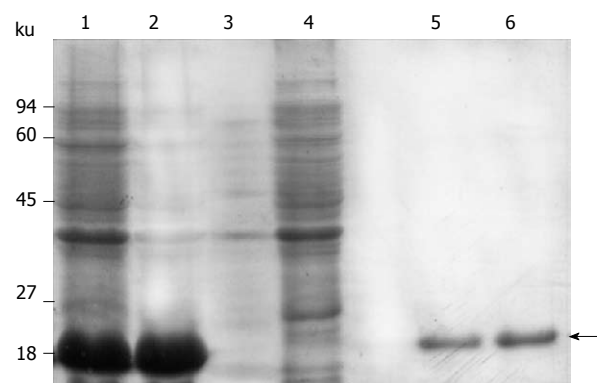


Figure 3 Soluble analysis and purification. 1: Samples induced with IPTG (0.4 mmol/L) for 4 h at 37°C; 2: Precipitate from the lysate of pET-28b/matrix metalloproteinase-7 (MMP-7) induced by IPTG; 3: The lysate supernatant of pET-28b/MMP-7 induced by IPTG; 4: Uninduced bacterial lysate; 5-6: Purified recombinant protein.

ited due to the overexpression of cellular proteins, such as Her2^[18], by the expression of mutated forms of cellular protein, such as mutated p53^[19], or by the aberrant expression of tissue-restricted gene products, such as cancer-testis antigens^[20] by cancer cells. These autoantibodies are raised against these specific antigens from the cancer cells; therefore, the detection of these antibodies in patients' sera can be exploited for cancer diagnosis in these patients. Furthermore, the immune system is especially well adapted for the early detection of cancer, because it can respond to low levels of an antigen by mounting a very specific and sensitive antibody response. Autoantibodies against cancer-specific antigens have been identified in cancers of the colon^[21], breast^[22], lung^[23], ovary^[24], prostate^[25], and head and neck^[26]. Thus, the use of the immune response as a biosensor for early detection of cancer through serum-based assays holds great potential as an ideal screening and diagnostic tool^[27,28].

MMP-7 is closely related to tumor invasion and metastasis: many studies have shown MMP-7 to be overexpressed in colorectal cancer^[29], esophagus^[30], stomach^[31], pancreatic cancer^[32], breast cancer^[33], prostate cancer^[34], and renal cell carcinoma^[35]. In addition, recent studies have shown that MMP-7 could be detected in the serum of cancer patients, including patients with ovarian^[36] and colorectal cancer^[17]. However, there has been no report regarding the diagnostic values of the serum autoantibodies against MMP-7 for any kind of cancers. In this study, we chose ESCC and gastric cancer as two typical gastrointestinal cancers to evaluate the diagnostic values of autoantibodies against MMP-7. Our results clearly suggested that serum autoantibodies against MMP-7 have the potential to be a tumor marker for ESCC and gastric cancer. MMP-7 is overexpressed in many cancers; therefore, elevated levels of autoantibodies against MMP-7 may also be present in other cancers as a nonspecific cancer biomarker. The samples in this study were mostly late stage serum samples; therefore, it is necessary to further validate these results using a large cohort of well-characterized patient samples, especially with early stage patient samples. It might be also

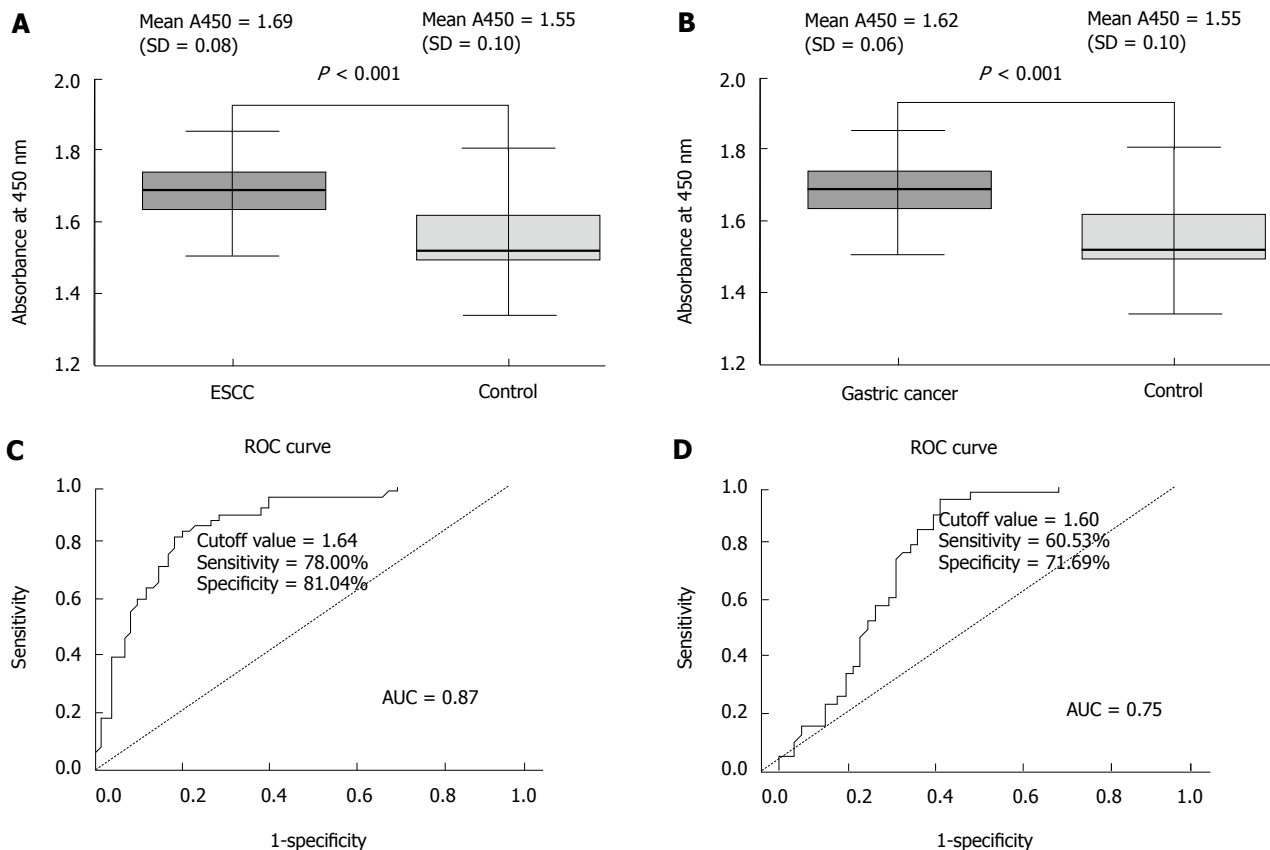


Figure 4 Comparison of the specificity and sensitivity of matrix metalloproteinase-7 autoantibodies. A: matrix metalloproteinase-7 (MMP-7) autoantibodies were detected by enzyme-linked immunosorbent assay (ELISA) in sera from patients with esophageal squamous cell carcinoma (ESCC) and control samples (Control); B: MMP-7 autoantibodies were detected by ELISA in sera from patients with gastric cancer (Gastric cancer patients) and controls (Control); C: ROC curve for ESCC serum antibodies against MMP-7; D: ROC curve for gastric cancer serum antibodies against MMP-7.

a good approach to further analyze the MMP-7 autoantibody diagnostic values in combination with the current tumor markers in ESCC and gastric cancer.

In conclusion, the results in this study are encouraging, even though further investigations are needed to evaluate its usefulness in population screening for ESCC. Moreover, the assay is easy to set up, because the recombinant proteins expressed and purified from *E. coli* are cost-effective and easy-to-standardize as serological reagents^[37].

COMMENTS

Background

Early diagnosis of esophageal squamous cell carcinoma (ESCC) has resulted in a significant reduction in morbidity and mortality, and new diagnostic markers could improve the results of screening. Matrix metalloproteinase-7 (MMP-7) is closely related to tumor invasion and metastasis. Increased MMP-7 levels have been found in ESCC. The present study aims to determine whether serum autoantibodies against MMP-7 in patients with ESCC could be used as biomarkers for diagnosis of the disease.

Research frontiers

New serum-based markers that could improve the accuracy of early detection of cancer are being sought. Recent findings suggested that MMP-7 is aberrantly expressed in ESCC. However, a prospective consecutive study of the evaluation of serum autoantibodies against MMP-7 as a diagnostic marker for ESCC has not been established.

Innovations and breakthroughs

Autoantibodies as biomarkers for early cancer detection have been recently

studied in other cancers. This is the first report to show that autoantibodies against MMP-7 could be a potential biomarker for ESCC diagnosis.

Applications

Serum autoantibodies against MMP-7 could become an important biomarker for ESCC early detection in clinics if this observation is further validated with a large cohort of patient samples.

Terminology

The Humoral Immune Response: is the aspect of immunity that is mediated by secreted antibodies (as opposed to cell-mediated immunity, which involves T lymphocytes) produced in the cells of the B lymphocyte lineage (B cell). B Cells (with co-stimulation) transform into plasma cells that secrete antibodies. Humoral immunity is so named because it involves substances found in the humors, or body fluids. Autoantibody: antibody that reacts with antigens found on the cells and tissues of an individual's own body. Autoantibodies can cause autoimmune diseases.

Peer review

The research described is very interesting. The manuscript is well written.

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Metal stenting to resolve post-photodynamic therapy stricture in early esophageal cancer

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Abstract

Photodynamic therapy (PDT) is an established endoscopic technique for ablating Barrett's esophagus with high-grade dysplasia or early-stage intraepithelial neoplasia. The most common clinically significant adverse effect of PDT is esophageal stricture formation. The strictures are usually superficial and might be dilated effectively with standard endoscopic accessories, such as endoscope balloon or Savary dilators. However, multiple dilations might be required to achieve stricture resolution in some cases. We report the case of stricture that recurred after dilation with a bougie, which was completely relieved by a self-expandable metal stent.

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Key words: Photodynamic therapy; Esophageal stricture; Metal stent

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INTRODUCTION

Photodynamic therapy (PDT) has been approved as an endoscopic treatment for Barrett's esophagus with high-grade dysplasia, and appears to be effective in the treatment of early esophageal cancer^[1-3]. The most common clinically significant adverse effect of PDT is esophageal stricture formation^[2]. In some published series, > 30% of the patients treated with PDT developed esophageal strictures^[3,4]. In the context of benign esophageal disease, stents have been used to seal esophageal perforations as a result of postoperative complications, endoscopic dilation procedures for achalasia^[5], and those associated with dilation of post-radiation strictures^[6]. Self-expandable metal stents (SEMSs) have been used for this indication.

We describe our experience with the successful resolution of an intractable post-PDT stricture using an SEMS in early esophageal cancer.

CASE REPORT

A 67-year-old man visited our hospital with dysphagia that involved solid and liquid food. Two months earlier, he had undergone PDT for early esophageal cancer. Initial endoscopy showed a flat, reddish lesion in the mid-esophagus. This lesion did not stain with Lugol's solution (Figure 1). Endoscopic biopsy revealed squamous cell carcinoma. Chest computed tomography (CT) showed no definite distant metastasis or lymph node enlargement. The patient refused surgical treatment, therefore, PDT was performed. The patient was given porfimer sodium (Photofrin II; Axcan Pharma, Quebec, Canada) intravenously at a dose of 2 mg/kg, 48 h before endoscopic photoradiation. Light was delivered from a laser (Ceralas PDT 633; CeramOptec, Bonn, Germany) that produced 630-nm light, with an

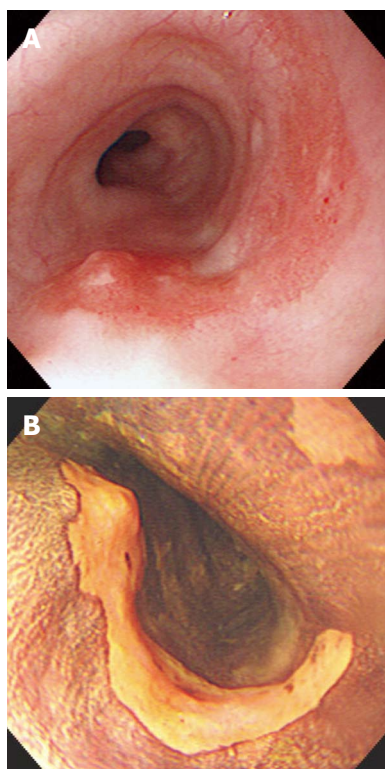


Figure 1 Gastroscopy before photodynamic therapy. A: Gastroscopy showed a flat and reddish lesion in the mid-esophagus (a biopsy showed squamous cell carcinoma); B: Gastroscopy showed an unstained lesion after spraying Lugol solution in the mid-esophagus.

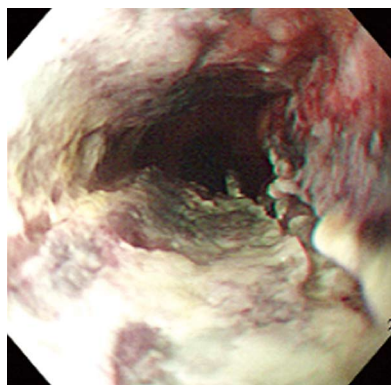


Figure 2 Two days after Photodynamic therapy. Endoscopy showed circumferential coagulation necrosis with an ulcer in the photodynamic therapy-treated lesion.

adjusted power output of 400 mW/cm, through a fiber that delivered a total energy of 180 J/cm fiber energy to the lesion. Two days after PDT, endoscopy showed circumferential coagulation necrosis with an ulcer involving the PDT-treated lesion (Figure 2). Two months after PDT, the patient complained of severe dysphagia. At that time, endoscopy showed luminal narrowing with fibrous scarring of the PDT-treated lesion (Figure 3). The patient underwent dilation three times with a Savary dilator (Cook Medical, Bloomington, IN, USA). However, the post-PDT stricture recurred within 1 mo. Consequently, a SEMS (Choo stent, 18 mm × 100 mm; MI Tech, Seoul, Ko-

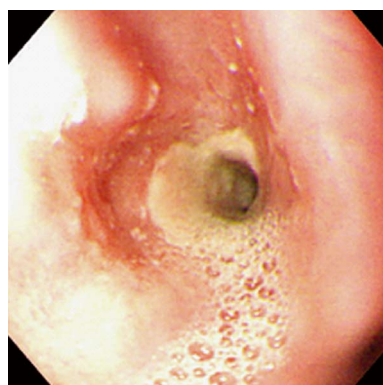


Figure 3 Two months after Photodynamic therapy. Endoscopy showed luminal narrowing with fibrous scarring changes in the photodynamic therapy-treated lesion.

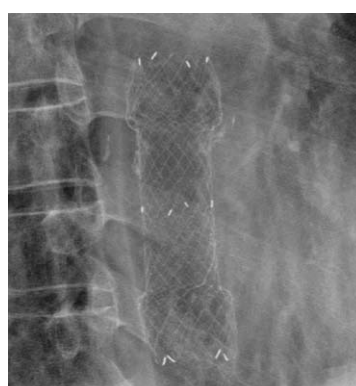


Figure 4 Fluoroscopic image showed a metal stent at the stricture site in the esophagus.

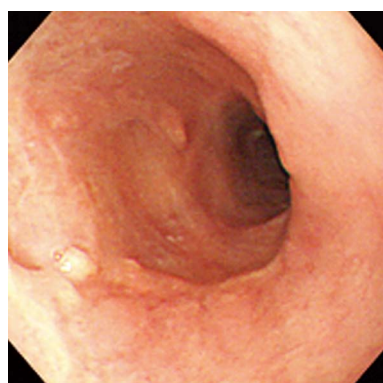


Figure 5 Endoscopy showed improvement of the previous stricture site 2 mo after stent removal.

rea) was placed through the stricture site (Figure 4). Two months after stenting, we removed the stent with grasping forceps, with no complications. After removing the stent, endoscopy showed a wide opening of the previous stricture and a biopsy revealed chronic inflammation with no tumor (Figure 5).

DISCUSSION

A stricture is the most common clinically significant ad-

verse effect of PDT^[2]. Patients who develop strictures after porfimer PDT typically present with symptomatic dysphagia 3 wk after treatment. The mechanism of stricture formation after PDT with porfimer sodium is unknown. It has been hypothesized that the deep, circumferential tissue injury and resultant inflammatory reaction produced by porfimer sodium PDT induces a fibrotic response that produces structuring^[4]. Other significant predictors of stricture formation are endoscopic mucosal resection before PDT, prior history of esophageal stricture, and multiple treatments of the same esophageal segment^[3,7,8].

Can the incidence of post-PDT strictures be reduced or even prevented? One study has shown that the use of oral prednisone beginning at the time of PDT light delivery did not prevent development of porfimer PDT strictures^[9]. One of the most important ways to prevent esophageal stricture may be to prevent circumferential mucosal injury of the esophagus during PDT.

The strictures are usually superficial and might be dilated effectively with standard endoscopic accessories, such as endoscope balloon or Savary dilators^[10]. However, multiple dilations might be required to achieve stricture resolution in some cases. The clinical course of post-PDT strictures appears to differ from that of other benign esophageal strictures. In a series of patients with peptic esophageal strictures, the median number of dilations needed for complete relief of dysphagia was only one^[11]. Compared with this, Prasad et al have reported that the median number of dilations for post-PDT strictures was four (range: 1-42). Another study has reported the need for multiple dilations in 11 of 34 patients^[8].

To predict which type of stricture is most likely to recur and benefit from stent placement, it is important to differentiate between esophageal strictures that are simple and those that are more complex^[12]. Simple esophageal strictures are focal, straight, and most have a diameter that allows passage of a normal-diameter endoscope. These strictures can successfully be treated with bougie or balloon dilation. Complex esophageal strictures are long, tortuous, or have a narrow diameter that does not allow the passage of any size of endoscope. The most common causes include caustic ingestion, anastomotic stricture, and severe peptic injury^[13]. Some post-PDT strictures are complex. These strictures are more difficult to treat, requiring at least three sessions, and are associated with high recurrence rates. If these strictures cannot be dilated to an adequate diameter, recur within a short time interval, or require ongoing dilation, they are considered to be refractory. Various stent designs, both SEMSs and self-expandable plastic stent (SEPSs), have been used to dilate these types of strictures.

As the long-term clinical success rate of both SEMSs and SEPSs in refractory benign strictures is well below 50%^[13], it is important to analyze which factors have played a part in these disappointing results. Factors related to long-term success were type of stricture, with post-radiation strictures being more successfully treated than peptic, anastomotic or achalasia strictures^[14], and length

of stricture, with shorter strictures being at lower risk of re-stricturing^[15]. However, there has been no report of long-term outcome of SEMSs for post-PDT esophageal stricture.

In the present case of stricture that recurred after dilation with a bougie, a SEMS completely relieved the post-PDT stricture. The stricture has not recurred during follow-up for > 1 year. The optimal timing of stent removal has been the subject of debate. Generally, when managing benign disease, the stent should be removed within 2 mo so that late stent-related problems are avoided^[15,16]. Tissue hyperproliferation at the ends of the stents represents a major limitation to long-term stent placement. Hyperplastic tissue ingrowth or overgrowth is the cause of stent-induced stricture or failure to remove a stent. Strictures that are longer or tighter require longer duration of stent placement^[16,17]. However, further study is needed with a larger number of patients and long-term follow-up to demonstrate a role for SEMSs in the treatment of post-PDT esophageal stricture.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatria 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



Instructions to authors

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In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S,** Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

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

Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 $24.5 \mu\text{g/L}$; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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**EDITORIAL**

- 1383 Molecular cross-talk between *Helicobacter pylori* and human gastric mucosa
Ricci V, Romano M, Boquet P
- 1400 Nodular regenerative hyperplasia: Evolving concepts on underdiagnosed causes of portal hypertension
Hartleb M, Gutkowski K, Milkiewicz P

TOPIC HIGH LIGHT

- 1410 Portal ductopathy: Clinical importance and nomenclature
Bayraktar Y

REVIEW

- 1416 Crohn's disease: Evidence for involvement of unregulated transcytosis in disease etio-pathogenesis
Pravda J

ORIGINAL ARTICLE

- 1427 Colorectal cancer and 18FDG-PET/CT: What about adding the T to the N parameter in loco-regional staging?
Mainenti PP, Iodice D, Segreto S, Storto G, Magliulo M, De Palma GD, Salvatore M, Pace L
- 1434 Proteomic analysis of pancreatic intraepithelial neoplasia and pancreatic carcinoma in rat models
Wang L, Liu HL, Li Y, Yuan P
- 1442 Serial observations on an orthotopic gastric cancer model constructed using improved implantation technique
Li Y, Li B, Zhang Y, Xiang CP, Li YY, Wu XL
- 1448 How we can improve patients' comfort after Milligan-Morgan open haemorrhoidectomy
A ba-bai-ke-re MMTJ, Huang HG, Re WN, Fan K, Chu H, Ai EHT, KE Li-Mu MMTTEX, Wang YR, Wen H

BRIEF ARTICLE

- 1457 Impaired gluconeogenesis in a porcine model of paracetamol induced acute liver failure
Dabos KJ, Whalen HR, Newsome PN, Parkinson JA, Henderson NC, Sadler IH, Hayes PC, Plevris JN

- 1462** Two cameras detect more lesions in the small-bowel than one
Triantafyllou K, Papanikolaou IS, Papaxoinis K, Ladas SD
- 1468** Factor analysis identifies subgroups of constipation
Dinning PG, Jones M, Hunt L, Fuentealba SE, Kalanter J, King DW, Lubowski DZ, Talley NJ, Cook LJ
- 1475** Anterior resection for rectal carcinoma - risk factors for anastomotic leaks and strictures
Kumar A, Daga R, Vijayaragavan P, Prakash A, Singh RK, Behari A, Kapoor VK, Saxena R
- 1480** Proton pump inhibitor step-down therapy for GERD: A multi-center study in Japan
Tsuzuki T, Okada H, Kawahara Y, Takenaka R, Nasu J, Ishioka H, Fujiwara A, Yoshinaga F, Yamamoto K
- 1488** Prevalence and impact of musculoskeletal pain in Japanese gastrointestinal endoscopists: A controlled study
Kuwabara T, Hiyama T, Urabe Y, Tanaka S, Shimomura T, Oka S, Yoshihara M, Chayama K
- 1494** Long-term result of endoscopic Histoacryl® (N-butyl-2-cyanoacrylate) injection for treatment of gastric varices
Kang EJ, Jeong SW, Jang JY, Cho JY, Lee SH, Kim HG, Kim SG, Kim YS, Cheon YK, Cho YD, Kim HS, Kim BS
- 1501** Clinicopathologic significance of HER-2/neu protein expression and gene amplification in gastric carcinoma
Yan SY, Hu Y, Fan JG, Tao GQ, Lu YM, Cai X, Yu BH, Du YQ
- 1507** Epigallocatechin gallate inhibits HBV DNA synthesis in a viral replication - inducible cell line
He W, Li LX, Liao QJ, Liu CL, Chen XL

CASE REPORT

- 1515** Spontaneous resolution of multiple lymphangiomas of the colon: A case report
Lee JM, Chung WC, Lee KM, Paik CN, Kim YJ, Lee BI, Cho YS, Choi HJ

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APPENDIX I Meetings
I-VI Instructions to authors

ABOUT COVER Kang EJ, Jeong SW, Jang JY, Cho JY, Lee SH, Kim HG, Kim SG, Kim YS, Cheon YK, Cho YD, Kim HS, Kim BS. Longterm result of endoscopic Histoacryl® (N-butyl-2-cyanoacrylate) injection for treatment of gastric varices: focus on primary prophylaxis. *World J Gastroenterol* 2011; 17(11): 1494-1500
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Molecular cross-talk between *Helicobacter pylori* and human gastric mucosa

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative microaerophilic, spiral bacterium that specifically colonizes the gastric mucosa, and it is the most common bacterial infection worldwide. Typically acquired during childhood, the infection can persist in the gastric ecosystem throughout the life span of the host, if untreated^[1]. Colonization of the stomach by *H. pylori* causes chronic gastritis that, during the decades that follow initial infection, can remain silent, due to the dynamic equilibrium between the bacterium and its human host, or evolve into more severe diseases, such as atrophic gastritis, peptic ulcer, lymphoma of the mucosa-associated lymphoid tissue or gastric adenocarcinoma^[2]. Gastric cancer, despite its declining incidence rate, remains the fourth most common cancer, the second leading cause of cancer-related death, and the 14th most common cause of death overall worldwide, which kills > 700 000 people each year^[3,4]. Early stages of the disease are often clinically silent, with patients having advanced stage disease at the time of diagnosis, and reported 5-year survival rates are approximately 20%^[5]. *H. pylori* infection is the strongest known risk factor for gastrointestinal malignancies that arise within the stomach, and epidemio-

Abstract

Helicobacter pylori (*H. pylori*) has co-evolved with humans to be transmitted from person to person and to colonize the stomach persistently. A well-choreographed equilibrium between the bacterial effectors and host responses permits microbial persistence and health of the host, but confers a risk for serious diseases including gastric cancer. During its long coexistence with humans, *H. pylori* has developed complex strategies to limit the degree and extent of gastric mucosal damage and inflammation, as well as immune effector activity. The present editorial thus aims to introduce and comment on major advances in the rapidly developing area of *H. pylori*/human gastric mucosa interaction (and its pathological sequelae), which is the result of millennia of co-evolution of, and thus of reciprocal knowledge between, the pathogen and its human host.

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logical studies have determined that the attributable risk for gastric cancer conferred by *H. pylori* is approximately 75%^[6]. While *H. pylori* infection increases the risk of developing both types of gastric cancer (i.e. diffuse and intestinal), chronic inflammation is not a prerequisite for development of diffuse-type cancer, thus suggesting that different mechanisms underlie the ability of *H. pylori* to induce gastric malignancies. Also, it is likely that *H. pylori* influences early stages in gastric carcinogenesis, as suggested by the demonstration that eradication of the infection significantly decreases the incidence of gastric cancer only in patients without premalignant lesions at the time of diagnosis^[7].

Development of gastric adenocarcinoma occurs in < 1% of *H. pylori*-infected subjects^[8]. Also, incidences of gastric carcinoma in *H. pylori*-infected individuals may vary dramatically among different geographical areas^[9]. This might be accounted for by *H. pylori* strain diversity within different geographical areas and/or within different individuals^[10], and further suggests that factors other than the bacterium may be involved in the carcinogenic process.

Evidence increasingly indicates that *H. pylori*-related gastric carcinogenesis is likely to be the result of a well-choreographed interaction between the pathogen and host, which is in turn, dependent on strain-specific bacterial factors, host genotypic traits and permissive environmental factors.

The present editorial thus aims to introduce and comment on major advances in the rapidly developing area of *H. pylori*/human gastric mucosa interaction (and its pathological sequelae), which is the result of millennia of co-evolution of, and thus of reciprocal knowledge between, the pathogen and its human host.

HOST FACTORS

The basic process that mediates *H. pylori*-induced damage is gastritis with its associated humoral and cell-mediated immune mechanisms. The outcome of *H. pylori* infection depends on the severity and the anatomical distribution of the gastritis induced by the bacterium. Individuals with corpus-predominant gastritis (so-called “gastric cancer phenotype”), which accounts for almost 1% of infected subjects, are more likely to develop hypochlorhydria, gastric atrophy, and eventually, gastric cancer; those with antrum-predominant gastritis (so-called “duodenal ulcer phenotype”), which accounts for up to 15% of infected subjects, have excessive acid secretion and are more likely to develop duodenal ulcer. Finally, subjects with mild, mixed antrum and corpus gastritis (so-called “benign gastritis phenotype”), which accounts for up to 85% of infected subjects, have almost normal acid secretion and, generally, no serious disease. These clinical outcomes seem to be mutually exclusive, and are largely influenced by a genetically regulated inflammatory response of the host gastric mucosa to the infection^[5,6].

A combination of polymorphisms in the host interleukin-1 (*IL1*) gene cluster (i.e. in the *IL1B* gene, which

encodes IL-1 β , a pro-inflammatory cytokine and a powerful inhibitor of gastric secretion, and in *IL1RN*, which encodes IL-1ra, the naturally occurring receptor antagonist of IL-1), and in the genes that encode tumor necrosis factor (TNF)- α , and IL-10, which result in elevated levels of IL-1 β and TNF- α and in low levels of IL-10 (which inhibits production of pro-inflammatory cytokines), confer a 27-fold increased risk of developing gastric cancer^[11]. Also, it has recently been demonstrated that polymorphisms in the promoter of *IL-8* gene, which enhance the transcriptional activity in response to IL-1 β or TNF- α , are associated with increased risk of gastric cancer in patients with *H. pylori* infection^[12,13]. This chemokine belongs to the CXC family and is a potent chemoattractant for neutrophils and lymphocytes, and has effects on cell proliferation, migration and tumor angiogenesis.

H. pylori infection is first handled by receptors of the innate immune response, and it is therefore conceivable that functionally relevant polymorphisms in genes of this arm of the immune system could affect the magnitude and subsequent direction of the host's response against the infection. In particular, toll-like receptor (TLR)4 is a member of a family of pattern recognition receptors that activate pro-inflammatory signaling pathways in response to microbes or pathogen-associated molecular patterns^[14]. TLR4 transduces signals that promote transcription of genes that are involved in immune activation, including nuclear factor (NF)- κ B and mitogen-activated protein (MAP) kinase pathways^[15]. A functional polymorphism at position +896 in exon 4 of the *TLR4* gene has been demonstrated^[16], which renders carriers hyporesponsive to lipopolysaccharide challenge by disrupting transport of TLR4 to the cell membrane, or impairing ligand binding or protein interactions^[16]. The defective signaling through TLR4 leads to an exaggerated inflammatory response with severe tissue destruction that causes gastric atrophy and severe hypochlorhydria. Two independent case-control studies have demonstrated that *TLR4*+896G carriers have an almost eightfold increase in OR for hypochlorhydria and gastric atrophy, and a 2.5-fold increase for gastric cancer^[17].

It is likely that subjects with an overall pro-inflammatory genetic makeup based on a combination of markers from the adaptive and innate immune systems, respond to *H. pylori* infection by creating an environment within the stomach that is chronically inflamed and with markedly reduced acidity. These environmental conditions favor the growth of other bacteria within the gastric milieu, which leads to sustained inflammation and decreased levels of vitamin C in gastric juice. This facilitates the formation of mutagenic N-nitroso compounds and reactive oxygen species (ROS)^[6,11], which ultimately leads to increased oxidative/genotoxic stress. Moreover, the profound inhibition of acid secretion that is associated with these pro-inflammatory genotypes favors a shift from an antrum-predominant to corpus-predominant gastritis with the onset of gastric atrophy and intestinal metaplasia (i.e. precancerous lesions).

H. PYLORI-INDUCED CELL SIGNALING IN GASTRIC CARCINOGENESIS

The host response to *H. pylori* infection may contribute to gastric carcinogenesis by promotion of a chronic inflammatory response that contributes to mucosal cell damage, or by interference with the mechanisms of proliferation and/or survival that regulate epithelial cell homeostasis^[2,6,10].

H. pylori proteins and induced responses play a major role in the increased disease risk associated with the infection, but they are not absolute determinants of gastric carcinogenesis. The chronic inflammation that develops in response to this organism greatly contributes to transformation. In this respect, bone-marrow-derived cells (BMDCs) have been demonstrated to home to and engraft the inflamed gastric mucosa of mice infected with *Helicobacter felis*. This phenomenon takes place within foci in which tissue injury induces excessive apoptosis and overwhelms the population of endogenous tissue stem cells^[18]. Subsequently, BMDCs in the inflamed gastric environment degenerate into adenocarcinoma, thus suggesting that gastric adenocarcinoma originates from BMDCs.

It is generally accepted that *H. pylori* infection results in a Th1-dominant response and that gastric inflammation largely depends on Th1 cell response with increased production of IL-1 β , TNF- α and IL-8, but not IL-4 and IL-10^[2,6,10,19,20]. A novel subset of effector T cells, identified by secretion of IL-17, has been defined as Th17 cells, which are distinct from Th1 and Th2 cells in their differentiation and function^[21]. TNF- α and IL-6 from activated macrophages/dendritic cells (DCs) are required for Th17 cell differentiation, whereas IL-12 and interferon- γ promote Th1 cell development, and IL-4 primes Th2 cell differentiation. Recently, it has been suggested that *H. pylori* infection mainly leads to a specific Th17/Th1 immune response that plays a major role in *H. pylori* infection, which promotes mucosal inflammation and contributes to bacterial colonization^[22]. In fact, *H. pylori* burden and inflammation are both reduced when IL-17 activity is blocked *in vivo* or IL-17^{-/-} mice are used^[22]. The dynamics of Th cell immune responses to *H. pylori* suggest that Th17 cell responses are induced earlier than Th1 cell responses, thus implying that Th17 and Th1 cells promote inflammation at different stages. It is likely that the Th17/IL-17 pathway modulates Th1 cell responses, and Th17 and Th1 cells may act synergistically to induce gastritis during *H. pylori* infection, by triggering the recruitment of inflammatory cells, including Th1 cells, into the gastric mucosa through the induction of chemokines. Also, the activated Th17/Th1 pathway may destroy gastric tissue by inducing matrix metalloproteinase (MMP) production, which favors subsequent pathogen dissemination and persistent infection. This might have implications also for the carcinogenic process associated with *H. pylori* infection. In fact, Th17 cells have been reported to contribute to gastric cancer pathogenesis^[23].

A concomitant helminthic infection that triggers a

Th2 cell response may blunt the Th17/Th1 cell responses associated with *H. pylori* infection, thus limiting the pathological consequences of *H. pylori* gastric colonization; in particular, gastric atrophy. This might partially explain why in African countries, where the prevalence of *H. pylori* infection acquired during childhood is close to 80%, the prevalence of gastric cancer is very low and accounts for < 2% of all malignant tumors^[24].

Variation in the ability of *H. pylori* strains to trigger the production of chemokines from gastric epithelium depends on the presence of a functional type IV secretion system (TFSS), which is encoded by the *cag* pathogenicity island (PAI). Although the *cag* PAI facilitates the translocation of CagA, the effect of this bacterial protein on cytokine synthesis is controversial; the majority of reports show no effect of CagA on cytokine synthesis, thus suggesting that other effectors are involved in the epithelial cytokine and chemokine response to *H. pylori* infection^[10,25,26]. Indeed, it has been shown that induction of pro-inflammatory responses in epithelial cells infected by *H. pylori* is mediated by the host protein CARD4 (also known as Nod1), an intracellular pathogen-recognition molecule, and that the effect is dependent on the delivery of peptidoglycan to host cells by the TFSS^[27]. Consistent with involvement of CARD4 in host defense, *Card4*-deficient mice are more susceptible to infection by *H. pylori* strains that contain the *cag* PAI than are wild-type mice^[27]. However, two studies have demonstrated that CagA directly induces IL-8 release from gastric mucosal cells^[28,29].

Although *H. pylori* elicits innate and acquired immune responses, the host is unable to eliminate the organism from the gastric mucosa, and chronic infection is the usual outcome^[2,6,30]. *H. pylori* antigenic variation (i.e. modification of bacterial antigenic determinants due to mutation or intragenomic homologous recombination) and mimicry of host antigens (i.e. bacterial expression of antigens similar to those expressed by the host)^[31], as well as induction of apoptosis by *H. pylori* in DC precursor monocytes^[20] and the intracellular persistence of the bacteria in gastric epithelial progenitor cells^[32], might be crucial for evasion of the immune response. Also, it has recently been demonstrated that *H. pylori* evades TLR5 innate immunity^[33]. In fact *H. pylori*, although being a flagellated organism, does not release flagellin, and recombinant *H. pylori* flagellin is much less active than *Salmonella typhimurium* flagellin in activating TLR5-mediated IL-8 secretion^[33].

Although it fails to eliminate the organism, the inflammatory response induced by *H. pylori* increases cellular damage. Activation of the TNF receptor by TNF- α results in the induction of apoptosis and mucosal cell damage. Increased apoptosis through direct or cytochrome-c-mediated activation of caspases is also contributed to by CD95/FAS and VacA. Recently, we have shown that *H. pylori* infection upregulated IL-21 levels in gastric epithelial cells *in vitro*, as well as in the gastric mucosa of *H. pylori*-infected humans^[34]. IL-21 overexpression is associated with increased production of MMP-2 and

MMP-9, through an NF- κ B-dependent mechanism. Increased MMP-2 and MMP-9 levels might contribute to chronic gastric damage and inflammation by degrading extracellular matrix proteins and by favoring the recruitment of circulating cells into inflamed tissue^[35,36].

Other ways in which pro-apoptotic pathways are induced during *H. pylori* infection include superoxide production by infiltrating neutrophils, elevated nitric oxide production by inducible nitric oxide synthase (iNOS), which is over-expressed in infected gastric mucosa^[2,6,37], and generation of ROS by bacterial secretion of γ -glutamyltranspeptidase (γ -GT) in the presence of glutathione (GSH) and transferrin, as a source of iron^[38]. In fact, it has been shown that γ -GT is important for *H. pylori*-mediated apoptosis of AGS (a cell line derived from a human gastric adenocarcinoma) gastric epithelial cells^[39]. It is also interesting to note that inflammatory stimuli that activate cytokine receptors and p38 (a stress-activated MAPK) can induce apoptosis. Conversely, activation of cytokine receptors and p38 might also inhibit apoptosis through NF- κ B and c-Jun activation^[6].

Inhibition or induction of apoptosis could both be relevant to *H. pylori*-related carcinogenesis. Induction of apoptosis might favor the development of atrophic gastritis and gastric gland recruitment of bone-marrow-derived precursor cells that might ultimately develop into intraepithelial cancer^[18]. Inhibition of apoptosis, on the other hand, represents the loss of a physiological safeguard against perpetuating the acquisition of DNA damage that can lead to the malignant transformation of cells^[2,6,10].

Proliferative response

Several signal transduction pathways are activated during the proliferative response of gastric epithelial cells to *H. pylori*-induced cell damage^[2,6]. The compensatory hyperproliferative response of gastric epithelial cells during *H. pylori* infection might be sustained by hypergastrinemia, increased expression of epidermal growth factor (EGF)-related peptides, and activation of the EGF receptor (EGFR) signal transduction pathway in gastric epithelial cells^[2,6,40-42]. In addition, translocation of CagA into gastric epithelial cells induces a growth-factor-like-response through activation of the Ras-MAPK pathway^[26]. Finally, it has been shown that *H. pylori* upregulates the expression of cyclooxygenase (COX)-2, the inducible isoform of the enzyme that is responsible for prostaglandin production, in human gastric epithelial cells *in vitro* and in human gastric mucosa *in vivo*^[37,43].

EGFR-related pathway is upregulated in a number of malignancies and is an important target for treatment of several neoplasms of the gastrointestinal tract. *H. pylori* has been shown to upregulate amphiregulin and HB-EGF, members of the EGFR ligands family, and to activate EGFR in MKN 28 cells^[40]. Subsequently, it has been demonstrated that EGFR transactivation by *H. pylori* is mediated through metalloproteinase-dependent cleavage of HB-EGF. The required metalloproteinases are likely to be members of a disintegrin and metalloproteinase (ADAM)

family. In particular ADAM17 is the ideal candidate enzyme for the regulation pathway^[42].

H. pylori-induced upregulation of COX-2 and EGF-related peptide expression in human gastric epithelial cells depends on the bacterial production of γ -GT^[38]. Activation of phosphatidylinositol-3'-kinase (PI3K)-dependent and/or p38-dependent pathways is responsible for *H. pylori* γ -GT-induced upregulation of COX-2 and EGF-related peptide expression in gastric mucosal cells^[38]. However, another study has demonstrated that upregulation of HB-EGF by *H. pylori* in human gastric epithelial cells is dependent on MAP kinase kinase activation, thus suggesting that EGF-related peptide expression might be regulated by different signal transduction pathways^[42]. In keeping with this, it has been demonstrated that COX-2 expression in gastric epithelial cells is also regulated by extracellular signal-regulated kinase (ERK)/MAPK activation^[43,44].

The upregulation of growth factor and COX-2 expression might increase the mitogenic activity of *H. pylori*-infected gastric mucosa, and protect cells from *H. pylori*-induced cell damage, which might therefore be regarded as early events in the development of *H. pylori*-associated gastric carcinogenesis^[2,6]. Upregulation of COX-2 and iNOS expression might also contribute to the high levels of oxidative DNA damage seen during *H. pylori* infection, and this could increase the mutation rate in infected hyperproliferative gastric mucosa^[2,37]. More recently, blockade of EGFR activation by HB-EGF neutralizing antibody or by abrogating ADAM17 expression, has been shown to protect gastric epithelial cells from *H. pylori*-induced apoptosis. The anti-apoptotic effect of EGFR activation seems to depend on PI3K-dependent activation of the anti-apoptotic factor Akt, increased expression of the anti-apoptotic factor Bcl-2, and decreased expression of the pro-apoptotic factor Bax^[45]. Because EGFR activation is linked to increased proliferation, reduced apoptosis, disruption of cell polarity and enhancement of cell migration, transactivation of EGFR by *H. pylori* might represent an attractive target for studying early events that may precede transformation. Also, the EGFR-related pathway might be regarded as a molecular target for treatment of gastric cancer^[46]. However, surveys of human gastric cancer specimens for evidence of overexpression or mutations of EGFR have found both events to be rare^[47].

The activation of a pathway mediated by EGFR, MAPK and COX-2 is also responsible for the induction of vascular endothelial growth factor (VEGF) expression in *H. pylori*-infected gastric epithelial cells^[44]. This effect is specifically related to the VacA toxin of *H. pylori*^[44], and is associated with a significant increase in blood vessel formation, suggesting that neoangiogenesis might contribute to tumor growth in *H. pylori*-related gastric carcinogenesis^[48]. *H. pylori* infection also promotes gastric epithelial cell invasion by inducing the production of MMP-7 through MAPK activation^[49], and MMP-9 and VEGF expression through an NF- κ B- and COX-2-mediated pathway^[50].

Another host effector that is aberrantly activated during *H. pylori*-induced gastric carcinogenesis is β -catenin, a ubiquitously expressed molecule that regulates the expression of several genes, including *c-myc*, the cyclin D genes, *MMP7* and *PTGS2* (which encodes COX-2)^[51]. Membrane-bound β -catenin is a component of adherens junctions that link cadherin receptors to the actin cytoskeleton. Cytoplasmic β -catenin is a downstream component of the Wnt signal transduction pathway. In the absence of Wnt ligand, the inhibitory complex composed of axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3 β (GSK3 β) induces the degradation of β -catenin, and maintains low steady-state levels of free β -catenin in the cytoplasm or nucleus. After binding of Wnt to its receptor Frizzled, Dishevelled is activated, thus preventing GSK3 β from phosphorylating β -catenin. This allows β -catenin to translocate to the nucleus and activate the transcription of target genes that are involved in carcinogenesis. Increased β -catenin expression or *APC* mutations are present in up to 50% of gastric adenocarcinomas^[52]. Moreover, an oncogenic *H. pylori* strain can induce nuclear translocation of β -catenin and activation of the LEF/TCF transcription factor that regulates the expression of β -catenin-responsive genes^[51]. β -catenin activation is dependent on the translocation of CagA into host epithelial cells, which reinforces the evidence that *cagA*⁺ *H. pylori* strains induce stronger activation of the signal transduction pathways that regulate the proliferation, invasion and survival of gastric epithelial cells^[51]. Recently, activation of PI3K and Akt has been shown to induce phosphorylation and inactivation of GSK3 β , which permits β -catenin to accumulate in the cytosol and nucleus^[53]. Sustained induction of PI3K/Akt signaling in response to *H. pylori* infection with subsequent β -catenin activation has been demonstrated to be due to the interaction of CagA with Met, the hepatocyte growth factor receptor, *via* CRPIA (for conserved repeat responsible for phosphorylation-independent activity), a conserved motif in the C-terminal region of CagA^[53]. Also, EGFR transactivation by *H. pylori* leads to activation of PI3K/Akt signaling, which ultimately leads to β -catenin activation^[45,54].

H. pylori has co-evolved with humans to be transmitted from person to person and to colonize the stomach persistently. A well-choreographed equilibrium between the bacterial effectors and host responses permits microbial persistence and health of the host, but confers a risk for serious diseases including gastric cancer. During its long coexistence with humans, *H. pylori* has evolved complex strategies to limit the degree and extent of gastric mucosal damage and inflammation, as well as of immune effector activity. Severe disease, associated with bacterial colonization, might reflect loss of this control^[51]. In this respect, we have recently demonstrated that Hp(2-20), a cationic α -helical peptide that has been isolated from the N-terminal region of the *H. pylori* ribosomal protein, L1, by interacting with formyl peptide receptors (FPRs), stimulates migration and proliferation of gastric epithelial cells *in vitro* and accelerates gastric mucosal healing *in vivo*^[55].

This raises the intriguing possibility that *H. pylori*, through the production of Hp(2-20) and its interaction with FPRs is also able to modulate the capacity of gastric mucosa to maintain or recover its integrity.

H. PYLORI INVASIVENESS AND INTERACTIONS WITH NON-EPITHELIAL CELLS: *IN VIVO* VERITAS

H. pylori is commonly considered an essentially extracellular, non-invasive bacterium. In an infection, 80%-90% of the bacteria freely swim into the mucus layer of the stomach, while the residual 10%-20% are in intimate contact with surface epithelial cells^[54,56]. As a consequence, a prominent immune-inflammatory response is invariably mounted in the underlying lamina propria. An intact epithelium should form a structural barrier that prevents direct contact between the bacterium on the luminal side and reactive inflammatory cells on the stromal side. Therefore, to explain how a strong mucosal and systemic reaction may be elicited, *H. pylori*-induced functional changes in the epithelium have been considered, such as a bacterial activation of the accessory immune competence inherent in the gastric epithelium, which in turn may modulate underlying stroma cells. Among pertinent epithelial changes so far documented are *de novo* or increased expression of pro-inflammatory cytokines, proteases (like cathepsins E, B, L, S and D) known to be involved in antigen processing, or HLA-DR and co-stimulatory molecules like B7-1 and B7-2 that are involved in antigen presentation^[57]. However, a few early light and electron microscopy studies have suggested the presence of *H. pylori* cells inside the gastric mucosa, either in epithelial cells and intraepithelial intercellular spaces, or in the underlying lamina propria^[57-59]. Nevertheless, the scientific community has exhibited a widespread reluctance to accept this published evidence. By contrast, a lot of *in vitro* studies have been carried out to investigate epithelial cell invasion by *H. pylori*, as well as roles and molecular mechanisms of direct interactions between *H. pylori* or *H. pylori* products/virulence factors and immune cells such as T or B lymphocytes, DCs, macrophages, monocytes, and mast cells^[57,59,60]. However, despite this interest, the importance of these *in vitro* studies has been questioned because of a lack of convincing evidence that *H. pylori* actually invades the gastric mucosa and interacts with non-epithelial cells, thus it is highly questionable whether any of these observations have clinical relevance^[60]. In this respect, Lu et al^[60] have strongly stressed the fact that *in vitro* studies are in theory designed to allow deeply detailed molecular study of *in vivo* events, whereas it is easy but meaningless to generate *in vitro* data irrespective of whether the experiment has an *in vivo* correlation. Similarly, other leading investigators^[61,62] have underlined the problem that *in vitro*-derived findings and considerations may make sense in terms of cell biology, but it remains to be established how and to what extent they apply to the *in vivo* situation,

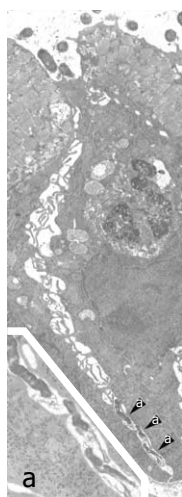


Figure 1 *Helicobacter pylori* penetration in human gastric epithelium *in vivo*. Three *Helicobacter pylori* (*H. pylori*) organisms (enlarged in a; 16 800 ×) lying in the deep intercellular intraepithelial space, just above the basal membrane. Note also luminal bacteria (top) overlying an apparently preserved tight junction, dilation of the underlying intercellular space, filled with lateral membrane plications, and an intraepithelial granulocyte (middle right, 6300 ×). Reprinted from Necchi *et al*^[57], with permission from Elsevier.

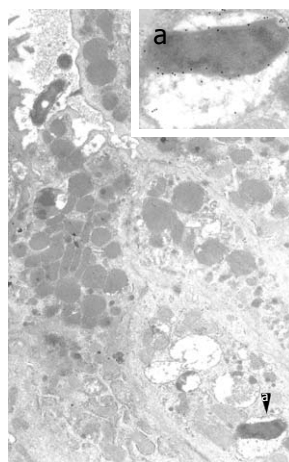


Figure 2 Intracellular *Helicobacter pylori* in human gastric epithelium *in vivo*. A well-preserved *Helicobacter pylori* (*H. pylori*) organism in a cytoplasmic vacuole, enlarged in a (55 200 ×; 15 nm gold particles) to show immunoreactivity with anti-VacA antibody. Note two *H. pylori* in a luminal cleft (top left, 13 500 ×). Reprinted from Necchi *et al*^[57], with permission from Elsevier.

which is now needed to be investigated in detail and in quantitative terms. Thus, only *in vivo* veritas!

Indeed, recently there has been a re-emerging interest for *in vivo* investigations of *H. pylori*/human gastric mucosa interaction. By microscopy and molecular investigations, Semino-Mora *et al*^[63] have demonstrated the presence of intracellular and interstitial *H. pylori* in preneoplastic as well as in neoplastic lesions of human gastric mucosa, with persistent intracellular expression of *H. pylori* virulence genes. These findings may substantially expand current concepts on the role of the bacterium in gastric carcinogenesis.

In a transmission electron microscopy study, Necchi *et al*^[57] have unequivocally detected *H. pylori* in intraepithelial, intercellular and stromal sites of the majority of human gastric biopsies, and have shown bacteria on their luminal side (Figures 1 and 2). In keeping with *in vitro* demonstration that *H. pylori* can functionally disrupt tight junctions, possibly through intraepithelial delivery of CagA^[64], CagA has been found to accumulate over and around tight junctions of colonized gastric epithelium, as well as ultrastructural alterations of the tight junctions that cover intercellular spaces that have been penetrated by *H. pylori* cells^[57]. These *in vivo* findings support the hypothesis that functional and structural alterations of the tight junctions may open the way to bacterial penetration into deep intercellular spaces, up to the underlying lamina propria. Consistent transepithelial penetration of *H. pylori* into infected gastric mucosa may help understand how the colonized mucosa invariably mounts a prominent local immune-inflammatory response, as well as a systemic immune reaction and, under special genetic conditions, even extra-gastric autoimmune pathology. In fact, direct contact (including intracellular penetration in some cases) between *H. pylori* or its remnants and immune-inflammatory stroma cells has been observed^[57].

The morphological and cytochemical detection of *H. pylori* (often well preserved, VacA- and CagA-storing, and apparently vital) inside intact epithelial cells of human gastric mucosa (Figure 2) has confirmed the intracellular pathogenic potential of the bacterium. This

observation confirms and extends previous *in vitro* observations that *H. pylori* exhibits an invasion frequency similar to that of *Yersinia* and greater than that of *Shigella*^[65]; both *bona fide* invasive pathogens. This is also in keeping with the expression of Nod1, a known intraepithelial pathogen-recognition molecule, by *H. pylori*-colonized epithelial cells^[27], as well as with the predominantly Th1 type of immune response that is elicited. In addition, confirmation of the intracellular occurrence of *H. pylori* is crucial when choosing appropriate antibiotics for bacterial eradication, as well as when monitoring their *in vivo* effectiveness after completion of treatment.

Of high interest is the presence of *H. pylori* in intestinal metaplasia (IM). Indeed, this finding substantially extends the possible interaction between *H. pylori* and IM in gastric carcinogenesis, from an early preneoplastic step that corresponds to epithelial progenitors^[32] and IM genesis, to the whole process of its progression to neoplasia. Indeed, it supports a persistent *in vivo* activity of several *H. pylori*-activated molecular mechanisms of gastric carcinogenesis such as CagA-mediated intracellular disruption of growth regulation^[26,66], inflammation-elicited NF-κB transcription factor activation^[67], and silencing of DNA mismatch repair genes^[68]. The demonstration by Necchi *et al*^[57] of *H. pylori* occurrence in dysplastic as well as in neoplastic growths, in agreement with that of Semino-Mora *et al*^[63], further substantiates and extends the *H. pylori* contribution to gastric carcinogenesis, and may explain some beneficial effects of bacterial eradication on IM-related cancer risk, as well as on cancer recurrence^[57].

Another *in vivo* study^[69] has recently shown that, in human superficial-foveolar gastric epithelium and its metaplastic or dysplastic foci, *H. pylori* products/virulence factors (sometimes coupled with bacterial bodies or remnants) accumulate in a discrete cytoplasmic structure that is characterized by 13-nm-thick cylindrical particles of regular punctate-linear substructure. This structure resembles the proteasome complex in size and structure, while being different from VacA-induced vacuoles, phagosomes, aggresomes or related bodies. Inside this novel cell compartment, named PaCS (for particle-rich cytoplasmic structure), co-localization of VacA, CagA,

urease and outer membrane proteins (OMPs) with NOD1 receptor, ubiquitin-activating enzyme E1, polyubiquitinated proteins, proteasome components and potentially oncogenic proteins like SHP2 and ERKs has been demonstrated^[69]. These findings suggest that PaCS is a novel, proteasome-enriched structure arising in ribosome-rich cytoplasm at sites of *H. pylori* product accumulation. As a site of selective concentration of bacterial virulence factors, the ubiquitin-proteasome system and interactive proteins, PaCS is likely to modulate immune-inflammatory and proliferative responses of the gastric epithelium of potential pathological relevance; also taking the mounting evidence for a role of the ubiquitin-proteasome system in cancer origin or progression^[70].

A hotly debated question waiting for a definitive answer is whether human gastric DCs are able to send cell processes that cross the epithelium, reach the lumen and directly contact and engulf *H. pylori*, as shown to occur for DC-mediated pathogen-sensing at the intestinal level. The dendritic, epithelial, granulocytic or macrophagic nature of the cell that first interacts with the bacterium seems to be important, given the different type and cellular distribution of microbial product receptors shown by different immunocompetent cells, and the different chemokines and interleukins that they release when activated^[27,62,71]. In fact, DCs have been found to respond differently when interacting directly with bacteria rather than secondarily to epithelium-bacteria contact, with IL-12 secretion and Th1 response preferentially activated only in the former case^[72].

By ultrastructural immunocytochemistry on endoscopic biopsy samples, clear-cut *in vivo* evidence has been provided^[73] of direct DC contact with *H. pylori* in the human gastric mucosa (Figure 3), which greatly extends the relevance of previous *in vitro* studies carried out on purified DCs. DCs have been shown to be present inside superficial-foveolar epithelium of *H. pylori*-infected (but not *H. pylori*-free) human gastric mucosa, and to send cytoplasmic extensions to the lumen, to which bacteria preferentially adhere (Figure 3). In addition, intraepithelial DCs are found to accumulate bacterial products like VacA, urease and OMPs in their cytoplasm. The importance of intraepithelial, lumen-contacting DCs lies in the well-known crucial role of these cells as sensors of pathogens, and as the first line of antibacterial immune defense of both innate and adaptive type^[74]. In fact, DCs have been shown to act as main processing and presenting cells of internalized antigens, and major regulators of cells involved in the mucosal inflammatory response. Depending on their mode of activation, they may secrete pro-inflammatory or anti-inflammatory cytokines and chemokines that dictate the composition of the cellular infiltrate, in addition to activating NK and T cells with either Th1 or Th2 effector and T regulatory cell responses^[73]. Thus, the direct interaction of DCs with *H. pylori* during active gastritis may be of key relevance. Also worth noting is the finding of a close adherence of DCs to surrounding epithelial cells along all or most of

their confronting membranes, with involvement of lateral folds and formation of focal interdigitating complexes. It has been speculated that this pattern, possibly related to the capacity of immature DCs to produce tight and adherens junction proteins, may be finalized to preserve the barrier function of the epithelium^[73].

Granulocytes have also been seen to contact and heavily phagocytose luminal *H. pylori*, while macrophages remain confined to basal epithelium, although taking up bacteria and bacterial products^[73]. The inhibitory role of VacA on phagolysosome formation and acidification with resulting persistence of engulfed *H. pylori* has been outlined^[75]; this may well allow macrophages and DCs to retain and translocate vital *H. pylori* to gastric lymph nodes, from which they have been cultivated^[76]. The substantial restriction of intraepithelial DCs to active, granulocyte-rich gastritis seems of interest, especially considering the dominant role of granulocytes in early, first-contact *H. pylori* gastritis. Although no direct morphological interactions have been observed between DCs and granulocytes, both have shown coexistence in the same mucosa and direct interaction with *H. pylori*, resulting in phagocytosis and intracellular accumulation of bacterial virulence factors^[73]. DC intracellular accumulation of *H. pylori* virulence factors like VacA, urease or OMPs, as documented in this paper, may also be of relevance for DC function and integrity; either as a source of antigenic material promoting the immune response or of cytotoxic damage. Indeed, signs of cellular damage have been observed in several intraepithelial DCs, including focal mitochondrial swelling, cytoplasmic edema and vacuolation and formation of autophagic vacuoles. These findings raise the issue of a possible impairment of DC function by accumulated bacterial toxins, which may reduce the effectiveness of the immune response and favor persistence of bacterial infection. Thus, this complex *in vivo* interaction of DCs, granulocytes and macrophages with *H. pylori* and *H. pylori*-infected epithelium is likely to have an important role in the origin, type specification, and outcome of the innate and adaptive immune responses.

VacA TOXIN AND ITS FUNCTIONAL INTERACTION WITH CagA: THE ART OF PRESSING THE BRAKE AND GAS PEDALS AT ONCE

VacA, the “vacuolating cytotoxin”, and CagA, the product of the cytotoxin-associated gene *A*, are virulence factors playing a pivotal role in *H. pylori*-induced pathogenesis. While VacA is defined as an A-B toxin (even though no enzymatic activity has been so far identified for any of its domain), CagA is usually defined as an effector protein: a bacterial protein delivered into host cells by a specialized bacterial machine so as to modulate a variety of cellular functions. Indeed, CagA is supposed to be directly injected into host cells by a TFSS. Many excellent reviews have been recently published on these two *H. pylori* virulence

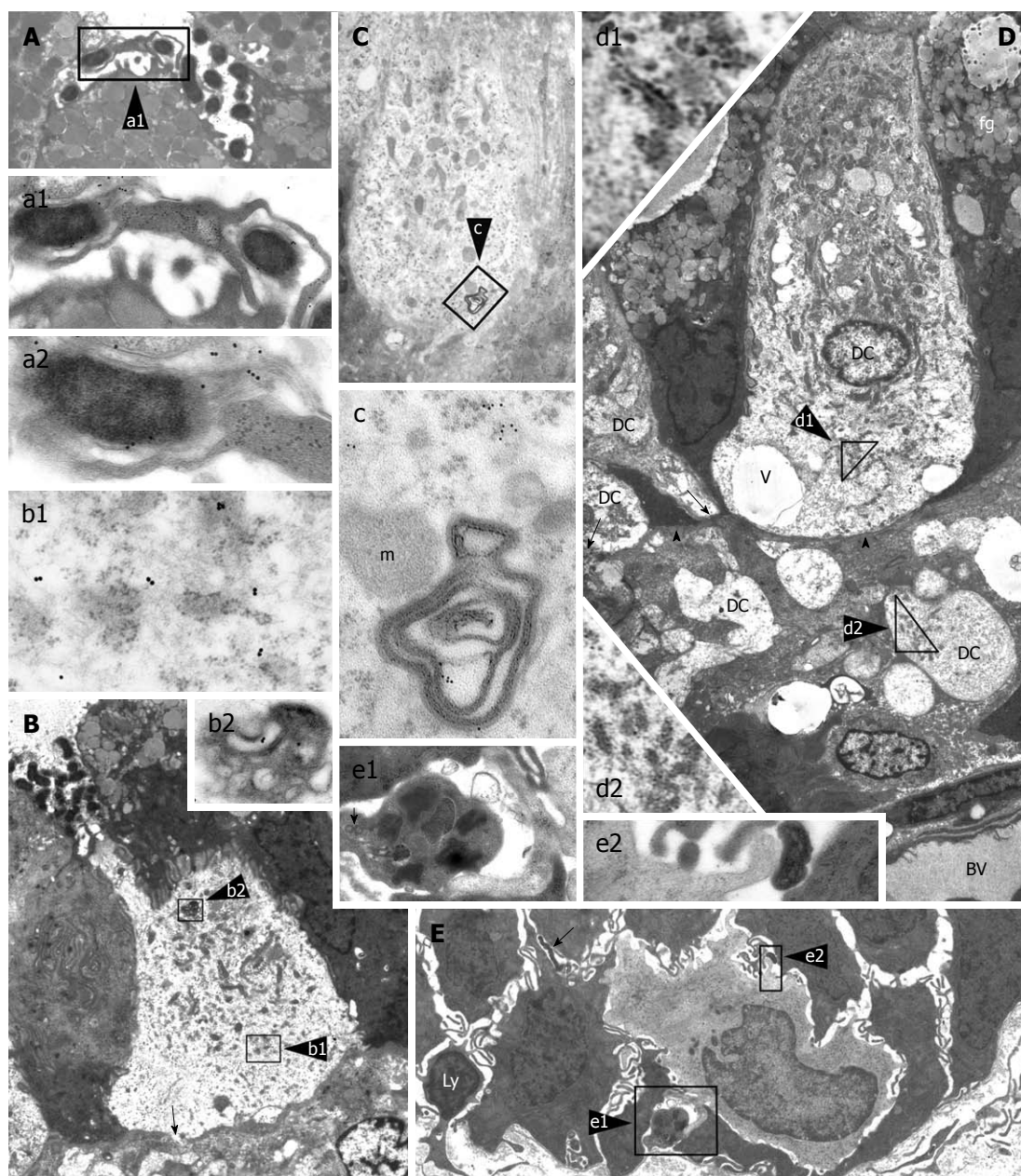


Figure 3 Intraepithelial dendritic cells in *Helicobacter pylori*-positive human gastric biopsies with active inflammation. A (5000 \times): Luminal ending of a dendritic cell (DC) process abutting on a collection of *Helicobacter pylori* (*H. pylori*); enlarged in a1 (16 000 \times) to show envelopment of a bacterium by caliceal veils (right); note in a2 (28 000 \times ; detail of a1), close adherence of a clubbed process (bottom left) to another bacterium showing VacA immunoreactivity of outer membrane and flagella (upper right); B (3000 \times): Intraepithelial DC with a narrow luminal process directly contacting bacteria; note close membrane adhesion to surrounding epithelial cells, focal interruption of the basal lamina (arrow), and outer membrane protein (OMP) immunoreactivity of cytoplasmic vacuoles (b1, 32 000 \times) and a multivesicular late endosome/lysosome body (b2, 22 000 \times); C (8000 \times): DC with clear cytoplasm, close adherence to surrounding epithelial cells, numerous mitochondria, sparse ribosomes and small rough endoplasmic reticulum (RER) cisternae; cytoplasmic vesicles and membranous remnants of an intracellular bacterium, enlarged in c (42 000 \times), show VacA immunoreactivity; D (2000 \times): Nucleated DC with abundant supranuclear mitochondria, no secretory granules, small tubular RER cisternae (enlarged in d1, 18 000 \times), several cytoplasmic vesicles, scattered vacuoles (v), and close adherence to epithelial cells. On the left, two DC processes (arrows) are contacting the basal membrane (arrowheads). Several cross or longitudinal sections of partly swollen cell processes are observed in the lamina propria; the largest of which (enlarged in d2, 12 000 \times) shows ultrastructural homology with DC cytoplasm, including scattered, small RER cisternae and vesicles; the precise cells of origin of such lamina propria processes could not be assessed. BV: Blood vessel; fg: Foveolar granules; E (2000 \times): Base of foveolar epithelium showing an immature monocytoic cell (DC precursor?) with kidney-shaped nucleus, scattered ribosomes, a few juxtanuclear lysosomes, and no vesicles or granules; note a caliceal process embracing a mast cell (enlarged in e1, 15 000 \times ; scroll bodies, arrow), a clubbed process adhering to *H. pylori* (enlarged in e2, 55 000 \times), a lymphoid cell (Ly) crossing the epithelium basal membrane and another intercellular bacterium (arrow). Reprinted from Necchi *et al.*^[73], with permission from John Wiley.

factors^[77-80], thus, we do not intend to discuss the well-known characteristics of these proteins in detail. Here, we focus on the most recent experimental data that shed new light on their structure/activity relationships, on their

internalization and trafficking in the host cells, and finally, on the apparent paradox of why *H. pylori* simultaneously produces two different virulence factors counteracting the effect of each other on host cells.

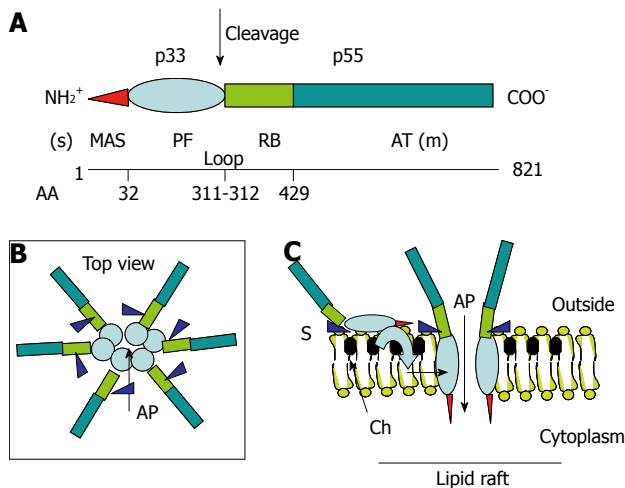


Figure 4 Structure-function relationships in the VacA toxin. A: VacA is an 88-kDa protein that can be cleaved into two subunits designated p33 (red and light blue) and p55 (light green and dark green). The cleavage between the subunit occurs in a flexible loop between residues 311 and 312. The two subunits are probably attached by non-covalent bonds between the p33 carboxy terminus and the N-p55 terminus. The amino-terminal part of p33 consists of 32 hydrophobic amino-acid stretch that is involved in the recognition of mitochondria (red) (MAS) (where the “s” toxin subtype site is located) followed by the p33 “core” subunit that forms, upon entry into the lipid membrane and oligomerization, an anionic channel (PF) (light blue). The toxin nicking site is located in the flexible loop domain. The amino-terminal part of p55 contains the cell receptor domain (about 110 amino acids) (RB; light green) followed by the type Va auto-transporter domain (AT; dark green) that is involved in the secretion of the toxin by the bacterium (where the “m” toxin subtype site is located); B and C: VacA binds as a monomer to its cell surface receptor sphingomyelin (S) with low affinity, then the p33 core progressively is embedded (light blue curve arrow) in the lipid membrane bilayers, at the level of lipid rafts [containing saturated lipid such as sphingomyelin and cholesterol (Ch)] and forms an anionic channel (AP) by oligomerization of p33. This multiplies by 6 the number of toxin cell receptors associated with the oligomerized VacA, thus increasing greatly the toxin affinity for the target cells.

VacA toxin

VacA has three well-confirmed cell activities, namely: cell vacuolation, apoptosis, and CD4⁺ T-lymphocyte activation and proliferation^[77,81]. This toxin was discovered as a secreted protein of *H. pylori* (at that time known as *Campylobacter pylori*) that induces a massive cytoplasmic vacuolation *in vitro*^[82]. About 55% of isolates of *H. pylori* are able to induce that cytopathic effect^[82]. VacA was shown later to form anionic channels into artificial lipid membranes, as well as cell membranes^[77]. VacA channel drives first an hyperacidification of late endocytic compartments by inducing, *via* the transport of anions into the lumen of these organelles, overactivity of the V-ATPase. Accumulation, by protonation, of weak bases such as ammonia (produced by *H. pylori* urease activity) into these compartments creates an osmotic imbalance that results in their swelling^[77,81].

Deep-etch electron microscopy of VacA preparations has revealed that the toxin forms regular oligomers with either six- or sevenfold radial symmetry^[77]. Oligomerization of the toxin molecules creates a cavity in the center of the oligomer and mimics the structure of a pore-forming toxin (Figure 4) such as the α hemolysin

of *Staphylococcus aureus*^[83]. In its mature state, VacA is a 88-kDa protein that can be cleaved into two subunits (Figure 4). According to their molecular mass, the N-terminal subunit is denominated “p33”, whereas “p55” is the C-terminal one (Figure 4). The N terminus of the mature p33 toxin subunit consists of a stretch of 32 uncharged hydrophobic amino acids (found in the VacA s1 subtype^[77,80]). The presence of additional residues to p33 (found in the s2 subtype), ablation deletions within the hydrophobic residues, or specific point mutations of amino acids in this stretch inhibit the vacuolating activity of VacA. From these results, it has been concluded that VacA channel formation involves the N-terminal 32 amino acids of p33^[77,80,84,85]. Other observations have led to the hypothesis that, in addition to the p33 N-terminal part and p33 itself^[85], a toxin domain localized in about the first 110 residues of the p55 is also required for the proper oligomerization of the toxin, for channel formation. This is supported by the fact that the minimal intracellular active domain of VacA contains the full p33 domain and about 110 residues of the N-terminal part of p55^[86,87]. In these experiments, the p33 toxin subunit, produced alone within cells, does not induce vacuolation^[86,87].

In addition to its vacuolating activity, VacA exhibits apoptotic activity^[88-90]. Importantly, these studies have revealed that the p33 VacA subunit is targeted to mitochondria and only is required for apoptosis^[88], and that a toxin mutant without vacuolating activity is non-apoptotic^[89]. Collectively, these data indicate that VacA induces, through the p33 subunit alone, apoptosis by acting on mitochondria, and most likely involves the toxin channel activity. Recently, it has been demonstrated that p33 oligomerizes without the need of p55 and forms an anionic channel with characteristics identical to that of the whole VacA molecule^[91]. Furthermore, ablation of the 32 N-terminal residues of p33 does not alter the pore-forming activity of the VacA subunit, but impairs the mitochondrial targeting activity of p33^[91]. Altogether, these new data indicate that the portion of p33 without its first 32 N-terminal residues (that we call the “core p33”) oligomerizes and contains all the channel activity of VacA. Domańska *et al*^[91] have clarified the following, formerly puzzling, observations: (1) that a mutant toxin with a major deletion in the p33 N-terminal hydrophobic amino acids (VacA delta 6-27), albeit inhibiting the vacuolation process, nonetheless induces anionic channels in artificial bilayers, even though exhibiting a longer delay of formation than the wild-type toxin^[85]; and (2) that a single deletion (δ 49-57) in the p33 core domain inhibits the oligomerization of the toxin and the vacuolation^[92]. Indeed, in the light of the study of Domańska *et al*^[91], these observations are well explained by the role of the p33 core without the N-terminal hydrophobic amino-acid stretch in forming the toxin channel by oligomerization.

As depicted in Figure 4, the structure-activity of VacA would thus be the following: starting from the N terminus, the 32-amino-acid stretch consists of a mem-

brane/mitochondrial targeting motif (which however does not participate in the toxin channel formation)^[91]. Then, the p33 channel core forms the channel activity, either in endosomal membranes or in the inner mitochondrial membrane (IMM)^[91]. At the end of the p33 subunit, there is the loop domain (58 residues) in which the toxin-nicking site is located (between residues 311 and 312). We speculate that a peptide motif just after the nicking site (i.e. residing in the 110 residues of the N-terminal part of p55 subunit) is implicated in the recognition of the toxin cell receptor. This is at odds with the current view that the toxin receptor domain is located in the so-called mid-region (m1/m2; i.e. residues 470-662) of p55^[77,80]. How to reconcile the recent data of Domańska *et al.*^[91] with previous results indicating that the intracellular minimum portion of the VacA molecule active in the cytosol requires, in addition to p33, the 110 N-terminal residues of p55 for VacA oligomerization^[84,86,87]? Taking in account that the p33 toxin subunit oligomerizes alone and supports all the pore-forming ability of VacA^[91], why does expression of this toxin subunit alone into cells not lead to cell vacuolation^[86,87]? We speculate that the different intracellularly expressed (by cDNA transfection) toxin domains might induce their vacuolating activities not after intracellular synthesis, but rather only after leaking out of the producing cells, followed by endocytosis *via* the VacA cognate receptor. This would point to the 110 residues present at the N-terminal part of p55 as containing the VacA cell receptor motif (Figure 4). This position would indeed be ideally suited to recognize a cell surface molecule close to the lipid bilayers (Figure 4). The 3D structure of the p55 VacA subunit has been recently resolved^[93] and shows that a large part of this toxin subunit has the classical fold of autotransporter structures of the bacterial *Va* secretion system (by which the toxin is transported), which is mostly implicated in the bacterial secretion of VacA. This is supported by previous experiments showing that several deletions in the p55 subunit abolish the production of VacA by the bacterium^[85]. Gangwer *et al.*^[93] have proposed that a conserved pocket, located in the m1/m2 regions of p55, is the receptor domain of VacA, which becomes fully accessible to the cell receptor when the toxin is assembled in either a single- or double-layered oligomeric structure. However, it is well known that it is necessary to disrupt the oligomeric structure of VacA (by acidic or alkaline treatments) to render it able to bind to its cell receptor. Furthermore, the mutant toxin δ 49-57, which does not form oligomeric structures, does not require to be acid-activated to bind the VacA cell receptor^[92]. This demonstrates that the toxin receptor domain is probably not fully accessible in the VacA oligomeric form. More recently, González-Rivera *et al.*^[94] have shown that a mixture of purified p33 and p55 does not form oligomeric structures in aqueous solvent, but only monomeric structures, whereas, upon addition of a detergent that mimicks a lipid membrane environment, there is formation of oligomeric structures. This result suggests that the hydrophobic environment induces the oligomer-

ization, most likely by the p33-dependent formation of the channel. Importantly, it has also been shown that the p33 subunit markedly increases the cell binding of p55^[94]. Thus, VacA as a monomer first binds one cell receptor with low affinity, while upon oligomerization, bound to six receptors, it exhibit a very high affinity (Figure 4).

An important breakthrough, seminal for the identification of the VacA receptor, has been the finding that the toxin requires the integrity of lipid rafts and glycosylphosphatidylinositol-anchored proteins (GPI-APs) for its full vacuolating activity, which suggests that VacA follows the GPI-AP pathway of endocytosis by a lipid raft-dependent, clathrin-independent pathway^[95]; later described in detail by Sabharanjak *et al.*^[96]. The first vacuolar organelles collecting the incoming GPI-APs involve new intracellular compartments that have been named GPI-AP-enriched early endosomal compartments (GEECs), which differ from classical early endosomes^[96]. This endocytic pathway, which also requires the small Rho GTPase Cdc42^[96], is currently named the GEEC endocytic pathway^[97,98], and has been confirmed to be exploited by VacA for its internalization^[99-102] (Figure 5). It was later shown that the GEEC pathway requires the presence, in the cell plasma membrane, of the unsaturated lipid sphingomyelin, and that the length of the sphingomyelin acyl chain determines its endocytic intracellular trafficking^[103]. This prompted an investigation that has demonstrated the role of sphingomyelin as the VacA cell receptor^[103]. Accordingly, it has been demonstrated that the length of the sphingomyelin acyl chain also determines the VacA intracellular trafficking^[102]. Normal cells contain in their lipid membrane a high proportion of long acyl chain sphingomyelin (C18), and VacA bound to this lipid follows the GEEC pathway^[102]. In contrast, when cells are artificially enriched in short-acyl-chain sphingomyelin (C2), the toxin does not follow the GEEC pathway but is recycled back to the plasma membrane in a Cdc42-independent fashion^[102].

Upon VacA treatment, transformed human CD4⁺ T lymphocytes (Jurkat cells) block their constitutive production of IL-2^[104]. It is well established that, in T lymphocytes, the nuclear factor NFAT, upon processing by the calcium-activated calcineurin protease, activates the transcription of the *IL-2* gene. Massive entry of calcium, through the voltage-dependent Ca²⁺ release-activated Ca²⁺ (CRAC) channels, is required to activate calcineurin. However, a VacA mutant without vacuolating activity has no effect on NFAT inhibition in Jurkat cells^[105]. Possibly, inhibition of NFAT by VacA is due to the electric depolarization of the lymphocytic plasma membrane, induced by the toxin anionic channel, which inhibits CRAC channels. Human primary T lymphocytes are, however, not sensitive to the deactivation of NFAT induced by VacA^[106]. Treatment of primary human T lymphocytes by phorbol myristate acetate, which induces their migratory activity, together with the expression of the CD18 β -integrin at their surface, restores the VacA-induced inhibition of NFAT^[107]. Furthermore, the direct

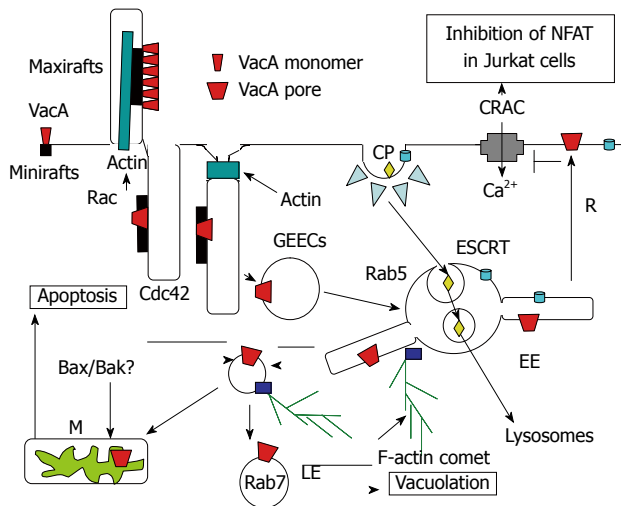


Figure 5 Endocytosis and intracellular trafficking of VacA. Monomeric VacA may bind sphingomyelin on small rafts. Then, by formation of membrane extensions by actin polymerization via Rac activation, VacA is clustered in macrafts where the p33 subunit oligomerizes and forms a channel that enters the membrane lipid bilayers. By a Cdc42-dependent process, VacA bound to sphingomyelin associated to lipid rafts is transferred into cell membrane invaginations (tubules?), which are then pinched out from the membrane, with the help of F-actin filament, which leading to the formation of the glycosylphosphatidylinositol-anchored protein-enriched early endosomal compartment (GEEC) compartment that contains VacA. The toxin is then transferred to Rab5-positive early endosomes (EEs). In EEs, VacA is selectively addressed to EE tubular extensions that are formed by an F-actin process. These tubular extensions are pinched out from the EEs and form highly motile vacuoles that are propelled by F-actin comets. These motile vesicles then fuse with mitochondria (M) or late endosomes (LEs), where VacA induces apoptosis or vacuolation. The proapoptotic channels Bax and Bak may be brought to mitochondria by binding on VacA-containing motile vacuoles. Some VacA molecules may be recycled (R) back to the plasma membrane where the channel activity of the toxin, by altering the electric transmembrane potential, inhibits the voltage-dependent Ca^{2+} release-activated Ca^{2+} (CRAC) channel. This blocks entry of calcium that activates the calcineurin protease, which is required for processing of the nuclear factor (NFAT), and therefore inhibits the transcription of the interleukin 2 (IL-2) gene in Jurkat T-lymphocytes. Ligands entering the coated-pit pathway (CP) are directed, by the endosomal sorting complex required for transport (ESCRT) complex, towards internal vesicles of EEs and form the multivesicular body (MVB). MVBs are directed to lysosomes, by being propelled along microtubules, where the contents of EE internal vesicles are transferred and degraded.

expression of CD18 into primary human T lymphocytes renders them able to respond to VacA^[107]. It has therefore been concluded that CD18 is the receptor for VacA in immune cells^[107]. This result is puzzling because, for all the different toxins studied so far, the cell binding activity always depends on a unique molecule (or a family of closely related molecules). However, a few observations do not support the role of CD18 as the sole VacA receptor in immune cells. Indeed, it has been demonstrated that, although human primary lymphocytes do not deactivate the nuclear factor NFAT, they respond perfectly to VacA by blocking their induced proliferation (i.e. blockage of their cell cycle) due to a mitochondria-induced depletion of ATP^[106,108,109]. This fact clearly indicates that VacA binds and penetrates into primary lymphocytes without needing CD18 (probably using the sphingomy-

elin receptor). The fact that VacA, entering *via* sphingomyelin in primary T cells, does not inactivate NFAT can be explained by the possibility that the toxin channel is probably not recycled back (or only weakly recycled) to the plasma membrane, and therefore, is unable to block calcium cell entry through CRAC channel inhibition. We speculate that CD18 may not be a real receptor for VacA, but rather activates a signaling pathway (perhaps *via* the direct activation of CD18 by VacA, because association of VacA with CD18 has been reported^[107]) that modifies the intracellular trafficking of the toxin. For instance, stimulation of the migratory activity of T lymphocytes by CD18 signaling might activate Cdc42 boosting of the entry of VacA^[99], and increasing its recycling to the cell surface. It has recently been reported that the GEEC pathway is pivotal during cell migration^[98]. Another possibility would be that CD18-driven signaling drastically changes the lipid membrane content of long-acyl-chain sphingomyelin molecules toward short ones forcing the toxin to recycle back to the plasma membrane as recently described^[102].

How is VacA transferred from early endosomes to mitochondria and/or late endosomes to induce apoptosis and vacuolation? It is well known that ligands or membrane receptors taken up and routed for degradation into lysosomes are endocytosed by the clathrin-dependent pathway and targeted to the luminal vesicles of endosome-forming multivesicular bodies (MVBs) by the endosomal sorting complex required for transport (ESCRT) complex^[110] (Figure 5). Propelled along microtubules, MVBs then move to lysosomes where the content of MVB internal vesicles is selectively delivered for degradation. Several lines of evidences indicate that MVBs and lysosomes are not the cell compartments that undergo vacuolation by VacA activity: (1) no internal vesicles are observed by electron microscopy in VacA-induced vacuoles and the enlarged compartment does not have the structure of lysosomes^[95,111]; (2) the vacuolated compartment contains markers of both late endosomes and lysosomes^[112]; and (3) disruption of microtubules does not block VacA-induced vacuolation^[113]. Thus, the organelles that are vacuolated by VacA are likely post-lysosomal compartments that are required to recycle the membrane of MVBs from lysosomes. VacA is probably transferred directly to that compartment by an F-actin-dependent mechanism of endosome motility^[101]. Indeed, upon entry of VacA into early endosomes *via* the GEEC pathway, VacA-containing early endosomes exhibit F-actin comet tails associated with their cytosolic membrane surface, and are rapidly moving in the cytosol^[101]. Inhibition of these F-actin structures blocks the VacA transfer into the late endosomal compartment and vacuole genesis^[101], but also VacA-induced apoptosis and the localization of toxin molecules in mitochondria^[114]. These findings suggest that VacA is addressed to late endosomes and mitochondria by the same mechanism that relies on F-actin-driven vesicular motility. F-actin structures have recently been shown to take place at the level of the early endosomal

surface to induce budding of tubular structures followed by their cleavage to yield vesicles that retain F-actin tails^[115]. It is thus tempting to speculate that VacA might be first transferred from GEECs to the early endosomal limiting membrane, then into these tubular extensions, and finally into the membrane of the ensuing F-actin motile vesicles (Figure 5). These vesicles are then brought to late endosomes and/or mitochondria *via* stochastic fusion events by F-actin-driven vesicular motility (Figure 5). In this model, the transfer of VacA (or only of its p33 subunit) from the donor membrane to the recipient membrane (late endosomes or mitochondria) would be achieved by the membrane/mitochondrial N-terminal 32 residues of p33^[91] that may stick out of the membrane (Figure 4). This would explain why point mutations, or deletion or modification of the length of this amino-acid stretch might modulate the vacuolating process without altering the pore-forming capacity of the toxin^[91]. Importantly, according to this model, the toxin would never be released free in the cytosol^[90], which differs from the enzymatic subunits of canonical A-B toxin. By remaining associated with lipid membranes, out of reach of degradative processes, the VacA channel would keep its full activity for a long time, as observed several years ago^[111].

Inside mitochondria, the p33 subunit (or the full VacA molecule) induces, probably by the rupture of IMM integrity^[91], the release of cytochrome *c* (cyt *c*) and induction of apoptosis^[88]. However, pro-apoptotic channels must be activated or transferred into the outer membrane of mitochondria to ensure the release into the cytosol of cyt *c*, which, by activation of the Apaf1 molecule, induces the stimulation of the caspase cascade that results in cell death. Accordingly, the pro-apoptotic channels Bax and Bak have been shown to be activated during VacA-induced apoptosis^[116,117], and VacA is unable to induce apoptosis in Bax- and Bak-deficient mouse embryonic fibroblasts^[117]. It has been recently proposed that Bax and Bak might be directly recruited from the cytosol by endosomes containing functional VacA channels in their membranes, which allows their juxtaposition with mitochondria^[117]. This would be an ingenious way to combine the transfer of VacA channels to the IMM (and thereby the release of cyt *c* in the mitochondrial inter-membrane space) and the leakage of cyt *c* in the cytosol.

Thus VacA is probably not really a multifunctional toxin, as previously defined^[77]. The diversity of VacA activities observed may be simply explained by the channel formed by the p33 subunit that is addressed to different cell membranes by the intracellular trafficking of the toxin, which may be different in different cell types or during cell differentiation (Figure 5).

Functional relationship between VacA and CagA

The gene encoding VacA is present in all the *H. pylori* strains, although only 55% express an active vacuolating toxin. This is due to either additions or deletions of peptide sequences in the toxin that probably impair the VacA intracellular trafficking or its binding to the cell surface,

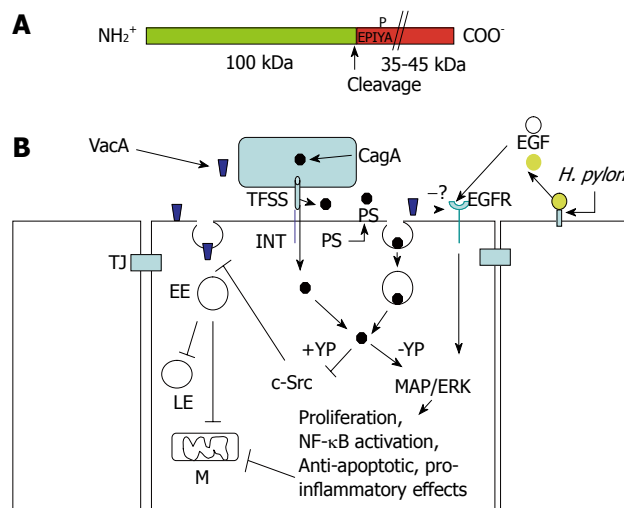


Figure 6 Structure of CagA and signaling cross-talk between VacA and CagA. A: CagA encompasses two fragments: the N-terminal 100-kDa fragment may contain the cell binding domain [to phosphatidylserine (PS)?]. Cleavage of CagA may take place just at the beginning of the first EPIYA motif, which can be tyrosine-phosphorylated by the c-Src tyrosine kinase. The C-terminal portion of CagA, which contains all the signaling activity of the molecule, may have a different molecular mass (up to 45 kDa) due to the repetition of the EPIYA-containing domain; B: CagA produced within the bacterium is transferred in the external medium by the type IV secretion system (TFSS) machinery. Two possibilities for CagA transfer into the target epithelial cell: (a) by binding to an integrin (INT), the TFSS punches the cell membrane and injects CagA; or (b) the TFSS induces the flipping of PS on the outer cell surface. By its 100-kDa N-terminal fragment, CagA binds PS and, upon endocytosis, which is transferred into the gastric cells. In the cytosol, the CagA signaling domain can be tyrosine-phosphorylated (+YP) and inhibits the c-Src kinase activity that is required to allow the transfer of VacA from glycosylphosphatidylinositol-anchored protein-enriched early endosomal compartments (GEECs) to early endosomes (EEs). This blocks vacuolation in late endosomes (LEs) and mitochondria (M)-dependent apoptosis induced by VacA. In an unphosphorylated (-YP) state, CagA activates mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) and nuclear factor (NF)-κB anti-apoptotic and pro-inflammatory pathways, which also counteract VacA-induced apoptosis. VacA interferes with epidermal growth factor (EGF) receptor (EGFR) activation and endocytosis, thus impairing the signaling pathway that is triggered by this receptor. Free EGF ligands (EGF) are liberated from the cell-surface-bound molecules *via* cleavage triggered by *Helicobacter pylori* (*H. pylori*). TJ: Tight junction.

as we have described above. It is now well-established that the most severe gastric pathology (i.e. peptic ulcer and gastric cancer) is restricted to *H. pylori* type 1 strains, which contain in their chromosome a PAI named *cag* PAI, and which secrete an active vacuolating VacA toxin^[10]. The *cag* PAI encodes for the CagA protein and for the TFSS machinery that is implicated in the transport of CagA out of the bacterium, and its transfer to the host cell cytosol^[78,79]. CagA is a 135-145-kDa protein that contains its full intracellular signaling activity in its 35-45-kDa C-terminal part^[78,79,118] (Figure 6). Nevertheless, it is still unclear how and where the *cag* TFSS recognizes and target gastric epithelial cells so as to transfer CagA into their cytosol. Recently, two mechanisms have been proposed. One points to the recognition and binding of the TFSS to an integrin^[119,120], then, through a mechanical sliding due to bound integrins, the TFSS might punch the cell surface and deliver CagA into the cytosol^[120]. One caveat

for this mechanism is that *H. pylori* might deliver CagA at the apical pole of colonized epithelial gastric cells, whereas integrins are localized on the basolateral pole. On the other hand, it has been proposed that the TFSS does not punch the cell surface but induces the flipping out of the lipid phosphatidylserine (PS) from the inner (where it normally resides) to the outer leaflet of the target cell membrane^[121]. In this model, CagA, which is secreted constitutively by the TFSS, binds to PS through a lipid-binding peptide motif that is present in its 100-kDa N-terminal part^[121]. Following this binding step, a novel type of endocytosis (not yet better characterized nor defined) is induced that carries CagA, associated with PS, into epithelial gastric cells^[121]. If this is true, CagA should no longer be defined as an effector protein, but rather as a new type of A-B toxin that is released only in close proximity to the target cells.

Inside the cell, CagA may be tyrosine phosphorylated by the p60 c-Src kinase^[118]. Phosphorylation occurs on the 35-45-kDa C-terminal part of CagA on specific five-amino-acid motifs called EPIYA, which can be repeated several-fold and increase the size of this fragment^[26,78]. CagA can be split into two fragments by proteases^[122,123]. This generates a 100-kDa N-terminal fragment and a C-terminal 35-45-kDa one that contain all the cell signaling activity of CagA^[114]. Proteolytic cleavage of CagA does not require its tyrosine phosphorylation, and the nicking site is localized just at the onset of the first EPIYA motif (Boquet and Ricci, unpublished data). The 100-kDa N-terminal part of CagA might be involved in the cell recognition and transfer of the 35-45-kDa C-terminal part to the cytosol, as suggested by recent data^[121]. Therefore, CagA would indeed behave as a typical A-B toxin.

Two main intracellular activities of CagA are now well established. In its tyrosine phosphorylated state, CagA may act, either directly or upon association and activation of the tyrosine phosphatase SHP2^[78], to inhibit the c-Src kinase and therefore to affect many regulating proteins that depend on this enzyme, among which are those involved in the actin cytoskeleton assembly/regulation^[124]. In its non-tyrosine-phosphorylated form (or independent of the phosphorylation state), CagA activates the MAP/ERK and NF- κ B pathways^[53,125], which induce cell proliferation, inhibition of apoptosis, a typical cellular elongation called "hummingbird phenotype"^[66], and inflammatory response^[28] (Figure 6).

An increasing body of evidence suggests that CagA and VacA have several opposing cellular effects. It has been shown that, while CagA activates the NFAT nuclear factor, VacA inhibits it^[126]. Then, it has been demonstrated that CagA decreases VacA-induced vacuolation, while in turn, VacA reduces CagA-induced hummingbird phenotype formation^[127]. It has also been shown that VacA can downregulate CagA effects on epithelial cells by interfering with activation and endocytosis of EGFR, thus impairing the signaling pathway triggered by this receptor, and which plays a pivotal role in cell proliferation and hummingbird phenotype formation^[128]. Taken together,

these findings raise the hypothesis that VacA and CagA may downregulate the cell effects of the other, allowing *H. pylori* interaction with epithelial cells, while avoiding excessive cell damage.

We have recently shown that CagA interferes with VacA action by two complementary mechanisms^[114]. In its tyrosine-phosphorylated state, CagA inhibits the c-Src kinase and, in doing so, blocks VacA in the GEECs and thus its delivery to early and late endosomes (inhibition of the vacuolation) or mitochondria (inhibition of apoptosis). In its unphosphorylated state, CagA stimulates the NF- κ B pathway and, in doing so, induces anti-apoptotic activity (mediated possibly by the anti-apoptotic factor Bcl2) that also blocks VacA-induced apoptosis. We hypothesize that, once bacteria have colonized the gastric niche, the apoptotic action of VacA might be detrimental for the survival of *H. pylori* that are adherent to the mucosa, and thus the CagA counteracting action gives a rationale for the association of these two virulence factors in the most pathogenic *H. pylori* strains^[114]. This would be a new, highly ingenious mechanism by which a bacterium locally protects its ecological niche against the action of one of its own virulence factors. However, while exerting a beneficial role for survival and growth of the bacterium by counteracting VacA toxin, CagA injection in the gastric epithelial cells triggers pro-inflammatory and anti-apoptotic responses that are detrimental for the human host in the long-term, because they favor the development of ulcer and cancer.

Because it simultaneously delivers to its host two independent virulence factors that antagonize each other, *H. pylori* appears to act like a driver that presses the brake and gas pedals at once. This apparently paradoxical behavior is now emerging as an intriguing strategy to achieve the best fit between the bacterium and the hostile gastric environment that represents its ecological niche.

CONCLUSION

With regard to the pathogen, illness is often inadvertent; the result of exquisite tricks learned long ago and played on human cells to achieve the paramount goal of the microorganism: conservation of the species^[56,129]. Taken together, all the new findings described above strongly reinforce the notion that *H. pylori* is a skilled bacterium that has been smart enough to: (1) learn about the physiology of its host; (2) keep this information in its genetic library; (3) share the gene book with other organisms; (4) be "open minded" about acquiring new knowledge and discarding obsolete information; and, of key importance; and (5) know how to communicate with its human host, realizing that killing the host would oblige it to find a new one very quickly to ensure its reproductive success^[56,129,130]. *H. pylori* makes a long and hard, but also successful, journey in the human stomach, given that *H. pylori* is widespread and has probably been part of the human biota since time immemorial^[31,56,130]. Although *H. pylori* seeking survival may be sometimes achieved at the expense of the well-being

of the stomach and the host, human physiology and immunology have co-evolved in the presence of persistent gastric *H. pylori* colonization, and so are disrupted by its absence^[130]. As emphasized by Atherton *et al.*^[130], this imbalance of a long-lasting pathogen/host equilibrium, caused by progressive disappearance of *H. pylori* from some populations, may however result in an increased incidence of other diseases such as reflux esophagitis and esophageal carcinoma, as well as obesity, type 2 diabetes, and allergic disorders.

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Nodular regenerative hyperplasia: Evolving concepts on underdiagnosed cause of portal hypertension

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Abstract

Nodular regenerative hyperplasia (NRH) is a rare liver condition characterized by a widespread benign transformation of the hepatic parenchyma into small regenerative nodules. NRH may lead to the development of non-cirrhotic portal hypertension. There are no published systematic population studies on NRH and our current knowledge is limited to case reports and case series. NRH may develop *via* autoimmune, hematological, infectious, neoplastic, or drug-related causes. The disease is usually asymptomatic, slowly or non-progressive unless complications of portal hypertension develop. Accurate diagnosis is made by histopathology, which demonstrates diffuse micronodular transformation without fibrous septa. Lack of perinuclear collagen tissue distinguishes NRH from typical regenerative nodules in the cirrhotic liver. While the initial treatment is to address the underlying disease, ultimately the therapy is directed to the management of portal hypertension. The prognosis of NRH depends on both the severity of the underlying illness and the prevention of secondary complications of portal hypertension. In this review we detail the epidemiology, pathogenesis, diagnosis, management, and prognosis of NRH.

INTRODUCTION

Nodular regenerative hyperplasia (NRH) belongs to the category of liver diseases responsible for non-cirrhotic intrahepatic portal hypertension (NCIPH)^[1], which include sinusoidal obstruction syndrome, perisinusoidal fibrosis, hepatoportal sclerosis and incomplete septal cirrhosis (Figure 1). In these conditions, the etiology is ascribed to an intrahepatic hypercoagulable state, possibly secondary to sinusoidal endothelial injury. In many Asian countries, the most frequent cause of NCIPH is schistosomiasis.

NRH was first defined by Steiner^[2] in 1959 as a condition characterized by diffuse benign transformation of the hepatic parenchyma into small regenerative nodules distributed evenly throughout the liver with minimal or no fibrosis in the perisinusoidal or periportal areas. This feature distinguishes NRH from other causes of NCIPH^[1]. The presence of fibrous septa between the nodules definitively excludes NRH. However, in rare cases, one patient may exhibit histopathologic features of both NRH and other NCIPH disorders. These observations suggest that similar etiological factors may induce various adaptive liver reactions.

Over the last two decades, multiple labels have been applied to describe what we now define as NRH. Terms such as “miliary hepatocellular adenomatosis”, “non-cirrhotic nodulation”, “hepatocellular adenomatosis”, or “adenomatous hyperplasia” have been previously used. Although our current knowledge is limited to single case reports and case series, the number of patients being given the diagnosis of NRH has dramatically increased in recent years. Up to the year 2000 approximately 200 patients had been reported, whereas in the last decade more than 260 new cases of NRH were reported worldwide.

EPIDEMIOLOGY

As stated previously, our understanding of the epidemiology of NRH is based upon case reports rather than systematic population studies. In the United States National Library of Medicine/National Library of Medicine (PubMed) database (<http://www.ncbi.nlm.nih.gov>) 375 case reports have been published since 1975, however, not all cases meet the strict histopathologic criteria for NRH. For example, earlier publications used the term “nodular regenerative hyperplasia” as a misnomer for large regenerative nodules (LRN), associated with post-sinusoidal obstructive conditions such as congestive cardiomyopathy or Budd-Chiari syndrome.

NRH comprises 27% of all cases of non-cirrhotic portal hypertension in Europe and about 14% in Japan^[3-5]. Autopsy studies indicate an overall incidence ranging between 0.72% and 2.6%^[6-8]. Timely clinical diagnosis of NRH is challenging, because the majority of patients do not present with symptoms of portal hypertension. In cases where the etiology of portal hypertension was unclear the histology disclosed NRH in less than 1%^[9,10]. While NRH is rare in comparison to other causes of portal hypertension, its presence is being increasingly recognized.

In the case reports we reviewed, the majority of patients were between 25 and 60 years old at diagnosis, with rare cases in children and even fetuses^[1]. According to autopsy studies, the risk of development of NRH and its potential complications increases with age. In 2500 autopsies the incidence of NRH after 80 years of age was 6%, seven times greater than in people under 60 years of age^[6]. One case series reported the prevalence of NRH in six siblings distributed in three unrelated families indicating the possibility of a family distribution of this disease^[11]. Sex and ethnicity seem to play no role in development of NRH.

ETIOLOGY

Portal vasculopathy

NRH appears to be a result of an adaptive hyperplastic reaction of hepatocytes. Normally, the mitotic activity of hepatocytes is very low; hyperplasia is considered to be a physiological response to injury. Increased oxygen and nutrient demand, chronic inflammation, hormone-mediated dysfunction or compensation for damage or disease

elsewhere play an important role in this process.

The pathogenesis of NRH seems to be related to abnormalities of portal hepatic blood flow akin to the “atrophy-hypertrophy complex”. Hemodynamic disturbances at the level of the hepatic microvasculature occur either secondary to a mechanical obstruction or functional blood flow alterations. One hypothesis is that local portal venous hypoperfusion leads to apoptosis and hepatocyte atrophy, coexisting with maintained or increased blood supply to adjacent acini cells. Local hyperperfusion leads, in turn, to elevated levels of cell growth activators which act as autocrine or paracrine peptides. This hypothesis has been supported by both histopathologic examinations of liver biopsies as well as animal experiments, which showed microvascular changes involving either portal vein radicles or less frequently, arterial or hepatic vein branches. Wanless coined a “portal obliterative venopathy” phenomenon of recurrent embolization of the portal venules by platelet aggregates or thrombi originating in the portal venous system or in the spleen. The ensuing vascular inflammation and fibrosis results in reduced luminal patency of portal vein radicles and local reduction in blood supply to the liver, confirmed in 64 autopsies^[6]. Nakanuma *et al.*^[5] also provided evidence of obliterated portal venules in 107 liver biopsies of patients with NRH. There are few case reports of NRH without vasculopathy^[12], e.g. diffuse carcinoid tumor, where multifocal liver ischemia is due to a functional and not an organic cause^[13].

NRH is commonly found in patients with Abernathy's Syndrome a condition that includes the rare anomaly of congenital absence of the portal vein. The intestinal and splenic veins drain directly into the inferior vena cava, bypassing the liver entirely. It is an extreme model of vascular pathology, where the entire liver relies upon high-pressure arterial perfusion. Rare cases of NRH were also reported in patients with thrombosis of portal vein trunk^[14,15].

Immunosuppressant and chemotherapeutic drugs

Immunosuppressive medications may induce NRH by damaging endothelial cells of small hepatic veins. There are several reports of NRH developing in response to prolonged treatment with thiopurines, including azathioprine (AZA), 6-mercaptopurine and 6-thioguanine (6-TG). NRH was found in a single case among 30 patients treated with thiopurines for Crohn's disease. Accumulation of toxic metabolites may be a factor, as NRH was found more frequently in post-transplant patients with impaired metabolism of AZA due to the thiopurine-methyltransferase mutation^[16]. In another study NRH was found on liver biopsy in three patients treated with AZA for inflammatory bowel disease for more than 1 year, who presented with elevated liver enzymes^[17]. Gane *et al.*^[18] demonstrated histological regression of NRH with normalization of liver enzymes in four patients after withdrawal of AZA, after being used for an average of 64 mo. While the etiology of this remains to be elucidated, it has been stated that 6-TG, used in treatment

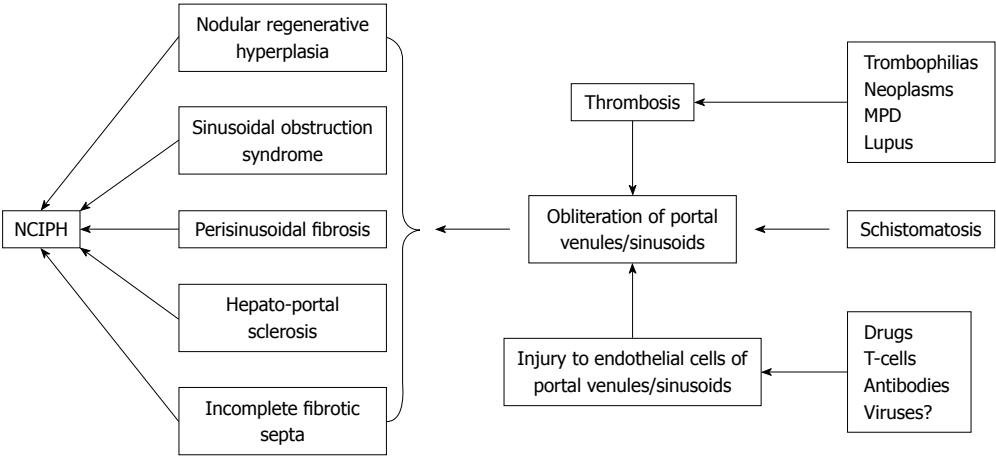


Figure 1 Development of nodular regenerative hyperplasia and other liver adaptive reactions causing non-cirrhotic intrahepatic portal hypertension. NCIPH: Non-cirrhotic intrahepatic portal hypertension; MPD: Myeloproliferative diseases.

of AZA-resistant forms of inflammatory bowel disease, may have a higher potential to induce NRH than other thiopurines^[19].

Other authors reported NRH in 8% of a human immunodeficiency virus (HIV)-positive cohort receiving highly active antiretroviral therapy (HAART)^[20]. Didanosine, a HAART drug responsible for liver injury and pulmonary hypertension, appears to induce NRH^[21].

An analysis of 334 liver biopsies from patients with metastatic colorectal cancer demonstrated that vascular pathology (as compared to chemotherapy-associated steatohepatitis) is a predominant histopathologic finding in chemotherapy-induced liver injury^[22]. There are numerous reports on NRH developing in patients with disseminated cancer after use of cytostatic drugs. Older reports associate NRH with use of busulphan, thioguanine or cyclophosphamide and, in more recent reports, with oxaliplatin-based therapies. Among 274 patients treated with oxaliplatin, sinusoidal obstruction syndrome and NRH were found in the histopathological study in 54% and 24.5% of patients, respectively. Peliosis and perisinusoidal or perivenular fibrosis were other vasculopathy-related liver diseases^[23]. Overall, about 70 cases of oxaliplatin-related NRH were reported in the literature (Table 1).

Underlying disease

NRH may develop as a result of underlying disease of autoimmune, inflammatory, neoplastic, or idiopathic origin (Table 1)^[143]. In patients with systemic lupus erythematosus (SLE), rheumatoid arthritis, and a host of other autoimmune diseases (including sarcoidosis or other granulomatous liver diseases)^[90], antibody reaction to the endothelial cells of small hepatic vessels combined with local hypercoagulation^[144] may predispose to NRH. Ziolk *et al.*^[145] found intrasinusoidal infiltrate composed of cytotoxic CD8+ T-lymphocytes in 32% of 44 patients with NRH. These T-cells were located near atrophic liver cell plates and were adjacent to endothelial cells exhibiting evidence of apoptosis. Moreover, in patients with SLE, anticardiolipin antibodies could be incriminated for por-

Table 1 Diseases and conditions coexisting with nodular regenerative hyperplasia¹

Disease	No. of cases	Ref.
Pulmonary hypertension	32	[11,24-44]
Rheumatoid arthritis/Felty's syndrome	30	[31,41,44-55]
Human immunodeficiency virus infection	20	[21,56-64]
Lupus erythematosus	12	[27,40,65-70]
Crohn's disease/ulcerative colitis	13	[17,28,56,71-76]
Celiac disease	9	[76-80]
Scleroderma/CREST	7	[25,32,81-85]
Antiphospholipid syndrome	11	[78,79,86-89]
Sarcoidosis	9	[90]
Post-transplant	18	[16,37,91-99]
Extrahepatic cancers	26	[26,59,100-104]
Lymphomas	12	[29,53,81,105-113]
Macroglobulinemia	5	[114,115]
Mixed cryoglobulinemia	3	[25,116,117]
ITP/aplastic anemia	4	[3,12,118,119]
Primary biliary cirrhosis	6	[25,82,120-122]
Krabbe disease	4	[123,124]
Congenital absence of portal vein	4	[15,125,126]
Portal vein thrombosis	2	[14,127]
Familial pulmonary fibrosis	4	[128]
Chronic glomerulonephritis	5	[51,117,129-131]
Cystinosis	2	[24]
Myasthenia	2	[132,133]
Polyarteritis nodosa	2	[134,135]
Common variable immunodeficiency syndrome	2	[136,137]
Turner's syndrome	2	[138,139]
Castleman's disease	2	[78,140]
Idiopathic eosinophilic syndrome	2	[141,142]

¹Only associations reported in the literature twice or more have been shown. ITP: Idiopathic thrombocytopenia. CREST: Calcinosis, raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia.

tal vasculopathy leading to NRH^[78,79,86], although this is not a universal finding in SLE^[68].

NRH has also been associated with hematologic disorders, especially myeloproliferative diseases and congenital thrombophilias, where the hypercoaguable state may induce a progressive splenic and portal vein thrombosis and subsequent portal hypertension^[2].

DIAGNOSIS

One should consider a diagnosis of NRH in all patients with clinical symptoms of portal hypertension (splenomegaly, esophageal varices, ascites) but with normal transaminases and no manifestations of cirrhosis (gynecomastia, palmar erythema, spider nevi). There are mildly increased liver enzymes, usually alkaline phosphatase, in 11%-25% of patients^[4,127,146]. It is estimated that NRH is complicated by clinically overt portal hypertension in at least 50% of cases, with an augmented hepatic venous pressure gradient confirming sinusoidal obstruction^[147,148]. Close surveillance of patients with predisposing conditions is important for early diagnosis, especially in situations where drug toxicity may play a role. In patients on AZA for autoimmune hepatitis, the occurrence of splenomegaly should alert the clinician to the development of portal hypertension secondary to NRH.

In all cases of NCIPH, more common treatable causes (viruses, alcohol, metabolic and autoimmune disorders) should be eliminated first, followed by an assessment of the usual exposures (acetaminophen, vitamin A, copper sulfate, vinyl chloride, arsenic salt). Portal and hepatic venous thrombosis may be excluded on radiographic imaging.

Imaging

Imaging methods have poor sensitivity and specificity for NRH. A diffusely heterogeneous hepatic parenchyma may be the only imaging abnormality. On ultrasound, regenerative nodules are usually not visible due to a small size or isoechogenicity. The presence of well-delineated hypoechoic or isoechoic tiny lesions with a sonoluscent rim are indistinguishable from metastases^[149]. Hyper-echoic nodules have been reported in very rare cases of NRH^[150]. On computed tomography (CT), regenerative nodules remain isodense or hypodense in both arterial and portal venous phases, distinguishing NRH from focal nodular hyperplasia and adenomas^[3].

The significance of magnetic resonance imaging in the diagnosis of NRH is still controversial, although because of its inherent propensity to resolve soft tissue details it may be superior to CT in visualization of regenerative nodules. NRH lesions appear hyperintense on T1-weighted images and iso- or hypointense on T2-weighted images^[67,151], with a sensitivity and specificity of 70%-80% when using gadolinium contrast^[152]; others found more disappointing results^[153].

Histopathology

Grossly, NRH presents as diffuse fine nodularity of the liver with 1-3 mm diameter nodules. Granularity of the hepatic surface may resemble micronodular cirrhosis^[77,144]. Rarely, nodules are larger^[7,144], and may coalesce into a large tumor^[154,155]. NRH nodules appear paler than the surrounding normal hepatic tissue. Mild hepatomegaly may be present.

The diagnosis of NRH is secured by histopathology demonstrating regenerative nodules without parietal thick-

ening of portal venules, and no or minimal perisinusoidal and portal fibrosis on reticulin staining. Hepatocytes commonly show feathery degeneration of cytoplasm suggesting impairments of bile production or transport. Two morphologically distinct populations of hepatocytes coexist within the nodules: hypertrophied hepatocytes centrally surrounded by atrophic hepatocytes peripherally. Hypertrophic cells may compress terminal hepatic venules, which frequently appear shrunken and may be undetectable. Heterogeneity may be explained by uneven perfusion. Dilated sinusoids and thrombosed portal vein radicles are occasionally present^[24,156,157].

If a dominant large regenerative nodule is sampled, adenoma-like features with discrete cytoplasmic and/or nuclear atypia can be seen. Therefore, sampling a single nodule may yield an incorrect diagnosis and multiple liver sampling becomes necessary. Dysplastic large hepatocytes were seen in 42% of NRH samples, without high-degree dysplasia^[9]. NRH should also be distinguished from LRN occurring in livers with disturbed hepatic venous outflow (e.g. Budd-Chiari syndrome, veno occlusive disease, or congestive pericarditis) but well developed compensatory arterialization and preserved hepatic venous collaterals.

It should be kept in mind that in small biopsy samples histologic features of NRH may be lacking or incomplete^[158]. The diagnosis can be made after careful examination by the experienced hematopathologist with a high index of suspicion. In justified cases a laparoscopy with open wedge biopsy should be done.

Overlap syndromes, involving both NRH and portal or pericentral fibrosis due to hepatitis C viral infection, alcoholic, or non-alcoholic liver disease, can occur and in such cases the diagnosis of NRH may be easily overlooked^[24,77,156]. Fibroscan or fibrotest panels may rule out cirrhosis, but have limited clinical value^[153].

TREATMENT

Treatment of NRH therapy is directed towards elimination of the causative factor, once established. Concomitant diseases should be treated appropriately and with attention to minimizing drug toxicity. Long-term anticoagulation treatment is usually indicated in the thrombophilias. Anticoagulation therapy was found to be beneficial in early stages of NRH induced by HAART in HIV-infected patients^[57].

In patients with NRH, the mainstay of management is directed primarily to prevention and treatment of complications related to portal hypertension, i.e. variceal bleeding, the main source of mortality. Treatment of portal hypertension is standard: low sodium diet, diuretics, and endoscopic banding of esophageal varices. Splenectomy is not indicated. A portosystemic surgical shunt or transjugular intrahepatic portosystemic shunt may offer a significant therapeutic benefit, especially in the case of severe recurrent esophageal variceal hemorrhage^[56]. Liver transplantation is rarely necessary and is reserved for patients with hepatic failure^[159-162].

NATURAL HISTORY AND PROGNOSIS

Generally, the prognosis of NRH is better than that of chronic liver disease and is related to the complications of portal hypertension and the severity of the associated diseases, if present. In most cases the disease is slowly progressive, although the rate of nodular growth may be accelerated for unknown reasons^[163]. The long-term prognosis is uncertain and considers the level of underlying myeloproliferative, thrombophilic, or autoimmune processes. There are several case reports demonstrating the presence of both NRH and hepatocellular carcinoma without underlying cirrhosis, although a neoplastic process has yet to be proven^[163-167].

CONCLUSION

NRH is a rare condition of NCIPH liver diseases. Our understanding of the epidemiology of NRH is based upon case reports rather than systematic population studies. Timely diagnosis is challenging, because the majority of patients do not present with overt signs of portal hypertension. The pathogenesis of NRH is not well established, but an adaptive hyperplastic reaction of hepatocytes appears to be related to abnormalities of hepatic venous perfusion. Immunosuppressive medications may induce NRH by damaging endothelial cells of small hepatic veins. NRH may also develop as a result of underlying autoimmune, inflammatory, neoplastic, or idiopathic disease.

The knowledge on tumorigenesis in NRH is virtually non-existent as compared with hepatocellular carcinoma, adenoma and even cirrhotic regeneration. Genetic studies indicate that *RASSF1A*, a gene acting in the proapoptotic pathway, is increasingly methylated in hepatocellular hyperplastic hepatocytes^[168]. Moreover, in ceramide synthase 2 null mice that are unable to synthesize very long acyl chain ceramides, an extensive hepatocellular anisocytosis with widespread formation of regenerative nodules composed of hyperplastic hepatocytes was found^[169]. This finding emphasizes the role of ceramide synthase 2 activity and altered hepatic sphingolipid profile for liver proliferative homeostasis.

Common causes of extrahepatic and intrahepatic portal hypertension should be excluded before making a diagnosis of NRH. Imaging may be helpful during the initial clinical encounter with liver biopsy as the gold standard for diagnosis. An accurate diagnosis can only be made on histopathology, which shows diffuse micronodular transformation without fibrous septa. Management is directed primarily for prevention and treatment of complications related to portal hypertension. Outcome and prognosis is related to the severity of both portal hypertensive complications and the underlying associated diseases. Further studies may be helpful to elucidate a molecular mechanism for the vasculopathy that appears to play a central role in the adaptive hyperplastic reaction universally seen in NRH.

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Yusuf Bayraktar, MD, Series Editor

Portal ductopathy: Clinical importance and nomenclature

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Abstract

Non-cirrhotic portal hypertension (PHT) accounts for about 20% of all PHT cases, portal vein thrombosis (PVT) resulting in cavernous transformation being the most common cause. All known complications of PHT may be encountered in patients with chronic PVT. However, the effect of this entity on the biliary tree and pancreatic duct has not yet been fully established. Additionally, a dispute remains regarding the nomenclature of common bile duct abnormalities which occur as a result of chronic PVT. Although many clinical reports have focused on biliary abnormalities, only a few have evaluated both the biliary and pancreatic ductal systems. In this review the relevant literature evaluating the effect of PVT on both ductal systems is discussed, and findings are considered with reference to results of a prominent center in Turkey, from which the term "portal ductopathy" has been put forth to replace "portal biliopathy".

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Key words: Portal hypertension; Portal vein thrombosis; Portal vein cavernous transformation; Congenital hepatic fibrosis; Non-cirrhotic portal hypertension; Portal ductopathy; Portal double ductopathy; Portal biliopathy

INTRODUCTION

Although liver cirrhosis is a major cause of portal hypertension (PHT), in 20% of cases PHT is classified as non-cirrhotic, occurring as a result of portal vein thrombosis (PVT), congenital hepatic fibrosis, idiopathic PHT and other rare disorders. The portal vein, which is 12 mm in diameter, carries blood from intra-abdominal organs to the liver at a rate of approximately 1200 mL/min. Thrombotic occlusion of the portal vein, whatever the cause, is rapidly followed by compensatory mechanisms such as attempts at re-canalization and the development of new collaterals around the occluded portal vein, bile ducts and gall bladder, aimed at reestablishing portal blood flow to the liver. The portal vein is eventually replaced by a "cavernoma" after what is now known as portal vein cavernous transformation (PVCT). Splenomegaly, esophageal and gastric varices, portal gastropathy, and rarely ascites, are well recognized and extensively studied complications of PHT due to PVCT. However, the effects of PVCT on the biliary tree and pancreatic duct are as yet to be unequivocally identified. Furthermore, a dispute remains regarding the nomenclature of common bile duct (CBD) abnormalities which occur as a result of PVCT. Till today, many of the published case series have described biliary abnormalities resulting from PVT, but only a few have focused on both duct systems, the biliary and pancreatic^[1,2].

In a prospective study published in 1992, abnormalities of the biliary tree in patients with PVCT which resulted in an appearance mimicking cholangiocellular carcinoma on endoscopic retrograde cholangiopancreatography (ERCP) were described^[3]. Meanwhile the descriptive terms “pseudosclerosing cholangitis”^[4] and “portal biliopathy” have also been introduced into the literature^[5]. Till today, the issue of proper nomenclature of this phenomenon has not been sufficiently discussed, and there is a dire need for clarification.

DEFINITION AND NOMENCLATURE

Since the introduction of the term “pseudocholangiocellular carcinoma sign” to describe radiologic abnormalities mimicking cholangiocarcinoma caused by the compression of bile ducts by the thrombosed portal vein and its collaterals^[3], several different terms have been coined, including “portal biliopathy”^[6], “cholangiopathy associated with portal hypertension”^[7], and “portal cavernoma-associated cholangiopathy”^[8]. Finally, Dhiman *et al*^[9] proposed the term “portal hypertensive biliopathy” to refer to abnormalities of the biliary tree, cystic duct and gall bladder in patients with PHT.

It would seem that “pseudo sclerosing cholangitis” and “portal biliopathy” do not appropriately represent or define abnormalities of the biliary system which occur as a result of PHT. Biliary strictures in patient with PVCT are smooth rather than irregular, making the term “pseudosclerosing cholangitis” an erroneous description^[4]. “Portal biliopathy” is also a misnomer as it implies abnormal content of bile, which has never been reported before in any of the studies describing abnormalities of the biliary tree. Although in cases of PVCT jaundice is a common clinical finding, bile composition is considered to be normal. Additionally, the term “biliopathy,” suggests that the pathology is limited to the biliary tree, whereas PVCT has been shown to also affect the pancreatic ducts in most patients. Moreover, ERCP findings of cholangiocarcinomas rarely resemble those associated with PVCT, which also renders the term “pseudo-cholangiocarcinoma sign” inadequate.

The pancreatic ducts of patients with PVCT have been thoroughly evaluated at Hacettepe University for the past two decades (since 1992), where 78 patients with PVCT have undergone ERCP procedures. Seventy of the 78 (90%) patients had involvement of the biliary tree, 54 of whom (70%) had both biliary and pancreatic duct involvement. Considering that PVCT results in “morphological” abnormalities in both ductal systems, it would be expected the nomenclature should reflect the ductal changes observed in these patients instead of misleading physicians to associate this entity with changes in biliary content. The term “portal ductopathy” may provide a more satisfactory means of depicting abnormalities seen in the biliary and pancreatic duct systems in patients with PVT.

PATHOGENESIS OF PORTAL DUCTOPATHY

PVT was first described by Balfour *et al*^[10] in 1964. The re-canalization of the thrombosed portal vein at the hepatic hilum leads to this clinical and radiological condition. Ohnishi *et al*^[11] demonstrated that after complete obstruction, the “venous rescue” begins immediately and is completed in about 5 wk. The newly formed small collaterals are mostly observed around the intrahepatic and extrahepatic biliary tract, cystic duct and around the gall bladder. There are two venous plexuses of the bile ducts and gall bladder; the so-called epicholedochal venous plexus of Saint^[12], and the paracholedochal veins of Petren^[13]. Saint’s plexus, which forms a fine reticular web located on the outer surface of the CBD and hepatic ducts, becomes dilated and causes fine irregularities in the biliary tract^[12-14]. Petren’s plexus, on the other hand, runs parallel to the CBD and is connected to the gastric, pancreaticoduodenal and portal veins and to the liver directly. When the portal vein is occluded by a thrombus or tumor, both plexuses become dilated and cause extrinsic compression of the CBD. External compression and protrusion of these newly formed vessels on the biliary tree have been shown to be responsible for portal ductopathy in the biliary tree. However, the reasons behind changes to the pancreatic duct are yet to be elucidated. Extension of newly formed vessels towards the pancreas may be implicated, although this remains largely speculative.

In spite of the well established role of newly developed vessels around the bile system in the development of biliary abnormalities (more appropriately called portal ductopathy), most studies have overlooked other important factors. Ischemia, fibrosis, direct compression of the thrombosed vessels and excessive connective tissue formation around the biliary system have a major impact on the formation of biliary abnormalities. These factors contribute to the formation of a “frozen portal hilum” so that even if the PHT is relieved by any effective means, the cholestasis usually does not improve^[15-17]. The entire process mimics the reaction of wound healing in which neovascularization, collagen formation and tissue turnover occur and re-cycle for a long period of time. Duration of PVT does not seem to have an effect on the extent of the radiological appearance of the ductal abnormalities. Biliary strictures leading to complete biliary obstruction may be caused by ischemia or by encasement within a solid tumor-like cavernoma^[18]. The mechanism of ischemia causing bile duct changes in patients with PVCT remains unexplained. Venous damage due to portal thrombosis results in ischemic necrosis of bile ducts by compressing the vascular supply at the level of capillaries and the arterioles^[9], resulting in biliary strictures and cholangiectasis^[19]. Segmental strictures and dilatations seen on ERCP may involve both intra- and extra-hepatic bile ducts, morphologically very similar to those seen in ischemic cholangiopathy after liver transplantation^[20].

In a prospective study^[21], the biliary tree, either intra- or extra-hepatic, was found to be affected in almost all patients with known PVCT. Additionally, pancreatic duct abnormalities were apparent in a large proportion of this patient group. Use of the term “portal double ductopathy” was suggested to describe involvement of both systems. Involvement of any of the ductal systems individually would be referred to as either “portal biliary ductopathy” or “portal pancreatic ductopathy”.

CLINICAL IMPLICATIONS OF PARTIAL BILIARY DUCTOPATHY

It is well known that PHT, whatever its etiology, results in many complications such as ascites, portal gastropathy, esophageal and gastric varices, hypersplenism and severe coagulopathy, which pose a great challenge for clinicians in daily medical practice. Although PVT has been associated with many biliary abnormalities, the majority of cases are asymptomatic and only a small percentage of this patient population develop signs and symptoms of biliary obstruction, presenting with jaundice, pruritus, fever and abdominal pain.

Cholestasis, one of the main clinical features of portal biliary ductopathy, may be explained by the mass effect of enlarged collaterals or chronic thrombus compressing on the intra- and/or extra-hepatic biliary lumen. Ensuing ischemia and fibrosis may also be implicated. In some cases compression may be so severe as to result in secondary biliary cirrhosis due to longstanding severe cholestasis. Fortunately this complication is rare; one which at Hacettepe University has been encountered in only 2 cases, both of whom eventually underwent liver transplantation. Both patients are still under follow-up and are healthy, productive members of society. Mild jaundice seen in these cases because of incomplete obstruction of CBD is not uncommon, usually leading to unnecessary investigative tests towards the cause of the direct hyperbilirubinemia. Cholestatic enzymes are generally elevated in parallel with bilirubin levels.

PVCT has been reported to result in an increase in the frequency of biliary stone disease and related complications. Regardless of age, sex and underlying etiology, an association between PVCT and an increased incidence of biliary tree diseases has consistently been reported in the literature^[4,6,7,15,22]. In such cases, direct bilirubin levels are quite elevated. Stone formation is facilitated by the chronic but incomplete obstruction caused by the above-mentioned factors. It is necessary to stress that incomplete obstruction at multiple levels of the intra- and extra-hepatic biliary system may exist simultaneously. The occurrence of fever and abdominal pain during follow-up of a patient with biliary stones associated with PVCT should raise a suspicion of cholangitis.

According to Dhiman *et al*^[9], choledocal varices were observed in 7.5% of cases with PVCT. It is important to note that these varices may bleed severely, thus complicating the clinical picture. Additionally, endoscopic pro-

cedures such as stenting and stone extraction may also result in bleeding from these otherwise silent varices^[23-25]. Endoscopic ultrasonography (EUS) with Doppler is a particularly useful technique in diagnosing bile duct varices and differentiating them from bile duct stones. Great care should be taken when undertaking interventional procedures such as stone extraction and sphincterotomy, as even gentle balloon dilatation may lead to bleeding from these small varices. Of note, such patients may already have thrombocytopenia and some degree of coagulopathy because of splenomegaly and tissue congestion caused by PHT. Liver function tests are typically normal in patients with PVT in the absence of an underlying disorder such as polycythemia vera or Behçet's disease. However, according to unpublished data at Hacettepe University, most patients have prolonged prothrombin times compared to healthy controls, without having any other signs of compromised liver function, an entity which as yet remains unexplained.

CLINICAL IMPLICATIONS OF PARTIAL PANCREATIC DUCTOPATHY

Chronic congestion due to PHT affects almost all intra-abdominal organs, including the pancreas. The effects of PVCT on pancreatic parenchyma and duct have not been fully established. In the only study to investigate pancreatic exocrine function in this patient group, Egesel *et al*^[21] demonstrated that the pancreatic ducts of PVCT patients tended to be smaller than normal controls. Additionally, in 15 of the 18 patients with chronic PVT who had pancreatic atrophy, they found that urinary excretion of para-aminobenzoic acid was significantly less than in control subjects. Moreover, the authors have attributed some uncertain symptoms in these patients, such as abdominal discomfort, abdominal pain and anorexia, to latent pancreatic insufficiency shown by bentiromide test. As the pancreas has a tremendous capacity to manipulate the body needs, the manifestation of symptoms occurs after a latent period, requiring significant pancreatic parenchymal pathology. More sensitive tests are needed to clarify the extent of exocrine and endocrine dysfunction of the pancreas associated with this disorder.

Three other studies have demonstrated significant changes in the pancreatic ducts of patients with PVCT^[1,2,21]. In a report published in 2008^[1], 31 of 36 (86.1%) patients with PVT had luminal narrowing throughout the pancreatic duct, local atrophy at the head of pancreas with moderate dilatation behind the narrowed segment and other unclassified pancreatic duct abnormalities. Since 2008, 22 more patients with PVCT due to portal thrombosis have been evaluated at Hacettepe University, 16 of whom (72%) had pancreatic duct abnormalities demonstrated by ERCP. In total, 78 patients with PVCT have been seen since 1992, all of whom underwent ERCP as part of a work-up for unexplained elevations in ALP, GGT and direct bilirubin levels. Approximately 70% of patients had pancreatic abnormalities. Although the clinical sig-

nificance of these ductal abnormalities has not been well delineated, as stated above, partial pancreatic insufficiency and some other patient complaints such as abdominal pain and dyspepsia may be explained by chronic congestion due to PHT. It is possible that PVT contributes to more severe pancreatic congestion when compared to cirrhotic causes of PHT, as extension of the thrombus to the splenic vein may hinder pancreatic venous drainage. As a result, pancreatic duct and parenchyma may be more significantly affected. Further studies are needed to investigate this phenomenon.

DIAGNOSIS OF PORTAL DUCTOPATHY

Biochemical tests

Characteristically, the majority of patients with PVCT have a predominant cholestatic pattern of elevated liver enzymes. This biochemical finding reflects the biliary duct changes secondary to PVCT. Despite the presence of PHT manifesting as massive splenomegaly and large esophageal varices, serum albumin levels are usually within normal limits, unless massive bleeding occurs. Mild ALP and GGT elevations occur at any one time during the follow-up period of such patients. Clinicians must bear in mind that portal ductopathy may be responsible for such mild elevations, in order to avoid further unnecessary testing towards a cause of the cholestatic picture. In the presence of biliary strictures or stones, more marked elevations in cholestatic enzymes and bilirubin levels may be observed.

Liver biopsy

Although not part of the diagnostic work-up for portal ductopathy, a liver biopsy is essential in establishing whether PVT is due to cirrhotic or non-cirrhotic causes. It is necessary to perform this procedure to rule out the presence of liver cirrhosis, particularly in patients with atrophic livers with heterogeneous parenchyma on sonographic examination. Usually liver biopsies show normal or nearly normal histology, sometimes with signs of portal vein dilatation in the portal tract, or segmental luminal narrowing of bile ducts with dilated small intrahepatic bile ducts. In congenital hepatic fibrosis, where histopathological examination is vital for making a diagnosis, portal vein abnormalities mimicking PVCT are relatively more common^[26,27].

Ultrasonography

Ultrasonography has traditionally been the most widely utilized modality for demonstrating biliary duct abnormalities in patients with chronic PVT. However, this technique has many shortcomings. For example, the presence of a high level of echoes in the porta hepatis may obscure the biliary system. Similarly, CBD may be hidden behind multiple collaterals demonstrating themselves as anechoic tubular and fibrotic structures. Color Doppler examination may help confirm the presence of multiple tortuous structures in the porta hepatis of patients with

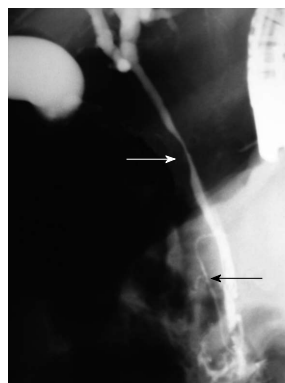


Figure 1 Endoscopic retrograde cholangiopancreatography showing typical portal ductopathy with external compression of the common bile duct (white arrow) leading to almost complete obstruction and dilatation of the intrahepatic bile ducts. The pancreatic duct is atrophic (black arrow).

PVCT. The nature of these tubular structures may not be correctly identified as blood vessels initially on grayscale. Real time and Doppler sonographic findings compatible with PVCT may prompt further evaluation by splenoportography, either with digital subtraction angiography or by computed tomography to confirm the diagnosis.

ERCP

This modality has established itself as one the most important procedures in diagnosing and defining the extent of involvement of the intrahepatic and/or extrahepatic biliary tree. As PVT may occur in either or both intra- and extra-hepatic portions of the portal vein, any part of the biliary system, including the gall bladder, may be affected by this thrombotic event. The development of cavernous changes, located at the portal hilum, may still affect the left and right intrahepatic biliary channels, usually manifesting as dilations. Changes described so far include undulation on the CBD along with narrowing and irregularity of various lengths and degree, sometimes leading to nearly complete obstruction (Figure 1), as well as segmental upstream and asymmetrical dilatation.

In contrast to obstruction of the CBD where both intrahepatic and extrahepatic bile ducts are proportionately dilated above the level of the obstruction, the most consistent radiologic finding that has been encountered at Hacettepe University is that the CBD tended to be narrower than the intrahepatic biliary ducts. In other words, the left or right hepatic ducts were usually dilated either alone or in combination with a dilated common hepatic duct.

Irregularities on the gallbladder wall may be seen, most probably because of intraluminal varices in a few cases. These varices may also be present in the CBD, manifesting themselves as filling defects, although rarely leading to bleeding, or so-called hemobilia.

There are mainly three types of pancreatic duct abnormalities: (1) Diffuse pancreatic duct abnormality in which the whole duct is narrowed with kinking, distortion of the normal anatomic pathway, thumb-printing type

of compression and with local luminal irregularities. The whole pancreatic duct is atrophic (Figure 1); (2) Proximal pancreatic duct abnormality in which the narrow part of the duct is limited to the level of head of pancreas, in contrast to the normal pancreatic duct in which the widest caliber is at the head of pancreas. Interestingly, the distal segment of these ducts appeared relatively dilated compared to the head region; and (3) unclassified abnormalities in which multiple small ducts connect to each other (different from pancreas divisum). Indentation, displacement and angulations occur.

Splenoportography with digital subtraction angiography

Although splenoportography is invasive with several reported complications, we have been utilizing this procedure in our hospital for a long time for the diagnosis of PVCT without the occurrence of any adverse effects. This technique is best for providing a clear image of the PVCT as well as other collaterals; however it does not evaluate the biliary system. Nowadays the use of CT portography as a less invasive imaging modality may be preferred.

Computed tomography with contrast

This procedure which helps to diagnose portal vein obstruction is particularly useful in identifying the presence of cavernous transformation, as well as for evaluating the dimension of bile duct abnormalities. Recently, CT portography has been introduced as an alternative to conventional angiographic splenoportography.

Magnetic resonance cholangiography with magnetic resonance portography

Not only is this technique non-invasive, but it is also more informative in that it allows for clear visualization of portal vein collaterals when confirming the presence of a cavernoma. However, in some cases ERCP is superior with regards to evaluation of the bile duct system. With the advent of high-resolution magnetic resonance (MR), MR cholangiography (MRCP) may eventually replace ERCP as the modality of choice for examining abnormalities of the intra-hepatic bile ducts, as it offers the advantage of being less invasive with fewer associated complications. If available, MRCP with MR portographic evaluation should follow real time or Doppler ultrasonography when investigating the bile duct system and portal vein.

EUS with Doppler

After conventional ultrasonography and Doppler ultrasound, the advent of EUS has been particularly useful in identifying CBD varices and/or bile duct stones, both of which may be the primary cause of biliary obstruction in patients with PVCT^[28]. Recognizing stones and differentiating them from varices is important as any intervention in the form of balloon dilatation or stone extraction may result in severe bleeding. In patients where an obstructive clinical picture is predominant, EUS with Doppler should

be performed to properly identify the cause of the obstruction, whether it is due to bile duct varices, stones, strictures or a tumor. In this perspective, EUS is a quite useful, even inevitable procedure.

TREATMENT

Most patients with portal ductopathy who are asymptomatic do not require any treatment. When symptoms due to stone formation, obstructive jaundice and cholangitis occur, treatment should be adjusted individually according to patient characteristics. Naturally, the occurrence of PVT warrants investigation into the cause, whether there is an underlying myeloproliferative disorder, deficiency of anticoagulant proteins, or an autoimmune disease. In the presence of an underlying thrombophilic condition, anticoagulant treatment may be indicated. On a different note, a proportion of patients may first present with variceal bleeding from the upper GI tract. Although the management of variceal bleeding is beyond the scope of this paper, it is important to stress that bleeding from gastric and esophageal varices may prove very challenging.

As mentioned before, PVCT results in a pathological condition involving ductular organs; CBD and pancreatic duct. Since bile composition is not an issue, all efforts should instead focus on the management of strictures, stones or sludge in the biliary tree. In cases such as these, endoscopic papillotomy and, if indicated, stone extraction and balloon dilatation, are the treatment modalities of choice. In some cases, concomitant presence of severe biliary stricture and biliary stones may be observed^[14]. This poses a challenge for the endoscopist, since after performing a sphincterotomy followed by stricture dilatation, great care should be taken while extracting any stone, since underlying thrombocytopenia due to hypersplenism and the presence of small or large varices in the peri-ampullary area and inside the CBD result in the risk of severe bleeding, further complicating an already complex and delicate clinical condition. In fact, some cases may even require the use of a mechanical lithotripter to crush large stones into small pieces.

Liver transplantation should be reserved for patients who develop secondary biliary cirrhosis or severe liver failure. At Hacettepe University, only 2 female patients developed secondary biliary cirrhosis due to biliary strictures and stone formation, both of whom underwent successful liver transplantation. To date, both patients are under follow-up with no significant restrictions in their daily activities.

CONCLUSION

Several disorders result in thrombosis of the portal vein which eventually undergoes cavernous transformation. The intra- and extra-hepatic bile ducts are affected by these changes in almost all cases, with relatively less frequent involvement of the pancreatic duct. Although sev-

eral terms such as "portal biliopathy" and "pseudocholangiocarcinoma sign" have been postulated to describe these changes, to allay any doubts regarding abnormalities in bile composition, use of the term "portal ductopathy" may be more appropriate. Involvement of both duct systems may be further described as "portal double ductopathy". Clinical implications of portal ductopathy consist of cholestasis, stone formation, and consequently cholangitis.

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Crohn's disease: Evidence for involvement of unregulated transcytosis in disease etio-pathogenesis

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Abstract

Crohn's disease (CD) is a chronic inflammatory bowel disease. Research has identified genetic predisposition and environmental factors as key elements in the development of the disease. However, the precise mechanism that initiates immune activation remains undefined. One pathway for luminal antigenic molecules to enter the sterile lamina propria and activate an immune response is *via* transcytosis. Transcytosis, although tightly regulated by the cell, has the potential for transepithelial transport of bacteria and highly antigenic luminal molecules whose uncontrolled translocation into the lamina propria can be the source of immune activation. Viewed as a whole, the evidence suggests that unregulated intestinal epithelial transcytosis is involved in the inappropriate presentation of immunogenic luminal macromolecules to the intestinal lamina propria. Thus fulfilling the role of an early pre-morbid mechanism that can result in antigenic overload of the lamina propria and initiate an immune response culminating in chronic inflammation characteristic of this disease. It is the aim of this paper to present evidence implicating enterocyte transcytosis in the early etio-pathogenesis of CD.

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Key words: Crohn's; Transcytosis; Endocytosis

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INTRODUCTION

Crohn's disease (CD) is a chronic, lifelong, and unrelenting inflammatory bowel disease that mainly strikes young people in the prime of their productive life^[1]. There are over 600 000 individuals with CD in North America, with up to 40 000 new cases being diagnosed each year, and double that amount at risk, based on a monozygotic concordance rate of 50%^[1-3]. The effects on the patient and their family are devastating, with lifelong medication and repeated abdominal surgeries to relieve obstructed bowel, intestinal bleeding, or abdominal pain. Studies have shown that the age adjusted mortality risk from CD is over 50% greater than in the general population^[2,4].

Current treatment for CD consists of inhibiting the immune response with powerful immunosuppressive agents. These medications have serious side effects and can eventually lose their therapeutic effect^[5]. More importantly, immunosuppressive agents do not alter the natural history of this disease, suggesting that the immune response in these individuals is a secondary reaction to an, as yet

undiscovered, primary cause, which results in chronic immune activation within the intestinal wall^[15,6].

The etiology of CD is currently unknown. It is generally attributed to a faulty immune system; however, despite decades of research, no antecedent immune abnormality has been identified in these individuals. A closer review of pertinent studies reveals that the immediate causal mechanism of CD may have its origin in a process of unregulated transcytosis of intestinal luminal contents by intestinal epithelial cells.

The data presented in this paper suggests that a primary inherited cell membrane defect may be responsible for the initiation and perpetuation of a pathological transcytotic state, allowing bacteria and other intestinal contents to persistently penetrate into the bowel wall and initiate a chronic life-long inflammatory response characteristic of this disease.

The aim of this paper, therefore, is to present evidence that unregulated intestinal transcytosis may have a significant role in the initiation of CD, and to provide data implicating an antecedent cell membrane abnormality as a primary inheritable factor capable of initiating this pathologically altered transcytotic state.

WHAT IS TRANSCYTOSIS?

Transcytosis is the transport of macromolecules (cargo) from one side of a cell to the other within a vesicular cell membrane-bound carrier. Conceptually, transcytosis can be divided into three distinct processes; absorption of molecules (endocytosis), conveyance of cargo through the cell (transcellular transport), and extrusion of cargo at the other side of the cell (exocytosis). During endocytosis, extracellular macromolecules converge upon the cell as a portion of the plasma membrane is invaginated at the point of contact and pinched off forming a cytoplasmic membrane-bounded vesicle called an endosome, which contains the engulfed cargo. Material packaged within the endosome may undergo processing by the cell, after which the cytoplasmic vesicle can move to the opposite side and fuse with the plasma membrane, releasing its contents to the extracellular space during the process of exocytosis (Figure 1).

Cytoplasmic material can also be extruded from the cell, forming plasma membrane bound extracellular vesicles called exosomes, in a similar, but reverse process, to endocytosis^[7,8].

Endocytosis is required for a vast amount of cellular functions that are paramount to the survival and wellbeing of the cell. Internalizing thousands of molecules, and up to five times its surface area per minute, the cell membrane is a seething cauldron of continuous endocytotic invaginations involved in the non-stop absorption of nutrients and water, in addition to cell surface receptors, antigens, cell signaling molecules, protein homeostasis, and maintenance of plasma membrane lipids^[9-14].

While inside the cell, the cargo is enclosed in a vesicle formed by the invagination of membrane lipids, called an

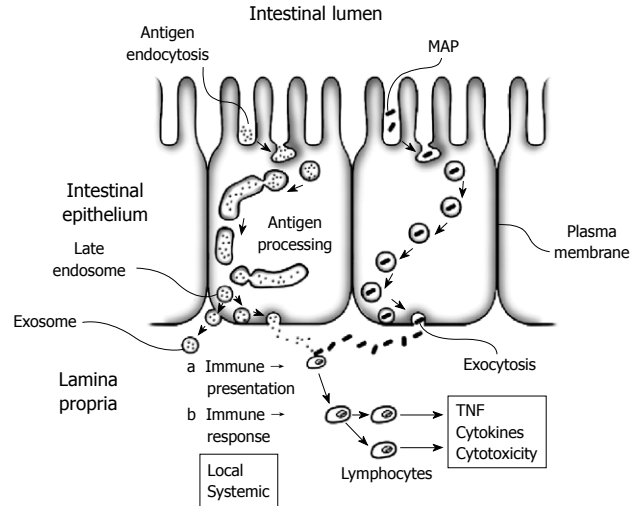


Figure 1 Antigenic exposure (a) of luminal antigens to the immune system can occur through either transcellular (through cells) or paracellular (between cells) pathways. Transcellular transport is mediated via transcytosis of processed antigen within discrete intracellular vesicles, termed endosomes, which are transported from apical to basal membrane prior to undergoing exocytosis, where immune recognition may occur within the lamina propria. Bacteria and macromolecules are transported in this fashion as they are too large to penetrate the tight intercellular space (paracellular pathway) maintained by tight junctional proteins. An immune response (b) can ensue subsequent to antigenic penetration into the lamina propria. Continuous unregulated transcytosis of luminal antigens and *Mycobacterium avium paratuberculosis* (MAP) into the lamina propria can initiate a chronic immune reaction. Inhibition of immune response (b) with immunosuppressive agents may temporarily restore epithelial integrity, but will not prevent transcytosis of luminal antigen, which continues upon discontinuation of immunosuppressive therapy. Mitigation of luminal antigenic exposure via restrictive diets or parenteral therapy cannot prevent subsequent transcytosis and relapse once normal dietary activity is resumed. A three pronged approach for acute therapy consisting of: (1) reduction of antigenic exposure via temporary dietary restriction; (2) temporary immunosuppression to inhibit immune mediated tissue damage and speed epithelial healing; and (3) Inhibition of transcytosis via endocytosis blocking agents to prevent future immune activation, may offer a therapeutic paradigm on which to base clinical decision making. Anti-MAP antibiotics may additionally be utilized to speed systemic removal of this bacterium and hasten downregulation of the immune response. Limited evidence suggests that inhibition of transcytosis may reduce intestinal antigenic exposure sufficiently to allow long term dietary activity with minimal restrictions. Maintenance therapy with endocytosis inhibiting agents may provide a safe and effective means of inducing long-term remission and interrupting the natural history of Crohn's disease. TNF: Tumor necrosis factor.

endosome. The endosome can be destined for transport to areas within the cell or undergo transcellular transport to the opposite side, where fusion with the cell membrane results in exocytosis and deposition of the cargo (in the intestinal epithelium) into the lamina propria^[15-17].

A multitasking membrane machine, the intestinal epithelium simultaneously integrates the transcytosis and processing of hundreds of distinct nutrient molecules into the lamina propria, while providing a single cell thickness physical barrier to luminal antigens that maintains sub-epithelial sterility, and does so while serving as a non-stop conveyor belt for the continual migration of new enterocytes to the surface epithelium.

Despite its highly dynamic physical state, the intestinal epithelium is very efficient at preventing translocation of luminal antigens into the lamina propria. This efficiency

however notwithstanding, the translocation of intact proteins through the intestinal epithelium has been demonstrated and cell mechanisms exist for the endocytosis of entire bacteria. At least ten mechanistically distinct endocytotic mechanisms have been described, from phagocytosis and macropinocytosis, employed for the absorption of cargo over 500 nm, to clathrin coated pits and caveoli mediated endocytosis for smaller particulate macromolecules^[9,15,18].

Endocytosis is highly dependent on the composition and organization of the cell membrane and experimentally induced changes in membrane properties can alter the cell's ability to appropriately engage extracellular particulate matter leading to alterations in one or more mechanisms of endocytosis^[9,15,19].

This implies that pathological endocytosis of luminal antigenic material (endo cytopathy) may result in transcytotic antigenic overload of the lamina propria, with subsequent immune activation and the establishment of a chronic inflammatory state. An endo cytopathic state would, in theory, present as an increased permeability of the intestinal epithelium prior to the start of inflammation, such as that observed in CD. It is therefore reasonable to speculate that an inherited endo cytopathy may be involved as an early etiopathogenetic mechanism in CD, with the participation of one or more endocytotic mechanisms contributing to the disease process.

The following section examines studies of early lesions of Crohn's epithelium for evidence of endocytopathy.

IS ABERRANT TRANSCYTOSIS INVOLVED IN CD?

Transcytosis is a normal endosomal generating process that is regulated by the cell. Pathologically triggered, unregulated, transcytosis can differ from its normal physiological counterpart by an increased endosomal generation rate; manifesting as an excess amount of cellular endosomes when compared to normal cells. Thus, a defining characteristic of unregulated transcytosis can be an increased cellular endosomal load.

This aspect of cellular function was evaluated by several studies, which documented multiple endosomes in 19 out of 19 cases of CD. No endosomes were observed in six control cases. Nor were they observed in radiation ileitis, celiac disease, experimental Yersinia enteritis, or in all of 155 cases of diagnosed ulcerative colitis^[20]. Normal colonocytes had no visible endosomes.

The cellular location of endosomes in apical, basal, and lateral portions of the cell suggests a process of active transcytosis. Although diminished exocytosis could account for increased endosomes, the presence of concurrent inflammation suggests ongoing deposition of antigenic material into the lamina propria, along with active exocytosis. The absence of endosomes in both negative and active control groups suggests that the endosomal structures observed in all CD study patients are the result

of a pathological transcytotic state, triggered by inappropriate endocytosis.

A more detailed analysis of the role of transcytosis in the pathogenesis of CD was undertaken in a study that examined the effects of autologous infusion of intestinal contents into the excluded ileum of three individuals who had undergone a resection of the terminal ileum with the creation of a temporary loop ileostomy. In this surgical procedure, the two openings from a bisected loop of ileum are brought through the abdominal wall to the surface^[21]. After 3-6 mo of diversion, the patients were infused, *via* a catheter, with 60cc of ileal effluent (collected from the proximal limb) into the distal limb four times daily for 7 d.

Distal ileal biopsies obtained prior to infusion were histologically normal without evidence of inflammation. Biopsies obtained 1 d after the last infusion (day eight) revealed a moderate increase of mononuclear cells, eosinophils, and polymorphonuclear cells in the lamina propria. Neutrophils were also noted in the small vessels and epithelium without cryptitis or crypt abscesses.

Epithelial electron micrographs revealed dilation of rough endoplasmic reticulum (ER) and Golgi apparatus (GA), in addition to basally located transport vesicles (endosomes). Mitochondria appeared damaged and dilated.

These ultrastructural changes are characteristic of biosynthesis [i.e. major histocompatibility complexes (MHC) peptides] and antigen processing during transcytosis prior to presentation of antigen-MHC complexes on the cell surface^[17,22]. Basal transport vesicles suggest late endosomes prior to exocytosis and exposure of antigen within the lamina propria (Figure 1).

Dilated mitochondria suggests sudden elevated metabolic activity, which is consistent with high ATP demand resulting from protein synthesis and the processing of a large influx of antigen by TAP (transporter associated peptide) during intracellular conjugation of processed antigen to MHC molecules. This process is estimated to consume 50 000 molecules of ATP per second/cell in prokaryotes during normal antigen processing, and is likely to consume more in eukaryotic (human) cells that may be undergoing excessive antigenic processing^[17,23-25]. The appearance of damaged mitochondria suggests that excess reactive oxygen species (i.e. superoxide, hydrogen peroxide, and hydroxyl radicals), generated during an acute excessive demand for ATP, had overwhelmed mitochondrial reductive (antioxidant) capacity, with resultant activation of the mitochondrial permeability transition pore leading to the observed structural mitochondrial and epithelial cell damage. The presence of minute collections of lymphocytes within the lamina propria (aphthous lesions), which are not associated with superficial erosions or lymphoid follicles in CD, is compatible with local antigenic presentation to the lamina propria^[26].

The above studies suggest that excessive transcytosis of luminal antigens is an early concomitant in the pathogenesis of CD intestinal inflammation. However, are luminal antigens actually transcytosed in CD and is this

transcytosis pathological and able to account for the initiation of inflammation in CD?

An elegant study designed to evaluate enterocyte transcytosis of luminal antigens in CD was performed on mucosal biopsies taken from ileal mucosa after *in-vivo* incubation with lumenally applied ovalbumin (OVA) in patients undergoing ileoscopy^[27]. The authors found OVA associated with MHC within cytoplasmic late endosomes (the stage prior to exocytosis) and with exosomes in the intercellular space (Figure 1). OVA was also found in the lamina propria. Importantly, OVA cytoplasmic trafficking and antigen processing showed no qualitative differences between CD patients (active disease or histologic remission) and controls.

This study indicated that a luminal antigen can be transcytosed by intestinal epithelial cells and the intracellular antigenic processing in both CD and normal controls is qualitatively similar, regardless of disease state. The absence of a significant demonstrable difference between normal and CD enterocyte antigenic processing suggests that a disorder may exist during the initiation of transcytosis at the level of the cell membrane. This is suggested by studies documenting multiple endosomes in CD enterocytes which were not present in normal controls implying a quantitative difference in antigenic transcytosis rather than a qualitative one^[20].

This interpretation is supported by studies in individuals undergoing small intestinal allograft transplantation as a result of short bowel syndrome subsequent to surgery required to treat CD^[28]. In a series of four CD patients receiving small intestinal allografts, early characteristic CD lesions were documented in two patients, *via* allograft biopsy, at 3 and 5 wk after transplantation. None of the four transplant recipients developed clinical or endoscopic recurrence during the follow-up period of 20-40 mo. No similar histological findings were observed in any of 57 non-CD patients receiving small intestinal allografts at this institution.

The appearance of lamina propria lymphoplasmacytosis and inter-epithelial neutrophil infiltration suggests antigenic presentation by the allograft to the recipient's immune system. This would not appear to be the result of aberrant immune processing by allograft tissue, since allograft donors are carefully screened prior to transplantation, and studies have documented similar enterocyte antigenic processing in both CD and normal controls (above). Likewise, there is no indication of abnormally increased intestinal permeability in allograft donors and no antecedent immune abnormality has been identified in CD to account for this immune reaction in the allografts.

An allograft immune reaction so soon after transplantation suggests a normal constitutive process of antigen presentation to the recipient's immune system and studies have shown the presence of a constitutive apical internalization pathway in enterocytes^[16,27]. This suggests that the allograft immune reaction in CD recipients is due to a heightened immune response to normal intestinal antigenic presentation as a result of normal constitutive en-

docytoysis in CD individuals hypersensitized as a result of previous excessive exposure to intestinal luminal antigens.

Therefore, if normal antigenic transcytosis from a normal transplanted gut can cause characteristic histological CD lesions in allograft recipients, then it is reasonable to speculate that excessive luminal antigenic exposure *via* unregulated transcytosis may play a significant role in early pathogenic events leading to the development of CD.

Why individuals with CD may have unregulated transcytosis is examined in the next section.

WHAT CAUSES UNREGULATED TRANSCYTOSIS?

The cell membrane is the gateway that mediates all interaction with the external environment. This is largely accomplished *via* membrane coated vesicles that constantly bud off from the cell membrane and enter the cytoplasm, while others arrive from the cytoplasm and fuse with the cell membrane (transcytosis). The composition, integrity, and 3-dimensional arrangement of plasma membrane components are crucial to control of the vesicular fission/fusion process, which in turn contributes to maintaining the composition of the plasma membrane as vesicles are recycled back into the cell membrane^[10,15,29].

Luminal antigenic sampling by enterocytes depends upon a controlled transcytotic antigenic presentation to the immune system; therefore, any alteration in the composition, properties, or structure of enterocyte cell membranes can have a significant impact on the immunological functionality of the entire gastrointestinal tract. Too little antigenic presentation and the gut cannot fulfill its role of immune surveillance; too much presentation can lead to a heightened immune response and chronic inflammation^[9].

Studies have shown that alterations in cell membrane properties can have a profound effect on endocytosis and transcytosis. Treatment of cells with the cell membrane intercalating agent phorbol ester can initiate spontaneous endocytosis^[15,30]. Enhanced endocytosis of non-targeted membrane enzymes has been observed during fat absorption, suggesting that this physiological process, which increases cell membrane fatty acid content, disturbs local membrane organization^[16,31]. A change in cell membrane fatty acid composition can modify membrane properties and its interaction with cytosolic proteins involved in endocytosis^[18].

Analysis of synthetic liposome transcytosis across cell membranes revealed that cell membrane fluidity is the most important factor influencing transcytosis, followed by surface charge^[19]. Other significant cell surface properties affecting transcytosis, such as lipid composition and surface density, have been described by other researchers in the field^[19]. These studies indicate that biochemical and biophysical properties of cell membranes are major factors controlling cellular uptake and transcytosis. Combined

with data suggesting unregulated intestinal transcytosis in CD, it is reasonable to consider the possibility of enterocyte cell membrane abnormalities as a contributing factor to unregulated transcytosis in the early pathogenesis of CD.

The next section will explore this aspect.

ARE CELL MEMBRANE ABNORMALITIES PRESENT IN CD?

Significant decreases in cell membrane fluidity, in addition to disturbances in cell membrane lipid composition, were observed in a study conducted on erythrocytes of individuals with active and inactive CD^[32]. A separate study examining the mucosal fatty acid profile in uninvolved (never inflamed) colonic mucosa in individuals with CD demonstrated altered lipid composition compared to healthy controls^[33].

Studies regarding cell membrane properties and lipid composition in CD are rare. Abnormal lipid profiles in uninvolved (never inflamed) colonic mucosa raises the possibility of an antecedent metabolic defect. Erythrocyte cell membrane fluidity abnormalities in CD are consistent with the possibility that membrane abnormalities may be present in other tissues, including the intestinal epithelium, resulting in deleterious effects on enterocyte transcytosis.

Further basic scientific data are necessary to identify potential cell membrane abnormalities and their role in enterocyte transcytosis. However, if unregulated transcytosis is involved in the pathogenesis of CD, then endocytosis blocking agents should have a beneficial therapeutic effect on the clinical parameters and progression of disease.

In the next section we evaluate the therapeutic effect of endocytosis blocking agents upon CD.

DO ENDOCYTOSIS BLOCKING AGENTS REDUCE INFLAMMATION IN CD?

Therapeutic measures available for the treatment of CD can be divided into three general categories; dietary measures, antibiotics, and immunosuppressive agents. No mechanism of action employed by these therapeutic interventions, either singly or in combination, has been proven to modify the natural history of CD. This suggests that an unrecognized pathogenetic mechanism is involved in the development of this disease. Clinical reports of complete remission in refractory CD after administration of non-conventional agents have been documented. One of these agents, thalidomide, a known endocytosis blocker, has been shown to be effective for induction and long term maintenance of remission in both intestinal and extraintestinal CD^[34-44].

Studies have demonstrated a reduction in inflammation and inflammatory parameters in CD using the cholesterol lowering agent atorvastatin^[45,46]. These agents inhibit

the biosynthesis of cholesterol, a critical membrane lipid constituent required for the formation of endosomal vesicles^[29].

The polyene antibiotic Nystatin, which inhibits endocytosis by cholesterol sequestration within cell membranes, has been used in combination therapy to reduce inflammatory activity in CD^[29,47].

Azithromycin, a macrolide antibiotic, was observed to markedly inhibit endocytosis and has also been used in therapeutic regimens to reduce inflammation in CD^[48-50].

Macrolide antibiotics, including azithromycin, are considered among the most effective therapeutic agents for the treatment of CD^[51,52]. The rationale for the use of endocytosis blocking agents in CD is to prevent enterocyte transcytosis of luminal antigens into the lamina propria. A reduction in intestinal transcytotic antigenic load is also consistent with the mucosal healing and decrease in inflammatory cytokines, mucosal permeability, and clinical disease activity observed with the use of specific dietary exclusion measures in the treatment of active CD^[53-56].

To date, no study has evaluated the effect of endocytosis blocking agents in the treatment of CD. The limited amount of data in which therapeutic agents with adjunct endocytotic blocking activity show a beneficial effect in the treatment of CD is consistent with the involvement of transcytosis in the pathogenesis of this disease. Further research is required to determine if specific endocytosis blocking agents are beneficial in the treatment of CD.

Certain questions, however, remain unanswered. For instance, what is the role of *Mycobacterium avium* paratuberculosis (MAP), a bacterium that has been uniquely associated with CD, and what is the genetic nature of the putative membrane abnormality proposed for CD?

A transcytosis mechanism for CD suggests answers to these questions, which are explored in following section.

THE ROLE OF MAP IN CD?

CD is the result of a complex interaction between the body's immune system and environmental (luminal) factors, played out at the gastrointestinal epithelial interface. The data presented in this paper suggests that unregulated transcytosis of luminal antigens plays a significant role in the early pathogenesis of this disease.

The adult mammalian intestinal epithelium is normally very selective regarding the absorption of luminal molecules, with macromolecules being degraded prior to entering the bloodstream. The neonatal intestinal epithelium, however, has a greater capacity for non-selective absorption, and undergoes a gradual change in permeability that restricts the uptake of macromolecules. This process of decline in intestinal permeability to immunologically recognizable molecules is called intestinal closure and, for most species, is complete by the end of the perinatal period 1 to 4 wk after birth^[16,57,58].

The process of intestinal closure is age dependent, and is accompanied by developmental changes and re-

modeling in membrane phospholipids, which become a potential regulator of intestinal transport^[57]. Thus, alterations in the composition of cell membrane lipids can alter membrane properties, such as hydrophobicity, molecular structure, and fluidity. These alterations can lead to dysfunction of cell membrane dependent processes, such as intestinal epithelial transcytosis resulting in enhanced uptake of immunologically active molecules or luminal organisms.

One organism that stands out regarding its association with CD is *Mycobacterium avium* paratuberculosis (MAP). Since its initial isolation from CD patients in 1984, detection studies have shown that up to 95% of CD patients harbor this bacterium^[59-61]. The association with CD is unique to MAP, and has not been described for other species of mycobacteria.

MAP is ubiquitously present in the environment, the water supply, and the human food chain^[60,62,63]. It infects many wild and domesticated animals, including up to 68% of milk producing dairy herds in any geographical area. Infected animals can develop chronic diarrhea and wasting called Johne's disease^[52,64]. Although MAP can be acquired from drinking water, contaminated vegetables, and aerosol droplets, the principal reservoir of MAP for transmission to humans is the intestinal tract of infected cattle, which serves as a distribution point for dissemination of MAP into dairy products such as milk, cheese, and other dairy by-products^[60,63,65].

Studies suggest that, once in the GI tract, the interaction of MAP with CD patients is specific and not the result of a random generic process^[66-69]. In other words, once contact is made, unique characteristics inherent in both Crohn's intestinal epithelium and the MAP cell wall favor continued adherence and transcytosis into the lamina propria, leading to an immune reaction.

Exposure of normal human, 12 wk old, fetal intestinal epithelia to MAP revealed almost no internalization of MAP by enterocytes, with uptake limited to goblet cells^[70]. This is consistent with resistance of normal human intestinal epithelium to MAP transcytosis, with a transitory gestational permeability effect on goblet cells, since oral Crohn's lesions can appear on stratified squamous gingival mucosa, which is devoid of both dendritic and goblet cells. Goblet cells have not been observed to serve as unique foci of inflammation in early Crohn's lesions^[21,71-76]. This suggests that specific inherited cell membrane abnormalities in Crohn's intestinal epithelium interact with unique MAP cell wall constituents that are not present in other mycobacterial species, conferring upon MAP the role of a transcytosis triggering agent when in contact with Crohn's intestinal epithelium. Once processed within intestinal epithelial cells, luminal derived antigens are deposited within the lamina propria, which evokes an immune response characteristic of CD. This implies a predisposing disease genotype whose phenotypic expression is associated with cell membrane composition; a consideration that is further developed below.

WHAT ARE THESE DISTINCTIVE CELL WALL COMPONENTS AND WHAT CELL WALL PROPERTIES MIGHT ENHANCE MAP TRANSCYTOSIS BY CROHN'S INTESTINAL EPITHELIUM?

MAP is unique in having a cell wall that differs significantly from that of other bacteria^[77,78]. The cell wall is composed of a thick layer of extremely hydrophobic lipid molecules. Long chain α branched lipids (mycolic acids) and species-specific glycopeptidolipids contribute to the extreme hydrophobicity of this organism^[77,78].

MAP is also highly negatively charged, and studies have demonstrated a greater attachment of MAP to like-charged particles compared to similarly charged bacteria because of the extreme hydrophobic nature of the MAP cell wall^[77]. Additional studies have demonstrated that MAP is highly predisposed to surface adherence, being the primary colonizers on a variety of external surfaces, the degree of which varies with the characteristics of the surface material being colonized^[79].

Thus, the combination of uniquely strong hydrophobicity and high electronegative charge present on the MAP outer surface can aid in its adherence to a genetically altered Crohn's intestinal epithelium, facilitating its transcytosis and ultimate dispersal from a negatively charged basal surface epithelium into the lamina propria, where an immune reaction can ensue^[80]. Studies have shown that cell surface hydrophobicity may play an important role in the rate of internalization of bacteria^[81].

Consequently, MAP can be considered an environmental response modifier that interacts with the biological expression (phenotype) of certain susceptibility genes, whose genotype contributes to the composition of cell membranes^[82-84]. To be consistent with this interpretation, alterations in cell membrane function should elicit a Crohn's inflammatory phenotype. This has been observed with epidemiological studies linking the ingestion of aspirin, a nonsteroidal anti-inflammatory drug (NSAID) with increased risk of developing CD^[85]. Aspirin, which is strongly lipophilic, binds to, and accumulates within, cell membranes altering their microviscosity, molecular structure, physical state, and biological function^[86,87]. Aspirin, and other NSAIDs, have also been demonstrated to induce disorders of cell membrane lipid assembly, as well as rearrangements in membrane protein patterns^[88,89].

NSAID-induced enteropathy is reported to have close similarities to CD and is not infrequently reported by the pathologist as consistent with CD^[90,91]. NSAID-induced alterations in model cell membranes provoke membrane fusion, suggesting the possibility that pre-existing cell membrane anomalies may also contribute to the high occurrence of intestinal fistulas in CD^[92]. NSAIDs increase the risk of developing *de-novo* CD, induce an enteropathy that can be histologically indistinguishable from CD, and alter cell membrane properties known to be involved in

transcytosis (fluidity, viscosity, composition). It is reasonable, therefore, to speculate that the genetic predisposition in CD is an inherited membrane abnormality that gives rise to unregulated enterocyte transcytosis, which ultimately leads to chronic intestinal inflammation.

IF A CELL MEMBRANE ABNORMALITY DOES EXIST IN CD; IS THERE ANY EVIDENCE THAT MIGHT PROVIDE SOME IDENTIFYING CHARACTERISTICS FOR A CANDIDATE GENE?

It has been known for some time that smoking increases the risk of developing CD, and will worsen existing disease^[93,94]. A clue to a potential underlying mechanism is the clinical observation of significant disease worsening above a threshold of 10-15 cigarettes per day^[93,95]. A phenotypic threshold effect is characteristic of mitochondrial involvement in disease pathogenesis^[96,97].

Smoking has been documented to cause considerable mitochondrial dysfunction, with up to 80% inhibition of electron transport chain activity and significant decreases in ATP production and availability^[98-105]. Conversely, smoking cessation is associated with both normalization of mitochondrial function and clinical improvement in CD activity, suggesting a cause and effect relationship between mitochondrial bioenergetics and CD severity^[93,95,106]. This unveils the possibility that an energy (ATP)-requiring enzyme participating in a cell membrane lipid biosynthetic pathway may be involved in CD pathogenesis.

A candidate enzyme fulfilling these criteria is long chain acyl Co-A fatty acid synthetase isoform 6 (ACSL6, E.C.6.2.1.3). The gene for this enzyme is located on 5q31, which has been designated as IBD susceptibility locus 5 (IBD5)^[107-109].

These isoenzymes, located on the peroxisomal surface membrane, have a crucial role in plasma membrane phospholipid turnover, *de novo* lipid biosynthesis, fatty acid catabolism, and remodeling of biological membranes. They also use ATP to convert fatty acids into an activated form that can be incorporated into cell membranes^[110-116].

Studies have shown a decrease in peroxisomal frequency in Crohn's mucosal biopsies^[117]. This suggests a mechanism in which a genetically dysfunctional or depleted ACSL6 enzyme may be further compromised by smoking-induced depletion of ATP, leading to cell membrane abnormalities that can enhance transcytosis of luminal antigens, resulting in development or worsening of the disease.

Smoking, however, does not affect all individuals with CD^[93]. This suggests the involvement of other, non-ATP-requiring enzymes involved in cell membrane biogenesis, such as LPCAT2 (Lysophosphatidylcholine acyltransferase2, E.C. 2.3.1.67)^[118]. The gene for this enzyme is located on 16q12, which has been designated IBD susceptibility locus 1 (IBD1); a locus that also contains the NOD2/CARD15 gene, which has been linked to CD.

LPCAT2 is involved in the biosynthesis of membrane lipids, suggesting the possibility of membrane abnormalities as a result of a compromised LPCAT2 enzyme, that may disrupt cell membrane properties regulating transcytosis leading to enhanced antigen deposition in the intestinal lamina propria and a chronic immune reaction^[119].

Finally, studies demonstrating that up to 50% of healthy individuals harbor MAP in their blood suggest a spectrum of intestinal mucosal affinity for MAP, in addition to variations in MAP exposure and interindividual immune response, as determining factors contributing to disease development and severity (Figure 1)^[120,121].

CONCLUSION

Evidence presented in this paper provides a reasonable basis for the premise that unregulated transcytosis may be fundamentally involved in the development and early pathogenesis of CD. Unregulated transcytosis is compatible with the repeated clinical observation that mucosal healing does not alter the fundamental disorder present within the intestinal epithelium, leading to disease relapse upon discontinuation of treatment^[5,122]. Present at birth, a genetic predisposition towards unregulated transcytosis (transcellular defect) can increase local concentrations of inflammatory cytokines that are known to trigger tight junctional barrier (paracellular) defects, which have been reported in CD patients and healthy first-degree relatives^[123]. The presence of MAP in a significant percentage of mucosal biopsies and blood of children with CD is consistent with an inherited congenital mucosal anomaly favoring mucosal adherence and transcytosis of MAP^[124].

Within this pathogenetic chronology, the immune-mediated paracellular permeability defect present in CD arises as a consequence of a primary inherited pathological transcellular transport of luminal antigens, resulting in a vicious cycle of paracellular antigenic overload, which is exacerbated by continuous unregulated transcytosis of immunogenic luminal macromolecules. Increased intestinal permeability to polyethylene glycol (a transcellular permeability probe) has been demonstrated in CD^[125-128].

Supporting this interpretation is a report of prodromal symptoms, including fever, occurring from 7-10 years prior to a diagnosis of CD in almost 28% of 29 patients enrolled in a questionnaire based study. In contrast, none of the 15 ulcerative colitis patients enrolled in the study reported fever occurring prior to diagnosis^[129].

This is compatible with a continuous stream of transcytosed immunogenic macromolecules resulting in systemic subclinical immune activation. Unregulated transcytosis is also consistent with other studies that implicated an inherent cell membrane permeability defect independent of inflammation^[130,131].

Since the term "natural history" was introduced for CD in 1965, there has been no hard evidence for change in disease outcome^[4,6]. The urgent need for a natural-history modifying therapy remains a priority. Although immune-mediated tissue damage is clearly evident, in a

complex condition such as CD it may not be what you see when you look, but how you look at what you see that provides the answer.

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Colorectal cancer and 18FDG-PET/CT: What about adding the T to the N parameter in loco-regional staging?

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Abstract

AIM: To evaluate whether FDG-positron emission tomography (PET)/computed tomography (CT) may be an accurate technique in the assessment of the T stage in patients with colorectal cancer.

METHODS: Thirty four consecutive patients (20 men and 14 women; mean age: 63 years) with a histologically proven diagnosis of colorectal adenocarcinoma and scheduled for surgery in our hospital were enrolled in this study. All patients underwent FDG-PET/CT preoperatively. The primary tumor site and extent were evaluated on PET/CT images. Colorectal wall invasion was analysed according to a modified T classification that considers only three stages (\leq T2, T3, T4). Assessment

of accuracy was carried out using 95% confidence intervals for T.

RESULTS: Thirty five/37 (94.6%) adenocarcinomas were identified and correctly located on PET/CT images. PET/CT correctly staged the T of 33/35 lesions identified showing an accuracy of 94.3% (95% CI: 87%-100%). All T1, T3 and T4 lesions were correctly staged, while two T2 neoplasms were overstated as T3.

CONCLUSION: Our data suggest that FDG-PET/CT may be an accurate modality for identifying primary tumor and defining its local extent in patients with colorectal cancer.

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Key words: Cancer; Colorectal; Positron emission tomography/computed tomography; Staging

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INTRODUCTION

In patients with colorectal cancer, accurate preoperative staging is essential for the planning of optimal therapy considering the many therapeutic options available.

Correct evaluation of the local extent (T) of the tumor and of the regional lymph nodes (N) is crucial since it influences the local surgical approach as well as the therapeutic management of the distant metastases (M). Various treatments of the primary tumor are available including radical or limited resection, palliative derivative surgery, local excision, laparoscopic surgical approach, preoperative neoadjuvant chemotherapy and/or radiotherapy: for example the laparoscopic surgical approach is used preferentially for T1 and T2 staged tumors; preoperative neoadjuvant therapy for advanced rectal cancer (T3, T4); limited resection or palliative derivative surgery for diffusely invasive cancers^[1-7]. Moreover, only if the whole primary tumor mass can be completely removed, the surgical option for liver, lung or non-regional lymph nodes metastases is considered^[8-10].

In daily practice, tumor size and infiltration of adjacent structures in colorectal cancer are assessed initially by contrast-enhanced computed tomography (ceCT)^[11-14]. Magnetic resonance imaging (MRI) and endorectal ultrasound (US) represent an accurate diagnostic option for rectal cancer^[15-19].

Non-enhanced FDG-positron emission tomography (PET)/CT^[20-21] and more recently contrast-enhanced FDG-PET/CT (PET/ceCT)^[22-24], are gaining a progressively more important role in the evaluation of the N and the M stages in the staging and follow-up of colorectal cancer, however, the performance of this modality in the evaluation of the T parameter has not been extensively investigated. A single study has recently proposed the evaluation of TNM staging by PET/CT colonography in patients with colorectal cancer and reported good assessment of the T parameter^[6-8]; the PET/CT colonography protocol requested previous bowel preparation with a solution containing polyethylene glycol-electrolytes, the iv injection of N-butyl scopolamine, administration of a rectal water enema, a water-based negative oral contrast agent assumption and the iv injection of iodinated contrast medium. This protocol may not be tolerated well by some patients, can be time consuming, is not recommended in patients with impaired renal function and requires additional costs.

The aim of this study was to evaluate whether FDG-PET/CT, without iv contrast medium or colonography technique, may be considered a proper diagnostic tool in the assessment of local primary tumor extent (T) in patients with colorectal cancer.

MATERIALS AND METHODS

Patients

The study protocol was approved by our institutional review board and informed consent was obtained from all patients.

The study population consisted of 34 consecutive patients (20 men and 14 women; age range, 29-81 years; mean age: 63 years) with a histologically proven diagnosis (the histological specimen was obtained during conventional colonoscopy) of colorectal adenocarcinoma and

scheduled for surgery in our hospital. Exclusion criteria were refusal to participate in the study.

Before surgery all patients underwent FDG-PET/CT in our Institution. Surgery was scheduled within 10 d of the examination, with the exception of three patients with rectal cancer who underwent neoadjuvant radio-chemotherapy after PET/CT and before surgery.

PET/CT technique

Dual modality imaging was performed with a PET/CT system (Discovery-LS, GE-Medical-Systems, Milwaukee, USA) consisting of a PET scanner and a four-row MDCT system.

All patients had been instructed to fast for a minimum of 6 h prior to the examination. Blood glucose levels were found to be in the normal range prior to 18FDG injection by blood sampling. PET/CT was carried out 60 min after iv administration of 370 MBq of 18FDG.

MDCT scans were acquired from the base of the skull to the upper thighs using the following parameters: 4 mm × 5 mm collimation (140 kV, 80 mAs), 0.5 s rotation time, a pitch of 6. 18FDG-PET data were acquired with the patient in the same position on the table at four bed positions (5 min for each bed position) covering the same field of view as CT.

Data obtained from the CT acquisition were used for attenuation correction of 18FDG-PET emission data. 18FDG-PET images were reconstructed with a 4.5 mm thickness.

18FDG-PET, CT and fused 18FDG-PET/CT images were reviewed on the dedicated workstation (Xeleris, GE Medical System).

Imaging interpretation

PET/CT examinations were interpreted before surgery by two pairs of observers, each pair composed of a radiologist and a nuclear medicine physician (each with > 5 years of experience). The images were interpreted jointly within each pair and independently by the two pairs of observers. In cases of disagreement, a consensus panel consisting of the original four observers plus a third blinded party (with 10 years experience) made the final decision.

PET/CT images were evaluated to determine the primary tumor site and extent and the lymph nodes status.

For determination of the primary tumor site and extent, and the lymph nodes status, each pair of observers could use the CT, the PET and the fused PET/CT images individually and simultaneously in no established order. This approach was dictated by the aim of our study which was to evaluate PET/CT exclusively as a single complex technique for local colorectal cancer staging.

For the localization of each lesion, the large intestine was divided into nine anatomic segments: rectum, rectosigmoid colon junction, sigmoid colon, descending colon, splenic flexure, transverse colon, hepatic flexure, ascending colon and caecum.

For the identification of each lesion, the tumor had to be depicted either morphologically or metabolically (a polypoid, annular, semiannular or flat lesion had to be

associated with focal abnormal FDG uptake, or focal abnormal FDG uptake had to be associated with a polypoid, annular, semiannular or flat lesion).

Abnormal FDG uptake was defined as focal increased activity higher than the background activity of soft tissues. The evaluation of each focal radiotracer uptake was qualitative and quantitative (the standard maximum uptake value (SUVmax) was calculated; the SUVmax was defined as abnormal when it appeared to be higher than the SUVmax of the background activity of soft tissues). Diffuse radiotracer uptake was assumed to represent normal or non-malignant bowel activity.

Colorectal wall invasion was analysed according to a modified T classification^[1,2] that considers only three stages ($\leq T2$, T3, T4). A parietal lesion concentrating the 18-FDG in the absence of extra-parietal radio-tracer uptake, was considered as a tumor confined to the bowel wall and defined as a $\leq T2$ lesion. A tumor either with a spiculated outer contour or with rounded or nodular advancing edges showing intra- and extra-parietal radio-tracer uptake was defined as a T3 lesion. A tumor infiltrating into adjacent organs as suggested by their increased glucose metabolism was defined as a T4 lesion.

Lymph node metastasis was evaluated in regional lymph nodes. The diagnosis of an abnormal lymph node on PET/CT was based on the presence of focal increased FDG uptake at a location that corresponded to a lymph node regardless of its size on CT scan. N1 was defined as focal FDG uptake in not more than three lymph nodes, while N2 was defined as focal FDG uptake in more than three lymph nodes.

On co-registered PET images, the SUVmax was calculated on the primary tumor as well as on contiguous organs appearing to be involved as well as on the lymph nodes with focal increased glucose metabolism.

Standard of reference

The standard of reference was represented by surgical findings and histopathological analysis of the surgical specimens.

Localization of the tumor was defined during surgical exploration.

Tumour invasion (T) and lymph node status (N) were based on the TNM classification of the surgical specimen.

Three patients with rectal cancer underwent neoadjuvant radio-chemotherapy after PET/CT and before surgery: considering the potential downstaging of the tumor, the T and the N evaluation obtained with both MRI and endorectal US was used as the standard of reference.

Statistical analysis

Data are expressed as mean \pm one SD or as proportion as appropriate. A commercial statistical software package was used (MedCalc®). Differences between continuous data were assessed using analysis of variance with post-hoc multiple groups comparison (Student-Newman-Keuls test). Categorical data were evaluated by χ^2 analysis, Fisher exact test, and McNemar test, as appropriate. Logistic

Table 1 T stage: positron emission tomography/computed tomography vs histology

	Histology			Total
	T ≤ 2	T3	T4	
PET/CT				
T ≤ 2	6	0	0	6
T3	2	21	0	23
T4	0	0	6	6
Total	8	21	6	35

PET/CT: Positron emission tomography/computed tomography. χ^2 : 58.9, ($P < 0.0001$). Accuracy: 94.3% (95% CI: 87%-100%).

analysis was used to evaluate significant determinants. A P value < 0.05 was considered significant.

RESULTS

Standard of reference

A total of 37 adenocarcinomas were found in the surgical specimens. Two synchronous lesions were found in 3 out of 34 patients (8.8%). Three adenocarcinomas showed a mucinous component on histopathological examination.

The regional distribution of the 37 tumors was as follows: rectum ($n = 6$), rectosigmoid colon junction ($n = 4$), sigmoid colon ($n = 15$), descending colon ($n = 3$), transverse colon ($n = 1$), hepatic flexure ($n = 3$), ascending colon ($n = 2$) and caecum ($n = 3$).

Five out of 37 (13.5%) tumors were classified as stage T1, 5 out of 37 (13.5%) as stage T2, 21 out of 37 (56.8%) as stage T3 and 6 out of 37 (16.2%) as stage T4. All three adenocarcinomas with a mucinous component were classified as T4.

Twenty one out of 37 (57%) lesions were classified as N- and 16 out of 37 (43%) as N+ (13/16 as N1 and 3/16 as N2).

Tumour identification and location

Thirty five out of 37 (94.6%) adenocarcinomas were identified and correctly located on PET/CT images. In two patients no lesions were disclosed on PET/CT images: the two lesions which were missed were located in the transverse colon and the sigmoid colon, respectively, and were flat and confined to the colonic wall resulting in T1 stage on histopathological examination, and measured 15 mm and 16 mm, respectively.

Tumour T staging

PET/CT correctly staged (Table 1) the T of 33/35 lesions identified, showing an accuracy of 94.3% (95% CI: 87%-100%). All T1, T3 and T4 lesions were correctly staged, while two T2 neoplasms, located in the sigmoid colon and rectum, respectively, were overstated as T3 (Figures 1 and 2).

For the six lesions correctly classified as T4, PET/CT showed infiltration of the uterus ($n = 1$), of the ovary ($n = 2$), of the small bowel ($n = 1$) and of the peritoneum ($n = 2$) (Figures 3 and 4).

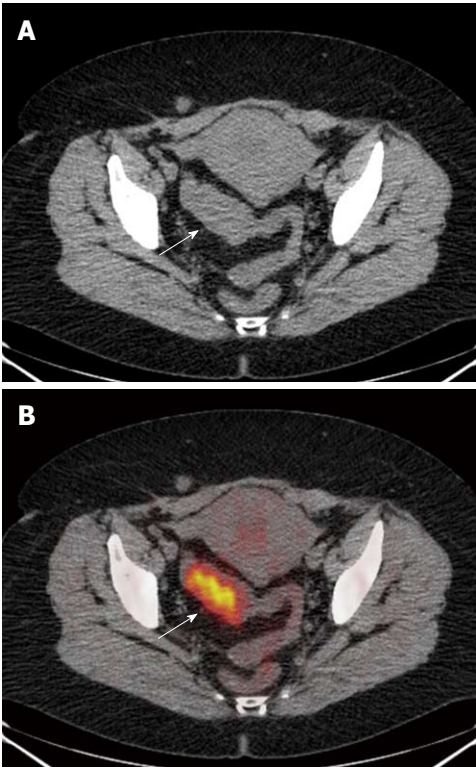


Figure 1 The computed tomography (A) and fused positron emission tomography/computed tomography (B) images. A: Neoplastic thickening of sigmoid colon walls, which show regular profiles with normal appearance of perilesional fat (arrow); B: Uptake of 18-FDG (arrow) (SUVmax 11) in the absence of extra-parietal radio-tracer uptake. The lesion was correctly classified as $\leq T2$.

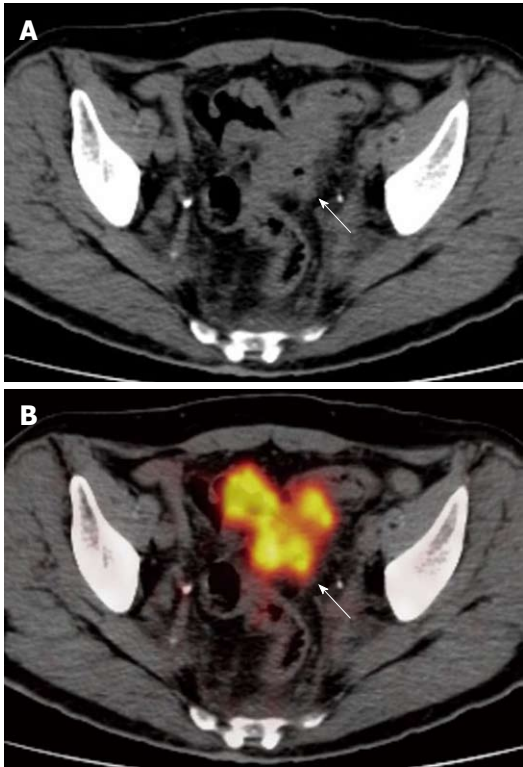


Figure 2 The computed tomography (A) and fused positron emission tomography/computed tomography (B) images show diffuse and irregular neoplastic thickening of sigmoid colon walls, which show rounded advancing margins with intense radiotracer uptake (arrows) (SUVmax 13). The lesion was correctly classified as T3.

Table 2 N stage: positron emission tomography/computed tomography *vs* histology

Istologia	Histology		Total
	N+	N-	
PET/CT			
N+	12	3	15
N-	4	15	19
Total	16	18	34

PET/CT: Positron emission tomography/computed tomography. χ^2 : 9.5, ($P < 0.005$). Accuracy: 79.4% (95% CI: 66%-93%).

The SUVmax of the identified lesions ranged from 1.8 to 27 with a mean of 14.3 ± 5.8 . Stratifying for T stages, the SUVmax of the T1 tumors ranged from 1.8 to 14 with a mean of 6.9 ± 6.4 , the T2 tumors ranged from 10 to 25 with a mean of 13.9 ± 6.3 , the T3 tumors ranged from 5.7 to 27 with a mean of 15.3 ± 5.5 , and the T4 tumors ranged from 9.4 to 21 with a mean of 14.7 ± 4.7 . No statistically significant difference between the SUVmax of each T group was found.

The SUVmax of the contiguous organs infiltrated ranged between 3.6 and 20 with a media of 11.5 ± 6.2 .

Tumour N staging

PET/CT correctly staged (Tables 2 and 3) the N of 27/34

Table 3 N stage: positron emission tomography/computed tomography *vs* histology with N1 and N2 stratification

	Histology			Total
	N0	N1	N2	
PET/CT				
N0	15	4	0	19
N1	3	9	0	12
N2	0	0	3	3
Total	18	13	3	34

PET/CT: Positron emission tomography/computed tomography. χ^2 : 43.6, ($P < 0.001$). Accuracy: 79.4% (95% CI: 66%-93%).

patients, showing an accuracy of 79.4% (95% CI: 66%-93%). There were three false positive and 4 false negative results. 15/18 N0, 9/13 N1 and 3/3 N2 lesions were correctly classified.

The SUVmax of the lymph nodes with focal increased radio-tracer uptake ranged from 1.6 to 10.3 with a mean of 5.5 ± 3.1 . No statistically significant differences between the SUVmax of each N group were observed.

DISCUSSION

Our data shows that FDG-PET/CT is a useful diagnostic tool in identifying primary tumor extent in patients with colorectal cancer: the T stage of 33 out of 35 (94.3%)

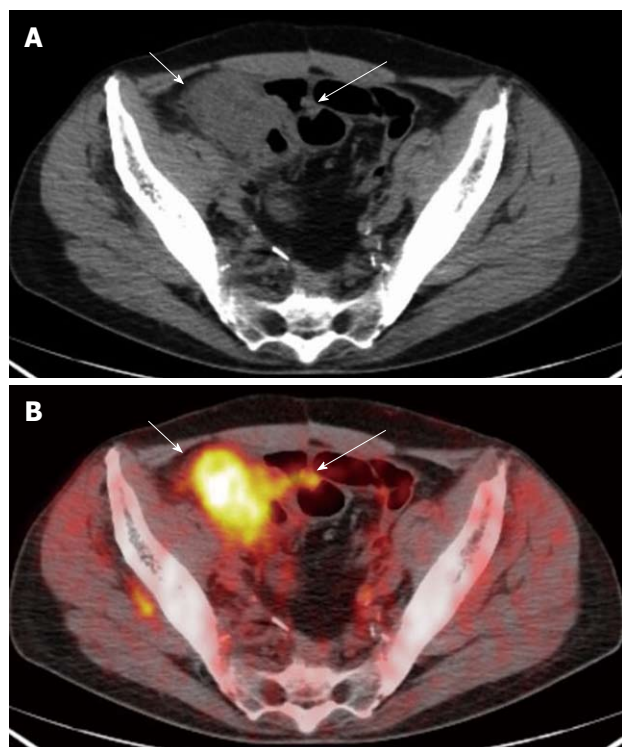


Figure 3 The computed tomography (A) and fused positron emission tomography/computed tomography (B) images show a neoplastic lesion of the caecum concentrating the radio-tracer (short arrows) (SUVmax 9.6). Focal radio-tracer uptake (SUVmax 3.6) of a contiguous ileal loop was disclosed on positron emission tomography/computed tomography (B) (long arrow) consistent with a short concentric wall thickening (A) (long arrow): the finding was suggestive of small bowel infiltration. The lesion was correctly classified as T4.

neoplastic colorectal lesions was correctly identified as well as the N stage of 27/34 (79.4%).

One of the major strengths of PET/CT as a cancer staging modality is its ability to identify systemic metastases. At any phase of cancer evaluation, the demonstration of systemic metastases has profound therapeutic and prognostic implications. Only in the absence of systemic metastases does nodal status become important, and only when unresectable nodal metastasis has been excluded does T stage become important. There are now accumulating data to suggest that PET/CT could be used as the first, rather than the last test to assess M and N stage in the evaluation of cancers^[25]. In this scenario, it would also be desirable for PET/CT to be accurate in the evaluation of the T stage. As a consequence, there is a great opportunity to use PET/CT as an all-in-one staging imaging modality in oncologic patients, and subsequently selecting and tailoring the performance of anatomically-based imaging modalities without or with iv contrast medium (US, MR, CT) to better define the abnormalities identified by PET/CT, when this information would be of relevance to management planning.

For these reasons, neither comparing the performance of PET/CT with other non-invasive imaging modalities nor investigating the added value of FDG information to CT data nor defining what was the contribution of

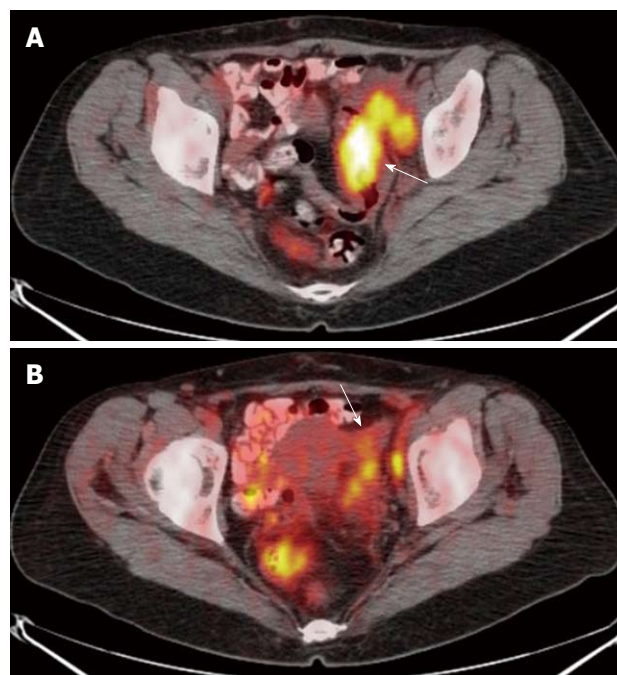


Figure 4 The fused positron emission tomography/computed tomography images show a neoplastic lesion of the sigmoid colon concentrating the radio-tracer (arrow) (SUVmax 14.6) (A) and focal radio-tracer uptake of the left ovary (arrow) (SUVmax 9) (B): the finding was suggestive of ovarian infiltration. The lesion was correctly classified as T4.

each component (PET, CT and fused PET/CT images) were our goals. On the contrary, the aim of our study was exclusively to evaluate PET/CT as a single complex technique in colorectal cancer T staging, comparing its performance directly with the gold standard of histological results.

In colorectal cancer, accurate assessment of the T stage and tumor size may aid in determining the correct way to access the lesion (local endoscopic excision, laparotomy, laparoscopy, or transanally), or the modality of surgery (radical or limited resection, palliative derivative surgery). Moreover, for rectal cancer, the T stage will be of major clinical relevance since its accurate preoperative assessment may help to select patients who will benefit from neoadjuvant therapy compared with resection alone.

In colorectal cancer, although various imaging modalities have been proposed for TNM staging, ceCT widely represents the first diagnostic step due to relatively low cost and widespread availability. For T staging, endorectal US and MRI are becoming mandatory in the management of rectal cancer^[15-19,26]. For N staging, malignant lymph node identification remains a problem and the use of the size criteria may lead to misdiagnosis: evaluation of the outline of the node and the features of signal intensity with MRI^[15-19,26] as well as the assessment of glucose metabolism with PET/CT^[22,23,27] have been shown to be more reliable. For M staging, particularly for liver metastases, the optimal imaging staging strategy has not yet been defined and the role of CT, MRI, PET/CT and US is still debated^[22,28-32].

As a result, if PET/CT is used as the all-in-one im-

aging modality in the staging of colo-rectal cancer, it is mandatory that it is demonstrated to be accurate in the evaluation of the T parameter, it adopts a minimal complex procedure, is well tolerated by patients, is less time consuming and is as inexpensive as possible.

Thus, the choice to perform PET/CT without the aid of iv contrast medium and colonography has been dictated by our interest in evaluating how accurate this modality, in its basal condition, is in T staging. The use of iv contrast medium allows better definition of the boundaries of structures and colonography permits easy identification of the primary tumor and a more accurate assessment of its local extent^[13,14]. However, the radio-tracer may play the role of “metabolic contrast agent” and is able to increase the contrast resolution of the structures, to characterize the perilesional tissues and to compensate for the absence of luminal distension on the unenhanced CT images, as demonstrated in our series in which 95% of adenocarcinomas were correctly identified and staged.

Moreover, a basal PET/CT protocol also offers the chance of colorectal cancer staging to those patients in whom the administration of iv contrast medium is contraindicated or not recommended, such as patients with impaired renal function or with an allergic history.

CT and PET/CT have limitations in distinguishing the wall layers of the colon, as a consequence, the differentiation of T1 and T2 tumors is not accurate^[13,14,22]. For this reason we decided to classify T1 and T2 tumors as a single group (\leq T2). Further technique developments concerning CT and PET resolution may improve their ability to differentiate the colonic wall layers and as a result T1 from T2 tumors. The distinction between T1 and T2 stage is not crucial for the therapeutic management of colorectal cancer because the mandatory information relates to whether the tumor is confined to the colonic wall or infiltrates the surrounding tissues.

In our series, a significant difference in SUVmax between each T group was not observed. As a result, it was not possible to identify a potential cut-off value of SUVmax for each T stage in our population.

It is well known that normal gastro-intestinal tract can accumulate FDG extensively, hindering pathological focal tracer uptake or simulating the presence of a tumor. The physiological FDG gastro-intestinal uptake was not the cause of misinterpretation in our series, as tumors were identified either morphologically or metabolically by the readers.

Neither bowel peristaltism nor respiratory motion resulted in mis-registered PET and CT datasets, hindering the interpretation of images in our population.

In our series, two lesions located in the transverse colon and in the sigmoid colon, respectively, were missed. In both cases, retrospective re-evaluation of the colonic segment involved allowed us to conclude that the flat morphology of the two lesions rather than their dimensions or the physiological bowel uptake hindered the identification of these lesions.

Although mucinous adenocarcinoma is a histopathological type of colorectal cancer known to have limited FDG PET sensitivity, the three mucinous adenocarcinomas were correctly identified and staged in our series.

Nodal status was correctly evaluated in 27/34 patients with an accuracy of 79.4%. The use of an un-enhanced PET/CT protocol did not invalidate the N stage as shown by our findings which are similar to those reported in other papers^[22,23]. Moreover, it has been demonstrated that contrast-enhanced PET/CT shows a trend towards more accurate N-staging of rectal cancer compared with non-contrast-enhanced PET/CT^[23]. Subcentimeter positive nodes are the major source of false negative results in nodal staging, being missed by both PET/CT with and PET/CT without contrast enhancement^[23]. As a result, the spatial resolution of PET/CT is not sufficient to detect small lymph node metastases and this limitation can not be obviated by the administration of iv contrast material.

In conclusion, our data suggest that FDG-PET/CT, without administration of iv contrast medium or colonography may be an accurate modality for identifying primary tumor and defining its local extent in patients with colorectal cancer. Further investigations using larger populations and PET/CT devices with improved spatial resolution need to be performed to confirm our observations.

COMMENTS

Background

In oncologic patients, the accurate evaluation of the T (tumor depth), the N (lymph node status) and M (distant metastases) of the primary neoplasm is a crucial point relative to therapeutic management. Although multiple imaging modalities, such as ultrasound (US), computed tomography (CT), magnetic resonance imaging and positron emission tomography (PET)/CT, are available for local tumor and distant staging, an all-in-one staging imaging modality would be a great opportunity to reduce the costs and the time of hospitalization.

Research frontiers

Un-enhanced FDG-PET/CT and more recently contrast-enhanced FDG-PET/CT (PET/ceCT) are gaining a progressively more important role in the evaluation of the N and the M stages in the staging and follow-up of colorectal cancer, however, the performance of this modality in the evaluation of the T parameter has not been extensively investigated.

Innovations and breakthroughs

Our findings suggest that FDG-PET/CT may be an accurate modality for defining local extent (T) of colorectal cancer.

Applications

If PET/CT accurately evaluated the T parameter, this would be a great opportunity to use PET/CT as an all-in-one staging imaging modality in colorectal cancer patients, and subsequently selecting and tailoring the performance of anatomically-based imaging modalities without or with iv contrast medium (US, magnetic resonance, CT) to better define the abnormalities identified by PET/CT, when this information would be of relevance to management planning.

Peer review

The article gives a new idea for the diagnosis and staging of colorectal cancer.

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Proteomic analysis of pancreatic intraepithelial neoplasia and pancreatic carcinoma in rat models

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Abstract

AIM: To detect the proteomic variabilities of pancreatic intraepithelial neoplasia (PanIN) and pancreatic carcinoma (PC) induced by 7,12-dimethylbenzanthracene (DMBA) in rat models and to identify potential biomarkers.

METHODS: Sixty adult male Sprague Dawley rats were randomized into three groups. The rats had DMBA implanted into their pancreas for one ($n = 20$) or two months ($n = 20$) or assigned to the normal group ($n = 20$). The rats were killed after one or two months, and were evaluated histopathologically. Three tissue samples from each group of rats with either normal pancreas, PanIN (PanIN-2) or PC were examined by 2D-DIGE. The different expression spot features were analyzed by matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI-TOF/TOF) tandem mass spectrometry. The expression of enolase 1, a differentially expressed protein, was identified by immu-

nohistochemistry.

RESULTS: There was significant difference in the proportions of neoplastic changes between the 1- and 2-month groups ($P = 0.0488$). There was an increase in the frequency of adenocarcinomas in the 2-month group compared with the 1-month group ($P = 0.0309$). No neoplastic changes were observed in any of the animals in the normal group. Enolase 1, pancreatic ELA3B, necdin, Hbp23, CHD3, hnRNP A2/B1, Rap80, and Gnb2l1 were up-regulated in the PanIN and PC tissues, and CEL, TPT1, NME2, PCK2, an unnamed protein product, and glycine C-acetyltransferase were down-regulated in the PanIN and PC tissues. The immunohistochemical results showed that enolase 1 expression was up-regulated in the pancreatic cancer tissues of rats and humans.

CONCLUSION: The pancreatic protein expression changes induced by DMBA suggest potential molecular targets for the early diagnosis and treatment of PC.

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Key words: 7,12-dimethylbenzanthracene; Pancreatic intraepithelial neoplasia; Pancreatic carcinoma; Proteomics

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Wang L, Liu HL, Li Y, Yuan P. Proteomic analysis of pancreatic intraepithelial neoplasia and pancreatic carcinoma in rat models. *World J Gastroenterol* 2011; 17(11): 1434-1441 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i11/1434.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i11.1434>

INTRODUCTION

Pancreatic carcinoma (PC) is one of the most lethal hu-

man cancers, and 80%-90% of PCs are pancreatic ductal adenocarcinomas. Pancreatic cancer is characterized by a late presentation, and for all stages of this cancer, the five-year survival rate is less than 5%. It is currently the fourth most common cause of cancer death for both men and women^[1]. This poor prognosis relates to the uniformly advanced disease stage at the time of diagnosis and its profound resistance to the existing therapies. An early detection of pancreatic cancer is required to improve its poor prognosis.

As the clinical diagnosis of pancreatic cancer is mostly at advanced stages, to study the mechanisms of PC and screen tumors for early diagnostic markers, it is important to establish animal models of precancerous pancreatic lesions and the early stages of PC with pathological characteristics resembling that of human PC. Pancreatic intraepithelial neoplasia (PanIN) has been identified as the precursor of pancreatic ductal adenocarcinoma^[2,3], and animal models of PanIN and pancreatic ductal adenocarcinoma can provide opportunities to investigate earlier pancreatic cancers that are rare in human samples. Animal models involve successful chemical carcinogens such as 7,12-dimethylbenzanthracene (DMBA). DMBA implantation into the pancreas is known to induce ductal PanIN and PC in mice and rats through pathways that consistently involve K-ras gene mutations and the activation of Notch signaling, as in human pancreatic cancer^[4-10]. At present, DMBA-induced pancreatic carcinogenesis models have been used in the identification of possible promoters or suppressors of PC^[8,11,12], stable isotope glucose-tracer studies^[13] and molecular analyses^[10].

Proteomic techniques are emerging as important tools for studying the mechanisms of disease and finding potential biomarkers and new therapeutic targets, which enable investigators to determine whether a particular protein level is increased or decreased when comparing two different conditions (e.g. a diseased state and a non-diseased state)^[14,15]. Although proteomic studies of PC in human samples have been reported, most of the samples were acquired from advanced PC but not precancerous pancreatic lesions or the early stages of PC. The protein expression changes from normal pancreas to PanIN and early stages of PC remain unclear. In the present research, we studied the proteomic variabilities of rat models of DMBA-induced PanIN and PC using proteomic techniques, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrometry, to reveal new potential biomarkers for PanIN and PC.

MATERIALS AND METHODS

Animal treatment

Sixty adult male Sprague Dawley (SD) rats (100-110 g) were randomized into three groups. The rats in the 1- ($n = 20$) and 2-mo groups ($n = 20$) had DMBA (10 mg/100 g) directly implanted into their pancreas, according to a previously established protocol^[9], and the surviving rats were killed after one month and two months, respectively.

Table 1 Sample markers and gel distribution in 2D-DIGE analysis

Gel	Cy2	Cy3	Cy5
Gel1	Internal standard	A1	B3
Gel2	Internal standard	B3	C2
Gel3	Internal standard	C1	A2
Gel4	Internal standard	B2	C3
Gel5	Internal standard	A3	B1

ly. A normal group of 20 rats was killed after two months. The carcinogen implant site was separated from the rest of the pancreas, the pancreatic nodules were fixed in formalin and embedded in paraffin, and multiple 5-mm sections were prepared and stained with hematoxylin and eosin for routine histological examinations. The pancreas slides were reviewed by a single pathologist and evaluated histologically according to the PanIN classification system^[2,3].

2D-DIGE and image analysis

Three rat models with either normal pancreas (group A), PanIN (group B, PanIN-2), or PC (group C) were established, and three samples per group were collected. Approximately 300 mg of each tissue sample was divided into 3-mm³ pieces and then homogenized with a Dounce's homogenizer on ice in 1 mL lysis solution containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS and a protease inhibitor mixture (Bio-Rad). The sample lysates were then placed into Eppendorf tubes and sonicated on ice for 10 s, and this procedure was repeated eight times. The sample lysates were centrifuged at 14000 rpm for 60 min, and the supernatants were collected. The total protein concentration in each sample was determined by the Bio-Rad method.

The protein concentration in each sample was dissolved to 5 µg/µL with lysis buffer, and equal amounts of protein from each individual sample were pooled to make the internal standard. The pH of all the samples and the internal standard was adjusted to 8.0-9.0. Five DIGE gels were included in the experimental design, as shown in Table 1. Two different samples in each gel contained 50 µg protein labeled with 1 µL green (Cy3) or red (Cy5) fluorescent dyes (Amersham Biosciences). The third sample contained 50 µg internal standard labeled with yellow (Cy2) fluorescent dye. The labeling reaction was performed on ice for 30 min in the dark and quenched with 1 µL of 10 mmol/L lysine for 10 min.

One-dimensional isoelectric focusing was carried out on an Amersham Biosciences Ettan IPGphor IEF system. The samples (100 µg) were loaded using 13-cm pH-3-10 IPG strips. Isoelectric focusing was performed at 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 8 h and 500 V for 4 h. After IEF, the IPG strips were equilibrated with an equilibration buffer containing 50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 2% SDS, and 1% DTT for 15 min at room temperature, followed by 2.5% iodoacetamide instead of 1% DTT in equilibration buffer for another 15 min. The equilibrated strip was transferred to the top of 1 mm of a 12.5% SDS-polyacrylamide gel and

fixed with 0.5% agarose. The second dimension of SDS-PAGE was performed at a constant current of 15 mA/gel for 30 min and 30 mA/gel until the bromophenol blue fronts reached 0.5 cm from the bottom of the gel.

The fluorescent dye-labeled proteins in each gel were scanned with a Typhoon 9400 fluorescence scanner (Amersham Biosciences) at different wavelengths specific for Cy2 (488/520 nm), Cy3 (532/580 nm) and Cy5 (633/670 nm). Image analysis of 2D-DIGE was performed with Decyder 5.0 software (GE Healthcare) according to the manufacturer's recommendations. Differential protein analysis of the three groups was carried out using one-way ANOVA. Differentially expressed protein spots ($P < 0.01$) were marked.

Protein identification

Two-dimensional electrophoresis of preparative gels containing 1 mg protein was performed like 2D-DIGE, and the gels were stained with Coomassie brilliant blue. The protein spots to be identified were manually excised from the preparative gels. The gel pieces were de-stained in 3% acetic acid and 100 mmol/L ammonium bicarbonate, and in-gel digestion was performed for 20 h at 37°C in 40 mmol/L ammonium bicarbonate containing 0.01 µg/µL trypsin. The tryptic peptides were extracted with 0.1% TFA (60% ACN from the supernatants), and the extracts were lyophilized and saved at -80°C.

The lyophilized peptide mixtures were re-dissolved in 0.1% TFA, the peptide solution was washed with 0.1% TFA, and the 60% ACN was mixed with HCCA (5 mg/mL). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and MALDI-TOF/TOF mass spectrometry were obtained using a Bruker-Daltonics Autoflex TOF-TOF Lift mass spectrometer. Peptide mass fingerprints (PMFs) were acquired for each differentially expressed protein in positive-reflection mode (20 kV accelerating voltage, 23 kV reflecting voltage). The PMFs were searched in the database of the National Center for Biotechnology Information (NCBI) of *Rattus* using the software Mascot. Carbamidomethyl was specified as fixed modification, and the oxidation as variable modifications, and the peptide mass tolerance was ± 100 ppm. The statistically significant protein scores were found ($P < 0.05$), and, if more than one protein was identified, the single protein with the top score was matched to the protein spot.

Immunohistochemistry

Three pairs of rat normal pancreas and DMBA-induced PC tissues and 30 pairs of PC and adjacent non-carcinoma tissues from humans in a tissue microarray (Shanghai Outdo Biotech Co. Ltd, OD-CT-DgPan03-002, 19 male and 11 female) were used for immunohistochemical staining. All of the samples were formalin-fixed and embedded in paraffin. The sample sections were deparaffinized and successively rehydrated in xylene and alcohol and washed three times in phosphate buffered saline (PBS). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 10 min, and the sections were then incubated with 0.05% trypsin for 30 min

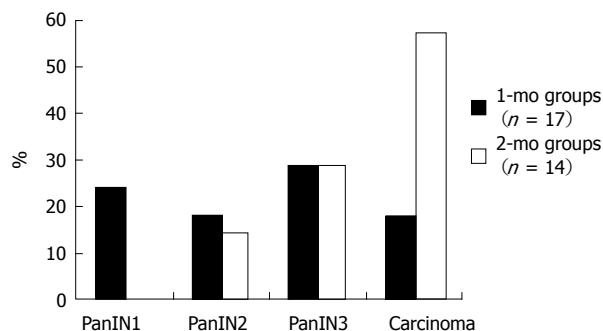


Figure 1 Pathological diagnoses in 1- and 2-mo groups after 7,12-dimethylbenzanthracene implantation. The difference between two groups was statistically significant ($P = 0.0488$), and there was an increase in the frequency of adenocarcinomas in the 2-mo group compared with the 1-mo group ($P = 0.0309$). PanIN: Pancreatic intraepithelial neoplasia.

at 37°C. After three washes with PBS, nonspecific binding was blocked with 10% BSA in PBS for 30 min at 37°C. The sections were then incubated overnight at 4°C with an enolase 1 antibody at a dilution of 1:400, and sections incubated with PBS instead of the enolase 1 antibody, served as control. DAB (1:50) was used to detect the enolase 1 with the deposition of a brown reaction product in the nuclei and cytoplasm of the positive cells. The proportion of positively stained cells in each section was averaged from three high-magnification images.

Statistical analysis

Differences between groups were analyzed by two-tailed Chi-square tests and two-tailed Fisher's exact tests. The significance level was defined as $P < 0.05$.

RESULTS

Rat models of DMBA-induced PanIN and PC

In the 1-mo group, 15% (3/20) of the rats died in the postoperative period, whereas 30% (6/20) died in the 2-mo group. No statistically significant difference in the death rate was observed between the groups ($P = 0.4489$). Pathologic evaluation revealed that 71% (12/17) of the rats had PanIN lesions and 18% (3/17) of the rats had adenocarcinomas in the 1-mo group. Of these, 24% (4/17) had PanIN1 lesions, 18% (3/17) had PanIN2 lesions and 29% (5/17) had PanIN3 lesions. In the 2-mo group, 14% (2/14) had PanIN2 lesions, 29% (4/14) had PanIN3 lesions, and 57% (8/14) had adenocarcinomas (Figure 1). The difference in the proportions of neoplastic changes was statistically significant between the two groups ($P = 0.0488$), and there was an increase in the frequency of adenocarcinomas in the 2-mo group compared with the 1-mo group ($P = 0.0309$). No neoplastic changes were observed in any of the animals in the normal group.

2D-DIGE analysis of differential proteomic expression of DMBA-induced PanIN and PC

After spot quantification and statistical analysis using the 2D-DIGE approach described above, 1445, 1469, 1380,

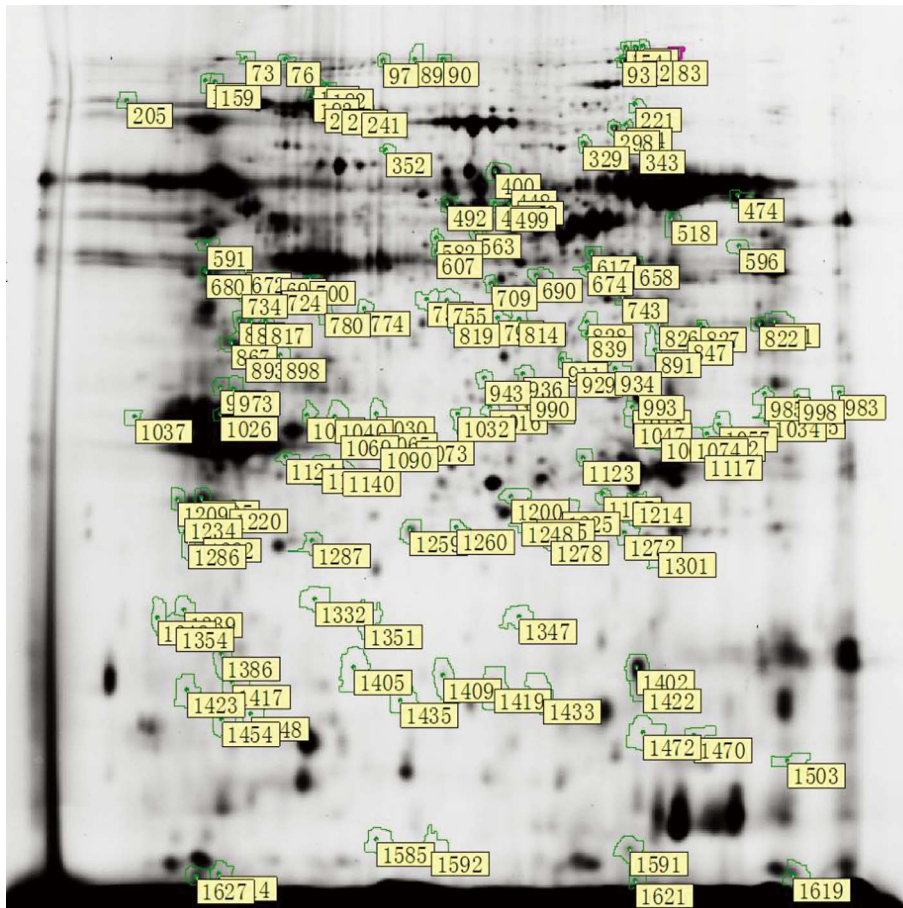


Figure 2 Map of 155 protein spots with significantly differential expression ($P < 0.01$) in normal pancreatic (group A), pancreatic intraepithelial neoplasia-2 (group B) and pancreatic carcinoma (group C) tissues. Thirty-one protein levels progressively increased, and 17 protein levels progressively decreased from normal pancreatic tissue to 7,12-dimethylbenzanthracene-induced pancreatic intraepithelial neoplasia and pancreatic carcinoma.

1512, and 1637 spots were identified in gel1, gel2, gel3, gel4 and gel5, respectively. After background subtraction and radiometric normalization, matched spots from all of the gels were used for statistical analysis. By using the criterion of $P < 0.01$, 155 spots were significantly differentially expressed by their relative abundances in groups A, B and C (Figure 2). Furthermore, 31 protein levels progressively increased from normal pancreas to pancreas with DMBA-induced PanIN and PC. Additional 17 protein levels progressively decreased, and 21 spots were selected for identification using a MALDI-TOF/TOF mass spectrometer. We found that the abundances of enolase 1, pancreatic elastase 3B (ELA3B), necdin, Hbp23, chromodomain helicase DNA-binding protein 3 (CHD3), heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1), retinoid X receptor-interacting protein (Rap80), guanine nucleotide-binding protein (G protein), and beta polypeptide 2-like 1 (Gnb2l1) progressively increased with the severity of the disease. The abundances of carboxyl ester lipase (CEL), tumor protein translationally controlled 1 (TPT1), expressed in non-metastatic cells 2 (NME2), phosphoenolpyruvate carboxykinase 2 (PCK2), an unnamed protein product, and glycine C-acetyltransferase progressively decreased with DMBA-induced disease severity (Table 2).

Immunohistochemical analysis of enolase 1

Immunohistochemical staining showed that the expression level of enolase 1 was highest in the nucleus and cytoplasm; membranous immunoreactivity was also detected. The enolase 1 expression levels in DMBA-induced PC and normal rat pancreatic tissues were compared, and the staining results showed that the rat PC tissue had a higher enolase 1 expression level ($P = 7.5633E-014$). In the rat PC tissue, an average of 83% of cells expressed enolase 1, whereas only 31% of the cells in rat normal pancreatic tissues expressed enolase 1. The expression of enolase 1 was compared in pancreatic cancer and adjacent non-carcinoma tissues from 30 patients, and the average proportion of enolase 1-positive cells in the human pancreatic cancer tissue was 91%, and only 37% in adjacent non-carcinoma tissues. The expression of enolase 1 was also significantly increased in human PC tissues ($P = 3.3514E-015$) (Figure 3).

DISCUSSION

Animal models of PanIN and PC that resemble the human disease are very important for research. The characteristics of these models should include the following: (1)

Table 2 Differentially expressed proteins identified by matrix-assisted laser desorption/ionization-time of flight/time of flight tandem mass spectrometry in normal pancreatic, pancreatic intraepithelial neoplasia-2 and pancreatic cancer tissues

Spot position	Protein number in NCBI	Protein name	MW	PI	Mascot PMF score	P
230	Mixture,gi 6753406 + gi 74205924	Carboxyl ester lipase+unnamed protein product	70	6	136	4.10E-5
780	gi 158186649	Enolase 1	40	5.5	76	5.40E-5
1205	gi 6678437	Tumor protein translationally controlled 1	25	5	98	7.90E-5
1402	gi 55926145	Expressed in non-metastatic cells 2	20	8	185	0.000
1132	gi 149024340	Pancreatic elastase 3B	25	5.5	91	0.000
1066	gi 56676354	Necdin	30	8	63	0.000
1272	gi 6435547	Hbp23	25	7.5	62	0.001
226	gi 6753406	Carboxyl ester lipase	90	5.5	118	0.001
1405	gi 109488364	Chromodomain helicase DNA-binding protein 3	20	5.5	72	0.001
755	Mixture,gi 149033753 + gi 149039895	Albumin+retinoid X receptor-interacting protein	40	6.5	181	0.001
1259	gi 12832572	Unnamed protein product	25	6	38	0.001
298	gi 149063967	Phosphoenolpyruvate carboxykinase 2	66	7.5	229	0.002
1057	gi 4504447	Heterogeneous nuclear ribonucleoprotein A2/B1	30	8.5	62	0.002
993	gi 5174447	Guanine nucleotide binding-protein (G-protein), beta polypeptide 2-like 1	30	7.5	146	0.003
1040	gi 5174447	Guanine nucleotide binding-protein (G-protein), beta polypeptide 2-like 1	30	5.5	142	0.004
658	gi 66730435	Glycine C-acetyltransferase	40	8	211	0.008

NCBI:National Center for Biotechnology Information; PMF:Peptide mass fingerprint; MW: Molecular weight ; PI: Isoelectric point.

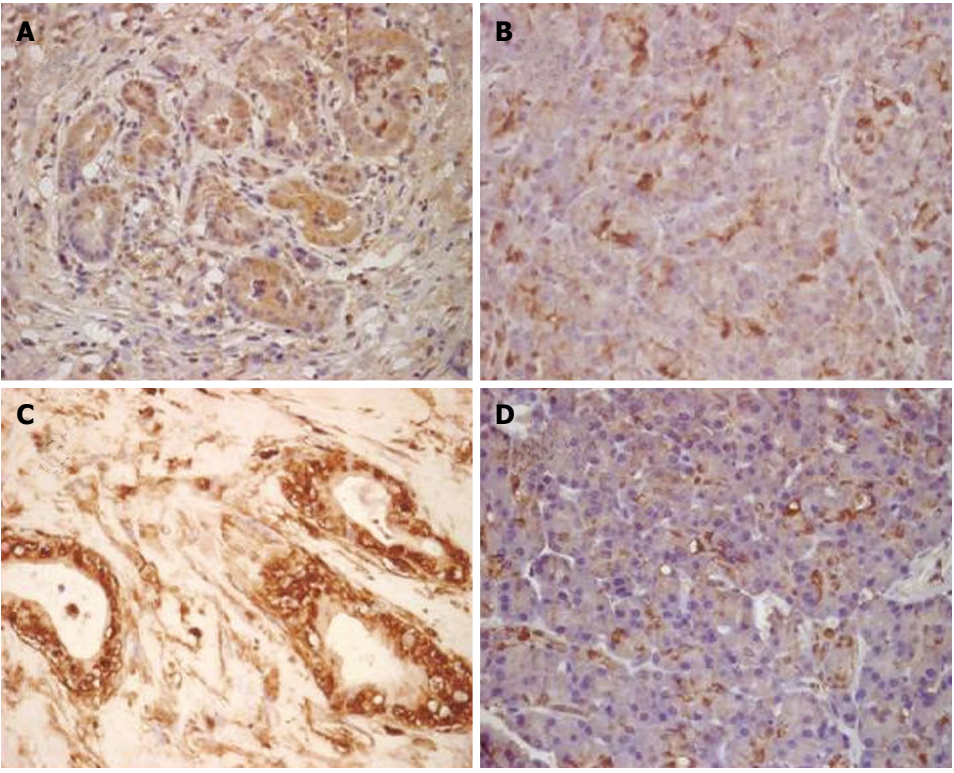


Figure 3 Enolase 1 expression was significantly increased in human and rat pancreatic carcinoma (immunohistochemistry, magnification × 400). A: Positive expression in rat pancreatic cancer; B: Weakly positive expression in the normal rat pancreas; C: Positive expression in human pancreatic cancer; D: Weakly positive expression in human adjacent non-carcinoma tissues.

A pancreatic ductal phenotype that arises from pancreatic ductal cells; (2) Extensive reactive proliferation of the

connective tissue; (3) Obstruction of the bile duct and stomach during disease progression; (4) Early neural, peritoneal, and liver metastases; and (5) High rates of K-ras, *P16* and *P53* gene mutations^[16]. The experimental models of PanIN and PC in the present study are chemical carcinogenesis models. DMBA-induced pancreatic carcinogenesis models resembled human PC in several previous studies. Rivera *et al*^[6] found that DMBA was one of the three different carcinogens reliably producing PC histology that is similar to human ductal adenocarcinoma. DMBA was either implanted directly into the pancreas or infused into the pancreatic ducts of SD rats, and the development of ductal hyperplastic, atypical, and dysplastic changes preceding and accompanying the invasive pancreatic ductal adenocarcinoma could be observed in this experimental model. Jimenez *et al*^[7] demonstrated that DMBA-induced rat tubular complexes and pancreatic adenocarcinomas strongly expressed ductal cell markers (keratin, cytokeratins 19 and 20) but not acinar cell markers (chymotrypsin), suggesting that these tumors arose from ductal cell transformation. K-ras mutations were significantly more frequent in DMBA-induced PC tissues than in normal tissues, with a prevalence of up to 91%^[8]. Pancreatic carcinogenesis induced by DMBA implantation in mice was re-evaluated according to the PanIN classification system after its establishment, and extensive pathological changes characteristic of PanIN could be observed one month after carcinogen implantation^[9]. We used the PanIN classification system in the histological analysis, and our pathologic evaluation showed that 71% (12/17) of the rats had PanIN lesions and 18% (3/17) had adenocarcinomas in the 1-mo group. In the 2-mo group, 43% (6/14) had PanIN lesions and 57% (8/14) had adenocarcinomas. The frequencies of PanIN and PC in our rat models were similar to a previous study in mice^[9]. Our models were satisfactory because the development of precursor lesions (PanIN) and an invasive adenocarcinoma were observed in all groups. There was an increase in the frequency of adenocarcinomas, and the PanIN classification increased with DMBA induction, reflecting the dynamic development of a normal pancreas into a cancerous pancreas.

There are several techniques available for protein separation, both gel- and non-gel-based. Gel-based techniques include traditional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and, more recently, 2D-DIGE. 2D-DIGE usually contains three samples labeled with three distinct fluorescent dyes: Cy2, Cy3 and Cy5. The Cy2 dye is typically used to label an internal standard; the strength of the internal standard helps map the spots/proteins between the gels, thus rendering different gels more comparable^[17-19]. In the current study, we used 2D-DIGE and MALDI-TOF/TOF tandem mass spectrometry to profile pancreatic proteins in rat models of DMBA-induced PanIN and PC and compared these profiles with those of normal rats. Our 2D-DIGE data showed that 155 spots were significantly differentially expressed based on their relative abundances in the three groups. Of these, 31 protein levels progressively increased from normal to PanIN and then to PC tissue, whereas 17 protein lev-

els progressively decreased. These results demonstrated synchronous dynamic changes in the pancreatic protein expression profile after DMBA induction. A number of novel proteins were identified that may be involved in important aspects of mRNA transcription, DNA damage repair, chromatin remodeling, oxidative stress, regulation of tumor growth and metastasis, glucose metabolism, and the synthesis and secretion of pancreatic enzymes.

Enolase 1, also known as α -enolase or non-neuronal enolase (NNE), is an isoenzyme of enolase, which is a glycolytic enzyme catalyzing the conversion of 2-phosphoglycerate into phosphoenolpyruvate. Recent researches have shown that enolase 1 plays an important role in several biological and pathophysiological processes. Enolase 1 is thought to play a potential role in tumorigenesis, cancer invasion and metastasis. Previous proteomic studies reported that enolase 1 was up-regulated in several cancers, such as hepatocellular carcinoma^[20-22], non-small lung cancer^[23,24], esophageal adenocarcinoma^[25], prostate cancer^[26], colon cancer^[27], oral epithelial and squamous cell carcinoma^[28]. In our study, we found a significant up-regulation of enolase 1 in rat models of DMBA-induced PanIN and PC with further verification by the immunohistochemical analysis of human and rat tissues. These results agree with other proteomic researches on human PC. Mikuriya *et al*^[29] found that the expression levels of glycolytic enzymes, including enolase 1, increased in the cancerous pancreatic tissues of 10 patients compared with the paired non-cancerous tissues, as determined by proteomic profiling using two-dimensional electrophoresis and liquid chromatography-mass spectrometry/mass spectrometry. Shen *et al*^[30] used a proteomic approach using two-dimensional gel electrophoresis and mass spectrometry to identify differentially expressed proteins in six PC cases, two normal adjacent tissues, seven cases of pancreatitis, and six normal pancreatic tissues. Alpha-enolase was also specifically overexpressed in tumors compared with normal and pancreatic tissues.

The hnRNP A2/B1 protein was shown to be up-regulated in our models. This protein plays an important role in the biogenesis and transport of mRNA. The over-expression of hnRNP A2/B1 indicates that normal transcriptional regulation is altered. A previous study also found high levels of hnRNP A2/B1 expression in a limited number of human pancreatic adenocarcinomas from smokers and two pancreatic tumor cell lines, HPAF-11 and SU 86.86^[31]. CEL is one kind of pancreatic exocrine enzyme, and we found that it was down-regulated after DMBA induction. Reuss *et al*^[32] verified strong CEL gene expression in the acinar cells of the normal pancreas, and adenocarcinomas showed no expression, which agrees with our results. The other differentially expressed proteins that we identified are rarely reported in PC, and their relationships with PC await further clarification.

In summary, our data have shown DMBA implantation into the pancreas is an effective method to establish rat models of PanIN and PC, and the protein changes observed in DMBA-induced PanIN and PC, such as enolase 1 up-regulation, demonstrate the feasibility of

identifying potential molecular targets for the early diagnosis and treatment of PC.

COMMENTS

Background

The early detection of pancreatic cancer (PC) is still difficult, but proteomic techniques are emerging as important tools to find potential biomarkers and new therapeutic targets for PC and precancerous pancreatic lesions.

Research frontiers

7,12-dimethylbenzanthracene (DMBA) implantation into the pancreas is known to induce PC and pancreatic ductal intraepithelial neoplasia (PanIN) in mice and rats that resemble human PC with K-ras gene mutations and the activation of Notch signaling.

Innovations and breakthroughs

Precancerous pancreas lesions and the early stages of PC among clinical diagnoses are rarely found. In the present research, the authors studied the proteomic variabilities of rat models of DMBA-induced PanIN and PC.

Applications

The protein changes in DMBA-induced PanIN and PC reported in this article, such as enolase 1 up-regulation, demonstrate the feasibility of identifying potential molecular targets for early PC diagnostics and therapeutics.

Terminology

Two-dimensional fluorescence difference gel electrophoresis is a gel-based technique that has recently been used for protein separation. It contains three samples labeled with three distinct fluorescent dyes: Cy2, Cy3 and Cy5.

Peer review

This is a good study to define the stages of pancreatic carcinoma in experimental models and its correlation with various markers.

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Serial observations on an orthotopic gastric cancer model constructed using improved implantation technique

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Abstract

AIM: To establish a gastric cancer nude-mouse model with improved orthotopic implantation and investigate its biological characteristics at different time points.

METHODS: Human gastric cancer SGC-7901 cell suspensions were injected subcutaneously into a nude mouse to develop solid tumors, and the tumor tissue pieces were implanted under the serous coat. The nude mice were then euthanized in group every two weeks to observe the primary tumor growth and metastases.

RESULTS: Within 2-4 wk, there were no obvious changes

about the primary tumor in stomach. At the sixth week, the primary tumor began to grow fast, resulting in incrasation of the gastric wall and stenosis of the gastric cavity, and metastases into the liver and lymph nodes were detected. The tumor, which compressed the adjacent organs, gradually became bigger and bigger followed by stenosis or vanishment of the gastric cavity from 8 to 12 wk. There were massive metastases, and the rate of metastasis was 58% in lymph nodes, 78% in liver, 39% in kidney, and 81% in peritoneum or septum.

CONCLUSION: A gastric cancer model is established, which can simulate the clinical tumor behavior and provide experimental carrier for clinical trials of gastric cancer treatment.

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Key words: Gastric cancer; Orthotopic implantation; Mouse model; Metastasis; Cell line

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INTRODUCTION

Metastasis is not only still the barrier to tumor therapy, but also linked with the prognosis of patients. Patients with gastric cancer hardly have symptoms in their early stage

so that they are always found with metastases after clinical examinations. Therefore, novel tumor models are needed to study the metastasis mechanism as well as therapeutic approaches. It is well known that orthotopic transplantation technique has gain popularity in the field of the animal model^[1-12]. Orthotopic tumor models not only mimic clinical cancer course, but also promote metastasis^[13]. In recent years, the procedure of orthotopic implantation has been improved from the “sewing” method to the “adhering” one^[2-4,14]. The progress greatly shortens the course of surgical operation and decreases the mortality of animals. To our knowledge, gastric cancer models of orthotopic implantation with intact tumor tissue have been well established^[1-3,5-8]. However, serial examination of tumor development and metastasis needs to be made in order to tailor the treatment strategies. Based on the previous researches, we intend to establish an orthotopic model of gastric cancer using tissue glue and observe tumor progress and metastasis consecutively to detect an appropriate therapeutic target for clinical trials.

MATERIALS AND METHODS

Cell line

Human gastric cancer cell lines SGC-7901 (poorly differentiated) was used for this study. The cell was obtained from the Centre of Cell Cultures of Chinese Academy of Medical Sciences, Shanghai, China, and cultured at 37°C in a humidified air with 5% CO₂ in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate.

Animals

Five to six-week-old male Balb/c nu-nu mice weighing 18-20 g were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Medical Sciences, China. All mice were maintained in a pathogen-free environment (temperature 25-27°C, humidity 45%-50%) and supplied with food and water *ad libitum*. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Chongqing Medical University, China, and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Orthotopic implantation in stomach

SGC-7901 cells were collected at the log phase and injected subcutaneously into the mice at 10⁷/0.2 mL. Two weeks later, tumors (about 2.0 cm × 2.0 cm × 1.0 cm) were harvested from the mice under anesthesia and minced into small pieces (1mm³) in RPMI-1640 basal medium. All procedures were then performed under anesthesia with Sumianxin II (0.02 mL per animal; China Academy of Military Medical Sciences). For implantation, the mouse stomach was gently exteriorized *via* a left-side upper abdominal incision, and one small tissue pocket was formed in the

middle wall of the greater curvature using a microscissor. One tumor fragment was placed into the pocket and fixed with a drop of medical tissue glue (gifts from Shunkang Corporation of Biological Adhesive, Beijing, China). The quantity of the tissue adhesive should be strictly controlled to avoid adhering to adjacent normal tissues. The stomach was then relocated into the abdominal cavity followed by the abdominal closure with 4-0 absorbable sutures.

Evaluation of tumor growth and metastasis

All mice were divided into 6 groups of 6 animals each after orthotopic implantation. One group was sacrificed every two weeks. At autopsy, the primary tumor, lymph nodes, and other organs were examined in detail. The samples were fixed in 10% formalin for paraffin sections, and stained with hematoxylin and eosin for microscopic examination.

Immunohistochemistry

Paraffin sections were examined histologically with the Cytokeratin 20 (CK20) mAb KS20.8 and Epithelial Membrane Antigen (EMA) mAb GP1.4 (MAB-0057, MAB-0061; Maixin Inc., Fuzhou, China), which are usually combined to diagnose the gastrointestinal adenocarcinoma. Ultrasensitive streptavidin-peroxidase (SP) kit (KIT-9710; Maixin Inc., Fuzhou, China) and DAB kit (DAB-0031; Maixin Inc., Fuzhou, China) were used in the immunohistochemical stain according to the manufacture's instructions. CK20 and EMA expression was defined as positive if the stained region of tumor cells was in the cytoplasm.

Assessment of primary tumor growth

The primary tumor volume was calculated by the following formula: $V = 0.4 \times ab^2$ (a: maximum diameter; b: minimum diameter)^[15]. Then the tumor growth curve was depicted reflecting the tumor growth trend.

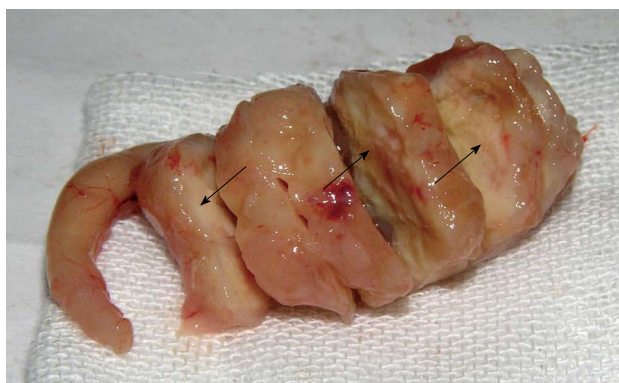
RESULTS

Macroscopic examination

From 2 to 4 wk, the primary tumor developed without obvious volume change, and showed insufficient blood supply. The gastric cavity of tumor-bearing mice appeared almost normal in size. At the 2nd wk after orthotopic implantation, metastatic infiltration was not found in all detected organs. However, tumor invasion into the liver was found at the 4th wk. Six weeks after transplantation, the tumor *in situ* grew increasingly in size, with sparse blood vessels. The stomach wall of mice was thickened and gastric cavity decreased, accompanied with enlargement of lymph nodes from gastric area and hilus pulmonis. The tumor invaded into the liver, resulting in metastatic nodules formation. At the 8th wk, the stomach tumor was characterized by large-size, irregular volume and rich blood supply. The resulting tumor squeezed the adjacent organs and deformed the stomach contributing to distal stenosis. The lymph nodes of the gastric area, hilus pulmonis and mesenterium presented enlargement caused by tumor metastasis. Metastatic lesions were

Table 1 Macroscopic observation on SGC-7901 gastric cancer model at different time points

Items	2 wk	4 wk	6 wk	8 wk	10 wk/12 wk
Primary tumor					
Shape	Oval	Oval	Globular	Irregularly lobular	Irregularly lobular
Color	Gray-white	Gray-white	Gray-white	Grayish yellow	Grayish yellow
Texture	Stiff	Stiff	Stiff	Stiff	Stiff
Vascularity	Sparse	Sparse	Rich	Richer	Richer
Section of stomach tumor					
Gastric cavity	No change	Mildly stenosis	Obvious stenosis	Obvious stenosis	Narrow or vanished
Greater and lesser curvature	Distinguishable	Distinguishable	Distinguishable	Distinguishable	Undistinguishable
Pylorochesis	No	No	Partly	Partly	Totally
Effects on adjacent organs					
Abdominal cavity	No effects	No effects	No effects	Occupying upper abdomen	Occupying whole abdomen
Adhering extent	Adhering to liver lobes	Adhering to liver lobes	Adhering to liver lobes	Adhering to liver lobes or posterior abdominal wall	Adhering to liver lobes or posterior abdominal wall
Compressed status	No compression	No compression	No compression	Partly oppressed	Partly oppressed
Ascites					
Liquid quantity	Zero	Zero	Zero	Little	Little
Property	-	-	-	Clear and yellowish	Clear and yellowish
Metastasis					
Lymph nodes	No	No	Yes	Yes	Yes
Other organs	No	Yes	Yes	Yes	Yes

**Figure 1** Macroscopic examination of the primary tumor in SGC-7901 model. The gastric cavity occupied by primary tumor was found vanished, and pylorus was obstructed totally (arrows).

found in the organs such as liver, kidney, peritoneum and diaphragm. From 10 to 12 wk, the tumor grew fast and huge, occupying almost the upper abdomen and squeezing severely the surrounding organs. The necrosis from central tumor areas was macroscopically visible, indicating that the tumor outgrew the sufficient blood supply. In most mice, the gastric cavity became narrow, even vanished, and pylorus was obstructed partly or totally (Figure 1). The stomach of some mice was so severely disfigured that the greater and lesser curvature failed to be distinguished. Enlargement of many lymph nodes was visible in the gastric area, hilus pulmonis and mesenterium. The metastatic infiltration into the liver and kidney was always detected, with characteristics of grey-white neoplastic nodules. At autopsy, a little clear and yellowish ascites was found (Table 1).

Histology

Tumor cells spread with schistose, nest-like, or cable-like structure, with characteristics of nuclear polymorphism,

nuclear hyperchromatism, much red nucleolus, and rich pathological karyokinesis. Mucus secreted in the cytoplasm, which resulted in mucus lake, was visible in part of tumor cells (Figure 2A). Glandular differentiation and rich vascularity were present in tumor areas. The stomach tumor invaded the gastric wall following the disruption of the integrity of the mucous layer or muscularis mucosae (Figure 2B). The lymph nodes were infiltrated and destroyed by metastatic tumor, characterized by the narrowness or diminishment of the lymph sinus (Figure 2C). Metastases were also detected in the liver (Figure 2D) and the kidney (Figure 2E), occasionally in the paranephros or pancreas (not shown). Metastatic lesions were always separated from adjacent normal tissues by fibrous integument, and lymphoid infiltration was found in the peripheral tumor areas. Tumor metastasized to the lung and surrounded bronchia or bronchiole (Figure 2F). Smear of cast-off cells from ascites confirmed the malignant cells from the primary adenocarcinoma (Figure 2G).

Immunohistochemistry

All primary tumors and metastases from other organs by immunohistochemical staining showed the positive expression of CK-20 (Figure 3A and B) and EMA (Figure 4A-C) protein.

Varying volume of primary tumor

The tumor volume gradually increased from the 6th wk after implantation, and reached a peak when nude mice died at the 12th wk (Figure 5). From 10 to 12 wk, the model mice declined in their general conditions, accompanied by cachexia and ascites.

Incidence of metastasis

After all mice were sacrificed, the metastasis incidence of involved organs was analyzed and the following results were obtained: lymph nodes 58% (21/36), liver

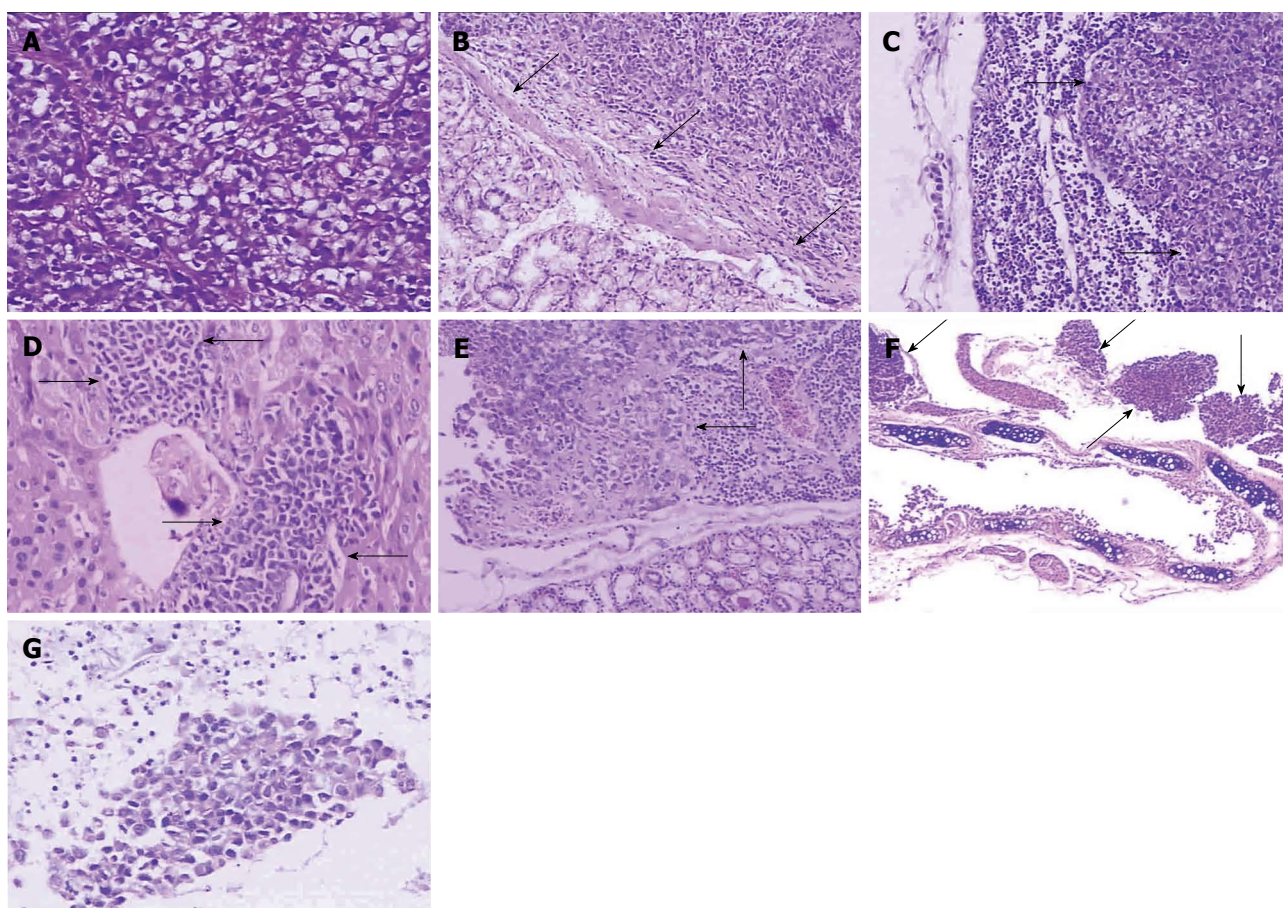


Figure 2 Histopathologic structure of SGC-7901 tumors by HE staining. A: Tumor cells showed nuclear polymorphism, nuclear hyperchromatism, and much mucus in the cytoplasm ($\times 200$); B: The primary tumor destroyed muscularis mucosae (arrows) ($\times 100$); C: The lymph nodes were infiltrated by metastatic tumor (arrows) ($\times 100$); D: Metastases (arrows) infiltrated into the liver ($\times 200$); E: Metastatic tumor (arrows) invaded the kidney ($\times 100$); F: Tumor (arrows) metastasized to the lung and surrounded bronchia or bronchiole ($\times 40$); G: Cast-off tumor cells were detected in ascites ($\times 200$).

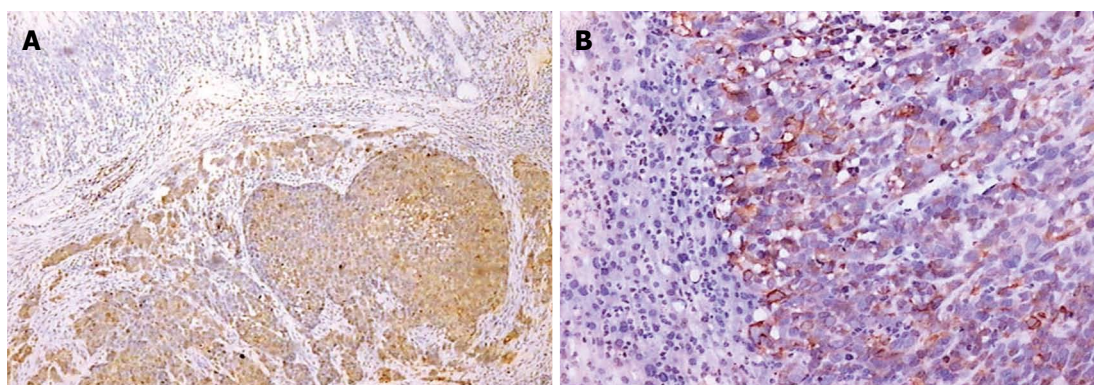


Figure 3 Immunohistochemistry of SGC-7901 tumor by streptavidin-peroxidase method. Expression of Cytochrome 20 (CK20) protein was positive, which was stained brown in the cytoplasm in the tumor of stomach (A $\times 40$) and liver (B $\times 100$).

78% (28/36), kidney 39% (14/36), and peritoneum and diaphragm 81% (29/36).

DISCUSSION

There are little proofs supporting serial observations on gastric cancer models although many researchers have constructed orthotopic models using intact tumor tissues. This makes it difficult to learn the whole process of

tumor growth and metastasis occurrence. In this study, we presented in detail such evidences as the process of primary tumor growth, the time point of metastasis, and the sites and incidence of metastasis.

The patients with gastric cancer hardly have symptoms in their early stage, whereas they are always tortured by gastrointestinal symptom and metastasis in their terminal stage. In the present study, an animal model of stomach cancer was established to replicate the tumor

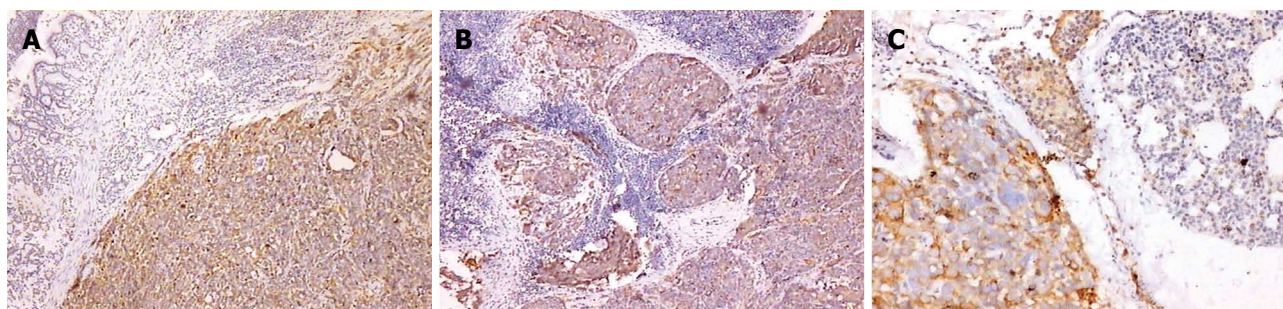


Figure 4 Immunostaining of SGC-7901 tumor using streptavidin-peroxidase method. Epithelial membrane antigen signal stained brown in the cytoplasm was positive in the tumor of stomach (A × 40), lymph node (B × 40) and lung (C × 100).

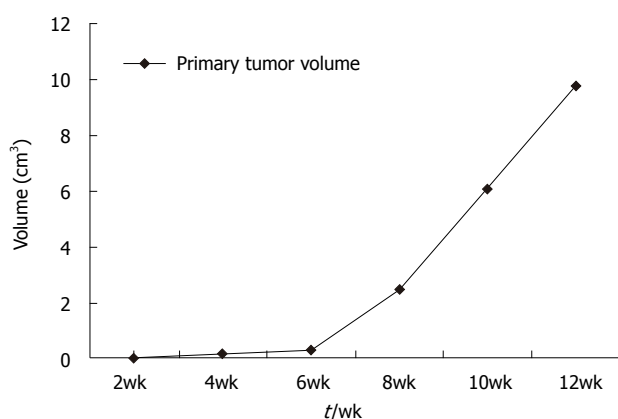


Figure 5 The curve of tumor growth at different time points. The tumor volume gradually increased from the 6th wk after implantation, and reached a peak at the 12th wk.

behavior of clinical patients. It showed that the primary tumor grew into the log phrase from the 6th wk after implantation and thereafter the tumor volume gradually increased. From the 6th wk, lymph node metastases and distant metastases were detected, accompanied by the increase of involved organs with the tumor progress. The tested mice revealed obvious cachexia from the 10th wk, and systemic failure at the 12th wk or so.

There are several pathways such as direct infiltration, lymphatic metastasis, vascular spread, and implantation dissemination which play important roles in metastasis of gastric cancer. Previous studies recapitulated the metastatic incidence of systematic organs, but did not mention the metastatic pathways of gastric cancer models. Moreover, there are uneven metastasis rates among different laboratories resulting from different experimental conditions, animal species and cell lines^[1,2,3,10,11,14]. The SGC-7901 cell line used in our study was derived from metastatic lymph node of poorly differentiated human gastric carcinoma. We could detect the metastatic lesions in the lymph nodes from gastroepiploic plexus, hilus pulmonis and mesentery, which accorded with the feature of this cell line. It is well known that liver is the most sensitive organ to vascular metastasis. In the present study, we found that the liver was not only invaded by the neoplastic cells from blood pathway but also directly infiltrated by the primary tumor. In addition, the fringe of kidney also showed metastatic

spread, yet only under renal capsule rather than in parenchyma. Hereby, we consider that metastatic tumor may float in the ascites by breaking through the stomach serous coat and then invade straightly the kidney, or metastasize to the lymphatic system of renal capsule *via* lymphatic route, instead of invading kidney through blood vessel.

The model constructed in our study could simulate the clinical tumor behavior and provide basic theory on gastric cancer models. However, orthotopic implantation models still have some limitations such as failure in clinical staging. It is well known that gastric cancer stage (early, median, late) is judged by tumor growth and infiltration and metastasis rather than disease course, i.e. TNM staging published by Union for International Cancer Control (UICC). The morphological changes of gastric carcinogenesis are as follows: diffuse hyperplasia → focal proliferation and metaplasia → benign tumor → grade I, II or III dysplasia → carcinoma in situ → invasive carcinoma, which reveals that the pathological changes of gastric cancer originate from mucous layer and progress layer-by-layer towards serous coat^[16]. However, orthotopic implantation was performed with tumor fragments under the stomach serous coat, which is unable to mimic the histopathological mechanism of gastric cancer. In fact, the procedure of orthotopic implantation mimics the artificial advanced stage which is characterized by tumor growth from serous coat to mucous layer or outside membrane serosa. The histological origin of primary tumor was not consistent with the principle of TMN stage although lymph node metastasis or distant dissemination was also detected. So the orthotopic model of gastric cancer is not suitable for clinical staging.

Some researchers had established the models of stomach cancer with chemical carcinogens which could simulate the histological origin of gastric carcinoma^[16-20]. But there are some disadvantages such as long period and accidental animal death. Some authors employed transgenic techniques in attempt to replicate the mechanism of gastric cancer^[18,21,22]. However, transgenic method is limited due to expensive cost, long period and advanced technical requirements for molecular biology. Compared with other methods for constructing gastric cancer model, the surgical orthotopic implantation is still a desired technique characteristic of short period, low cost and simple practice.

Some experimental skills for this animal model should

be noticed. For example, necrotic area should be removed from tumor tissues used for orthotopic implantation; the quantity of medical glue should be strictly controlled to avoid adhering to adjacent organs; and the middle part of the greater gastric curvature is the first choice for orthotopic implantation because of the operational convenience and rich blood supply.

In conclusion, we can succeed in establishing a gastric cancer model if only we master the experimental skills above and avoid the adverse factors.

COMMENTS

Background

Metastasis is still the barrier to the treatment of patients with gastric cancer. Gastric cancer models of orthotopic implantation with intact tumor tissues have been well established. Moreover, the procedure of orthotopic implantation has been improved from the "sewing" method to the "adhering" one. However, there are little evidences regarding serial observations on gastric cancer models to tailor treatment strategies.

Research frontiers

It is well known that orthotopic transplantation technique plays an important role in establishing animal models of various tumors. Gastric cancer model of orthotopic implantation could mimic clinical cancer process and contribute to metastasis occurrence. The procedure greatly facilitates surgical operation course and decreases the mortality of animals.

Innovations and breakthroughs

Orthotopic implantation with "glue paste technique" is a popular and new method in recent years which is characterized by easy performance, low cost and short operation course. This study makes it possible to help researchers learn the whole process of tumor development and metastasis, thus choosing the appropriate therapy target for clinical trials.

Applications

Serial observations on the models can provide evidences for researchers to treat stomach cancer and test the therapeutic effects.

Peer review

The study is interesting and well done. The model used by the Authors well mimics the dynamics of local growth and spread of gastric cancer cells at both lymph-nodes and distant organs.

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How we can improve patients' comfort after Milligan-Morgan open haemorrhoidectomy

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Abstract

AIM: To demonstrate the value of Diosmin (flavonoidic fraction) in the management of post-haemorrhoidectomy symptoms.

METHODS: Eighty-six consecutive patients with grades III and IV acute mixed hemorrhoids admitted to the

Anorectal Surgical Department of First Affiliated Hospital, Xinjiang Medical University from April 2009 to April 2010, were enrolled in this study. An observer-blinded, randomized trial was conducted to compare post-haemorrhoidectomy symptoms with use of Diosmin flavonoidic fraction *vs* placebo. Eighty-six patients were randomly allocated to receive Diosmin flavonoidic fraction 500 mg for 1 wk ($n = 43$) or placebo ($n = 43$). The Milligan-Morgan open haemorrhoidectomy was performed by a standardized diathermy excision method. Pain, bleeding, heaviness, pruritus, wound edema and mucosal discharge were observed after surgery. The postoperative symptoms and hospitalization time were recorded.

RESULTS: The mean age of the Diosmin group and controls was 53.2 and 51.3 years, respectively. In Diosmin group, haemorrhoid piles were of the third degree in 33 patients and the fourth degree in 10; and in the control group, 29 were of the third degree and 14 were of the fourth degree. There was no statistically significance in age, gender distribution, degree and number of excised haemorrhoid piles, and the mean duration of haemorrhoidal disease between the two groups. There was a statistically significant improvement in pain, heaviness, bleeding, pruritus from baseline to the 8th week after operation ($P < 0.05$). Patients taking Diosmin had a shorter hospitalization stay after surgery ($P < 0.05$). There was also a significant improvement on the proctoscopic appearance ($P < 0.001$). However, there was no statistical difference between the two groups in terms of wound mucosal discharge. Two patients experienced minor bleeding at the 8th week in Diosmin group, and underwent surgery.

CONCLUSION: Diosmin is effective in alleviating post-operational symptoms of haemorrhoids. Therefore, it should be considered for the initial treatment after haemorrhoid surgery. However, further prospective randomized trials are needed to confirm the findings of this study.

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Key words: Flavonoidic fraction; Postoperative complication; Haemorrhoids

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INTRODUCTION

Hemorrhoid is one of the most common anorectal disorders. Although haemorrhoidectomy is considered as a minor inpatient procedure, it is usually associated with significant postoperative complications, including pain, bleeding, heaviness, pruritus, mucosal discharge and anal stenosis, resulting in a protracted period of recovery. The Milligan-Morgan open haemorrhoidectomy is the most widely practiced surgical approach for the management of hemorrhoids and is considered the "gold standard". Hemorrhoids are divided into 4 stages depending on symptoms and degree of prolapse. The 3rd and 4th stages are indicated for Milligan-Morgan open haemorrhoidectomy. Hemorrhoidectomy is usually associated with considerable pain, bleeding, and mucosal discharge after operation^[1], which seem to be multifactorial, such as individual tolerance, mode of anesthesia, postoperative analgesics, and surgical technique^[2].

Pain is a major postoperative complication after haemorrhoidectomy. Although Longo's procedure (the procedure for prolapse and hemorrhoids, PPH) has been widely used in recent years, it can also be confronted with the postoperative management dilemma after the procedure. A Meta-analysis comparing the PPH procedures and open haemorrhoidectomy did not show any significant differences in terms of post-operative pain. Although the post-operative bleeding and blood loss were significantly lower in the PPH group, there was no statistical difference in the aspect of other complications such as pain, pruritus, and mucosal discharge. In identifying approaches to reduce the symptoms after haemorrhoidectomy, published studies have mainly focused on the choice of surgical technique or the prevention of secondary infection in the wound^[3-8]. The superiority of stapled haemorrhoidectomy in terms of less post-operative pain and quicker recovery was confirmed by a more recent systematic review of 25 randomized trials that compared stapled haemorrhoidectomy and conventional haemorrhoidectomy^[9]. However, the control

of hemorrhoid symptoms is not striking. Both open and closed haemorrhoidectomy have been evaluated in terms of postoperative pain. Two predominant factors responsible for post-operative pain include discomfort from the surgical wound in the sensitive anoderm as well as perianal skin and edema from tissue inflammation around the wound^[10]. Alleviation of pain from the surgical incision should be achieved by minimizing tissue dissection and using different electrosurgical devices, such as diathermy, Harmonic scalpel[®], and ligature[™], which diminishes thermal injury to the subjacent tissues^[11]. For reduction of pain from the open wound of haemorrhoidectomy, various kinds of medication, including metronidazole, glyceryl trinitrate (0.2%), steroids, local anesthetics (bupivacaine), anti-inflammatory drugs, hemorrhoid creams, are being used with variable outcome^[11-13]. These studies indicated some limitations with these medications such as short duration of action and occurrence of serious side effects.

Postoperative bleeding is another important complication in hemorrhoids due to its frequency, which varies between 0.6% and 10%^[14,15]. Post-haemorrhoidectomy bleeding is commonly associated with the passage of a hard stool. Sometimes bleeding may be alarming, because it may cause anemia very rapidly in patients. The causes of post-operative bleeding are not easily explained: in some cases it should be attributed to falling off of a scar due to electrocoagulation, whereas in other cases it is due to the lack of a thrombus, its expulsion or its dissolution, concomitant with the falling or reabsorption of the transfixed stitch.

Diosmin, flavonoidic fraction, which is derived from some plants or the *flavonoid Hesperidin*, is promoted as a high-quality active ingredient in vein improvement supplements. Diosmin reduces inflammation and increases vein tonicity, two important factors that contribute to hemorrhoids. Researches indicate that Diosmin also appears to significantly shorten the duration of hemorrhoid bleeding as well as reduce the postoperative pain^[16]. A 2000 Italian study of 66 hemorrhoid patients reported that Diosmin decreased pain by 79% and bleeding by 67% during the first week of treatment, followed by an astonishing 98% and 86% reduction in these symptoms by the second week^[17]. After hemorrhoid surgery, flavonoids were found to relieve pain, bleeding and other symptoms more rapidly than standard antibiotic/anti-inflammatory treatment alone, with especially significant symptom relief during the first 3 d after surgery^[18].

This study was designed to evaluate the influence of Diosmin on reducing postoperative pain, bleeding, heaviness, pruritus, and mucosal discharge after the Milligan-Morgan open haemorrhoidectomy in a randomized, observer-blinded, placebo-controlled clinical trial.

MATERIALS AND METHODS

Study design

Protocol synopsis for this trial and supporting CONSORT checklist were used as supporting information

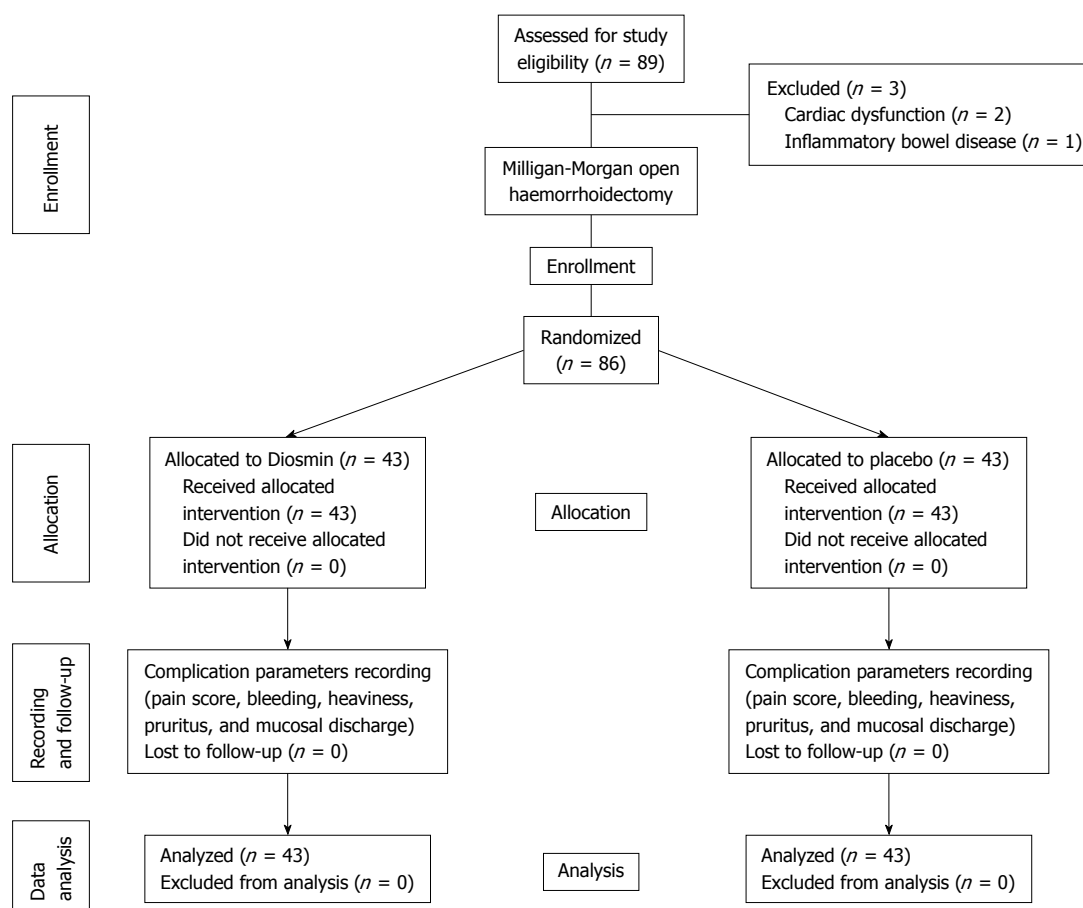


Table 1 Demographic data in Diosmin and control groups

	Diosmin group ¹ (n = 43)	Control group ² (n = 43)	P value
Mean age(yr)	53.2 (11.2)	51.3 (10.4)	0.6332
Male/female ratio	26/17	24/19	0.6620
No. of resected hemorrhoids	3.4 ± 0.4	3.8 ± 0.5	0.1522
Grades III/IV hemorrhoids	33/10	29/14	0.9247
Mean duration of hemorrhoidal disease (mo)	21.6 (12.6)	22.8 (14.5)	0.6821
Operating time (min)	15.8 (1.9)	16.9 (1.5)	0.1295
No. of constipation	28	26	0.8235

¹Patients treated with Diosmin; ²Patients treated with placebo drug.

(Figure 1). Diosmin clinical trial was a phase II randomized, prospective, observer-blinded, placebo-controlled clinical trial.

Inclusion/exclusion criteria

Patients aged 12-75 years with an indication for haemorrhoidectomy were eligible for the study, provided that they met the following inclusion criteria: symptomatic and prolapsing hemorrhoids Grade III or IV and informed consent. Patients complicated with fistula or anal fissure, inflammatory bowel disease, dermatitis, proctitis, pregnancy or severe cardiovascular state or pulmonary complication were excluded from the study.

Patients

The study was conducted using a computer-randomized design. A total of 86 consecutive patients with the grades III and IV acute mixed hemorrhoids admitted to Anorectal Surgical Department of First Affiliated Hospital, Xinjiang Medical University from April 2009 to April 2010, were enrolled in this study. Demographic data (age and gender), disease grades, preoperative constipation status, mean duration of disease, operating time and number of resected piles were recorded for each patient. There were no statistical differences between the two groups in these aspects (Table 1).

Medical and research ethics

The trial was conducted in accordance with the principles of the Declaration of Helsinki^[19], the Guidelines for Good Clinical Practice (GCP) for Trials on Pharmaceutical Products^[20]. The study protocol was approved by China governmental law and local regulations of Xinjiang Uygur Autonomous Region. This study was also conducted according to a protocol approved by the Medical Ethics Committee of First Affiliated Hospital of Xinjiang Medical University. Informed consent was obtained from all the patients or their relatives before the trial. The whole study consisted of two periods of observation (4 wk for each period). The last visit should be terminated in 90 d after operation.

Formal written informed consent was obtained from each patient after the preliminary assessment of patient's detailed history of the disease and general and systemic examination. The patients were subjected to a few baseline investigations (haemoglobin, bleeding time, clotting time, urine complete examination). They were randomly subjected to Diosmin or placebo depending on their choice, after discussing the advantages and disadvantages of both drugs with them. The study was "blind" and the observers evaluating the complication symptom parameters were unaware of the individual treatment schedules. Blinding and coding of the drugs were done by an independent monitor who was not an investigator after repacking the look-alike capsules by a pharmaceutical company in Xinjiang. The codes were broken only after completion of the study.

Surgery

Two fixed anorectal surgeons performed all the procedures with the patient in the supine lithotomy position or jackknife position. All patients underwent proctoscopy in order to exclude other diseases in the rectum before surgery. The operations were carried out under spinal anesthesia with 15 mL bupivacaine with 1:200 000 adrenalin. Further 5 mL of the same solution was used to dissect the haemorrhoidal nodules from the internal sphincter. Except 3 patients, who had a considerable rectal mucosal prolapse and were treated with stapled haemorrhoidopexy, all the patients underwent the standard Milligan-Morgan haemorrhoidectomy.

We removed the haemorrhoidal nodule using an upside-down V-shaped incision on the anal dermis, without widening the surgical wound while approaching the sphincters. This was done in order to maintain ample mucous membrane bridges. Possible secondary nodules were removed through submucosa. Ligature of the vascular pedicle was performed clear of the internal sphincter. The extent of surgical incision was tailored according to the number of haemorrhoidal complexes. Hemorrhoids were excised to the anorectal junction or dentate line. The intervening skin and anoderm bridges were preserved adequately. Coagulation with electrotome on the anal sphincters was avoided for all patients. The edges of the residual surgical wound have to be as sharp as possible. No packs were left in the anus postoperatively. After operation, patients in both groups were prescribed fiber supplements and naproxen sodium 550 mg tablets or intramuscular pethidine (1 mg/kg body weight) as required. All patients were also advised to gently shower their perianal wounds with lukewarm water twice daily, and after bowel movements. The data concerning the complications were compared between the two groups of patients. No antibiotic prophylaxis or any kind of analgesics has ever been administered.

Randomization

Computer-based sequential method was used for the randomization at the completion of surgery into one of the

two groups. This computer generated random codes used for envelopes containing the information "Diosmin" or "Placebo". These envelopes were prepared by a statistician who was not involved the patient's treatment or other work specific to the study. The computer randomization was completed in the Medical Statistical Center of Xinjiang Medical University.

Diosmin treatment

All patients were routinely discharged on the first postoperative day unless otherwise clinically indicated. Eighty-six patients each were either given Diosmin 500 mg or received placebo medication according to the computer-randomized result. Diosmin 500 mg was given at a dose of 3 tablets twice daily, after meals, for 3 d followed by 2 tablets twice daily from day 4 to day 7. Each complication symptom was recorded at hours 6 and 12 and on days 1, 2, 7 and 14 after operation. On the 7th day, the symptoms and any relief were recorded and the dose was further reduced to one tablet twice daily for the next 15 d. Consequent follow-ups were made on days 15, 30 and 90.

Assessment of postoperative symptoms

The Milligan-Morgan open haemorrhoidectomy was performed by a standardized diathermy excision method. Diosmin was started on the 6th day after surgery. A standardized questionnaire was completed which included postoperative information about pain, bleeding, heaviness, pruritus, wound edema and mucosal discharge. The evolution of these symptoms during the postoperative period was assessed by means of patient's self-questionnaires. Two predominant observatory parameters were postoperative pain and bleeding. Pain was assessed using verbal response and visual analog scale at hours 6 and 12 and on days 1, 2, 7 and 14, respectively after operation. The verbal response scales had four options: no pain, mild pain, moderate pain, and severe pain. The visual analogue scale consisted of a 10-cm line with the words "no pain" on the left hand side and "worst pain imaginable" on the right. Two types of pain were assessed, pain on defecation and pain during the preceding 24 h. The scales for pain on defecation were completed immediately after defecation and the scales for 24 h pain completed each evening. In order that pain could be assessed for seven postoperative days, patients were asked to complete the forms at hospital. Patients were discharged from hospital at the discretion of their consultants.

The use of narcotic drugs, antibiotics and laxatives, complication symptoms and hospital stay in all the patients were recorded after surgery. At the conclusion of the study, the codes were broken and the results were analyzed. The visual analogue scores were measured in cm, and the score for each 24 h was a single value. The score for pain on defecation was the mean value of scores during that day.

Statistical analysis

Before initiating the trial, sample size was calculated us-

Table 2 Postoperative course of Diosmin and control groups

	Diosmin group ¹ (<i>n</i> = 43)	Control group ² (<i>n</i> = 43)	<i>P</i> value
Median hospital stay (d)	6.1 (1.3)	7.3 (3.4)	0.0306
Median time to first bowel action (h)	48 (4.2)	56 (4.6)	0
Median No. of bowel actions in the first week	9 (2.3)	14 (3.1)	0

¹Patients treated with Diosmin; ²Patients treated with placebo drug. *P* value was the result of Mann-Whitney *U* test.

Table 3 Postoperative symptoms of Diosmin and control groups *n* (%)

	Diosmin group ¹ (<i>n</i> = 43)	Control group ² (<i>n</i> = 43)	<i>P</i> value
Minor bleeding			
At 2 wk	3 (6.97)	11 (25.58)	0.0409
At 8 wk	2 (4.65)	3 (4.64)	0.6449
Heaviness			
At 2 wk	4 (9.30)	14 (32.56)	0.0171
At 8 wk	2 (4.65)	8 (18.60)	0.0436
Pruritus			
At 2 wk	9 (20.93)	18 (41.86)	0.0365
At 8 wk	3 (6.97)	10 (23.25)	0.0351
Mucosal discharge			
At 2 wk	7 (16.28)	11 (25.58)	0.2890
At 8 wk	2 (4.64)	4 (9.30)	0.3972

¹Patients treated with Diosmin; ²Patients treated with placebo drug.

ing SPSS software 15.0 version. A power calculation estimated that 40 patients would be needed in each group to demonstrate a reduction of 20% pain with a power of 80% and at a 5% significance level. Discrete variables were analyzed using χ^2 test with Yates correction when appropriate. Continuous variables were analyzed by Wilcoxon signed tests for paired observations. Pain scores at each time interval were compared between groups with Wilcoxon's rank-sum test (nonparametric analysis of ranked data). A two-tailed Spearman's correlation coefficient was calculated where indicated. Statistical significance was assumed when $P < 0.05$. Statistical evaluation was done as intend-to-treat analysis. When not otherwise specified, data were presented as median and range.

RESULTS

After standard hemorrhoid surgery, 86 patients were allocated to receive Diosmin (experimental group, *n* = 43) and placebo capsules (control group, *n* = 43). None of the patients in either study group complained of any severe symptoms during the 90-d follow-up after treatment. The two groups were well matched for age, sex, disease grades, and number of piles. There were no statistical differences between the two groups in these aspects (Table 1).

The Diosmin group defecated earlier ($P = 0.00$), had more frequent bowel actions in the first postoperative week ($P = 0.00$), and had a shorter hospital stay ($P = 0.03$)

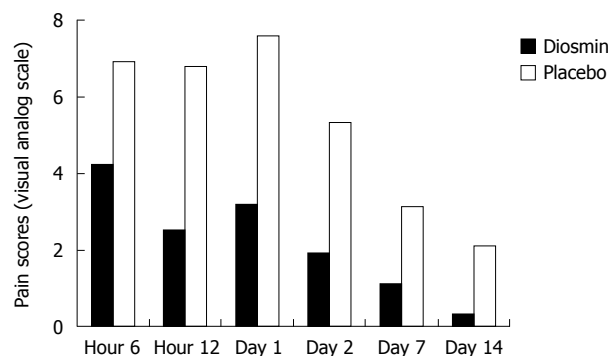


Figure 2 Postoperative pain scores in Diosmin and placebo groups. Pain scores ranged from 0 (no pain) to 10 (very severe pain).

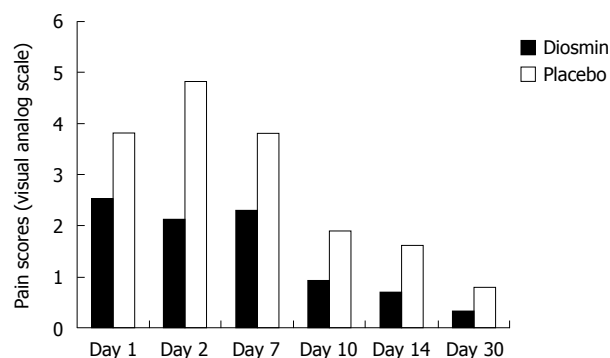


Figure 3 Pain on defecation in Diosmin and placebo groups. Pain scores ranged from 0 (no pain) to 10 (very severe pain).

compared with the placebo group (Table 2). No patient withdrew from the study because of any complaints. Significantly more placebo patients were troubled by minor rectal bleeding at 2 wk, although these rates were similar at 8 wk. The follow-up after 8 wk found that two patients experienced minor bleeding in Diosmin group, and therefore, underwent surgery. There was a statistically significant ($P < 0.05$) improvement in pruritus and heaviness at both 2 and 8 wk (Table 3). In addition, there was no statistical difference between the two groups in terms of wound mucosal discharge.

Patients in Diosmin group experienced significantly less pain at 6 and 12 h after surgery ($P = 0.03$, $P < 0.01$), and on days 1, 2, 7, 10, and 14 ($P = 0.02$, $P < 0.01$, $P = 0.02$, $P = 0.03$, $P < 0.01$) (Figure 2). There was also significant ($P < 0.001$) improvement on the proctoscopic appearance. Patients in the experimental group had significantly less defecation pain compared with the placebo group on days 1, 2, 7, 10, 14, and 30 ($P = 0.03$, $P < 0.01$, $P < 0.01$, $P = 0.02$, $P = 0.04$, $P = 0.04$) (Figure 3). No significant Diosmin-related complications or allergic reactions were reported by any patient. A similar number of patients reported burning with both treatments at the 2nd week after surgery (3 in Diosmin and 2 in placebo).

DISCUSSION

Haemorrhoid is a common disease affecting people of

all ages and both sexes. It is estimated that 50% of the people older than 50 years have hemorrhoid symptoms at least for a period of time. The causes of haemorrhoidal disease are multiple, but most are attributable to difficult passage of stool or constipation. Over the last few years, there has been increasing attention on surgical procedures to treat haemorrhoids. Several comparative studies have been performed to evaluate the available procedures to treat grades II, III, and IV hemorrhoids, and new surgical techniques, such as haemorrhoidectomy with Harmonic scalpel[®][21-23] and LigasureTM[24], Doppler-guided haemorrhoidal plexus ligation^[25,26] and the stapled haemorrhoidopexy^[27-31]. The most recent medium- and long-term studies on ample case series provide data on the efficacy, the results and complications of these techniques^[24-31]. None of them proved to reduce complications such as pain and bleeding^[21]. The ideal method should combine the high safety and efficacy of the treatment, yielding low postoperative pain and bringing comfort to the patients. Such considerations are related to the severity of the disease and can be addressed by evidence-based medicine by randomized, controlled trial. According to a recent meta-analysis of the Cochrane library^[32,33], conventional haemorrhoidectomy as first described by Milligan and Morgan, is still the most widely used, effective, and definite surgical treatment for patients with symptomatic grades III and IV haemorrhoids. However, it is associated with significant postoperative complications such as pain, bleeding and mucous discharge. Although there is a consensus on the treatment of grades III and IV haemorrhoids, there is still confusion regarding the ideal treatment for these complications after surgery.

In 1971, Daflon, consisting of 90% Diosmin and 10% Hesperidin (Daflon 500; Serdia Pharmaceuticals, India and Vinosmin; Elder Pharmaceuticals, India), was firstly introduced in France by Bensaude *et al*^[34] for the treatment of haemorrhoids and other capillovenous diseases. Diosmin mainly works on the inflammatory pathology of haemorrhoids by increasing the contraction of veins and local lymphatic drainage and decreasing the synthesis of prostaglandins such as PGE2 and thromboxane B2^[35-37]. The anti-inflammatory effects of Diosmin are reflected in the reduction of capillary hyperpermeability and fragility in controlled clinical studies. Damom *et al*^[38] found the same effects of Diosmin in increasing the duration of vascular contraction and prostaglandin components which are responsible for the inflammatory process. Diosmin also increases the local lymphatic drainage. Side-effects of the drugs are limited according to Meyer^[39] who was first used Diosmin to treat hemorrhoid symptoms. He reported mild gastrointestinal and autonomic disturbances in 10% cases. A fixed micronized combination of the citrus bioflavonoids Diosmin (90%) and hesperidin (10%) has been widely used in Europe to treat diseases of the blood vessels and lymphatic system since 2000. The combination also appears to be beneficial for chronic venous insufficiency and venous stasis ulcers. Extensive safety evaluations have found that Diosmin/hesperidin was free

from toxicological risk. From 2005, Diosmin was used to treat vascular diseases^[40-45]. The obtained evidence strongly supports its use in haemorrhoids in recent years although only several randomized controlled trials were available.

In this randomized trial, we concluded that Diosmin leads to the rapid cessation of haemorrhoidal bleeding, alleviation of the associated symptoms and gives objective relief from complications of post-haemorrhoidectomy. This result is similar to Mlakar's study^[46]. In their study, Flavonoids was found very effective in the first 30 d of treatment and led to the rapid relief of various associated symptoms of haemorrhoid surgery. Because, up to now, there have only a few randomized controlled studies to investigate the effectiveness and safety of Diosmin to treat symptoms after haemorrhoidectomy in the world, we could not perform meta-analysis for these studies. In our study, Diosmin was more effective to control postoperative pain than placebo capsules during the early phase of the surgery. This is a highlight in our study. The Diosmin group defecated earlier ($P < 0.05$), had more frequent bowel actions in the first postoperative week ($P < 0.05$), and had a shorter hospital stay ($P < 0.05$) compared with the placebo group. This is and a striking result compared with Mlakar's study^[46]. In spite of some minor bleeding, no patient withdrew from the study because of any kind of adverse events. This may be associated with proper drug usage after surgery, especially in the early phases. Significantly more placebo patients were troubled by minor rectal bleeding at 2 wk, although these rates were similar at 8 wk. However, during the follow-up after 8 wk, we found that two patients had minor bleeding in experimental group, therefore, they underwent surgery. Postoperative bleeding is a particularly important complication in hemorrhoids treatment due to its frequency varying between 0.6% and 10%^[15,47]. Sometimes bleeding may be alarming, because it may cause anemia very rapidly in the patients. Several randomized controlled studies evaluated the use of oral micronized, purified flavonoid fraction in the treatment of haemorrhoidal bleeding. In these studies, bleeding was relieved rapidly, and no complication was reported. This is somewhat conflict with our bleeding cases. However, we used 500 mg Diosmin capsules compared with 450 mg micronized purified flavonoid fraction in Yo YH's study^[16]. The most important reason of our poor result related with bleeding is that we included grades III and IV piles, but Yo YH's study included only grades I, II, and III piles. A similar study of 100 patients reported that acute bleeding had subsided by the third day of treatment in 80% of patients receiving micronized flavonoids, 2 d sooner than in patients receiving a placebo. But, the different points compared with our trial, which were also disadvantages of their studies, were associated with the difference of their study designs. They compared micronized flavonoids medication with hemorrhoid surgery itself. Although we think Milligan-Morgan open haemorrhoidectomy is the most widely practiced "gold standard" surgical approach and the stages III and IV are the clear indication for this procedure, it is not necessary to alter the

indication for hemorrhoid surgery to medication. Another point is the cost of medication. A limitation of the drug is the lack of patient compliance due to the long duration of treatment and the high cost of the drug. The safety of the drug has already been proved but more studies need to be done to see if the total dose of Diosmin can be increased so as to increase the response rate and decrease the duration of postoperative treatment. A decrease in the cost of the drug should also be considered.

Purified flavonoid fraction is a botanical extract from citrus. It exerts its effects on both diseased and intact vasculature, increasing vascular tone, lymphatic drainage, and capillary resistance; it is also assumed to have anti-inflammatory effects and promote wound healing. In another recent randomized controlled trial, postoperative use of micronized, purified flavonoid fraction, in combination with short-term routine antibiotic and anti-inflammatory therapy, reduced both the duration and extent of postoperative symptoms and wound bleeding after haemorrhoidectomy, compared with antibiotic and anti-inflammatory treatment alone^[18].

Postoperative pain is the most important unacceptability which was also our predominant observatory parameter. Post haemorrhoid pain is difficult to assess, though verbal response scales and visual analogue scales are recognized methods. Maxwell concluded that the *t* test is "very robust" when comparing differences between visual analogue scale scores^[48], and we therefore used this method of analysis. On two occasions, the verbal response scale in pain was a day less than the visual analogue scale. This may be because the discrete verbal response scale is less sensitive than the continuous visual analogue scale. Another highlight in our study was that patients in Diosmin group experienced significantly less pain at hours 6 and 12 ($P < 0.05$), and on days 1, 2, 7, 10, and 14 after surgery ($P < 0.05$). At the same time, patients in the experimental group had significantly less defecation pain compared with placebo groups on days 1, 2, 7, 10, 14 and 30 after surgery ($P < 0.05$). The exact cause of pain after haemorrhoidectomy has not yet to be defined. Various factors believed to be responsible for the pain including incarceration of smooth muscle fibers and mucosa in the transfixed vascular pedicle, epithelial denudation of the anal canal, and spasm of the internal sphincter^[3]. Another reason for pain could be the development of linear wounds extending up to the anorectal ring, which appear similar to those of a chronic anal fissure^[18]. Postoperative pain was also associated with bacterial fibrinolysis and defecation stress^[49]. In our study, postoperative pain in the placebo group can be explained by the traction of the nonsensitive sliding haemorrhoidal tissue at the highly sensitive anal skin. The diminished postoperative pain in the Diosmin group might be related to its capillary resistance and diminished tissue edema and anti-inflammatory process. There was significant difference in different postsurgical days and weeks. Based on these results, we suggested that Diosmin has a clear action against anorectal postoperative pain. Therefore, it should be considered

initially for patients presenting with haemorrhoidal symptoms after surgery. In addition, there was also a significant improvement on the proctoscopic appearance ($P < 0.001$).

Although there was a statistically significant improvement in heaviness and pruritus from baseline to the 8th week postoperatively, however, there were no statistical differences between the two groups in terms of wound mucosal discharge ($P < 0.05$). Our hypothesis was that our nonabsorbable suture used for internal mucosa ligation is responsible for this poor result.

In a 12-wk study of 50 pregnant women suffering from acute hemorrhoids, micronized Diosmin/hesperidin therapy was reported to be a "safe, acceptable and effective" treatment, and 66% obtained relief from symptoms within 4 d^[50]. However, we suggest not using Diosmin for pregnant women, considering Diosmin is a new alternative for hemorrhoids.

This study has shown that Diosmin can reduce the complications from haemorrhoidectomy, especially in the early phase. We therefore suggest that this regimen should be a part of the routine postoperative management of patients for haemorrhoidectomy.

In conclusion, Diosmin (flavonoid fraction) has shown to be effective in alleviating symptoms after haemorrhoidal surgery and improving the proctoscopic appearance. Therefore, it should be considered initially for patients presenting with haemorrhoidal symptoms after surgery. However, further prospective randomized trials and longer follow-up are needed to confirm the findings of this study and observe the side effects of this drug.

COMMENTS

Background

Over the past few years, there has been increasing attention on surgical procedures to treat hemorrhoids. The Milligan-Morgan haemorrhoidectomy is still a major surgical approach for hemorrhoids. This study was designed to evaluate the influence of Diosmin on reducing postoperative pain, bleeding, heaviness, pruritus, and mucosal discharge after the Milligan-Morgan open haemorrhoidectomy in a randomized, observer-blinded, placebo-controlled clinical trial.

Research frontiers

Phlebotropic activity, protective effect on the capillaries and the anti-inflammatory effect of Diosmin have been reported in several studies in recent years. More recent clinical studies showed that flavonoid fraction such as Dalfon (phlebotropic agent) can reduce postoperative pain, bleeding and heaviness after haemorrhoidectomy.

Innovations and breakthroughs

This clinical trial has confirmed that Diosmin (flavonoid fraction) can reduce postoperative pain, bleeding and heaviness after Milligan-Morgan open haemorrhoidectomy.

Applications

Diosmin (flavonoid fraction) has shown to be effective in alleviating symptoms after haemorrhoidal surgery and improving the proctoscopic appearance. Therefore, it should be considered initially for patients presenting with haemorrhoidal symptoms after surgery. However, further prospective randomized trials and longer follow-up are needed to confirm the findings of this study and observe the side effects of this drug.

Terminology

Diosmin is derived from some plants or used as a high-quality active ingredient in vein improvement supplements. Diosmin reduces inflammation and increases duration of vascular contraction that contributes to hemorrhoids.

Peer review

The authors engagingly described a pathomechanism of anal pain after haemorrhoidectomy and Diosmin's action. The article is worth publishing.

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Impaired gluconeogenesis in a porcine model of paracetamol induced acute liver failure

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Abstract

AIM: To investigate glucose homeostasis and in particular gluconeogenesis in a large animal model of acute liver failure (ALF).

METHODS: Six pigs with paracetamol induced ALF under general anaesthesia were studied over 25 h. Plasma samples were withdrawn every five hours from a central vein. Three animals were used as controls and were maintained under anaesthesia only. Using ^1H NMR spectroscopy we identified most gluconeogenic amino acids along with lactate and pyruvate in the animal plasma samples.

RESULTS: No significant changes were observed in the concentrations of the amino acids studied in the animals maintained under anaesthesia only. If we look at the ALF animals, we observed a statistically significant rise of lactate ($P < 0.003$) and pyruvate ($P < 0.018$) at the end of the experiments. We also observed statistically significant rises in the concentrations of alanine ($P < 0.002$), glycine ($P < 0.005$), threonine ($P < 0.048$), tyrosine ($P < 0.000$), phenylalanine ($P < 0.000$) and isoleucine ($P < 0.01$). Valine levels decreased significantly ($P < 0.05$).

CONCLUSION: Our pig model of ALF is characterized by an altered gluconeogenetic capacity, an impaired tricarboxylic acid (TCA) cycle and a glycolytic state.

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Key words: Lactate; Pyruvate; Branch chain amino acids; Aromatic amino acids

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INTRODUCTION

Acute liver failure (ALF) is a clinical syndrome defined by massive cell death in the absence of chronic liver disease, resulting in hepatic encephalopathy^[1]. Although brain oedema and brain herniation are common causes

of death in ALF multi organ failure is also common^[2,3]. Previous studies have shown that metabolic pathways are affected in ALF and systemic changes in metabolite levels could be the pathophysiological reason behind multi organ failure in ALF^[4-6].

Orthotopic liver transplantation remains the most widely accepted treatment for ALF^[1,2,7]. Chronic donor shortages however, motivate the search for alternative non-surgical therapies. In that quest animal models of ALF play an important role. Most studies have explored mouse models of ALF which although useful do not adequately address the issue^[4-6]. We have recently developed in our laboratory a porcine model of paracetamol induced ALF^[7]. It is characterized histologically by severe centrilobular necrosis with coagulative necrosis. The animals invariably develop acidosis, hypoglycaemia, coagulopathy and acute renal failure.

Acidosis could be explained by lactate production and energy depletion in the liver of our animal model^[5]. Coagulopathy is due to loss of production of factors of the coagulation cascade namely Factors V and VIII^[8]. Acute renal failure is a characteristic of paracetamol overdose. Often patients develop acute renal failure through toxic injury and require dialysis in that setting without serious liver injury^[9].

The hypoglycaemia observed in our model is a hallmark of disrupted hepatic glucose metabolism. Studies on other animal models of ALF and cirrhosis have shown that, despite a relative glucose homeostasis, there is a decrease in gluconeogenesis and tricarboxylic acid (TCA) cycle coupled with an increase in lactate production^[6,10-12].

We hypothesized that there was a loss of gluconeogenic capacity in our large animal model with an increase in lactate production, an inhibited TCA cycle and a switch to glycolysis during injury to compensate for energy demands.

MATERIALS AND METHODS

Animals

Large white pigs (median body mass 35 kg) were used for this study. Animal experiments were performed in accordance with the Home Office regulations under the Animal (Scientific Procedures) Act 1986 as per Project Licence 60/2389. All animals received humane care and study protocols complied with our institution's guidelines.

Our experimental model was described in detail elsewhere^[7]. Briefly animals were anaesthetized with ketamine and midazolam as induction agents and maintained with isoflurane and nitrous oxide. Animals were hydrated with normal saline and glucose. All animals received similar amounts of glucose. Haemodynamic variables and intracranial pressure were continuously monitored.

Animals were pretreated with phenobarbital 20 mg orally for 5 d to induce cytochrome P450 activity. In six animals intravenous paracetamol was administered while the three animals used as controls were monitored but did not receive any paracetamol. A loading dose of paracetamol was administered by intravenous infusion (0.1875 g/kg) followed by an infusion for 12 h (1.8 mg/kg per min). Ex-

periments lasted 28 h and at that time point any surviving animals were euthanized.

Sample preparation for NMR spectroscopy

Samples were prepared by adding a D₂O solution of (150 μ L) to plasma (600 μ L) thus providing an internal field frequency lock for the spectrometer. As a reference substance 20 μ L of sodium 3-(trimethylsilyl) 2, 2, 3, 3-2H₄-1 propionate (TSP) were added to the plasma. Chemical shifts were referenced internally to the singlet methyl resonance of TSP at zero ppm.

The following potentially gluconeogenic amino acids were quantified by NMR: leucine, isoleucine, valine, tyrosine, phenylalanine, histidine, methionine, alanine, threonine, glutamate and glutamine. Lactate, pyruvate and the gluconeogenic amino acids were measured to provide information on glycolysis and gluconeogenesis.

Proton NMR spectroscopy

¹H-NMR spectra were measured from plasma samples taken from a large central vein at 5 hourly intervals until the experiments were terminated at $t = 28$ h. Data were acquired on a Varian INOVA 600 NMR Spectrometer operating at 600 MHz for protons. All spectra were acquired at ambient probe temperature (298 ± 0.2 °K). For each sample 128 transients (FID's) were acquired into 32 K complex data points over a spectral width of 6 KHz. 300 pulses were applied with an acquisition time of 2.5 s to achieve better resolution followed by an additional pulse recycle time of 4 s to allow for complete T₁ relaxation. Water signal suppression was achieved by applying a gated secondary irradiation field at the water resonance frequency. Spectral assignments were made by reference to literature values of chemical shifts in various media and biological fluids and coupling constants^[13]. The coefficient of variation between samples was < 6% and reproducibility for the same sample was good with a difference of < 2% on the same sample. The CMPG (Carr-Purcell-Meiboom-Gunn) sequence was applied for data acquisition, as this sequence enabled observation of a flat baseline in our spectra from plasma samples by minimising the signals acquired from macromolecules present in the plasma such as proteins and lipoproteins^[14]. NMR spectra analysis was performed using the MNova platform for NMR analysis (Mestrelab, Santiago de Compostela, Spain).

Statistical analysis

To compare between groups in the initial sample the Student's *t*-test for parameters with non-missing values and the Mann Whitney *U* test for parameters with missing values were used. Values were expressed as mean (range and standard error). A *P* value of < 0.05 was taken as statistically significant (two-tail test of significance). Numeric results are expressed as μ mol/L. All analysis was done using the SPSS statistical package (Version 9.0).

RESULTS

On all samples studied we were able to identify the fol-

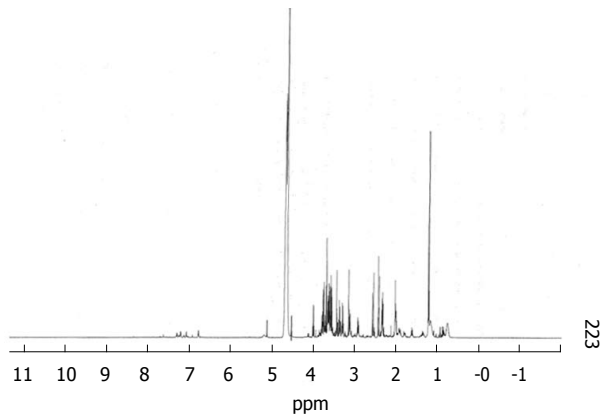


Figure 1 A representative ^1H -NMR spectrum of plasma taken at $t = 15$ h from one of the acute liver failure animals. Relevant peaks identified and used for analysis are as follows: Lactate CH_3 @1.33 ppm doublet Pyruvate CH_3 @ 2.38 ppm singlet; Alanine CH @1.48 ppm doublet Threonine CH_3 @ 1.34 ppm doublet; Glycine CH_2 @ 3.57 ppm singlet Leucine CH_3 @ 0.96 ppm triplet; Isoleucine CH_3 @ 1.01 ppm doublet Valine CH_3 @1.04 ppm doublet; Tyrosine H_3/H_5 @ 6.91 ppm Phenylalanine H_4 @ 7.38 ppm multiplet doublet.

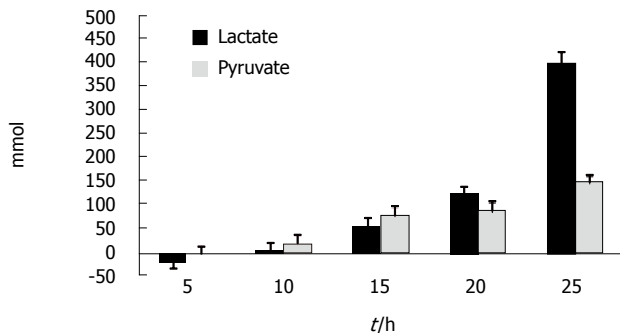


Figure 2 There was a net increase of lactate and pyruvate concentrations during the experiments.

lowing metabolites: lactate, pyruvate, leucine, isoleucine, valine, tyrosine, phenylalanine, histidine, arginine, glycine, alanine, threonine, glutamate and glutamine. Resonances from proline, methionine and ornithine were not suitably characterised and results from those amino acids are not available. Figure 1 shows a sample spectrum.

In control pigs there were no significant differences in the concentrations of the substrates studied at any time point sampled. Animals who received paracetamol showed statistically significant differences in the concentrations of lactate, pyruvate and the amino acids.

Lactate and pyruvate

Figure 2 shows the results for lactate and pyruvate. Increases in the concentration of lactate became significant at $t = 15$ h and at $t = 25$ h; compared to $t = 0$ an average increase of 405% was seen ($P < 0.003$). Increases in the concentration of pyruvate became significant at $t = 20$ h and at $t = 25$ h; compared to $t = 0$ an average increase of 150% was seen ($P < 0.018$).

Amino acids

Figure 3 shows the results for threonine, alanine and gly-

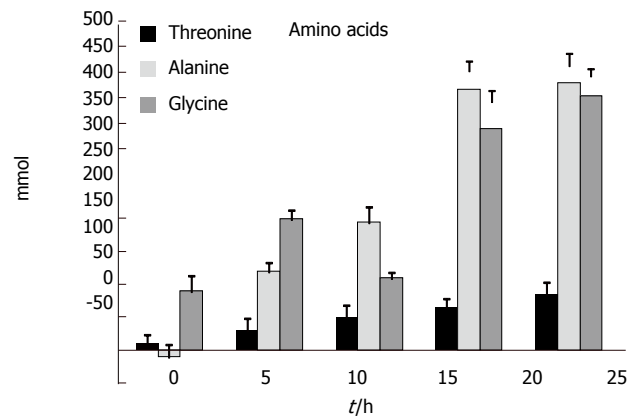


Figure 3 There was a net increase in the concentrations of alanine, threonine and glycine during the experiments.

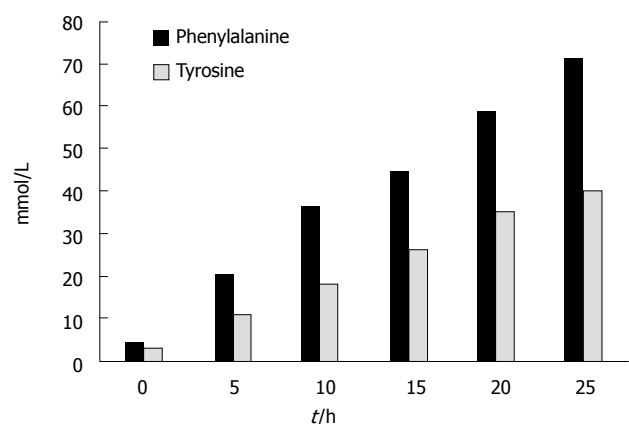


Figure 4 There was a net increase in the concentrations of tyrosine and phenylalanine during the experiments.

cine. Increases in the concentration of threonine became significant at $t = 20$ h and at $t = 25$ h; compared to $t = 0$ an average increase of 82% was seen ($P < 0.048$). Increases in the concentration of alanine became significant at $t = 10$ h and at $t = 25$ h; compared to $t = 0$ an average increase of 410% was seen ($P < 0.002$). Finally, increases in the concentration of glycine became significant at $t = 5$ h and at $t = 25$ h; compared to $t = 0$ an average increase of 390% was seen ($P < 0.005$).

Figure 4 shows the results for the aromatic amino acids. Tyrosine levels significantly increased at $t = 5$ h and at $t = 25$ h; There was an average increase of 1330% ($P < 0.000$). Phenylalanine levels also increased significantly at $t = 5$ h and at $t = 25$ h; There was an average increase of 1420% ($P < 0.000$).

Figure 5 shows the results for the branch chain amino acids. There were no statistically significant changes in the concentration of leucine between the beginning and the end of the experiments (0.17 ± 0.02 vs 0.175 ± 0.02). Isoleucine levels increased significantly at $t = 10$ h and at $t = 25$ h; an average increase of 250% was seen ($P < 0.01$). Valine levels, on the contrary, significantly decreased at $t = 20$ h and at $t = 25$ h; an average decrease of 150% was seen ($P < 0.05$).

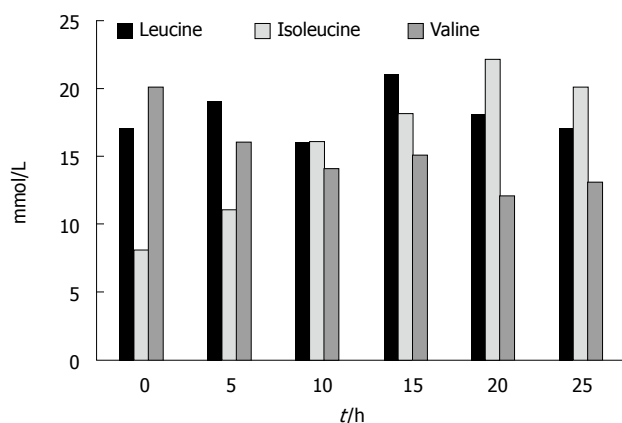


Figure 5 Isoleucine concentrations significantly increased during the experiments. Valine concentrations significantly decreased during the experiments. No changes were seen in the concentration of leucine.

No statistically significant differences were observed in the concentrations of glutamate, glutamine, arginine and histidine up to the end of the experiments.

DISCUSSION

In this study in a large animal model of paracetamol induced acute liver failure we have shown that glucose homeostasis is impaired with hypoglycaemia, increased concentrations of lactate and pyruvate and increased concentrations of most gluconeogenic amino acids.

It is common knowledge that patients with severe acute liver injury due to paracetamol overdose quickly develop hypoglycaemia. In contrast, most animal models of ALF do not develop hypoglycaemia. In ALF the hepatic production of glucose is inhibited but there is compensation by kidney gluconeogenesis^[4,5,14,15].

In our model the concentration of lactate, despite a temporary decline at the beginning of the experiments, increased quickly and was significantly increased at $t = 15$ h. Likewise, pyruvate concentration increased and that increase became significant at $t = 20$ h. This suggests that early on glycolysis is switched on in the liver. As paracetamol also affects the kidney and an early kidney toxic injury is usually seen in cases of paracetamol induced ALF^[16], attempted compensatory gluconeogenesis maybe occurring in the kidney but it is not sufficient to maintain normal glucose levels in this model of ALF.

We have also observed that major gluconeogenic amino acids were not taken up by the liver. Concentrations of alanine increased significantly throughout the study along with concentrations of threonine and glycine. Likewise the concentrations of the aromatic amino acids tyrosine and phenylalanine increased very early on and rose dramatically at the end of the experiments. This is in accordance with previous studies in mice that showed similar results^[4-6,17]. This is a good indication that gluconeogenesis in the liver is impaired and the TCA cycle is not functioning properly.

If we look at the branch chain amino acids the re-

sults are somehow different. No change was seen in the concentration of leucine and a decrease was observed in the concentration of valine. Significant increases at the end of the experiments were only seen in the concentrations of isoleucine.

ALF is characterized by the development of encephalopathy. In that respect Fischer's ratio (the ratio of branch chain to aromatic amino acids) is believed to be a good index^[18]. A decrease of the ratio is a good marker of severe hepatic encephalopathy. As we have shown previously in our model a significant decrease in the Fischer's ratio was observed by $t = 15$ h of the experiments^[7]. In a previous study we have shown that although in patients with non-paracetamol induced ALF all branch chain amino acids are increased, in patients with paracetamol induced ALF valine is an exception and decreases over time^[19]. We believe our results to be in accordance with this observation. We should point out though that the attempts to correct hepatic encephalopathy with correction of the Fischer's ratio by exogenous substitution of branch chain amino acids were not a success and the reason might lie in the fact that it is the aromatic and not the branch chain amino acids that are disturbed^[20].

An interesting finding was that we observed no significant changes in the concentrations of glutamate, glutamine, histidine and arginine, key metabolic components of the urea cycle. We unfortunately were unable to quantify aspartate but there is strong evidence that the urea cycle in this model remains largely unaffected by paracetamol poisoning. This is in accordance with other studies that have shown that ALF induced hyperammonemia is caused by gut production of ammonia, a by-product of the production of alanine from glutamate^[21]. Novel attempts to correct the metabolic abnormalities of hepatic encephalopathy by providing ornithine as a substrate for urea synthesis might be a good path^[22-25].

The major drawback of this study is that we performed a study that describes changes in amino acids that pertain to gluconeogenesis and that we assumed that the changes were provoked by the inhibition of gluconeogenesis by the acute liver injury. We had no means to exclude the possibility that some other metabolic function could be responsible for the observed amino acid disturbances.

In conclusion, in this large animal model of paracetamol induced ALF we have observed an inhibition of gluconeogenesis in the liver with subsequent dysfunction of the TCA cycle. This has led to ATP and energy depletion and the liver switching to glycolysis to compensate. Further studies in animals and also in humans are needed to fully characterize the metabolic abnormalities of liver failure and to provide a pathophysiological basis for the new emerging metabolically centred therapies for hepatic encephalopathy^[26].

COMMENTS

Background

Acute liver failure in humans is a deadly condition which could lead to multi-organ failure and death of the patient. Abnormalities in many metabolic

pathways are known to exist but there are very few large animal models where the authors are able to reproduce the acute liver injury. Recently the authors have developed such a porcine model in our laboratory.

Research frontiers

Paracetamol induced acute liver injury in humans and animal models is characterised by profound hypoglycaemia in a very short time from the liver injury. The exact mechanism of hypoglycaemia remains debatable.

Innovations and breakthroughs

In this article the authors were able to provide some evidence that gluconeogenesis is impaired in a porcine model of acute liver injury as manifested by a relative increase in the plasma concentrations of the gluconeogenic amino acids. Gluconeogenic amino acids are the main substrate for glucose production after the exhaustion of glycogen stock very early in the acute liver injury.

Applications

This paper provides some insight into the metabolism in acute liver failure. It shows that gluconeogenesis a key metabolic pathway in paracetamol induced acute liver failure fails, early on in the disease. This needs to be confirmed in human studies and it could have implications in the way these patients are managed.

Peer review

The article by Dabos *et al.* describes increased lactate, pyruvate and distinct amino acids in a pig model of paracetamol induced acute liver injury.

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Two cameras detect more lesions in the small-bowel than one

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Abstract

AIM: To explore the feasibility of dual camera capsule (DCC) small-bowel (SB) imaging and to examine if two cameras complement each other to detect more SB lesions.

METHODS: Forty-one eligible, consecutive patients underwent DCC SB imaging. Two experienced investigators examined the videos and compared the total number of detected lesions to the number of lesions detected by each camera separately. Examination tolerability was assessed using a questionnaire.

RESULTS: One patient was excluded. DCC cameras detected 68 positive findings (POS) in 20 (50%) cases. Fifty of them were detected by the "yellow" camera, 48 by the "green" and 28 by both cameras; 44% ($n =$

22) of the "yellow" camera's POS were not detected by the "green" camera and 42% ($n = 20$) of the "green" camera's POS were not detected by the "yellow" camera. In two cases, only one camera detected significant findings. All participants had 216 findings of unknown significance (FUS). The "yellow", "green" and both cameras detected 171, 161, and 116 FUS, respectively; 32% ($n = 55$) of the "yellow" camera's FUS were not detected by the "green" camera and 28% ($n = 45$) of the "green" camera's FUS were not detected by the "yellow" camera. There were no complications related to the examination, and 97.6% of the patients would repeat the examination, if necessary.

CONCLUSION: DCC SB examination is feasible and well tolerated. The two cameras complement each other to detect more SB lesions.

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Key words: Small-bowel capsule endoscopy; Dual camera capsule endoscope; Feasibility; Lesion detection; Diagnostic yield

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INTRODUCTION

Small bowel video capsule endoscopy is an established

method for examining the small-bowel (SB). However, a recent systematic review of 227 studies showed that the examination's detection rate of positive findings (POS) (that explain the symptoms for which the test was performed and assist the further management of the patient) varies from 55.3% to 60.5% (overall 59.4%)^[1]. To increase the examination's diagnostic yield, several methods have been proposed, including the use of cathartics and prokinetics, changing body posture, repeating a negative exam, with varying results^[2].

Recently, a dual camera capsule (DCC) endoscope (PillCam Colon, Given Imaging, Yoqneam, Israel) designed for colon examination has been introduced to the market. This DCC is 6 mm longer than the conventional SB capsule (PillCam Small Bowel 2, Given Imaging, Yoqneam, Israel). Its specific technological properties include: two cameras - the "yellow" and the "green", enabling the device to acquire video images from both ends, optics with more than twice the coverage area of the small bowel capsule, automatic light control, a frame acquisition rate of four frames per second (double the rate of the SB capsule), and a total operating duration of approximately 10 h. After initial capsule activation and several minutes of image transmission, the capsule enters a delay mode of approximately 2 h, after which it spontaneously restarts the transmission of images. Similarly to the SB capsule, the system includes a sensor array and data recorder connected to the patient during the procedure. The recorded data are downloaded into the Rapid Viewer (Given Imaging, Yoqneam, Israel) workstation for review of the video. The reviewer can review images captured from each individual camera and from both cameras simultaneously^[3].

Given these properties, this capsule has the theoretical potential to detect more lesions than the conventional SB capsule during SB capsule examination. Whether this advantage is relevant clinically remains to be proven. The aim of our study was to explore the feasibility and tolerability of DCC SB imaging and to examine if the capsule's two cameras can complement each other to detect more SB lesions.

MATERIALS AND METHODS

Ethics

The study was approved by the Ethics Committees of both Hospitals and each participant gave informed consent.

Patients

This prospective, non-randomized, feasibility study on consecutive outpatients was conducted in two Academic Departments in Athens, Greece, over a 5-mo period from September 2008 to January 2009. We included 41 patients with indications for SB capsule endoscopy. Exclusion criteria were: known or suspected gastrointestinal obstruction, stricture or fistula, conditions associated with delayed capsule endoscopy gastric/small bowel transit time (i.e. diabetes mellitus), gastrectomy, pregnancy, paralysis

or impaired mobility, medication use that could affect gastrointestinal motility (i.e. prokinetics, antidepressants, anticholinergics, and narcotics) or mucosa visibility (iron, sucralfate), and renal impairment in which sodium phosphate is contraindicated.

Bowel preparation and DCC procedure

All patients received detailed written instructions on their bowel preparation. They ingested only clear liquids the day before the procedure and they fasted for 8 h overnight, prior to testing.

Capsule examinations started at between 8 and 10 o'clock the next morning. After its initial activation, the DCC remained beside the patients during the delay mode period. Patients ingested the DCC, after its definite re-activation, approximately 2 h later. Thereafter, patients were checked every 10 min with the Real Time Viewer (Given Imaging, Yoqneam, Israel) until the entrance of the capsule into the duodenum. At this time, they drank 45 mL of sodium phosphate (Phospholaxat, Pharma Line, Athens, Greece) and were advised to drink two liters of water during the following 2 h^[4]. This type of SB preparation has been shown to improve mucosa visibility during SB capsule endoscopy in a prospective, randomized, double blind study^[4] and it is the preferred preparation strategy in our Departments. Patients ate a light snack 4 h after DCC ingestion and they dined when recording ceased. Before discharge, patients were asked to fill a questionnaire, answering the following questions: (1) "Will you repeat the examination, if needed?" (yes - no); (2) "How comfortable were you during the examination" (very comfortable - comfortable - neither comfortable nor uncomfortable - uncomfortable - very uncomfortable); and (3) "Did you experience any unwanted symptoms during the examination?" (yes - no, if yes, please describe). In cases where the DCC had not been expelled before discharge, we advised patients to search for the capsule in their feces during the following days. In cases of capsule retention, patients were followed clinically and radiologically until capsule excretion.

Video review

Downloaded videos were first read locally for patients' clinical management and thereafter, they were coded and submitted to two investigators for video review and data analysis.

Both investigators had experience of at least 200 capsule endoscopy studies (SB and colon), they were aware of the study indication, but they were blinded to the results of the local review. They examined the videos independently, in random order, using the Rapid Reader, version 5.1 (Given Imaging Ltd, Yoqneam, Israel) software, as outlined below: (1) they rated the overall quality of SB mucosa visibility using a four steps scale: bad - fair - good - excellent^[5]; (2) they counted the POS and the findings of unknown significance (FUS)^[6] detected by either the "yellow" or the "green" camera only, or by both cameras. POS are those that explain the symptoms for which the test was performed, assist the further man-

agement of the patient, or are subsequently confirmed by other diagnostic modalities. Findings of uncertain significance (red spots, small erosions, lymphangiectasias, *etc.*) are those that fail to completely explain the symptoms, thus necessitating further investigation^[6]; and (3) they provided a diagnosis for each examination according to the “yellow” and “green” camera’s findings. In cases of disagreement, a decision was made by a third independent experienced investigator.

The tolerability of the examinations was assessed by the completed questionnaires. Any serious adverse events were also recorded.

Statistical analysis

The main results of the study are reported in descriptive manner. Qualitative data are presented as absolute and value percent and quantitative data are presented as median value (IQR).

Agreement between investigators for the quality of SB mucosa visibility and between the two cameras regarding diagnosis was assessed by κ statistics. Correlations were assessed by regression analysis. For these comparisons, a *P* value of < 0.05 indicated statistical significance.

RESULTS

We prospectively enrolled 41 consecutive patients who met the study’s criteria. In one patient, the capsule remained in the stomach for 9 h, for no apparent reason. The capsule was excreted 2 d later but the patient did not consent to undergo another examination. One capsule failed to re-activate after entering the delay mode, but the patient received another capsule that worked properly. Therefore, we report the results of 40 DCC SB examinations. The patients’ baseline characteristics are shown in Table 1.

DCC reached the cecum in 36/40 (85%) patients. DCC’s gastric and SB transit times were 45.5 (IQR: 17.7-78.5) min and 200 (IQR: 117-277) min, respectively. There was moderate - good agreement between the two investigators regarding SB mucosa visibility ($\kappa = 0.66$, $P < 0.001$). Thirty two (80%) and 27 (67.5%) cases were rated with good-excellent mucosa visibility by investigators 1 and 2, respectively.

DCC cameras’ findings

There was excellent correlation ($r > 0.9$, $P < 0.001$) between the two investigators regarding the number of findings detected by DCC. Therefore, we analyzed the mean value of their measurements. Overall, DCC cameras detected 68 POS in 50% of the cases. The “yellow” and the “green” camera detected 50 and 48 POS, respectively, while both cameras detected 28 POS findings simultaneously. Figure 1 shows that the two DCC cameras detected different POS: 44% ($n = 22$) of the “yellow” camera’s POS were not detected by the “green” camera and 42% ($n = 20$) of the “green” camera’s POS were not detected by the “yellow” camera. More precisely, Figure 2 shows that the POS detected by the two capsule’s cam-

Table 1 Patients’ characteristics (mean \pm SD) ($n = 40$) n (%)

Male sex	29 (72.5)
Age (yr)	58 \pm 16
Height (cm)	168.1 \pm 9.3
Weight (kg)	73.6 \pm 12.8
Indication	
Iron deficiency anemia	13 (32.5)
Obscure gastrointestinal bleeding	15 (37.5)
Crohn’s disease	1 (2.5)
Chronic diarrhea	7 (17.5)
Other	4 (10)

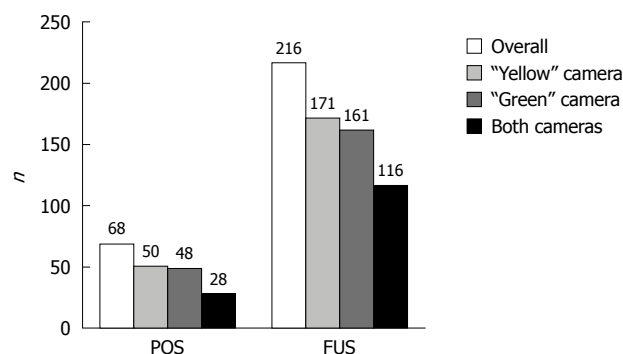


Figure 1 Positive findings and findings of uncertain significance detected in 40 dual camera small-bowel capsule endoscopy examinations; overall, by the “yellow” camera, by the “green” camera, and by both cameras simultaneously. POS: Positive findings; FUS: Findings of uncertain significance.

eras were identical only in 6 of the 20 cases. Moreover, there were two (0.05%) cases (case 18 and 20 in Figure 2) where only one camera -the “green” one- detected significant findings (three and one angiodysplasias, respectively).

All participants had FUS and the capsule’s cameras detected 216 FUS overall; 171 detected by the “yellow” camera, 161 detected by the “green” one, and 116 by both of them simultaneously (Figure 1). Moreover, 32% ($n = 55$) of the “yellow” camera’s FUS were not detected by the “green” camera and 28% ($n = 45$) of the “green” camera’s FUS were not detected by the “yellow” camera.

Diagnostic yield of the two cameras

There was agreement between the investigators in 39/40 cases regarding the diagnosis. Disagreement occurred for one chronic diarrhea case with erosions detected by DCC and the case was finally assigned to a diagnosis of Crohn’s disease by the third investigator. Overall, the diagnostic yield of DCC SB examination was 50%. The diagnostic yield of each DCC camera is shown in Table 2: 45% and 50% for the “yellow” and the “green” camera, respectively ($P = 0.987$). The agreement between the DCC cameras diagnosis was high ($\kappa = 0.92$, $P < 0.001$). However, there was numerical difference because of the two cases with angiodysplasia(s) missed by the “yellow” camera.

Examination’s tolerability

There were no serious adverse events related to the ex-

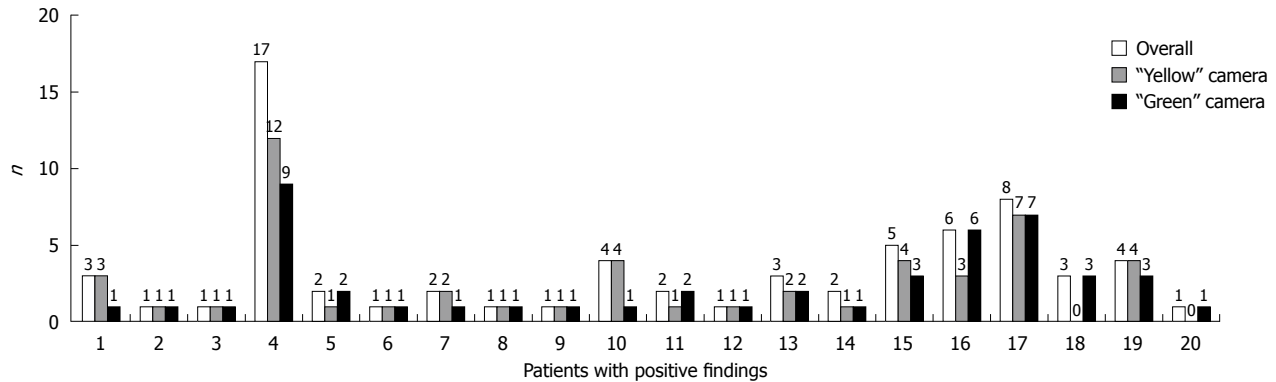


Figure 2 Positive findings detected in each of the 20 positive dual camera small-bowel capsule endoscopy examinations; overall, by the "yellow" and by the "green" camera, respectively.

Table 2 Diagnostic yield of the dual camera capsule small-bowel examinations ($n = 40$) according to the findings of the "yellow" and the "green" camera n (%)

Diagnosis	"yellow" camera	"green" camera	<i>P</i>
Normal examination	22 (55)	20 (50)	0.987
Angiodysplasia(s)	7 (17.5)	9 (22.5)	
Active bleeding (unidentified source)	2 (5)	2 (5)	
Crohn's disease	7 (17.5)	7 (17.5)	
Polyp(s)	2 (5)	2 (5)	

Table 3 Tolerability of the examination ($n = 40$)

	<i>n</i> (%)
"How comfortable were you during the examination?"	
Very comfortable	3 (7.5)
Comfortable	7 (17.5)
Neither comfortable nor uncomfortable	26 (65)
Uncomfortable	3 (7.5)
Very uncomfortable	1 (2.5)
"Did you experience any unwanted symptom(s) during the examination?"	
Yes ¹	8 (20)
Abdominal cramps	4
Abdominal bloating	7
Urgency	2
Nausea	1
No	32 (80)

¹Unwanted symptoms add up > 8 because some patients experienced more than one of them.

amination. There was no capsule retention. Forty (97.6%) of the 41 initially included participants stated that they would repeat DCC SB examination -if necessary. 90% of patients were not uncomfortable during the examination, while 20% of them reported mild adverse reactions related to bowel preparation (Table 3).

DISCUSSION

The diagnostic yield of SB capsule endoscopy varies across the indications of the examination^[1]. Capsule

endoscopy is expensive and time consuming; therefore efforts have been made to improve its performance^[2]. Meta analysis has shown that only purgative small bowel preparation increases the diagnostic yield of the examination^[7], while other modalities have shown conflicting results^[2]. The properties of the new DCC designed for colon examination (mainly the two cameras and the rate of capturing images) provide an opportunity to test its utility in SB imaging. Our study showed, for the first time, that SB capsule endoscopy with DCC is feasible, well tolerated, and that the two cameras have the potential to detect more SB lesions by complementing each other. While our study was underway, a study was published in abstract form examining the same hypothesis. Investigators from Portugal using DCC SB endoscopy detected 44 SB findings overall - 24 exclusively from the "yellow" camera, 13 from the "green", and only one from both - in 10 patients, concluding that using a device with two cameras would increase the diagnostic acuity of capsule endoscopy SB examination^[8]. Our study revealed that each DCC camera misses 40% of the significant and 30% of the FUS detected by the other one. It is not clear why this happens. Although the SB lumen is small enough in diameter to allow lesion detection by one camera, our study indicates that because the capsule endoscope is trembling and rotating in SB lumen, there might be blind spots along its passage that can be detected on a second camera recording. More importantly, there were two cases with significant SB examination findings detected by only one camera, raising the possibility that the correct diagnosis might have been missed if the small bowel had been explored with a single camera video capsule. This finding might extrapolate to one additional positive DCC SB examination for every 20 false negative conventional SB capsule explorations, in which case a formal comparison between the two capsules would confirm our study results. However, this assumption has to be proven in a controlled trial.

Two previous studies highlighted the results of sequential SB capsule endoscopy in patients with obscure gastrointestinal bleeding. In each study, 51^[9] and 40^[10] patients, respectively, underwent sequential SB capsule endoscopy with two different capsule endoscopy systems

(PillCam SB and Olympus EndoCapsule). Patients ingested the capsules in a randomized order and data showed that the DY of the two systems was similar. However in the German study^[10], the second capsule detected angiodysplasias in two (5%) more patients. The results of these studies differ from the results of studies that investigated the value of a second capsule endoscopy, using the same endoscopy system, in patients with obscure gastrointestinal bleeding or iron deficiency anemia with one previous non-diagnostic capsule investigation. Bar-Meir *et al*^[11] showed significant lesions in seven of the 20 included severe iron deficiency patients, Jones *et al*^[12] revealed positive finding in 18 of 24 obscure gastrointestinal bleeders, while Viazis *et al*^[13] established a diagnosis in 37 of 76 obscure gastrointestinal bleeders who underwent second look capsule endoscopy. Whether dual camera SB capsule endoscopy can reduce repeat SB examination (including capsule endoscopy) until a confirmed diagnosis remains to be investigated.

Apart from establishing a diagnosis, SB capsule endoscopy also estimates the extent of the disease in cases of multiple angiodysplasias and Crohn's disease, in which the involvement of different parts of the small bowel might dictate different therapeutic approaches. Therefore, missing lesions may underestimate disease extent. For example in case 3 with multiple small bowel ulcers/erosions shown in Figure 2, the "yellow" camera detected erosions throughout the recording, while all "green" camera's POS were detected by the end of the recording (terminal ileum).

In our study, mucosa visibility was good-excellent in 68% and 80% of the cases, as rated by each investigator, respectively, with a half dose colonoscopy preparation regimen given after the insertion of the capsule at the duodenum. While this purge is given as a boost in colon capsule endoscopy studies, in our departments we use it as the standard preparation scheme for SB capsule endoscopy to both avoid prolonging the capsule's gastric transit time and to improve mucosa visibility, specifically in the ileum^[4]. This preparation was both effective and well tolerated, causing mild adverse reactions in only 20% of the participants, which is similar to the complication rate published in the literature^[2,7].

Finally, we addressed patient's acceptance, which is one of the major issues for a successful diagnostic modality^[14]. The acceptance rate of DCC SB examination in our study was high: all participants (apart from one who was excluded initially) agreed to repeat the study if necessary. More importantly, there was no capsule retention and 90% of the patients did not feel uncomfortable during the examination.

Our study was a prospective feasibility trial aiming to find grounds for further formal investigation of DCC SB imaging. Therefore, the results should be interpreted with caution in clinical practice and some reservations should be noted. Firstly, the study design is not optimal for detecting firm conclusions and the sample size is small. The ideal trial to explore whether DCC endoscopy of the SB results in higher DY compared to the conventional

capsule, should include patients with one specific indication (e.g. obscure gastrointestinal bleeding) who would ingest a single camera capsule operating on a different frequency than the DCC initially and the DCC later, or vice versa. Secondly, the capsule is 6 mm longer and this might result in swallowing difficulties, in a higher retention rate of the capsule in the stomach, and in a higher rate of incomplete SB examinations^[3]. All our patients swallowed the capsule easily. However, in one case, the capsule remained in the stomach during the life span of the capsule's battery, without any predisposing factor. We did not detect increased DCC gastric and small bowel transit time, compared to those published with the conventional SB capsule^[7], and the rate of complete SB examinations with cecal visualization was 85%, similar to that reported in the literature with the conventional SB capsule^[7]. Furthermore, complete colon examination was observed in 42.5% of the cases without any further purgative boost; however, bad-moderate bowel cleansing in 79%-85% of the cases prevented any thorough colon examination (data not shown in the results). Thirdly, the longer time required to review the videos from each individual camera and from both cameras simultaneously might be an issue. However, we have not addressed this parameter. Lastly, it might be not acceptable by many patients to wait 2 h until the definite DCC activation. However for the purpose of our study, we have not tried the capsule's initial activation in advance.

In conclusion, we showed that DCC SB endoscopy is feasible and well tolerated. Moreover, the two capsule's cameras complement each other to detect more SB lesions. It remains to be determined whether DCC SB endoscopy can increase the diagnostic yield of capsule endoscopy SB examination and if it can improve the clinical outcome of patients undergoing the examination.

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Part of our study has been presented in the 2009 Digestive Diseases Week, Chicago, IL, USA (in poster form) and in the GASTRO 2009, London, UK (oral presentation).

COMMENTS

Background

Wireless video-capsule endoscopy is a non-invasive and patient friendly examination, which has revolutionized small-bowel (SB) exploration. It has been proved to be superior to any other radiographic or endoscopic modality for SB mucosa examination. However, it is expensive and its diagnostic yield varies across the indications. Until recently several methods have been introduced to improve the method's diagnostic yield, with conflicting results.

Research frontiers

Wireless capsule endoscopy is an evolving technology. Recently, a new capsule endoscope designed for colon examination has been introduced to the market. It is a dual camera capsule (DCC) that compared to the single camera conventional SB capsule has a theoretical advantage to detect more SB lesions.

Innovations and breakthroughs

This theoretical advantage has been tested in 41 consecutive patients with an indication for SB mucosa examination and DCC endoscopy was performed to examine whether the two cameras complement each other to detect more le-

sions than each of them alone. The results showed that each camera missed 40% of the findings that explain patient's symptoms detected by the other one. More importantly, there were two cases with significant SB examination findings detected by only one camera, raising the possibility that the correct diagnosis might have been missed if the small bowel had been explored with a single camera video-capsule. In conclusion, this study revealed that DCC SB endoscopy is feasible, well tolerated, and that the two capsule's cameras complement each other to detect more SB lesions.

Applications

Given that a feasibility trial can not reach firm conclusions, the hypothesis that DCC may increase the diagnostic yield of wireless capsule SB endoscopy must be tested in a formal way. If proven, DCC SB endoscopy will be the standard for SB mucosa exploration.

Terminology

Wireless video-capsule endoscopy is an endoscopy system equipped with a capsule endoscope that acquires images from the gut lumen and transmits them to a data recorder using wireless emission technology. Images are processed and reviewed in a video format using a commercially available computer.

Peer review

The trial of a colon capsule to view the small intestine is an innovative idea and the results indicate that it has considerable clinical importance. This is a well designed and clinically relevant study.

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Factor analysis identifies subgroups of constipation

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Abstract

AIM: To determine whether distinct symptom groupings exist in a constipated population and whether such grouping might correlate with quantifiable pathophysiological measures of colonic dysfunction.

METHODS: One hundred and ninety-one patients presenting to a Gastroenterology clinic with constipation and 32 constipated patients responding to a newspaper advertisement completed a 53-item, wide-ranging self-report questionnaire. One hundred of these patients had colonic transit measured scintigraphically. Factor analysis determined whether constipation-related symptoms grouped into distinct aspects of symptomatology. Cluster analysis was used to determine whether indi-

vidual patients naturally group into distinct subtypes.

RESULTS: Cluster analysis yielded a 4 cluster solution with the presence or absence of pain and laxative unresponsiveness providing the main descriptors. Amongst all clusters there was a considerable proportion of patients with demonstrable delayed colon transit, irritable bowel syndrome positive criteria and regular stool frequency. The majority of patients with these characteristics also reported regular laxative use.

CONCLUSION: Factor analysis identified four constipation subgroups, based on severity and laxative unresponsiveness, in a constipated population. However, clear stratification into clinically identifiable groups remains imprecise.

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Key words: Factor analysis; Constipation; Symptoms; Clusters; Laxatives

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INTRODUCTION

Constipation is a heterogeneous disorder, the most consistent generic descriptor for which is difficult or infrequent passage of stool^[1,2]. A fundamental aim in researching and treating any heterogeneous disorder is to subclassify

the condition into categories that are predictive of either pathophysiology or treatment outcome. Constipation is currently conceptualized in three broad categories: normal transit constipation (NTC), slow transit constipation (STC) and disorders of defecation or rectal evacuation^[3-5]. This distinction among subgroups, in some cases, has proven beneficial in planning treatment and in predicting therapeutic outcome^[4,6-12].

It remains unknown whether subtypes of constipation can be identified reliably on the basis of symptoms. The mathematical techniques of factor and/or cluster analysis, to determine whether certain symptoms and/or subjects do group together more than expected by chance, can provide empiric evidence of the existence of true syndromes. Studies applying these techniques have found conflicting results, with Mertz *et al.*^[13] identifying 3 subtypes while Eltringham *et al.*^[14] did not. These findings suggest that the *a priori* assumption that these subtypes are distinct or distinguishable from each other on the basis of symptoms may be incorrect, or that investigators have yet to combine the correct symptoms to identify pathophysiologically distinct abnormalities.

Utilizing a 53-item specific constipation questionnaire and colonic scintigraphy our aim was to identify symptoms or groups of symptoms that identify underlying severe constipation subgroups. Specifically, we hypothesized that within this constipation population at least three distinct subgroups, STC, evacuation disorder and irritable bowel syndrome (IBS), can be identified by groups of symptoms and that these symptom groups will be predictive of specific underlying objective physiological measures such as colonic transit time.

MATERIALS AND METHODS

Design of the questionnaire and assessment of symptoms

The Sydney Constipation Questionnaire (SCQ) was derived from the previously validated Bowel Disease Questionnaire^[15] and the Bowel Symptom Questionnaire^[16]. The SCQ comprises 53 items, including 42 symptom items and the validated Bristol stool scale^[17]. Additional constipation items were then added and the face validity was assessed by responses from 29 internationally recognised constipation experts who were asked to rate each question according to clinical relevance. The test-retest reliability of the final questionnaire was evaluated in 47 patients who repeated the questionnaire within 3 wk of initial testing^[18]. The design of the questionnaire took place long before the release of Rome III criteria for bowel^[19] and anorectal^[20] dysfunction, nevertheless the questionnaire does contain all of the symptomatic questions that define constipation, IBS and outlet obstruction by the Rome III criteria.

Population sample

Subjects were obtained through two sources: (1) *via* referrals from specialists in the Sydney area; and (2) by direct

advertisement that specified criteria approximating tertiary-referred subjects. The impact of subject source was considered in the statistical analysis. Overall, the sample was designed to reflect community members suffering serious constipation symptoms. Subjects were deliberately not selected based on Rome III constipation criteria, but rather on the basis of serious symptoms to avoid making assumptions and because we wanted also to examine the proportion of the study cohort and its clusters that would be positive for Rome III criteria for constipation or IBS.

Referrals to colorectal surgeons or gastroenterologists in Sydney metropolitan region, seeking treatment for constipation, were recruited over a 4-year period and were given a questionnaire if they fulfilled the following criteria: (1) had not undergone any form of colectomy, rectocele or rectal prolapse repair; and (2) their constipation was not deemed secondary to a metabolic or neurological disease or pregnancy. Additionally, the questionnaire was given to potential patients who responded to advertisements in Sydney newspapers recruiting subjects for a randomized control trial of sacral nerve stimulation for the treatment of constipation (in progress; to be reported in future). These potential patients were screened during an initial phone conversation and if they fulfilled the criteria listed above the questionnaires were mailed out to them. As these were chronically constipated patients, who for the most part had been taking laxatives for many years, they were not asked to report on what their symptoms are, were or might be, in the absence of laxatives. Studies have shown that the correlation between a patient's reported stool frequency and actual measures is poor^[21] and in our experience a patient's ability to recall symptoms from the years prior to laxative use is also poor.

Allowing 3 wk for the questionnaire to be completed and returned, each non-responder was then phoned and asked if they intended to return the questionnaire. A proportion of community responders had decided that their symptoms were not severe enough to warrant sacral nerve stimulation and these patients did not return the questionnaire. Allowing an additional 4 wk, all other non-responders were excluded from the study. All participants in this study gave written, informed consent and the study was approved by the Human Ethics Committees of the South Eastern Area Health Service, Sydney and the University of New South Wales.

A priori symptom groups

Three symptom groups were defined *a priori*: (1) STC was considered to comprise infrequent bowel movements (less than 2 defecations/wk), lack of defecation urge, excessive straining and hard stools^[22-24]; (2) Obstructed defecation was classified when all of the following symptoms were present: (a) an inability to initiate defecation following the urge to do so, or difficulty with stool evacuation; (b) excessive straining at stool more than 25% of the time or self-digitation to facilitate defecation more than 25% of the time; and (c) a feeling of incomplete evacuation after

defecation^[1,22,25-29]; and (3) IBS classified those patients with symptoms of abdominal pain and bloating that improve with defecation or in whom the onset of such symptoms is associated with a change in frequency or form of the stool^[19,22,24,30].

Transit studies

Colonic transit was measured using a standard nuclear medicine technique^[31,32]. Briefly, the subjects attended a Nuclear Medicine Department on a Monday morning and were given 4 MBq ¹¹¹In-DTPA orally at approximately 9:00 am. They returned to the department the same day at approximately 3:00 pm and on subsequent days at approximately 24, 48, 72 and 96 h following the oral administration. On each occasion, anterior and posterior abdominal images were obtained each for 10 min using a large field-of-view gamma camera and medium energy collimator. Background images were obtained for background correction. All laxative medications were stopped 3 d before and during the scan. No effort was made to control the patient's diet during the scan period. Method of the scintigraphic analysis is reported elsewhere^[33]. Briefly, at each time point, profiles of the activity along the colon were derived from a geometric mean image obtained from the anterior and posterior images. Total percent retention (T%R) and mean activity position (MAP) at each time were calculated for each subject. T%R indicates the retained activity in the colon using the value at 6 h as 100%. MAP indicates the geometric center of the activity, where position 0 is at the cecum, position 98 at the anus, and position 99 being excreted activity. The scintigraphic definition of delayed transit constipation was met if the study showed isotope retention of greater than 9% in the right (cecum to mid-transverse) or left colon (mid-transverse to distal descending colon) at 72 h^[31,32].

Data analysis

Since this has been a vexed question to date, a naturalistic approach was adopted in which multivariate statistical techniques were used to identify distinct clusters of individuals with respect to bowel symptom patterns and these clusters were then examined with respect to their symptom profiles to determine what, if any, clinically meaningful differences existed between them. Analysis proceeded in two steps; the first reduced the data dimensionality from 42 observed symptom variables to 15 latent variables which were then used in the second step in a cluster analysis to form internally homogeneous clusters of individuals: details follow. The first step identified independent dimensions of symptom profile through principle components analysis followed by orthogonal rotation of the factor space. Identifying independent dimensions of bowel symptoms has several advantages. Bowel symptom questionnaires typically ask several questions around a given bowel symptom to fully characterize patients' symptomatology. Despite the clinical value of these questions, they are typically strongly correlated, leading to statistical

redundancy amongst them. In addition, cluster analysis is prone to dominance by scales that are numerically large and factor scores calculated for individuals follow a unit of normal distribution (mean zero, standard deviation 1.0) and are therefore standardized. Factors were interpreted and used in the subsequent cluster analysis if the corresponding eigenvalue was > 1.0, which corresponds to approximately 2% explained variance in the original data. The variance in the original data explained by each factor is reported in Table 1, as well as the total explained variance across all 15 factors used. A score was calculated for each factor for each subject, which is in effect a weighted sum across all original data items deemed to load on a given factor. These are reported in Table 1, along with the rotated factor loading for each item, using a criterion of rotated factor loadings > 0.4 (in absolute value). Factor loadings can be interpreted as the correlation between a given original data item and its corresponding latent variable. In the second analytic step, these scores were used in a non-hierarchical (K-Means) cluster analysis. It is important to note that only symptom latent variables were utilized in cluster formation, not transit times. Based on an *a priori* expectation of a moderate number of distinct clusters, solutions between 1 and 6 clusters were considered. A single cluster solution would imply the subjects were not differentiated in any systematic fashion, while six clusters would represent a quite complex system of constipation subgroups. The choice of cluster solution adopted was a trade-off of within-cluster homogeneity and minimizing unnecessary complexity. In a K-Means analysis the algorithm assigns individuals to clusters such that the overall within-cluster variance is minimized and the between-cluster variance is maximized, given the pre-specified number of clusters. Euclidean distance was used to measure the distance between individual points and their cluster centroid.

Determination of the clinical value of the clusters identified was through a comparison of profiles of individual symptom items and by comparing rates of *a priori* criteria, Rome III-defined constipation, IBS and outlet dysfunction, and rates of slow transit measured as described earlier.

RESULTS

The questionnaire was given to a total of 326 individuals suffering from constipation. Of these, 246 were referrals to colorectal surgeons or gastroenterologists and 80 were recruited from the community by advertisements. Of these, a total of 223 responded, representing an overall response rate of 68% [191 (78%) response rate for clinic cases; 32 (40%) response rate for community cases]. Of the 223 returned questionnaires, 4 were not able to be utilized (2 incomplete; 2 were multivariate outliers) leaving *n* = 217 for final analysis. There was no significant age or gender difference between the clinic group (45 ± 17 years; range 18-81 years; 13 M:178 F) and community groups (56 ± 20 years; range 24-82 years; 5 M:27 F). The

Table 1 Factor loadings derived from the rotated factor matrix

	Factor loading	Percent variance
Factor 1: Straining		17.3
Strain hard: How often	0.82	
Straining: How bad usually	0.80	
Straining: How long	0.76	
Straining: How often	0.71	
How long to bowel motions take	0.59	
How often incomplete evacuation	0.55	
How often unsuccessful attempts	0.52	
Frequency of any bowel problems	0.47	
How troubling is constipation	0.41	
Change positions: How often	0.35	
Factor 2: Pain frequency and severity		8.4
Abdominal pain: How often	0.80	
Pain in belly: Past 3 mo	0.79	
Abdominal pain: Severity	0.79	
Abdominal pain: Length	0.65	
Rectal mucus: How often	0.41	
Rectal pain: How often	0.39	
Pain in belly: Past 12 mo	-0.58	
Factor 3: Duration of constipation		6.5
Constipation: How many years	0.89	
Straining: How many years	0.87	
Abdominal pain: First occurrence	0.57	
Factor 4: Irritable bowel syndrome symptoms		5.6
Abdominal pain: Improved after bowel motion	0.75	
Abdominal pain: Improved after passing gas	0.70	
Experience lower abdominal pain	0.63	
Bowel motions: Harder than usual past 12 mo	0.40	
Factor 5: Urge frequency		4.1
Urge for bowel motion: How often	0.72	
Perceive an urge before attempt to open bowels	0.68	
Visits to toilet: How often	0.56	
Urgency for bowel motion: How often	0.42	
Factor 6: Diarrhea frequency		3.8
Loose/watery stool: How often	0.75	
Pebble-like stool: How often	-0.42	
Rectal disimpaction: How often required	-0.45	
Hard/lumpy stool: How often	-0.63	
Factor 7: Alternating between diarrhea and constipation		3.4
Usually alternating	0.87	
Usually constipated	-0.86	
Factor 8: Bloating frequency		3.2
Stomach swelling in the last 12 mo: How often	0.71	
Felt bloated in the last 12 mo: How often	0.68	
Felt blocked: How often	0.47	
Factor 9: Laxative efficacy		2.9
Laxative use: Longest gap between taking and bowel motion	0.70	
Bowel motions: Fewer than usual past 12 mo	0.56	
Laxative use: How often	0.44	
Factor 10: Rectal urgency		2.7
Urge from rectum	0.89	
Urge from abdomen and rectum	-0.80	
Factor 11: Bowel motion frequency		2.6
Longest gap between bowel motions	0.81	
Usual bowel frequency	-0.56	
Factor 12: Change in bowel frequency		2.4
Bowel motions: More than usual past 12 mo	0.74	
Bowel motions: Looser than usual past 12 mo	0.62	
Factor 13: Abdominal urge		2.3
Experience an urge from abdomen	0.89	
Factor 14: Diarrhea predominance		2.2
Usually experience diarrhea	0.79	
Factor 15: Antecedent to constipation		2.0
An antecedent for the constipation	0.71	
Experience upper abdominal pain	0.51	
Total (1-15)		69.3

mean age of the entire group was 47 ± 17 years (range 18-82 years).

Colonic transit was performed in all patients who had access to a nuclear medicine facility; in total 100 patients (52%) underwent the procedure study.

Factor analysis

The factor analysis produced a rotated factor matrix comprising 15 factors. Table 1 lists these 15 factors and the labels that we have attached to each factor, along with the loadings of the associated questions. For brevity, we have only shown questions within each factor for which the absolute value of the loading was ≥ 0.35 . While this process is simply an intermediary step towards cluster analysis, there are a number of potentially relevant observations to be made from this matrix. An IBS-like factor emerged, indicating that IBS-like symptoms stand out as a distinct entity in this population. Despite the population being selected for its constipation, a diarrhea factor emerged, probably accounted for by laxative usage (see below).

Cluster analysis

Cluster 1: Patients in this cluster are less likely than average to report preservation of their defecatory urge (compared to cluster 4) despite 80% using laxatives regularly. This cluster is relatively laxative-responsive with 83% describing laxatives as somewhat or very effective. Although clusters 1 and 4 share some similarities, they differ dramatically in their responsiveness to laxatives. Cluster 1 patients experience less upper abdominal pain than patients in clusters 2 and 4. Patients in cluster 1 have more IBS-like features than those in other clusters and they gain pain relief following a bowel action.

Cluster 2: This group has features similar to those of cluster 1 but when compared with cluster 1, their pain is somewhat less severe and less prevalent and they describe a shift in the site of their pain from lower to the upper abdomen. They describe similar laxative usage and responsiveness to patients in clusters 1 and 3. These patients are least likely to visit the toilet daily (63%) despite the fact that they report the highest rate of "urge prior to attempting to defecate" (72%). They are relatively laxative-responsive with 76% reporting them "somewhat or very effective".

Cluster 3: Patients in this cluster have the lowest pain scores of all 4 clusters, have a short history of constipation and are more likely to report a weekly stool frequency within the Rome III defined range for constipation. They report the lowest laxative usage, but they are the most laxative-responsive of all patients. They rarely report a feeling of rectal blockage and are less likely to adopt self digitation to facilitate evacuation. These patients never report diarrhea and rarely report hard stool.

Cluster 4: These patients report the highest pain scores and are strikingly unresponsive to laxatives, despite re-

Table 2 Summary of the proportion of patients within each cluster who were positive for constipation¹ or irritable bowel syndrome on the basis of Rome III criteria, as well as proportions in each group with slow transit constipation

	Clusters (4-fold solution)			
	1 (n = 71)	2 (n = 44)	3 (n = 34)	4 (n = 43)
Rome III constipation				
Yes	84.50%	77.30%	58.80%	72.10%
Rome III IBS				
Yes	52.10%	27.30%	20.60%	53.50%
Outlet dysfunction				
Yes	52.10%	56.80%	52.90%	48.80%
Colonic transit	n = 43	n = 28	n = 16	n = 29
Delayed transit	74.40%	71.40%	62.50%	79.30%
Normal transit	25.60%	28.60%	37.50%	20.70%

¹With the exception that symptoms were assessed irrespective of laxative usage. IBS: Irritable bowel syndrome.

porting the highest laxative usage of all (98% use them > 50% of the time). Abdominal pain is described as more severe, more frequent and lasting longer than in the other clusters. Importantly, patients in this cluster report that a bowel motion does not relieve their pain. They are less likely to have an antecedent (e.g. hysterectomy, pregnancy) than those in the other clusters. In comparison with other clusters, these patients report increased frequency of defecatory urge with 30% reporting an urge to defecate more than 3 times/d, more frequent toilet visits (60% > once/d), frequent unsuccessful attempts at defecation (73%) and frequent sense of blockage (66%) during passage of stool.

Correlation with Rome III criteria for constipation or IBS

In this population with severe constipation, the positivity rate for Rome III constipation was 59%-85% across the 4 clusters (Table 2). In this severely constipated population, with the exception of cluster 3, 78%-98% were habitual laxative users. Clusters 1 and 4 are characterized by high rates of Rome III IBS (over 50%, Table 2) compared with 20%-27% in clusters 2 and 3 (Table 2). The majority (92%) of patients that met Rome III IBS criteria were also associated with heavy laxative use.

Correlation with symptomatically-defined obstructed defecation

A remarkably constant 49%-57% of patients reported symptoms that have traditionally been attributed to obstructed defecation. The prevalence of this pattern did not differ among the four clusters.

Correlation with scintigraphically confirmed slow transit

Between 63% and 79% of patients had slow transit (Table 2). The prevalence of slow transit was least in cluster 3 (63%), the cluster with the mildest symptoms, but this still represents a sizable majority of this group. Of the 25% of severely constipated patients that report > 3 bowel motions a day, 75% have demonstrable STC. The vast majority of

these patients are also heavy laxative users, report mainly liquid stool, and a feeling of incomplete evacuation.

DISCUSSION

The ability to subtype severely constipated patients based upon symptoms has merit because it focuses and systematizes epidemiological enquiry, and has the potential to dictate logical and cost effective investigation algorithms for clinicians, to influence management and to predict therapeutic outcome. However, this study highlights the difficulties in using symptoms as discriminators of severe constipation subtypes. Although four groupings were identified by cluster analysis, it is difficult to attach clearly recognizable pathophysiological labels to these clusters. The major finding of this study is the identification of a group (cluster 4) with long history of constipation, a profound lack of response to laxatives despite extremely high laxative usage, and the highest pain scores. In contrast, cluster 3 was characterized by low pain scores, low rates of co-morbidity and the lowest rate of delayed colonic transit. Overall, our data appear to suggest subtypes based on severity and chronicity of disease which is reflected in rates of, and responsiveness to, laxative therapy.

The positivity rate for Rome III-defined constipation was 59%-85% across the four clusters. Interestingly, of the patients that did not meet the Rome criteria, 63%-80% across the 4 clusters have demonstrable delayed colonic transit. In other words, Rome III criteria will not pick up a substantial proportion of people who are severely troubled by constipation and who clearly have markedly disturbed physiology as confirmed by delayed colonic transit. In addition, a large proportion of patients, particularly in clusters 1 (52%) and 4 (54%), met the Rome III criteria for IBS. In our experience this overall high prevalence of criteria satisfying the definition of IBS is in keeping with the situation commonly encountered by clinicians and has been reported previously^[34].

One of the potential problems with subtyping constipated patients into categories based on questionnaire data is the prevalence of laxative use. Given that the approach to constipation subtyping used in this study relied upon symptom patterns, laxative use is a potential confounder because these agents can induce symptoms of bloating and pain and alter stool frequency/consistency. While laxative use is mentioned in previous studies^[13,14,34-36], little or no attempt is made to discern their impact upon symptoms. For example, of those patients that met IBS criteria in this study, 92% were heavy laxative users. Asking patients to detail their symptoms in the absence of laxatives has been attempted^[14]. However, in our experience such questions are difficult to answer for a chronically constipated population who, for the most part, have been taking laxatives for many years. Indeed, previous studies have shown a poor concordance between a patient's recollection of events and actual measures^[21].

Furthermore, high rates of laxative use may also con-

found attempts to identify symptom-based distinct subtypes of constipation that correspond to pathophysiological subtypes. This becomes evident when examining the correlation between delayed transit and infrequent stool frequency. There is literature to support infrequent bowel movements in predicting delayed transit^[36-38]. Those studies found that < 3 bm/wk predicted delayed transit in 85%-100% of patients. Indeed, it is the practice of some groups to only evaluate transit formally in patients with infrequent stools^[39]. However, this study indicates that a high percentage (75%) of patients who use their bowel > 3/d have demonstrable slow colonic transit. Importantly, these patients also report high laxative use. Therefore, these data suggest that normal stool frequency, in the context of concurrent laxative use, certainly does not preclude STC.

Delayed colonic transit was found in the majority of patients in whom scintigraphy was performed, which is comparable with the reports of others^[38,40,41]. While it was lowest in cluster 3, a sizable 63% in this relatively mildly affected group had delayed transit; hence, our findings suggest that delayed transit cannot be predicted accurately on the basis of a combination of symptoms.

Using existing criteria for obstructed defecation based purely on symptoms^[1,22,25-28], this syndrome was both common and equiprevalent across all four clusters (49%-57%). This lack of discriminatory ability of clusters to co-localize with these symptoms supports the consistent findings of a number of investigators that symptoms are not predictive of pelvic dyssynergia demonstrated by anorectal manometry and balloon expulsion testing^[34-36,42] or demonstrated by defecography^[43]. Grotz *et al*^[34] found that only a sense of anal blockage correlated with proven pelvic floor dysfunction. However, as Grotz *et al*^[34] point out, the usefulness of this finding must be questioned because this symptom was present in 67% of pelvic floor dysfunction but was found in 50% of patients with STC and in 53% with NTC. Indeed, in their multivariate analysis, they could not identify any colonic symptoms as discriminators of constipation subtypes.

Clinical history remains a “blunt instrument” and while combinations of symptoms do point towards 4 subsets of constipation, we are some distance yet from defining those subsets in unequivocal and specific terms. In light of the overlap with IBS symptoms in this study, it behooves the clinician to consider severe delayed transit in patients presenting with constipation-predominant IBS as this may influence management and avoid mislabeling the patient. Currently, combinations of symptoms cannot predict accurately whether the patient that is categorized into one of these 4 clusters has normal or delayed transit. However, laxative use in severely constipated patients may influence the reported symptoms and this potential confounder needs to be taken into consideration when interpreting these results.

COMMENTS

Background

Constipation is often perceived as a benign, easily treated condition; however,

a number of studies confirm that constipation has a significant adverse effect on a patient's quality of life. Constipation is a heterogeneous disorder and a fundamental aim in researching and treating any heterogeneous disorder is to subclassify the condition into categories that will help guide treatment options. As a patient's symptoms are the first point of discussion with their doctor, the ability to subclassify on the basis of symptoms is a primary goal.

Research frontiers

The mathematical techniques of factor and/or cluster analysis have been used in an attempt to determine whether certain symptoms can predict constipation subtypes. However, studies applying these techniques have found conflicting results suggesting that the *a priori* assumption that these subtypes are distinct or distinguishable from each other on the basis of symptoms may be incorrect, or that investigators have yet to combine the correct symptoms to identify pathophysiologically distinct abnormalities. Utilizing a 53-item specific constipation questionnaire our aim was to identify groups of symptoms that identify underlying severe constipation subgroups, such as slow transit constipation, evacuation disorder and irritable bowel syndrome.

Innovations and breakthroughs

Factor analysis of 221 questionnaires yielded a 4 cluster solution with the presence or absence of pain and laxative unresponsiveness providing the main descriptors. Amongst all clusters there was a considerable proportion of patients with demonstrable delayed colon transit, irritable bowel syndrome positive criteria and regular stool frequency. Therefore, as with previous studies, we have demonstrated that significant overlap exists between mathematically defined clusters of symptoms and globally accepted sub-types of constipation.

Applications

Laxative use in severely constipated patients may influence the reported symptoms and this potential confounder needs to be taken into consideration when interpreting these results.

Peer review

This is an important study in which authors attempted to evaluate whether cluster of symptoms can help to understand pathophysiology of constipation.

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Anterior resection for rectal carcinoma - risk factors for anastomotic leaks and strictures

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Abstract

AIM: To determine the incidence and factors responsible for anastomotic leaks and stricture following anterior resection (AR) and its subsequent management.

METHODS: Retrospective analysis of data from 108 patients with rectal carcinoma who underwent AR or low anterior resection (LAR) to identify the various pre-operative, operative, and post operative factors that might have influence on anastomotic leaks and strictures.

RESULTS: There were 68 males and 40 females with an average of 47 years (range 21-75 years). The median distance of the tumor from the anal verge was 8 cm (range 3-15 cm). Sixty (55.6%) patients underwent handsewn anastomosis and 48 (44.4%) were stapled. The median operating time was 3.5 h (range

2.0-7.5 h). Sixteen (14.6%) patients had an anastomotic leak. Among these, 11 patients required re-exploration and five were managed expectantly. The anastomotic leak rate was similar in patients with and without diverting stoma (8/60, 13.4% with stoma and 8/48; 16.7% without stoma). In 15 (13.9%) patients, resection margins were positive for malignancy. Nineteen (17.6%) patients developed anastomotic strictures at a median duration of 8 mo (range 3-20 mo). Among these, 15 patients were successfully managed with per-anal dilatation. On multivariate analysis, advance age (> 60 years) was the only risk factor for anastomotic leak ($P = 0.004$). On the other hand, anastomotic leak ($P = 0.00$), mucin positive tumor ($P = 0.021$), and lower rectal growth ($P = 0.011$) were found as risk factors for the development of an anastomotic stricture.

CONCLUSION: Advance age is a risk factor for an anastomotic leak. An anastomotic leak, a mucin-secreting tumor, and lower rectal growth predispose patients to develop anastomotic strictures.

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Key words: Rectal carcinoma; Anterior resection; Anastomotic leak; Stricture

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INTRODUCTION

Anterior resection (AR), especially low anterior resection (LAR), for rectal carcinoma and colorectal anastomosis is a technical challenge to surgeons. The introduction of circular stapling devices has made more and more LARs technically feasible. The two most serious complications following AR/LAR, anastomotic leaks and stenosis, are causes of concern because these affect the long-term outcome and quality of life. The incidence of these complications has been variably reported in the literature because of the different definitions used. The objective of this study is to determine the incidence of anastomotic leaks and anastomotic strictures following AR/LAR, the factors responsible for these complications, and to define their management.

MATERIALS AND METHODS

Between January 1989 and December 2008, 280 patients were operated on for rectal carcinoma in the department of Surgical Gastroenterology, a tertiary level referral hospital in Northern India. One hundred and eight of these patients underwent AR/LAR. For the purpose of the study, information was collected both from medical records and a computerized database. Colonoscopy or sigmoidoscopy was performed on all patients to localize the lesion and for tissue sampling for histopathology. A barium enema was administered to some patients to assess the proximal colon for synchronous lesions in cases where a colonoscopy was either incomplete or could not be performed for technical reasons. Chest X-rays were performed on all patients to rule out lung secondaries. Contrast enhanced computed tomography (CECT) was performed to determine the extent of the tumor, to assess lymph nodes, and to detect liver secondaries. Neo-adjuvant chemoradiotherapy was given to patients with unresectable T4 lesions (infiltration to adjacent organs) for downstaging. Routine hemogram, liver function, and renal function tests were ordered as part of the pre-operative work up. All patients received mechanical bowel preparation with Polyethylene Glycol (PEG) solution the day before the planned procedure. Prophylactic antibiotics were administered at the time of induction. Antibiotics were continued for 5 d postoperatively. The procedures were performed either by consultants or registrars. The decision to perform hand-sewn *vs* stapled anastomosis (CDH, Ethicon, Johnson and Johnson, Illinois, USA), and the decision to add a proximal diverting stoma (either a loop ileostomy or a loop colostomy) were taken by the operating surgeon on a case-to-case basis. Suction drains were routinely left in the pelvis, near the anastomosis, in all patients and were removed when the drainage was serous in nature and the amount less than 50 mL/d. A contrast study was performed in patients with clinical suspicion of a leak.

An anastomotic leak is defined as either evidence of feculent drainage or a leak demonstrated on contrast imaging. An anastomotic stricture is defined as anastomotic

Table 1 Factors analyzed for their significance in anastomotic leaks

Factor	Leak (n = 16)	No leak (n = 92)	P value (univariate analysis)
Age (more than 60 yr)	7	13	0.01 ¹
Male sex	11	57	0.78
Distance of the tumor from anal verge (mean, cm)	8.2	8.9	0.5
Pre-operative hemoglobin (mean, g/dL)	10	10.2	0.6
Pre-operative serum albumin (mean, g/dL)	3.6	3.5	0.4
Stapled anastomosis	6	42	0.59
Doughnut incomplete	3	2	0.032
Duration of surgery (mean, h)	1.9	2.8	0.28
Blood loss (mean, mL)	157	168	0.9
Diverting stoma	8	52	0.78
R0 resection	12	65	1
Positive resection margin	2	13	1
Mucin secreting tumour	5	28	1

¹Factors found to be significant.

Table 2 Factors analyzed for their significance in anastomotic strictures

Factor	Stricture (n = 19)	No stricture (n = 89)	P value (univariate analysis)
Age (more than 60 yr)	3	17	1
Male sex	15	53	0.126
Distance from anal verge (mean, cm)	6.6	9.3	0.011 ¹
Pre-operative hemoglobin (mean, gm/dL)	10.7	10.1	0.23
Pre-operative serum albumin (mean, gm/dL)	3.5	3.5	0.92
Stapled anastomosis	11	37	0.21
Doughnut incomplete	2	3	0.63
Duration of surgery (mean, h)	2.25	2.76	0.43
Blood loss (mean, mL)	177	164	0.86
Diverting stoma	11	49	1
R0 resection	15	62	0.57
Positive resection margin	2	13	0.1
Anastomotic leak	8	8	0.001 ¹
Mucin secreting tumour	10	23	0.029 ¹

¹Factors found to be significant.

narrowing that does not allowing the passage of the distal inter-phalangeal joint of the index finger, or narrowing causing difficulty in the evacuation of stool. Patients with T3, T4 disease and/or lymph node positive disease, and/or positive histological margins received chemoradiation as an adjuvant treatment. The diverting stoma was closed after 8-12 wk when a contrast study revealed no anastomotic leak or stricture.

Various clinical, tumor related, and intra-operative factors, which might influence the development of leaks (Table 1) and strictures (Table 2), were analyzed. Univariate analysis was done using Pearson's χ^2 test, Fishers exact test, or Student's *t* test. Multivariate analysis was done

using the binary logistic regression method. SPSS 15 software was used for the analysis. Significance was calculated at the 95% CI and P value < 0.05 .

RESULTS

Among 108 patients who underwent AR/LAR, there were 60 males and 48 females with a median age of 47 years (range 21 to 75 years). The median distance of the tumor from the anal verge was 8 cm (range 3-15 cm). The tumor was situated within 5 cm from the anal verge in 30 patients, between 5 and 10 cm in 46 patients, and above 10 cm in 32 patients. Endoscopic biopsy revealed adenocarcinoma in 100 and adenoma with dysplasia in four. The biopsy was inconclusive in remaining four patients. No patient had lung metastasis on X-ray chest. Based on pre-operative staging, five patients received neo-adjuvant treatment.

LAR (anastomosis below the level of the peritoneal reflection) was performed in 93 patients (86%). Fifteen patients (14%) underwent anterior resection (anastomosis above the level of the peritoneal reflection). Sixty patients (55.6%) underwent hand-sewn anastomosis and 48 (44.4%) had stapled anastomosis. The median distance of the tumor from the anal verge in the hand sewn anastomosis group was 10.2 cm (range 4-15 cm) and it was 6 cm (range 3-15 cm) in the stapled group. Twenty-nine anastomoses were performed using CDH29, ten with CDH31, and three with CDH33. In the remaining six patients, no information regarding the size of the stapler was available. Information regarding anastomotic doughnuts was available in 39 patients. Doughnuts were complete in 34 patients and incomplete in five. Diverting stomas were created in 60 patients (55.6%); 33 (69%) with stapled anastomosis and 27 (45%) with hand-sewn anastomosis. The median duration of operation was 3.5 h (range 2.0-7.5 h). Final histopathology revealed adenocarcinoma in all patients. Resection margins were positive for malignancy in 15 (13.9%) patients. Seven patients with stapled anastomosis and eight with hand-sewn anastomosis had positive resection margins. The resections were R0 (no microscopic or gross residual disease) in 77 patients (71.3%) and R1/R2 (microscopic/macrosopic residual disease left) in 31 (28.7%) patients. Nineteen patients (17.6%) had evidence of distant metastases at operation (liver in 13, peritoneal in three, and both liver and peritoneal in three).

Fifty Six patients (51.8%) had post operative complications. Major complications included wound infection ($n = 27$, 25%), intra-abdominal bleed ($n = 4$, 3.7%), anastomotic leak ($n = 16$, 14.6%), anastomotic stricture ($n = 19$, 17.6%), and intestinal obstruction ($n = 16$, 14.6%). Of the 16 patients who had anastomotic leaks, eight had diverting stoma. Overall, 18 patients (16.7%) required re-exploration for the management of post-operative complications. Eleven patients with anastomotic leaks required re-exploration, and in seven of these, diverting stomas were created at second surgery. The remaining

Table 3 Significant factors determined by multivariate analysis

Factors	P value	Odds ratio
Factors affecting anastomotic leak		
Advance age (> 60 yr)	0.004	7.23
Factors affecting anastomotic stricture		
Anastomotic leak	0.000	13.6
Distance of growth from anal verge	0.011	6.5
Mucin secreting tumor	0.021	5.3

five patients with leaks were managed expectantly. There were two (1.85 %) postoperative deaths (one due to an intra-abdominal bleed and the other due to pneumonitis). Local pelvic recurrence developed in eight patients during the follow up (follow up duration: 1-15 years). Nineteen (17.6%) patients presented with anastomotic strictures at a median duration of 8 mo (range 3-20 mo) after surgery. Biopsy from these strictures revealed no evidence of malignancy in any of them. Seven of these strictures were managed with dilatation using Hegar's dilators under general anesthesia. In others, the dilatations were carried out on an outpatient basis. The median number of dilatation required was 1 (1-4). Diverting stomas were closed in all patients, except for four who had severe fibrotic strictures and did not respond to dilatations even after multiple sessions.

Advanced age (greater than 60 years) and incomplete doughnuts were found to be significant risks for anastomotic leaks on univariate analysis (Table 1). However, age was the only significant risk factor for anastomotic leaks on multivariate analysis (Table 3). On the other hand, increased distance of the tumor from the anal verge, a mucin secreting tumor, and an anastomotic leak were the factors found significant for the development of stricture, both on uni- and multivariate analysis (Tables 2 and 3).

DISCUSSION

Incidence of anastomotic leaks following AR/LAR has been reported to be 3%-21%^[1-7]. Various patient-related, tumor-related, and technique-related factors have been enumerated as predisposing factors for anastomotic leaks. Male sex has been reported to be one such factor because of their unfavorable pelvic anatomy^[1,2]. No significant difference in the number of leaks between males and females was observed in our study.

Distance of the tumor from the anal verge and the position of the anastomosis were found to be associated with the development of anastomotic leaks^[2-4]. The reported higher incidence of leaks as the anastomosis becomes lower may be because of the increasing technical difficulty and ischemia of the distal end. In our series, distance of the tumor from the anal verge was not associated with leaks on univariate analysis. A leak rate of 3%-18% following stapled anastomosis in AR has been reported by various authors^[1,2,5,6]. Law *et al*^[1] and Rullier *et al*^[2] demonstrated a higher leak rate following stapled anastomosis compared to hand-sewn. They attributed

this to the difficulty of the cases undergoing stapled anastomosis. A systematic review of nine randomized controlled trials could not find any significant difference in leak rates between the two groups^[8]. The leak rates of 16.7% for stapled anastomosis and 12.5% for hand-sewn anastomosis in our study were comparable to the published series^[8]. The high leak rates in our study may reflect the experience of operating surgeons (both trainee and consultants). We assume that, as the number of patients and experience increase, the leak rate will decrease.

Although the use of a protective stoma has not been shown to decrease the overall anastomotic leak rate, it reduces the rate of re-operation and postoperative mortality in the event of a leak^[9,10]. In the present series, creation of a protective stoma did not reduce the anastomotic leak rate. The majority of authors recommend a selective policy in providing covering stoma after AR/LAR, reserving it for patients with high risk for of leaks (anastomosis within 5 cm from the anal verge, male gender, and incomplete doughnut)^[4,7,11].

Although anastomotic leak and positive resection margin were implicated as factors promoting recurrence after AR^[12,13], the relation between the leak and a positive resection margin is not well studied. A positive resection margin was not a significant risk factor for anastomotic leak in our patients.

Over all, the anastomotic stricture rate after hand-sewn anastomosis varies from 5%-9%^[14]. The incidence of strictures after hand-sewn anastomosis that necessitated treatment, was 0.6% in Goligher's experience^[15]. The low stricture rate in Goligher's series compared to recent experience could be due to the lower number tumors close to the anal verge. Today, as more and more low rectal tumors are being submitted for LAR and ultra low AR, the stricture rate may also show a similar increase. Stricture rates of up to 20% have been reported^[16]. Some animal studies have suggested that healing by scarring of the of the exposed seromuscular layer with poor epithelial bridging might explain the significant incidence of strictures following stapled anastomosis^[17]. A meta-analysis of 13 randomized controlled trials showed increased stricture rates following stapled anastomosis compared to hand-sewn^[18]. In our series, 22.9% developed strictures after stapled anastomosis, which is higher than the 13.3% developed after hand-sewn anastomosis; however, it was not statistically significant. Waxmann *et al*^[19], in a review of 10 series, reported 6% incidence of strictures with stapled anastomosis. They also noticed a reduced stricture rate with Russian staples, which deliver a single row of staples, compared to the new generation staples, which deliver two rows of staples. The size of the stapler has also some bearing on the stricture rate, according to some published series. Miller *et al*^[20], in their report of 103 patients with stapled anastomosis, had a 4 % stricture rate, and the strictures were more common when a 28 mm diameter stapler was used as compared to one of 31 mm.

The high incidence of anastomotic leaks (14.6%) was another reason for the high stricture rate (17.6%), because a leak predisposes the patient to intense inflamma-

tion and scarring. The stricture rate will therefore invariably increase.

The need for permanent stoma because of anastomotic stricture has been variably reported (1%-9%)^[21-23]. Although the incidence of anastomotic stricture was high in our series (17.6%), it is noteworthy that 15/19 (78.9%) strictures were successfully managed by per-anal dilations. The incidence of anastomotic leaks and strictures are bound to be high in a teaching hospital where there will always be an influx of new trainees.

In conclusion, in our study, advance age was the only factor significantly associated with anastomotic leaks. On the other hand, anastomotic leaks, an aggressive tumor (mucin secreting), and growth in the lower rectum were predisposing factors for development of anastomotic strictures.

COMMENTS

Background

Two major complications of anterior resection (AR) and Low AR, anastomotic leaks and anastomotic strictures, were analyzed retrospectively. The effects of various factors that can lead to anastomotic leaks and anastomotic strictures were analyzed.

Research frontiers

Neoadjuvant Chemotherapy is under evaluation for locally advance tumors. Intersphincteric resections are under evaluation for patients with lower rectal tumors.

Innovations and breakthroughs

Introduction of total mesorectal excision was a major achievement in the development of surgery for lower rectal growth, leading to significant decreases in the complications rate and a significant decrease in local recurrence. Development of end-to-end staplers was also a significant breakthrough, which made anastomosis possible in the lower rectum, thus facilitating sphincter preservation.

Applications

As advance age is a significant risk factor for anastomotic leaks, so attention to detail is important while performing surgery on elderly patients. Similarly, special precautions are needed while performing anastomosis on low-lying growths, and mucin positive tumors; these patients should be operated on only by an experienced surgeon.

Peer review

This is a good paper, but the study is over a period of 20 years, and changes may have happened regarding management.

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Proton pump inhibitor step-down therapy for GERD: A multi-center study in Japan

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Abstract

AIM: To investigate the predictors of success in step-down of proton pump inhibitor and to assess the quality of life (QOL).

METHODS: Patients who had heartburn twice a week or more were treated with 20 mg omeprazole (OPZ) once daily for 8 wk as an initial therapy (study 1). Patients whose heartburn decreased to once a week or less at the end of the initial therapy were enrolled in study 2 and treated with 10 mg OPZ as maintenance therapy for an additional 6 mo (study 2). QOL was in-

vestigated using the gastrointestinal symptom rating scale (GSRS) before initial therapy, after both 4 and 8 wk of initial therapy, and at 1, 2, 3, and 6 mo after starting maintenance therapy.

RESULTS: In study 1, 108 patients were analyzed. Their characteristics were as follows; median age: 63 (range: 20-88) years, sex: 46 women and 62 men. The success rate of the initial therapy was 76%. In the patients with successful initial therapy, abdominal pain, indigestion and reflux GSRS scores were improved. In study 2, 83 patients were analyzed. Seventy of 83 patients completed the study 2 protocol. In the per-protocol analysis, 80% of 70 patients were successful for step-down. On multivariate analysis of baseline demographic data and clinical information, no previous treatment for gastroesophageal reflux disease (GERD) [odds ratio (OR) 0.255, 95% CI: 0.06-0.98] and a lower indigestion score in GSRS at the beginning of step-down therapy (OR 0.214, 95% CI: 0.06-0.73) were found to be the predictors of successful step-down therapy. The improved GSRS scores by initial therapy were maintained through the step-down therapy.

CONCLUSION: OPZ was effective for most GERD patients. However, those who have had previous treatment for GERD and experience dyspepsia before step-down require particular monitoring for relapse.

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Key words: Gastroesophageal reflux disease; Proton pump inhibitor; Omeprazole; Step-down therapy; Gastrointestinal symptom rating scale

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined as the reflux of gastric contents into the esophagus; it may lead to esophagitis, reflux symptoms sufficient to impair quality of life (QOL), and/or long-term complications^[1]. In Japan, GERD was, in the past, considered to be a rare disease, but is now one of the most common chronic disorders. One systematic review of Japan^[2] reported that 15.4% (725) of 4723 patients had heartburn twice or more per week; 42.2% had symptoms of heartburn, including those who had heartburn once or less per week; and 16.7% (602) of 3608 patients had reflux esophagitis. Some papers have suggested that the increasing prevalence of GERD is a result of rising acid secretion in the general Japanese population, caused by the westernization of lifestyle and diet (to include dairy products) and decline in *Helicobacter pylori* (*H. pylori*) infection^[3].

Proton pump inhibitors (PPIs) provide the highest levels of symptom relief, healing of esophagitis, and prevention of relapse and complications^[4]. They are the first choice for therapy in most GERD patients with or without esophageal mucosal injury^[5,6]. However, PPIs cost more than do other acid-inhibiting agents. Some papers suggest that long-term PPI therapy, particularly at high doses, is associated with increased risks of community-acquired pneumonia^[7] and hip fracture^[8]. Therefore, if possible, PPIs should be administered at the lower dose. Full-dose PPI first “step-down” therapy is superior, both to administering histamine-2-receptor antagonist (H₂RA) first and low-dose PPI first “step-up” strategy, with regard to both efficacy and cost-effectiveness^[9,10]. However, some patients experience a recurrence after step-down^[11,12].

On the other hand, there is increasing interest in evaluating the QOL of patients with GERD. It is widely accepted that patients with GERD have a decreased QOL compared with the general population^[13-15], similar to that seen in patients with other chronic diseases^[16]. Consequently, patient-reported symptoms and QOL are important in assessing treatment outcome.

In this article, we describe 2 series of studies devised to investigate the predictors of success in step-down PPI therapy and to assess symptom-related QOL as determined by a questionnaire administered during GERD therapy.

MATERIALS AND METHODS

Study 1 (PPI initial therapy for GERD)

Subjects: This multicenter trial was conducted at Okaya-

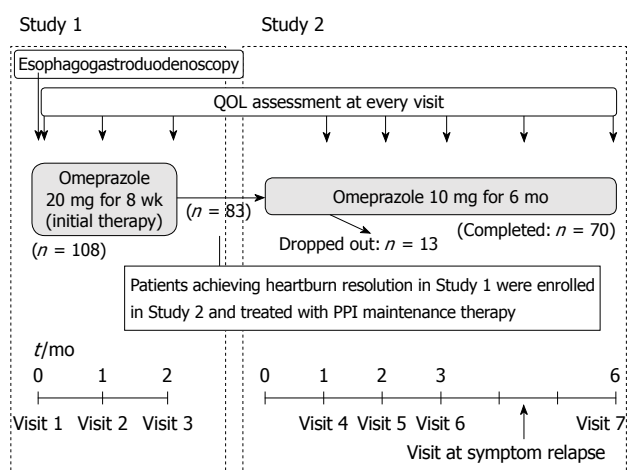


Figure 1 Overview of the study design. QOL: Quality of life; PPI: Proton pump inhibitor.

ma University Hospital and 20 affiliated hospitals in Japan. The study protocol was approved by the local institutional review boards of Okayama University Hospital and of each of the affiliated hospitals. Written informed consent was obtained from each patient before their enrollment in this study.

The major eligibility criteria were the following: heartburn that occurs twice a week or more; age 20 years or older; and either erosive esophagitis (EE) or non-erosive reflux disease. The exclusion criteria were as follows: use of PPIs within 1 mo of the start of the study; open gastric or duodenal ulcer; malignant neoplasm; serious systemic disease; and pregnancy, signs of pregnancy, or occurrence during a lactation period.

Study design: Patients who met the above-mentioned inclusion criteria were asked to complete the Gastrointestinal Symptom Rating Scale (GSRS) to assess gastrointestinal symptom-related QOL. Additional information, including demographic data, height and weight measurements used to assess body mass index (BMI), comorbid conditions, concurrent medication use, history of GERD symptoms, and previous GERD medication, was collected at the initial visit. Upper gastrointestinal endoscopy was performed to assess hiatus hernia, esophagitis, and upper gastrointestinal disorders before treatment.

PPI therapy was performed according to the following strategy (Figure 1): Patients were treated with 20 mg of omeprazole (OPZ) once daily for 8 wk as an initial therapy. GSRS results were evaluated before the initial therapy and after both 4 and 8 wk of treatment.

The primary analysis was to investigate the rate of success of initial PPI therapy. Success was defined as having heartburn once a week or less at the end of the initial therapy. Secondary analyses examined changes in QOL during the initial treatment of PPI.

Study 2 (PPI step-down therapy as maintenance therapy of GERD)

Subjects: Patients whose incidence of heartburn de-

creased to once a week or less at the end of study 1 (the initial therapy) were enrolled in this study. Study 2 subjects included patients who achieved complete remission and those who achieved partial remission of GERD symptoms.

Study design: Patients received PPI step-down therapy: namely, 10 mg of OPZ once daily for 6 mo (Figure 1). QOL related to gastrointestinal symptoms was evaluated at 1, 2, 3, and 6 mo after the beginning of maintenance therapy. If heartburn recurred twice a week or more during maintenance therapy, the treatment was stopped and the QOL at that time was evaluated.

The primary analysis was designed to identify predictors of subjects who were successful in PPI step-down therapy-defined as having no recurrence of heartburn 6 mo after PPI step-down. Secondary analyses examined changes in QOL during the maintenance treatment of PPI.

Endoscopic assessment

The severity of esophagitis was established by endoscopic examination using the modified Los Angeles (LA) classification of Grade A to D, with Grade M and N^[17]. This adds an additional grade N (defined as no apparent mucosal change) and grade M (defined as minimal changes in the mucosa, such as erythema and/or whitish turbidity). The modified LA system has recently become widely used in Japan^[18]. A diagnosis of hiatus hernia was made when the retroflexed endoscope, under the condition of gastric inflation, showed gaping esophageal lumen allowing the squamous epithelium to be viewed below^[19]. Atrophic gastritis was diagnosed by endoscopy using the Kimura-Takemoto endoscopic classification^[20], in which atrophic gastritis was divided largely into closed type (C-type) and open type (O-type) by the location of an atrophic border detected by endoscopy. C-type means that the atrophic border remains on the lesser curvature of the stomach, while O-type means that the atrophic border no longer exists on the lesser curvature but extends along the anterior and posterior walls of the stomach. In this study, atrophic gastritis was defined as only the O-type of the Kimura-Takemoto classification: the endoscopic diagnosis of C-type atrophic gastritis may be unreliable because of interobserver variation.

QOL assessment

Symptom-related QOL was assessed using the GSRS. The GSRS is a disease-specific 15-item questionnaire developed, based on reviews of gastrointestinal symptoms and clinical experience, to evaluate common symptoms of gastrointestinal disorders^[21,22]. Patients are asked to numerically score their subjective symptoms on a Likert-type scale of 1-7. The sum of the scores for all 15 items is regarded as the total GSRS score. Because each of the 15 questions can be scored from 1 to 7, the minimum total score obtainable is 15, and the maximum total score is 105. This total is then divided by 15 to obtain the overall

GSRS score, from a minimum of 1 to a maximum overall score of 7. Furthermore, the scores for the 5 symptom categories (reflux, abdominal pain, dyspepsia, diarrhea, and constipation) are obtained by calculating the means of the scores on the items for each symptom category. The higher the overall score, the more severe the symptoms^[23].

Statistical analysis

All categorical variables were analyzed using chi-square or Fisher's exact test.

Analyses of efficacy parameters were performed on 2 populations: the intention-to-treat (ITT) and the per-protocol (PP) populations.

The time to first symptom relapse after PPI step-down was assessed by Kaplan-Meier analysis. To determine the predictors of successful step-down, univariate and multivariable logistic regression analyses were planned. Variables found in the univariate analysis to be significantly associated with successful step-down were included in a multivariable logistic regression analysis. Changes in QOL were analyzed with paired *t* tests. An overall significance level of 5% was used in all tests. Statistical analysis was performed with SPSS version 11.0 using Windows.

RESULTS

Study 1

Between April 2004 and March 2007, 108 eligible patients were entered into study 1. For reasons that were apparently not causally related to the medication, 21 out of 108 patients dropped out of the study. The demographic characteristics of the patients are shown in Table 1. In the ITT analysis, 83 (76%) of 108 patients completed the 8 wk of initial therapy successfully. In the PP analysis, 83 (95.4%) of 87 patients were successful. The reasons for failure of the 4 patients in initial therapy were insufficiency of GERD symptoms in 2 patients and side effects of PPI in the other 2. The adverse reactions were diarrhea (one patient) and tinnitus (one patient). The reasons for dropout of 21 patients in study 1 were as follows: one patient withdrew her consent before the initial therapy, 12 patients never came to the hospital after the initial visit without excuse, 8 patients never came to the hospital after the second visit without excuse (3 of them still had symptoms of reflux at that time), the others got relief of reflux symptoms.

Symptom-related QOL analysis was performed for subjects who successfully completed the initial therapy. The changes in QOL during therapy are shown in Figure 2. The GSRS total score significantly improved from the baseline after 4-wk treatment ($P < 0.0001$). Of the 83 patients who had heartburn once a week or less at the end of the initial therapy, 55 patients (66%) had a reflux score of one, indicating no symptoms, 23 patients (28%) had a score of two, and the others had a score of three. In other words, 78 (94%) of 83 patients having heartburn

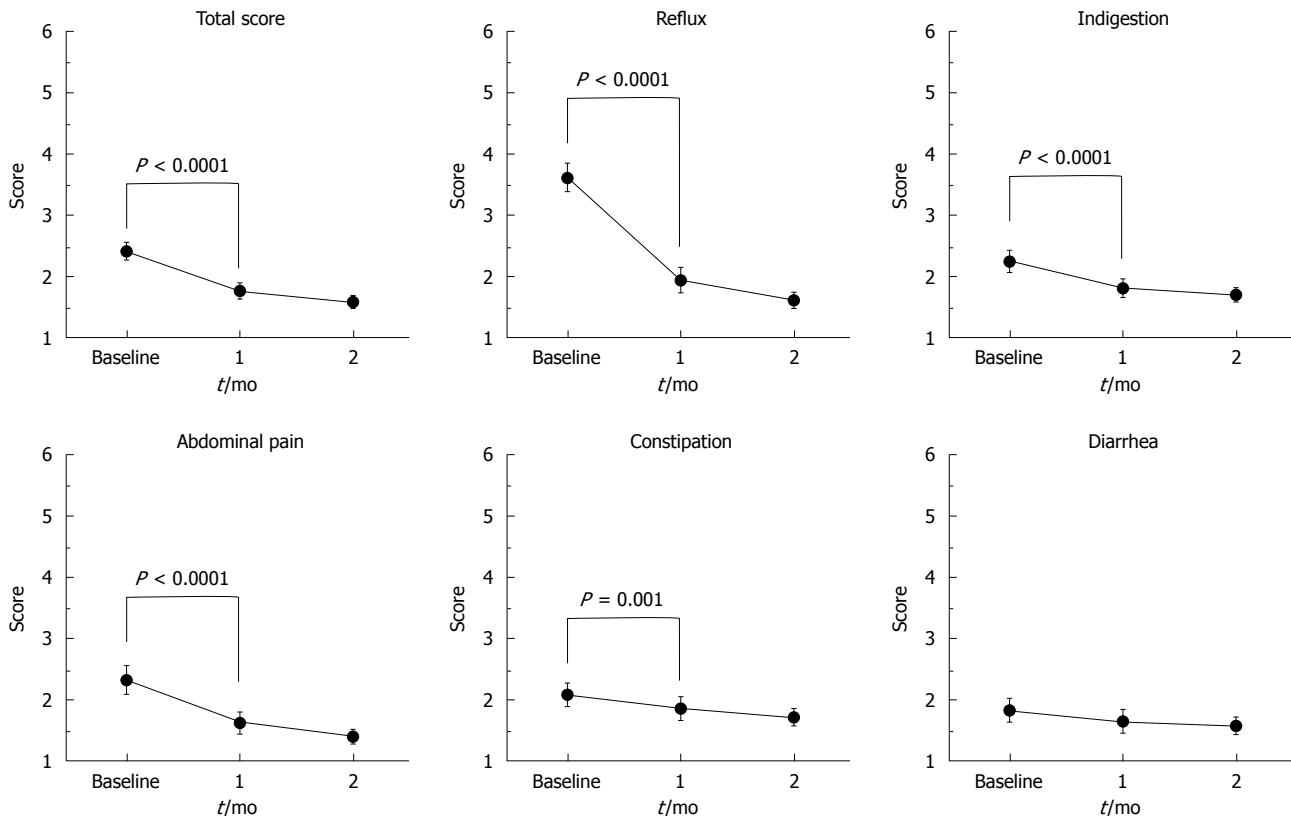


Figure 2 Gastrointestinal Symptom Rating Scale scores during the initial treatment (study 1).

Table 1 Population characteristics of study 1 *n* (%)

Characteristic	ITT population (<i>n</i> = 108)
Gender	
Male/female	62 (57.4)/46 (42.6)
Age (yr)	
Range	20-88
Median	63
BMI (kg/m ²)	
≤ 24.9 (normal or below normal)	77 (71.3)
25-29.9 (overweight)	28 (25.9)
≥ 30 (obese)	3 (2.8)
Previous treatment for GERD	38 (35.2)
Duration of GERD symptoms before initial treatment (mo)	
< 1	21 (19.4)
1-11	47 (43.5)
≥ 12	40 (37.0)
Tobacco use	24 (22.2)
Alcohol use	44 (40.7)
<i>Helicobacter pylori</i> infection	31 (28.7)
The modified Los Angeles Classification	
N/M	20 (18.5)/17 (15.7)
A/B	32 (29.6)/32 (29.6)
C/D	5 (4.6)/2 (1.9)
Atrophic gastritis ¹	18 (16.7)
Hiatus hernia	57 (52.8)
Ischemic heart disease	9 (8.3)
Diabetes mellitus	11 (10.2)
Bronchial asthma	4 (3.7)

¹Atrophic gastritis was defined as only the O-type of the Kimura-Takemoto classification^[20]. BMI: Body mass index; GERD: Gastroesophageal reflux disease; ITT: Intention-to-treat.

once a week or less complained of little or no symptoms of reflux at the end of the initial therapy. Not only the GSRS reflux scores but also the abdominal pain, indigestion, and constipation scores showed significant improvements ($P < 0.0001$).

Study 2

Eighty three eligible patients who had heartburn resolution after the initial 8 wk of therapy were recruited for study 2-step-down treatment as maintenance therapy. Thirteen patients dropped out during the maintenance therapy without recurrence or adverse reactions; 70 patients completed the study 2 protocol. Of 13 dropout patients, one patient experienced exaggerated symptoms of reflux before dropout, nine experienced complete resolution of reflux symptoms before dropout, and the others never came to the hospital after starting the step-down therapy.

Demographic and baseline characteristics of the study 2 population are summarized in Table 2. In the ITT analysis, 56 (67.5%) of 83 patients did not suffer a recurrence during maintenance therapy. In the PP analysis, 56 (80%) of 70 patients were successful in step-down therapy (Figure 3). Of 14 failures, 10 occurred within the first 3 mo of maintenance therapy. The mean time to failure was 56 d (range, 16-180 d).

In the univariate analysis of the ITT population, there were no significant predictors for successful step-down. In the univariate analysis of the PP population ($n = 70$), the significant predictors for successful step-down were:

Table 2 Population characteristics of study 2 *n* (%)

Characteristic	ITT population (<i>n</i> = 83)
Gender	
Male/female	50 (60.2)/33 (39.8)
Age (yr)	
Range	25-88
Median	64
BMI (kg/m ²)	
≤ 24.9 (normal or below normal)	60 (72.3)
25-29.9 (overweight)	20 (24.1)
≥ 30 (obese)	3 (3.6)
Previous treatment for GERD	27 (32.5)
Duration of GERD symptoms before initial treatment (mo)	
< 1	14 (16.9)
1-11	42 (50.6)
≥ 12	27 (32.5)
Tobacco use	21 (25.3)
Alcohol use	39 (47.0)
<i>Helicobacter pylori</i> infection	27 (32.5)
The modified Los Angeles Classification	
N/M	15 (18.1)/9 (10.8)
A/B	24 (28.9)/30 (36.1)
C/D	3 (3.6)/2 (2.4)
Atrophic gastritis ¹	15 (18.1)
Hiatus hernia	44 (53.0)
Ischemic heart disease	8 (9.6)
Diabetes mellitus	7 (8.4)
Bronchial asthma	3 (3.6)
GERD symptom before step-down	
Complete remission	55 (66.3)
Partial remission	28 (33.7)

¹Atrophic gastritis was defined as only the O-type of the Kimura-Takemoto classification^[20]. BMI: Body mass index; GERD: Gastroesophageal reflux disease; ITT: Intention-to-treat.

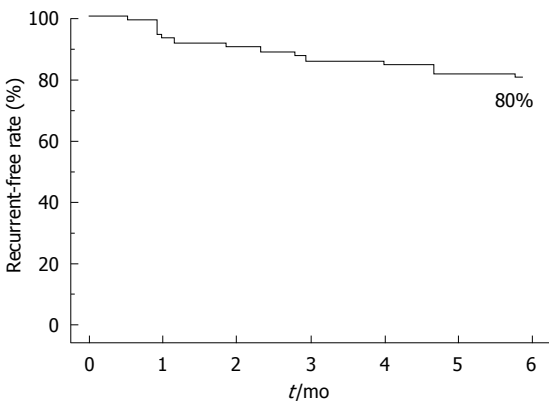


Figure 3 Proportion of subjects without recurrent gastroesophageal reflux disease symptoms after proton pump inhibitor step-down (Study 2 per-protocol population: *n* = 70).

complete remission of GERD symptoms before step-down ($P = 0.042$), no past history of GERD ($P = 0.019$), no H₂RA use at baseline ($P = 0.023$), and better GSRS total ($P = 0.045$) and indigestion ($P = 0.011$) scores at the beginning of step-down (Table 3). In a multivariate analysis of the PP population, no past history of GERD ($P = 0.047$) and a better GSRS indigestion score at the begin-

Table 3 Predictors of successful step-down (univariate analysis)

Variable	ITT population (<i>n</i> = 83)		PP population (<i>n</i> = 70)	
	OR	<i>P</i>	OR	<i>P</i>
Age	0.984	0.428	0.965	0.212
BMI	3.667	0.073	2.108	0.251
Past history of treatment for GERD	0.497	0.161	0.228	0.019
Duration of GERD symptoms	1.004	0.416	1.199	0.536
Alcohol use	1.182	0.732	1.486	0.487
Tobacco use	1.125	0.834	0.692	0.531
<i>Helicobacter pylori</i> infection	0.963	0.942	1.500	0.577
Erosive esophagitis	2.599	0.062	1.402	0.620
LA classification	1.431	0.070	1.238	0.360
Atrophic gastritis	0.930	0.906	0.568	0.411
Hiatus hernia	1.333	0.566	1.476	0.545
complete remission of GERD symptoms before step-down	1.355	0.539	3.676	0.042
GSRS total score (at the beginning of step-down)	0.771	0.647	0.208	0.045
Reflux score in GSRS (at the beginning of step-down)	0.917	0.803	0.477	0.072
Indigestion score in GSRS (at the beginning of step-down)	0.594	0.209	0.218	0.011

BMI: Body mass index; GERD: Gastroesophageal reflux disease; GSRS: Gastrointestinal Symptom Rating Scale; ITT: Intention-to-treat; PP: Per-protocol; OR: Odds ratio; LA: Los Angeles.

Table 4 Predictors of successful step-down (multivariate analysis, per-protocol population: *n* = 70)

Variable	OR	95% CI	<i>P</i>
Past history of treatment for GERD	0.255	0.066-0.980	0.047
Indigestion score in GSRS (at the beginning of step down)	0.214	0.063-0.731	0.014

GERD: Gastroesophageal reflux disease; GSRS: Gastrointestinal Symptom Rating Scale; OR: Odds ratio; CI: Confidence interval.

ning of step-down ($P = 0.014$) were predictive factors for successful step-down (Table 4).

Symptom-related QOL analysis was performed on subjects for whom maintenance therapy was successful. The changes in QOL during therapy are shown in Figure 3. The improved GSRS scores of initial therapy were maintained throughout step-down therapy (Figure 4).

DISCUSSION

In study 1, we investigated the effectiveness of OPZ (20 mg) for the initial treatment of GERD and changes in symptom-related QOL during the initial therapy. Previous studies of patients with GERD using PPIs showed that 64%-88% of patients with typical reflux symptoms respond to PPI therapy^[24-26]. The heartburn resolution rate of the present study (95.4%) was higher than that of previous studies. It is reasonable to assume that a less rigorous end-point of the present study, namely having heartburn once a week or less, would have resulted in a higher rate of symptom response.

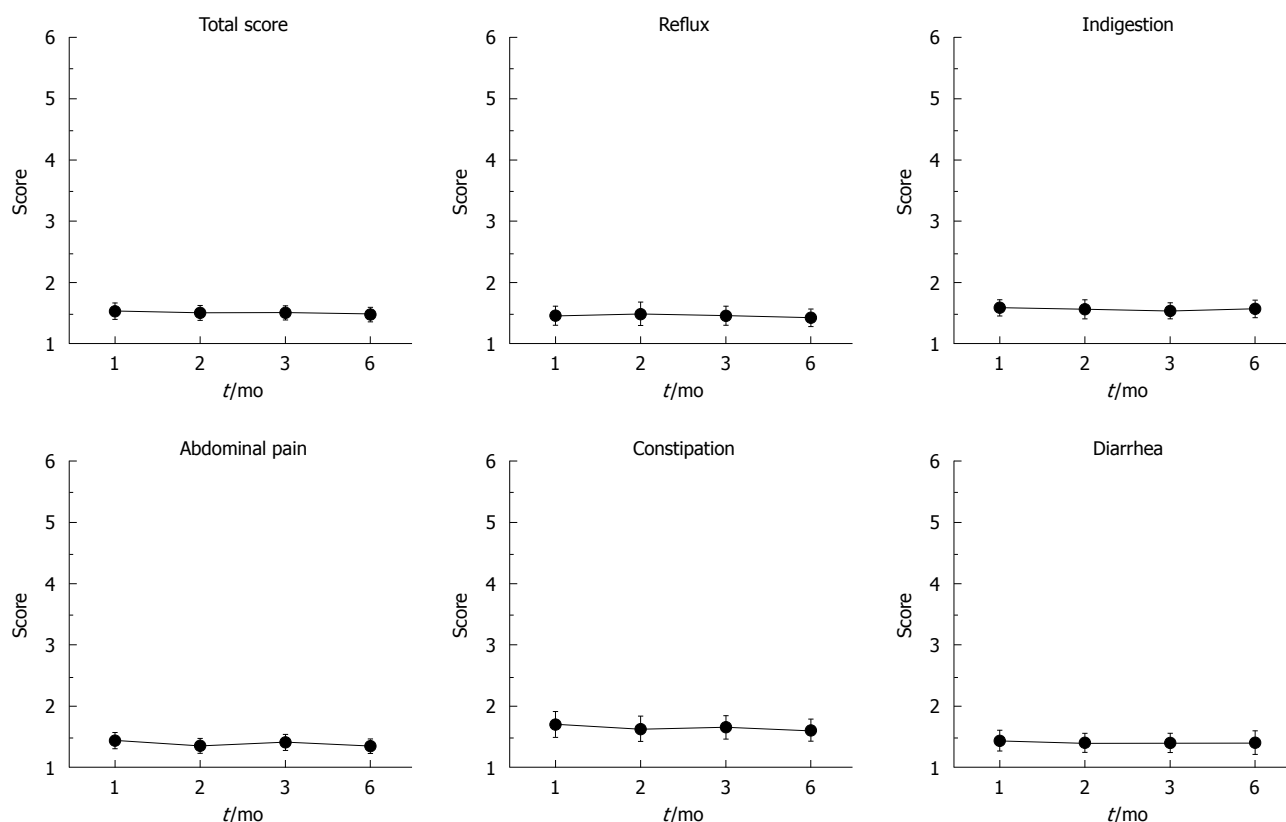


Figure 4 Gastrointestinal Symptom Rating Scale scores during maintenance treatment (study 2).

The assessment of symptom-related QOL in study 1 demonstrated that PPI therapy for GERD produced an improvement not only in reflux symptoms but also in other symptoms, such as abdominal pain and symptoms of indigestion. We could not exclude other factors possibly related to improvement of those symptoms in addition to the drug effect, for instance, regression toward mean, the nature of symptom fluctuation, Hawthorn effect, *etc.* This result confirms that most GERD patients have other gastrointestinal symptoms (dyspepsia and abdominal pain), which, if they are related to acid reflux, are improved by acid suppressants^[27-29].

In study 2, we determined the characteristics of GERD patients whose heartburn relief achieved by the initial therapy could be sustained through maintenance therapy with a half-dose of PPI therapy. The results showed that 80% of subjects whose symptoms were controlled with full-dose PPI could be successfully managed with a lower dose of PPI for 6 mo. The success of step-down was predicted only by no previous treatment for GERD and a better GSRS indigestion score at the beginning of step-down. Inadomi *et al.* showed that the success of step-down was predicted only by the duration of PPI use before the study^[30]. This result is similar to that of the present study in that both studies suggest that the other baseline patient factors likely to influence the efficacy of step-down therapy, such as BMI, *H. pylori* infection, LA classification and erosive esophagitis in endoscopic findings, and hiatus hernia were not predictors of successful step-down. There was no significant difference in

the therapeutic outcome according to LA classification, probably because there were a few patients with severe esophagitis; i.e. grade C or D, in the present study.

It is interesting that the indigestion score after initial therapy is associated with the success of step-down. This result means that patients who are in remission from GERD symptoms, but have dyspeptic symptoms after initial therapy, need stronger acid-suppression than do those who have no symptoms after a standard dose of PPI therapy. The patients with PPI non-responsive dyspepsia may have delayed gastric emptying, which could increase the frequency of transient lower esophageal sphincter relaxation and stimulate gastric acid secretion^[31,32]. This is probably the reason why stronger acid-suppression is necessary for heartburn control. These results demonstrate that physicians who treat GERD should ask patients about several conditions in addition to reflux.

There are several limitations to the present study. First, there were a relatively large number of dropouts during maintenance therapy. So, when analysis regarded dropouts as failures on the basis of the ITT principle, there were no predictors for successful step-down. When the pursuit period of study 2 was shortened from 6 mo to 3 mo, ITT analysis showed several statistically significant differences. In a univariate analysis, significant predictors for successful step-down were no H₂RA use at baseline ($P = 0.05$) and a better GSRS indigestion score at the beginning of step-down ($P = 0.009$). In a multivariate analysis, only the better GSRS indigestion scores at the beginning

of step-down ($P = 0.016$) were predictive factors for successful step-down. In the present study, most patients had mild esophagitis, which would probably benefit from the mild treatment or even by non-drug therapy, for instance, light and early dinner, inclined bed, and straight posture^[33]. This may explain why many patients dropped out. Indeed, most patients discontinued therapy because they had no further symptoms. Furthermore, it cannot be denied that some patients with successful step-down may have had good control of their GERD symptoms by non-drug therapy because we did not take into account the recommended life-style and dietary changes in our study protocol.

Second, in this study, many patients (75% of patients who received 8 wk of initial therapy) did not have the post-treatment endoscopic assessment. Some patients in clinical remission after the initial therapy did not have endoscopic remission. If all subjects underwent post-treatment endoscopy, the endoscopic findings could be the candidate predictor of successful step-down.

Third, cytochrome P450 2C19 genotypic differences, which are related to the cure rates for GERD with PPIs (OPZ and lansoprazole)^[11,34,35], are not considered in this study. This factor could also be the candidate predictor^[11,36]. The analysis of these factors may provide more information for GERD treatment.

In conclusion, our study revealed that the majority of patients whose heartburn decreased to once a week or less on full-dose OPZ therapy can be successfully stepped-down to a half-dose of PPI. The predictors of successful step-down are no previous treatment for GERD and a better indigestion score in the GSRS at the beginning of step-down. Improvement of symptom-related QOL was maintained throughout the step-down therapy.

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COMMENTS

Background

Gastroesophageal reflux disease (GERD) is one of the most common disorders not only in the Western world but also in Japan, with increasing prevalence and incidence in the last decades. Typical GERD symptoms include heartburn, acid regurgitation into the pharynx, and dysphagia. The GERD patients sometimes suffer from chest pain, dyspepsia, and other atypical symptoms including coughing, wheezing, hoarseness, etc.

Research frontiers

Proton pump inhibitors (PPIs) are the first choice of therapy for most GERD patients with or without esophageal mucosal injury. Full dose PPI-first "step-down" therapy is superior to histamin-2-receptor antagonist (H₂RA) or low dose PPI-first "step-up" strategy with regard to both efficacy and cost-effectiveness. However, there is no clear consensus regarding the predictors for successful step-down. Treatment of GERD aims not only at healing esophagitis but also at managing the symptoms of GERD associated with decreased quality of life (QOL). In this study, we investigated the predictors of success in step-down PPI therapy and assessed the symptom-related QOL.

Innovations and breakthroughs

Many studies evaluating the efficacy of PPI in terms of the symptoms of GERD patients in the Western world did not evaluate the grade of esophagitis with endoscopy. Recently, many studies in Japan have focused mainly on non-erosive reflux disease (NERD) patients. The data of this study originated from GERD patients (both erosive gastritis and NERD) who were diagnosed with endoscopy. Therefore, the results may reflect those of the GERD patients in the Japanese general population and can be considered useful for GERD treatment in the clinical practice.

Applications

By considering the clinical features of GERD patients with recurrence during the step-down therapy, this study's findings may suggest new strategies for additional PPI dose reduction including every-other-day administration, on-demand therapy, step-down to H₂RA, and drug-free management.

Peer review

The authors investigated the effect of omeprazole and subsequent step-down maintenance therapy in patients with GERD. This study must be a very interesting topic for readers in view of the rarity of PPI outcome studies in the Asia pacific region.

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Prevalence and impact of musculoskeletal pain in Japanese gastrointestinal endoscopists: A controlled study

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Abstract

AIM: To examine the frequency and prevention of musculoskeletal pain in Japanese gastrointestinal endoscopists and non-endoscopist physicians.

METHODS: Questionnaires were sent to 275 endoscopists and 173 non-endoscopists working in Hiroshima University Hospital and its affiliated hospitals.

RESULTS: The completed questionnaires were returned by 190 (69%) endoscopists and 120 (69%) non-endoscopists. The frequency of pain in the hand and wrist, and especially the left thumb, was significantly higher in endoscopists than in non-endoscopists (17% vs 6%, $P = 0.004$). Using multivariate analysis, the only significant factor associated with this pain was the age of the endoscopist (odds ratio 2.77, 95% confidence interval,

1.23-6.71, $P = 0.018$). Interestingly, endoscopists had made significantly fewer modifications to their endoscopic practices than non-endoscopists (12% vs 33%, $P < 0.0001$) to prevent pain.

CONCLUSION: Pain in the hand and wrist may be endoscopy-related. However, endoscopists made little modifications in practice to prevent such pain. More attention to prevention appears necessary.

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Key words: Endoscopy; Musculoskeletal pain; Pain prevention

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INTRODUCTION

Muscle and joint pain is a common complaint in individuals whose jobs require repetitive isometric maneuvers or awkward body positions^[1]. Such pain has been reported in individuals with various occupations such as bus drivers^[2], unskilled laborers^[3], musicians^[4], physical therapists^[5], and computer keyboard operators^[6]. Recently, ergonomic mechanisms related to the development of work-related musculoskeletal disorders have drawn substantial interest.

Endoscopy in clinical gastroenterology is becoming more important not only in Western countries but also in

Eastern countries including Japan^[7,8], and the number of endoscopic examinations and treatments has increased rapidly. In addition, procedures requiring long performance time such as endoscopic submucosal dissection^[7,9], endoscopic sphincterotomy, and endoscopic papillary balloon dilatation are commonly performed. The work burden of endoscopists has significantly increased.

Evidence suggests that endoscopists frequently report a variety of musculoskeletal problems including neck pain, low back pain, and thumb and hand pain^[10,11]. On the basis of previous reports, it has been suggested that the performance of endoscopy predisposes the operator to the development of musculoskeletal pain. However, there is little published evidence of this, and most of this evidence comes from Western countries; little evidence has come from Eastern countries including Japan. More attention needs to be paid to the impact of musculoskeletal pain and its prevention in Eastern countries. Therefore, we examined the frequency of musculoskeletal pain in Japanese gastrointestinal endoscopists and non-endoscopist physicians. The use of pain prevention strategies and physician requests to improve endoscopic design were also examined.

MATERIALS AND METHODS

General overview

Questionnaires were sent to endoscopists and non-endoscopist physicians working in Hiroshima University Hospital and its affiliated hospitals. The questionnaires were sent with an introductory letter explaining the study. The survey was conducted between March and May, 2010.

Subjects

Subjects consisted of endoscopists and non-endoscopist physicians in Hiroshima University Hospital and its affiliated hospitals. A control group was also included and consisted of a random sample of non-endoscopist physicians.

Questionnaire

The questionnaire for endoscopists consisted of questions on the endoscope model primarily used, mean number of endoscopic examinations and treatments per week, mean amount of time spent performing endoscopic procedures per week, location and description of pain present at the time of the survey, impact of pain on the endoscopist, type of treatment needed for the pain, and injury prevention strategies currently in place, if any. Non-identifying demographic information including age, sex, body mass index (BMI), hand dominance, physical activity level, and practice years was obtained. A modified survey was sent to the non-endoscopists.

Statistical analysis

Student *t*-tests were performed to evaluate differences in weight, height, and BMI between the groups. χ^2 tests were used to assess variables including age, sex, hand dominance, physical activity level, and practice years. To identify the factors associated with musculoskeletal pain,

Table 1 Non-endoscopist physicians who participated in this survey

	<i>n</i> (%)
Internist	65 (54)
Surgeon	17 (14)
Dermatologist	10 (8)
Psychiatrist	7 (6)
Radiologist	4 (3)
Resident	4 (3)
Pathologist	3 (3)
Pediatrician	1 (1)
Orthopedic surgeon	1 (1)
Gynecologist	1 (1)
Emergency physician	1 (1)
NI	6 (5)
Total	120 (100)

NI: Not informed.

Table 2 Subject characteristics

Characteristics	Endoscopists (<i>n</i> = 190)	Non-endoscopists (<i>n</i> = 120)	<i>P</i> value
Age (yr, mean \pm SD)	41.4 \pm 6.7	40.1 \pm 7.6	0.202
Years in practice (yr, mean \pm SD)	16.2 \pm 8.1	14.8 \pm 9.4	0.083
Sex			0.197
Male/female	164/26	97/23	
Dominant hand			0.077
Right/left/NI	179/9/2	108/12/0	
Activity level			0.046
Mild	131 (69%)	70 (58%)	
Moderate	50 (26%)	43 (36%)	
Remarkable	5 (3%)	5 (4%)	
NI	4 (2%)	4 (3%)	
Height (cm)	168.6 \pm 6.9	168.1 \pm 7.0	0.202
Weight (kg)	66.3 \pm 10.6	64.9 \pm 11.3	0.287
Body mass index (kg/m ²)	23.2 \pm 2.2	22.8 \pm 3.0	0.306

NI: Not informed.

Table 3 Number of endoscopic procedures completed per week (mean \pm SD)

Procedure type	No. per week
Esophagogastroduodenoscopy (<i>n</i> = 190)	15.0 \pm 8.7
Colonoscopy (<i>n</i> = 161)	6.7 \pm 3.7
Endoscopic retrograde cholangiopancreatography (<i>n</i> = 63)	2.1 \pm 1.8
Endoscopic ultrasonography (<i>n</i> = 48)	2.2 \pm 1.9

especially pain in the hand and wrist among endoscopists, univariate and multivariate logistic regression analyses were performed with JMP IN (Cary, NC, USA) software. All significance levels were set at $P < 0.05$.

RESULTS

Subject characteristics

questionnaires were sent to 275 endoscopists and 173 non-endoscopist physicians. One hundred ninety (69%) endoscopists and 120 (69%) non-endoscopists returned

Table 4 Environmental factors affecting endoscopic procedures

Factor	No. per week (<i>n</i> = 190)
Use of height-adjustable examination table (yes/no)	186 (98)/4 (2)
Position of monitor (directly in front of endoscopist, at eye level/other position)	133 (70)/57 (30)
Manufacturer of endoscope primarily used (Olympus/Fujifilm/NI)	138 (73)/47 (25)/5 (3)

NI: Not informed.

completed questionnaires. The details of non-endoscopist physicians who participated in this survey are shown in Table 1. The two groups were similar in terms of age, years in practice, sex, hand dominance, and BMI (Table 2). Physical activity level was significantly lower in endoscopists than in non-endoscopists ($P = 0.046$).

Endoscopy information

The number of esophagogastroduodenoscopies completed per week was 15.0 ± 8.7 (mean \pm SD) (Table 3). Of the 190 endoscopists, 161 (85%) performed colonoscopies, and the mean number of colonoscopies performed per week was 6.7 ± 3.7 (mean \pm SD). Sixty-three (33%) endoscopists performed endoscopic retrograde cholangiopancreatography (ERCP). Forty-eight (25%) endoscopists performed endoscopic ultrasonography (EUS). On average, the amount of time spent per week performing endoscopy was 11.9 ± 8.7 h (mean \pm SD).

Environmental factors affecting endoscopic procedures

Most (98%) endoscopists indicated that they used height-adjustable examination tables (Table 4). As for monitor position, 133 of 190 (70%) endoscopists replied that the monitor was directly in front of the endoscopist and at eye level. With regard to the manufacturer of the endoscope used, 138 (73%) endoscopists used Olympus, 47 (25%) used Fujifilm, and 5 (3%) gave no response.

Musculoskeletal pain in endoscopists

None of the endoscopists reported a history of preexisting musculoskeletal conditions. Eighty-one (43%) endoscopists had musculoskeletal pain at the time of the survey, and 21 (26%) of those reporting pain sought medical care as a consequence.

The most frequent sites of pain identified by the endoscopists were the lower back ($n = 50$, 26%), the neck ($n = 18$, 9%), the right shoulder ($n = 18$, 9%), and the left thumb ($n = 16$, 8%) (Table 5). Pain in the hand and wrist was reported by 32 (17%) endoscopists.

Musculoskeletal pain in non-endoscopists

None of the non-endoscopists reported a history of preexisting musculoskeletal conditions. Forty-nine (41%) non-endoscopists reported musculoskeletal pain at the time of the survey, and 7 (14%) of these physicians sought medical care as a consequence.

The most frequent sites of pain identified in the non-endoscopists were the lower back ($n = 24$, 20%), the right shoulder ($n = 14$, 12%), the neck ($n = 13$, 11%), and the left shoulder ($n = 12$, 10%) (Table 5). Pain in the hand and wrist was reported by 7 (6%) non-endoscopists.

Comparison of musculoskeletal pain between endoscopists and non-endoscopists

The frequency of overall musculoskeletal pain did not differ significantly between the endoscopists and non-endoscopists (81 of 190 (43%) *vs* 49 of 120 (41%), $P = 0.755$). However, the frequency of pain in the left thumb was significantly higher in the endoscopists than in the non-endoscopists (16 of 190 (8%) *vs* 0 of 120 (0%), $P = 0.001$). Pain in the hand and wrist was significantly higher in the endoscopists than in the non-endoscopists (32 of 190 (17%) *vs* 7 of 120 (6%), $P = 0.004$). There were no significant differences between the groups in the frequency of pain at other locations, such as the shoulder and lower back.

Univariate and multivariate logistic regression analyses for factors associated with thumb, finger, hand, and wrist pain in endoscopists

Results of univariate analysis for factors related to thumb, finger, hand, and wrist pain in endoscopists are shown in Table 6. The only significant factor was age of the endoscopist (> 40 years *vs* ≤ 40 years, $P = 0.011$). The mean number of colonoscopies per week and dominant hand tended to be significant (> 6.7 *vs* ≤ 6.7 , $P = 0.091$, left *vs* right, $P = 0.098$, respectively). Thus, endoscopists who performed more than 6.7 colonoscopies per week or who were left-handed tended to have more frequent pain in the hand and wrist.

Results of multivariate analysis for factors related to pain in the hand and wrist in endoscopists are shown in Table 7. Only age was found to be a significant factor associated with pain in endoscopists (> 40 years *vs* ≤ 40 years, odds ratio (OR) 2.77, 95% confidence interval, 1.23-6.71, $P = 0.018$) by multivariate analysis.

Pain prevention

Only 23 (12%) endoscopists had made modifications to their endoscopic practice in response to pain arising due to performing endoscopic procedures. The most common practice modifications endorsed are shown in Table 7. These modifications were stretching exercises ($n = 11$, 6%), taking breaks ($n = 11$, 6%), wearing athletic shoes ($n = 8$, 4%), and less work ($n = 5$, 3%). Interestingly, endoscopists had made significantly fewer modifications to their practice than non-endoscopists to address pain prevention.

Requests to improve endoscopic design from endoscopists

The requests made by Japanese endoscopists to improve endoscope design are shown in Table 8. The most frequent request was to make the operating part lighter ($n = 85$, 45%), followed by making the operating part

Table 5 Frequency of major types of musculoskeletal pain in Japanese endoscopists and non-endoscopists *n* (%)

Site of pain	Endoscopists (<i>n</i> = 190)	Non-endoscopists (<i>n</i> = 120)	<i>P</i> value
Neck (present/absent)	18 (9)/172 (91)	13 (11)/107 (89)	0.698
Right shoulder (present/absent)	18 (9)/172 (91)	14 (12)/106 (88)	0.536
Left shoulder (present/absent)	15 (8)/175 (92)	12 (10)/108 (90)	0.522
Right wrist (present/absent)	3 (2)/187 (98)	1 (1)/119 (99)	0.571
Left wrist (present/absent)	13 (7)/177 (93)	4 (3)/116 (97)	0.186
Right thumb (present/absent)	3 (2)/187 (98)	0 (0)/120 (100)	0.167
Left thumb (present/absent)	16 (8)/174 (92)	0 (0)/120 (100)	0.001
Right hand/fingers (present/absent)	5 (3)/185 (97)	1 (1)/119 (99)	0.263
Left hand/fingers (present/absent)	4 (2)/186 (98)	1 (1)/119 (99)	0.387
Lower back (present/absent)	50 (26)/140 (74)	24 (20)/96 (80)	0.204
Hand and wrist (present/absent)	32 (17)/158 (83)	7 (6)/113 (94)	0.004
Total (present/absent)	81 (43)/109 (57)	49 (41)/71 (59)	0.755

Table 6 Univariate analysis of factors associated with pain in the hand and wrist in endoscopists

Characteristics	<i>P</i> value
Age (> 40 yr <i>vs</i> < 40 yr)	0.011
Sex (male <i>vs</i> female)	0.378
Dominant hand (left <i>vs</i> right)	0.098
Activity level (moderate/remarkable <i>vs</i> mild)	0.338
Height (> 168 cm <i>vs</i> < 168 cm)	0.796
Weight (> 66 kg <i>vs</i> < 66 kg)	0.642
Body mass index (> 25 kg/m ² <i>vs</i> < 25 kg/m ²)	0.61
Mean number of esophagogastroduodenoscopies per wk (> 15 <i>vs</i> < 15)	0.215
Mean number of colonoscopies per wk (> 6.7 <i>vs</i> < 6.7)	0.091
Do ERCP (yes <i>vs</i> no)	0.871
Do EUS (yes <i>vs</i> no)	0.969
Mean time performing endoscopic procedures per week (> 12 h <i>vs</i> < 6.7 h)	0.636
Use of height-adjustable examination table (no <i>vs</i> yes)	0.836
Position of monitor (other position <i>vs</i> directly in front of endoscopist, at eye level)	0.8
Manufacturer of endoscope primarily used (fujifilm <i>vs</i> olympus)	0.28

ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasonography.

smaller (*n* = 41, 22%), and reducing the resistance of the angulation controller (*n* = 36, 19%). There were no significant differences between the frequency of requests and the manufacturers of the endoscope primarily used (data not shown).

DISCUSSION

Our data indicate that the frequency of pain in the hand and wrist, and especially in the left thumb, was significantly higher in endoscopists than non-endoscopists (17% *vs* 6%, *P* = 0.004). By multivariate analysis, the only significant factor associated with pain was the age of the endoscopist (OR 2.77, *P* = 0.018).

Previous studies have reported that the incidence of symptoms or injuries attributable to the performance of endoscopy ranges from 39% to 78%^[11]. These results suggest that musculoskeletal pain is prevalent in

Table 7 Modifications to prevent musculoskeletal pain

Modification	Endoscopists (<i>n</i> = 190)	Non-endoscopists (<i>n</i> = 120)	<i>P</i> value
No modifications	167 (88)	80 (67)	< 0.0001
Stretching exercises	11 (6)	26 (22)	< 0.0001
Taking breaks	11 (6)	8 (7)	0.754
Wearing athletic shoes	8 (4)	6 (5)	0.744
Less work	5 (3)	1 (1)	0.263

Table 8 Requests to improve endoscopic design from endoscopists *n* (%)

Request	No. of endoscopists (<i>n</i> = 190)
Make the operating part heavier/lighter	0 (0)/85 (45)
Make the operating part larger/smaller	1 (1)/41 (22)
Make the angulation controller larger/smaller	15 (8)/27 (14)
Make the resistance of the angulation controller heavier/lighter	0 (0)/36 (19)

endoscopists. In the present study, 43% of endoscopists had musculoskeletal pain, which is similar to that in previous reports. In addition, a U.S. study showed that the frequency of reported musculoskeletal pain was higher in endoscopists than non-endoscopists (74% *vs* 35%, *P* < 0.001)^[10]. In the present study, pain in the hand and wrist was significantly more frequent in endoscopists than in non-endoscopists, indicating that endoscopists may be at higher risk for musculoskeletal pain, especially in the hand and wrist, than non-endoscopists.

The most frequent sites of pain reported in our study were the lower back, neck, right shoulder, and left thumb. Previous studies have reported similar anatomic sites of pain associated with performing endoscopy, including a case report of de Quervain's syndrome or "endoscopists' thumb"^[11-14]. Buschbacker^[15] found that 27% of responders reported low back pain, 19% thumb pain, and 19% shoulder pain. Preliminary results from an American Society for Gastrointestinal Endoscopy web-based survey found that 43% reported hand or carpal tunnel injuries, 29% reported back pain, and 28%

reported neck pain^[16]. Specific aspects of performing endoscopy which may contribute to musculoskeletal pain are as follows: adjusting the tip of angulation controls, torquing with the right hand, and standing for prolonged periods of time. Manipulation of the tip angulation controls and torquing of the endoscope may lead to hand and wrist pain, whereas standing for prolonged periods of time may lead to back and neck pain.

In the present study, only age of the endoscopist was significantly associated with pain. Several researchers reported that age did not have an impact on the musculoskeletal pain identified. However, the location of pain has been reported to be different between a beginner group (duration of practicing endoscopy, < 39 mo) and an experienced group^[11]. The left thumb and fingers were the most common painful areas in beginners (43%), whereas the left shoulder was most painful in experienced endoscopists (33%). Furthermore, older age and sex were associated with many musculoskeletal disorders^[17]. Age may be an important factor associated with the musculoskeletal pain reported by endoscopists.

ERCP and EUS are both procedures requiring long endoscopy times with special devices. Some may think that these procedures are more likely to lead to musculoskeletal pain than other procedures. However, in the present study, performance of these procedures was not a significant factor associated with pain, whereas the mean number of colonoscopies performed per week and dominant hand tended to be significant by univariate analysis. In other words, the performance of colonoscopy may be a risk factor for the development of pain in the hand and wrist rather than the performance of ERCP or EUS. The torquing technique is very important in the performance of colonoscopy, and it is done frequently and for long periods of time. Furthermore, colonoscopy may require stronger torquing power than any other procedures, including ERCP and EUS. Therefore, torquing of the colonoscope appears to be another risk factor associated with pain. The colonoscope is torqued with the right hand, which is the weaker hand for left-handed endoscopists, and it is plausible that left-handed endoscopists tend to have more pain in the hand and wrist than right-handed endoscopists. A larger study is needed to investigate this issue.

Methods to prevent back pain include the use of a height-adjustable examination table, rubberized floor mats, and a short foot stool to alternate weight distribution^[18]. Monitor placement is an especially important determinant of torso and head posture. Monitors should be placed directly in front of the endoscopist while in the working position to avoid rotation and flexion of the cervical spine and should be adjusted to eye level^[18]. Most (98%) responders in the present study reported use of an adjustable examination table. However, 30% of responders reported that the position of monitors was inadequate. None of the endoscopists used rubberized floor mats or a short foot stool. There is much room for improvement in relation to these factors.

Improving posture, increasing physical activity, and stretching exercises before endoscopy have been suggested to prevent disability^[18]. Interestingly, in the present study, endoscopists made significantly fewer modifications to their endoscopic practices to prevent musculoskeletal pain, especially in regard to performing stretching exercises, than non-endoscopists. The reason for this is unknown. One possibility is that Japanese endoscopists may be busier than other non-endoscopist physicians and may not have enough time or willingness to exercise or stretch. Of course, selection bias may be undeniable. Education on methods to reduce endoscopic-related injuries must be carried out in a positive manner.

Endoscopic techniques that can help to prevent pain include minimizing torque and having an assistant apply torque when necessary. Ultimately, reevaluating the design of the endoscope with regard to ergonomics may be the best long-term strategy to reduce overuse injuries in an era of high-volume endoscopy. Endoscopists in the present study requested a lighter and smaller operating part and reduced resistance of the angulation controller. These requests may be useful for improving the design of the endoscope.

In conclusion, our data suggest that pain in the hand and wrist may be endoscopy-related. However, endoscopists made few modifications to their practices to prevent pain. More attention to pain prevention appears to be needed. Given the importance of endoscopy in the clinical setting, our results support a further controlled study on this subject in a much larger and more diverse population of endoscopists.

COMMENTS

Background

It has been suggested that the performance of endoscopy predisposes the operator to the development of musculoskeletal pain.

Research frontiers

There is little published evidence of this, especially from Eastern countries including Japan.

Innovations and breakthroughs

Frequency of occurrence of pain in the hand and wrist, and especially the left thumb, was significantly higher in endoscopists than in non-endoscopists (17% vs 6%, $P = 0.004$). By multivariate analysis, the only significant factor associated with the pain was the age of the endoscopist (odds ratio 2.77, 95% confidence interval, 1.23-6.71, $P = 0.018$).

Applications

The authors' data suggest that pain in the hand and wrist may be endoscopy-related. More attention toward pain prevention appears necessary.

Peer review

This is a study on an important issue, musculoskeletal pain in endoscopists. As authors described, more attention toward pain prevention in endoscopists should be paid.

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Long-term result of endoscopic Histoacryl® (N-butyl-2-cyanoacrylate) injection for treatment of gastric varices

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Abstract

AIM: To evaluate the long-term efficacy and safety of endoscopic obliteration with Histoacryl® for treatment of gastric variceal bleeding and prophylaxis.

METHODS: Between January 1994 and March 2010 at SoonChunHyang University Hospital, a total of 127 patients with gastric varices received Histoacryl® injections endoscopically. One hundred patients underwent endoscopic Histoacryl® injections because of variceal bleeding, the other 27 patients received such injections as a prophylactic procedure.

RESULTS: According to Sarin classification, 56 patients were GOV1, 61 patients were GOV2 and 10 patients were IGIV. Most of the varices were large (F2 or F3, 111 patients). The average volume of Histoacryl® per each session was 1.7 ± 1.3 cc and mean number of sessions was 1.3 ± 0.6 . (1 session-98 patients, 2 sessions-25 patients,

≥ 3 sessions-4 patients). Twenty-seven patients with high risk of bleeding (large or fundal or RCS+ or Child C) received Histoacryl® injection as a primary prophylactic procedure. In these patients, hepatitis B virus was the major etiology of cirrhosis, 25 patients showed GOV1 or 2 (92.6%) and F2 or F3 accounted for 88.9% ($n = 24$). The rate of initial hemostasis was 98.4% and recurrent bleeding within one year occurred in 18.1% of patients. Successful hemostasis during episodes of rebleeding was achieved in 73.9% of cases. Median survival was 50 mo (95% CI 30.5-69.5). Major complications occurred in 4 patients (3.1%). The rebleeding rate in patients with hepatocellular carcinoma or GOV2 was higher than in those with other conditions. None of the 27 subjects who were treated prophylactically experienced treatment-related complications. Cumulative survival rates of the 127 patients at 6 mo, 1, 3, and 5 years were 92.1%, 84.2%, 64.2%, and 45.3%, respectively. The 6 mo cumulative survival rate of the 27 patients treated prophylactically was 75%.

CONCLUSION: Histoacryl® injection therapy is an effective treatment for gastric varices and also an effective prophylactic treatment of gastric varices which carry high risk of bleeding.

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Key words: Gastric varix; Prophylaxis; Histoacryl® (N-butyl-2-cyanoacrylate); Histoacryl® injection; Treatment of gastric varix

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Kang EJ, Jeong SW, Jang JY, Cho JY, Lee SH, Kim HG, Kim SG, Kim YS, Cheon YK, Cho YD, Kim HS, Kim BS. Long-term result of endoscopic Histoacryl® (N-butyl-2-cyanoacrylate) injection for treatment of gastric varices. *World J Gastroenterol* 2011; 17(11): 1494-1500 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i11/1494.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i11.1494>

INTRODUCTION

Gastric varices occur in 18%-70% of patients with portal hypertension^[1,2] and are classified as gastro-esophageal varices (GOV) 1 (esophageal varix extending down to the cardia or lesser curve) or GOV2 (esophageal and fundal varices). Isolated gastric varices (IGV) may be located either in the fundus (IGV1) or elsewhere in the stomach (IGV2)^[1]. Although the incidence of bleeding from gastric varices is relatively low (10%-36%), when it occurs it tends to be more severe, to require more transfusion, and to have a higher mortality rate than esophageal variceal bleeding^[1,3-5] while acute bleeding is controlled. Gastric varices have a high rate of rebleeding (38%-89%)^[6-8]. Precisely what triggers a bleed from a gastric varix is not known. However a number of risk factors for gastric variceal bleeding have been identified: location in the fundus, advanced Child's stage, presence of red spots, and increasing size of the varices are the important factors in determining the risk of a first bleed from gastric varices^[9,10].

A number of treatment modalities for acute gastric variceal bleeding and prevention of bleeding are available. These include endoscopic treatment (sclerotherapy, band ligation, *etc.*), TIPS (transjugular intrahepatic portosystemic shunt), and B-RTO (balloon-occluded retrograde transvenous obliteration). Of these, the first-line treatment for gastric variceal bleeding is endoscopic obliteration with Histoacryl® (N-butyl-2-cyanoacrylate)^[11,12].

Though it has been used worldwide for the treatment for gastric varices, data regarding the long-term efficacy and safety of this procedure are still lacking. Indeed, although rare, fatal complications do occur^[13,14].

We evaluated the long-term efficacy and safety of endoscopic obliteration with Histoacryl® for treatment of gastric variceal bleeding in terms of the prevalence of complications, ability to predict rebleeding and survival rates. Furthermore, the efficacy, prevalence of complications and survival rates of prophylactic endoscopic injection sclerotherapy with Histoacryl for high risk gastric varices found incidentally also assessed.

MATERIALS AND METHODS

Patients

This study was a retrospective analysis of patients with cirrhosis who underwent endoscopic Histoacryl® injection. From January 1994 to March 2010, patients (127) who presented to our hospital with acute gastric varices as evidenced by hematemesis or melena, or who had gastric varices without bleeding evidence, were considered for enrollment. This included 100 patients with active gastric variceal bleeding and 27 with high-risk varices (large, located in the fundus, red color sign (RCS), Child C)^[10] who underwent primary prophylaxis.

Of the 127 patients, 97 (76.4%) were male and 30 (23.6%) female, and the mean age was 55.69 years. The most common etiology of gastric varices was hepatitis B-related cirrhosis in 50 patients (39.3%), followed by alcoholic liver cirrhosis in 48 (37.8%), unknown in 19 (15%) and hepatitis C virus in 10 (7.9%). Twenty-one patients (16.5%) had been

Table 1 Baseline characteristics-127 patients treated with Histoacryl® -injection therapy

Clinical characteristics of patients	Number	%
No. of patients (male/female)	127(97/30)	76.4/23.6
Mean age (yr)	55.69	
Etiology of gastric varices		
Liver cirrhosis	127	100
Hepatitis B	50	39.3
Hepatitis C	10	7.9
Alcoholic	48	37.8
Unknown	19	15
Association of hepatocellular carcinoma	21	16.5
Child-pugh classification (A/B/C)	42/59/26	33.1/46.4/20.5
Bleeding status		
< 24 h	48	37.8
> 24 h	52	41
Prevention	27	21.2
Sarin classification of gastro-esophageal varices		
GOV1	56	44.1
GOV2	61	48
IGV1	8	6.3
IGV2	2	1.6
Form of gastric varices		
F1	15	12
F2	56	44.4
F3	55	43.6

GOV: Gastro-esophageal varices; IGV: Isolated gastric varices.

diagnosed with hepatocellular carcinoma. The lesions of 42 patients (33.1%) were classified as Child-Pugh class A, 59 (46.4%) as Child-Pugh B, and 26 (20.5%) as Child-Pugh C. Endoscopic Histoacryl® injection was conducted within 24 h of gastric variceal bleeding in 48 patients (37.8%). Of the 127 patients, GOV1 was present in 56 (44.1%), GOV2 in 61 (48%), IGV1 in 8 (6.3%), and IGV2 in 2 (1.6%). Most varices ($n = 111$) were large (F2 or F3) (Table 1)^[9].

Twenty-seven patients with high risk gastric varices underwent endoscopic Histoacryl® injection as a primary prophylaxis. Of these, 21 (77.4%) were male and six (22.6%) female, and the mean age was 55.29 years. The most common etiology of gastric varices was hepatitis B-related cirrhosis (14 patients, 51.9%), followed by alcoholic liver cirrhosis in 6 (22.2%). Five of the 27 (18.5%) patients were diagnosed with hepatocellular carcinoma. Twelve (44.4%) were classified as Child-Pugh A and 11 (40.7%) as Child-Pugh B to Child-Pugh C. Of the 27, GOV1 was seen in 15 patients (55.6%) and GOV2 in 10 (37%). Most varices ($n = 24$) were categorized as size F2 or F3 (Table 2).

Treatment methods

Histoacryl® injection was performed using a GIFQ 230 or GIFQ 240 endoscope (Olympus C, Tokyo, Japan) and 23-gauge disposable injection needle catheter (1650 mm in length). Each shot contained 1.0-2.0 cc Histoacryl® and an equal volume of Lipiodol (Guerbet, Aulnay-Sous-Bois, France). The endoscopic suction channel was flushed with lubricant (olive oil) before insertion of the catheter. The preload sclerotherapy catheter was advanced through the endoscope into the stomach, and the needle was inserted directly in the gastric varix (Figure 1A and B). Immediately

Table 2 Baseline Characteristics-27 patients who underwent primary prophylaxis

Clinical characteristics	Number	%
No. of patients (male/female)	27(21/6)	71.4/28.6
Mean age (yr)	55.29	
Etiology of gastric varices		
Liver cirrhosis	27	100
Hepatitis B	14	51.9
Hepatitis C	3	11.1
Alcoholic	6	22.2
Unknown	4	14.8
Association of hepatocellular carcinoma	5	18.5
Child-Pugh classification (A/B/C)	12/11/4	44.4/40.7/14.9
Sarin classification of gastro-esophageal varices		
GOV1	15	55.6
GOV2	10	37
IGV1	2	7.4
IGV2	0	0
Form of gastric varices		
F1	3	11.1
F2	9	33.3
F3	15	55.6

GOV: Gastro-esophageal varices; IGV: Isolated gastric varices.

following injection of cyanoacrylate mixture into the gastric varix, the location of lipiodol in the gastric varix was assessed using fluoroscopic visualization (Figure 2). Lipiodol was given through the catheter to deliver the glue mixture into the varix, and the needle was then withdrawn while glue was still flowing to decrease the risk of needle embedment. The catheter was then flushed with sterile water. Typically 2 cc of a 1:1 mixture of lipiodol and Histoacryl® was administered per injection. Injections were repeated until gastric varices appeared to occlude, as judged by probing with a blunt probe.

Outcome measures

Successful hemostasis was defined as vital sign stability, no drop in hemoglobin and no rebleeding within 24 h of after sclerotherapy. Rebleeding was defined as hematemesis and/or melena, hypotension (a drop in systolic blood pressure of > 20 mm Hg from baseline), 2 mg/dL fall in hemoglobin and/or transfusion requirement of ≥ 2 units of packed red blood cells after 24 h.

Statistical analysis

Statistical interpretation of data was performed using the Statistical Program for Social Sciences (SPSS) version 14. Results were expressed as mean \pm SD for continuous variables and as numbers (percentage) for categorical data. Analysis was performed using the independent *t*-test, χ^2 test and Fisher's exact test, as appropriate. A *P*-value < 0.05 was considered statistically significant. Kaplan-Meier method and then log rank test were used to examine predictive factors of rebleeding rate within 1 year and survival rates.

RESULTS

Hemostatic outcomes

A total of 162 endoscopies were performed and the mean

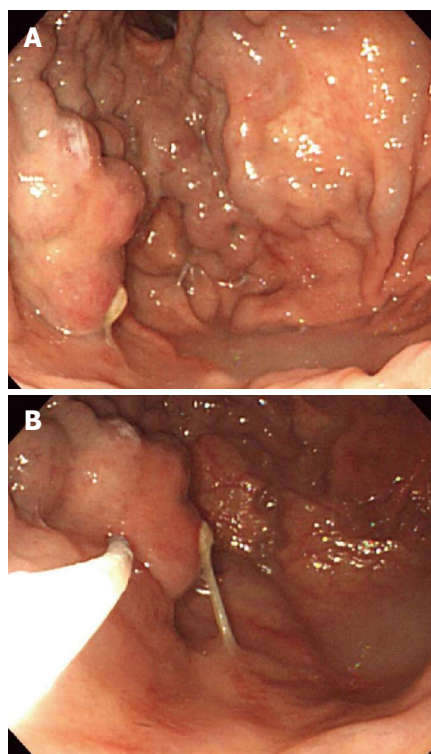


Figure 1 Endoscopic Histoacryl® injection for treatment of gastric varices. A: Endoscopic image showing a large gastric varix; B: Injection of Histoacryl® into the gastric varix using the catheter.



Figure 2 X-ray showing lipiodol in the gastric varix during the procedure.

number of sessions was 1.3 ± 0.6 . Obliteration of gastric varices was achieved with one session in 98 patients and two sessions in 25. Four patients required three or more sessions. The mean volume of Histoacryl® administered was 1.7 ± 1.3 cc. Initial hemostasis was achieved in 125 patients (98.4%). Total number of rebleeding episodes in 127 patients was 29 (22.8%). The mean follow-up period of rebleeding was 322.71 ± 551.14 d. Episodes of rebleeding from 1 d to 1 year post-therapy occurred in 23 (18.1%) subjects with recurrent bleeding. One patient of these was considered to have treatment failure, whereas the others (22, 73.9%) experienced successful hemostasis after rebleeding (Table 3).

Only one Histoacryl® treatment was needed in 21 of 27 subjects (77.8%) who underwent primary prophylaxis. The other 6 patients received several sessions of Histo-

Table 3 Results of 127 Histoacryl®-injection patients

Result of Histoacryl® injection	Number	%
No. of sessions		
1	98	77.1
2	25	19.7
3-5 (3/5)	4 (3/1)	3.2
Mean number of sessions	1.3 ± 0.6	
Mean injection volume (cc)	1.7 ± 1.3	
Initial hemostasis	125	98.4
Number of rebleeding	29	22.8
Duration of rebleeding		
< 5 d	4	3.1
< 6 wk	10	7.9
< 1 yr	9	7.1
Successful hemostasis of rebleeding	22	73.9

Table 4 Results of 27 patients with primary prophylaxis

Result of Histoacryl® injection	Number	%
No. of sessions		
1	21	77.8
2	4	14.8
3-5 (3/5)	2 (1/1)	3.7/3.7
Mean number of sessions	1.37	
Mean injection volume (cc)	2.1	
Number of patients	27	
Death	4	14.8
Hepatic failure	3	11.1
Hepatocellular carcinoma	1	3.7
Prophylactic treatment-related complication	0	0

acryl® injection therapy because of incomplete obliteration of varices. The mean volume of Histoacryl® administered to these individuals was 2.1 cc. Three patients died due to hepatic failure and one due to hepatocellular carcinoma (HCC). No prophylactic treatment-related complications were noted (Table 4).

Complications

Complications were observed in 73/127 patients. The most common was fever ($n = 44$), followed by mild abdominal pain ($n = 22$) which subsided within 24 h. Two patients developed post-treatment infections; *Klebsiella pneumonia* and *Escherichia coli* were identified through blood cultures. One developed pulmonary embolism (Figure 3A) and one patient suffered splenic infarction (Figure 3B); two patients developed adrenal abscesses (Figure 4A). Adrenal abscesses were drained through the catheter percutaneously, and both individuals recovered fully (Figure 4B). A conservative treatment strategy for both pulmonary embolism and splenic infarction was conducted (Table 5).

Predictive factors of rebleeding

Rebleeding occurred in 9 of 21 patients with HCC ($P = 0.000$). Also it occurred in 15 patients with GOV2 and 8 patients with GOV 1 ($P = 0.009$). The presence of HCC or GOV2 by Sarin classification was significantly associated with rebleeding within 1 year of the initial Histoacryl® injection (Figure 5A and B).

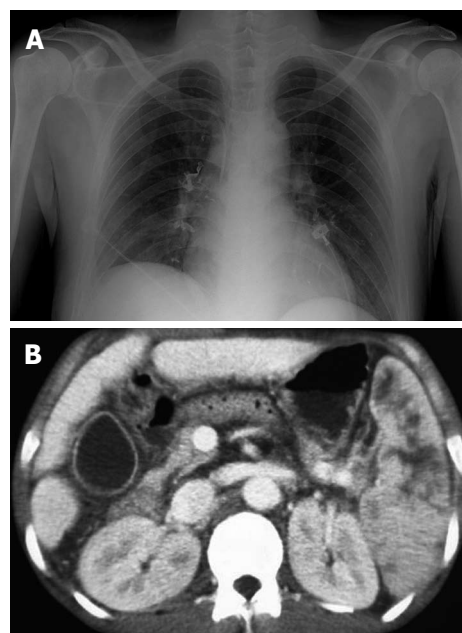


Figure 3 Embolic complications of Histoacryl® injection. A: Chest radiography showing pulmonary embolism after Histoacryl® injection; B: Computed tomography showing the presence of lipiodol in the splenic vein after Histoacryl® injection.

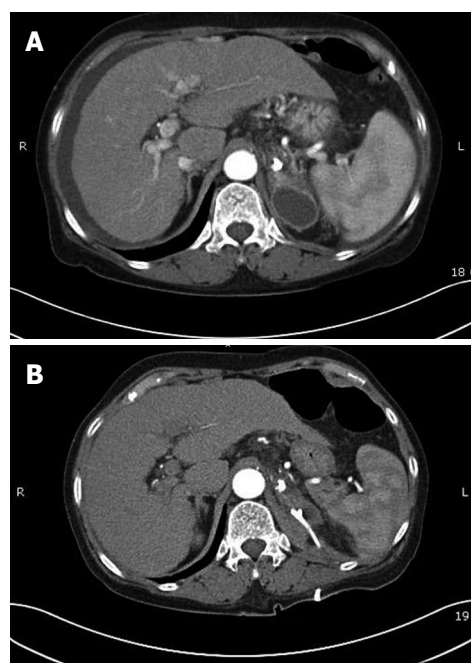


Figure 4 Adrenal abscess after Histoacryl® injection. A: Computed tomography scan showing adrenal abscess after Histoacryl® injection; B: Adrenal abscess was resolved after PCD insertion.

One year rebleeding rates were not associated with sex ($P = 0.597$), the cause of cirrhosis ($P = 0.707$), forms of varices ($P = 0.269$), time from bleeding to hemostasis ($P = 0.815$) or CTP grade ($P = 0.230$) (Table 6).

Survival

The median survival duration was 50 mo (95% CI 30.5-69.5). The cumulative survival rates of the 127 patients at 6 mo

Table 5 Complications of Histoacryl® injection

Complications	Number
Pulmonary embolism	1
Adrenal abscess	2
Splenic infarction	1
Bacteremia	2
Fever	44
Abdominal pain	22

Table 6 Predictive factors of rebleeding within 1 year

Clinical characteristics	Rebleeding	Non rebleeding	P
Sex			0.597
Male	17	80	
Female	6	24	
Etiology			0.707
Hepatitis B	11	39	
Hepatitis C	2	8	
Alcoholic	6	6	
Unknown	4	4	
Association of hepatocellular carcinoma			0.000
Yes	9	12	
No	14	92	
Sarin classification of gastro-esophageal varices			0.009
GOV1	8	48	
GOV2	15	46	
Form of gastric varices			0.269
F1	1	14	
F2	11	45	
F3	11	44	
Duration			0.815
< 24 h	17	80	
> 24 h	6	24	
CTP grade			0.230
A	6	36	
B	9	52	
C	8	16	

GOV: Gastro-esophageal varices.

and at 1, 3, and 5 years were 92.1%, 84.2%, 64.2%, and 45.3%, respectively (Figure 6A). Four of the 27 patients who underwent primary prophylaxis died of hepatic failure or HCC (Table 4). The 6-mo cumulative survival rate of these patients was 75% (Figure 6B).

DISCUSSION

Gastric varices and their association with portal hypertension were first described in 1931^[15]. The overall incidence of gastric varices in patients with portal hypertension is 18%-70%^[1,2]. The incidence of bleeding from gastric varices is relatively lower, ranging from 10%-36%, compared with bleeding from esophageal varices^[1]. Mortality associated with a first variceal bleed appears to have improved in recent years but remains as high as 20% within 6 wk of the index bleed^[3,4].

Endoscopic injection of Histoacryl® is the currently recommended in recent consensus and guidelines as the initial treatment for acute gastric variceal bleeding^[11,12]. Endoscopic injection of tissue glue for gastric variceal

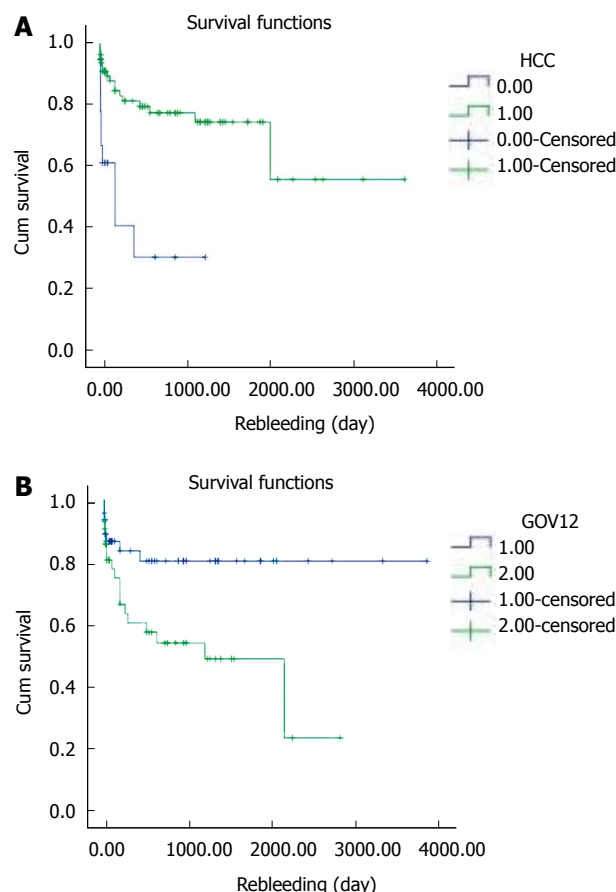


Figure 5 The 1-year rebleeding rate using the Kaplan-Meier method and then log rank test. A: Comparing the groups with hepatocellular carcinoma (HCC) or without HCC; B: Comparing GOV1 and GOV2. GOV: Gastro-esophageal varices.

bleeding was first reported in 1986 by Soehendra *et al*^[16]. However, the rebleeding rate remains high, at 22%-34% and episodic complications such as systemic embolism have been the main issues^[13]. Cerebral stroke, portal vein embolization, splenic infarction, coronary embolism, and nonfatal pulmonary emboli in 4.6% of cases were reported as complications of tissue adhesive use^[14,17-19]. But the complication rate was relatively low and most of the complications were not severe. The common adverse effects of Histoacryl® injection therapy include fever and abdominal discomfort. In this study, unusual adverse effects, such as pulmonary embolism, splenic infarction, and adrenal abscess were noted. We already reported two cases of adrenal abscess after NBC injection (Gut and Liver, article in press). The routes for gastric variceal drainage are gastrorenal shunt or gastro-caval shunt. In our cases, the adrenal abscesses developed 4-6 mo after the Histoacryl® injection. The reason why adrenal abscesses after Histoacryl® injection were so delayed is that insufficient drainage and stasis of adrenal vein blood by the Histoacryl® material made a significant contribution to abscess formation. In theory, using undiluted Histoacryl® may prevent distal embolization because the polymer solidifies rapidly upon contact with blood^[20]. D'Imperio *et al*^[21] studied undiluted Histoacryl® in 80 patients with bleeding gastric, esophageal, and duodenal varices. Distal embolization to distant abdominal

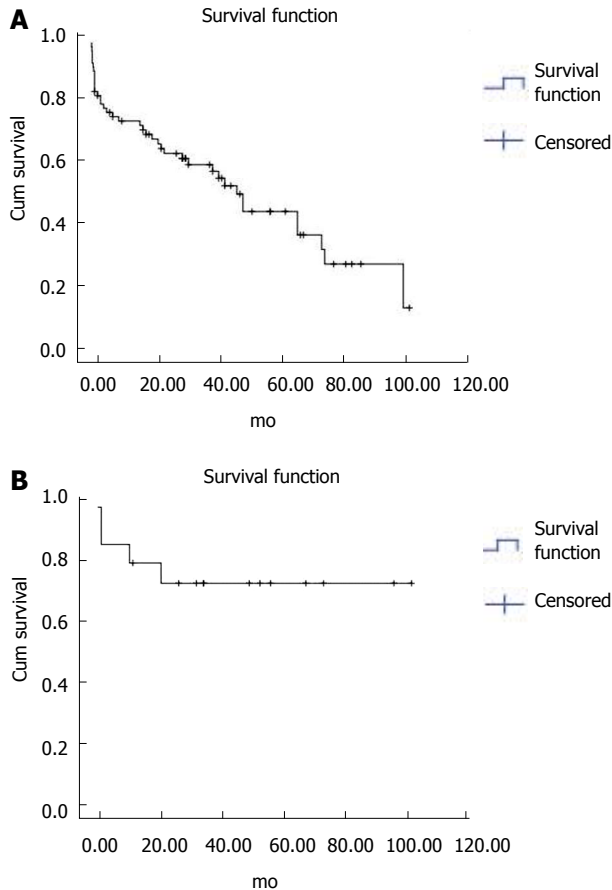


Figure 6 Survival of Histoacryl® injected patients. A: Survival of 127 Histoacryl®-injected patients using the Kaplan-Meier method. The cumulative survival rates of the 127 patients at 6 mo, and 1, 3 and 5 years were 92.1%, 84.2%, 64.2%, and 45.3%, respectively; B: Survival of the 27 patients who underwent primary prophylaxis, using the Kaplan-Meier method. The 6 mo cumulative survival rate of these patients was 75%.

sites occurred in two patients. Therefore, using undiluted Histoacryl® does not preclude the risk of embolisation. In our cases, all subjects recovered fully; no symptoms such as cough and chest pain caused by migration of Histoacryl® from the variceal location to the pulmonary vein were noted. PCD was inserted into the adrenal abscess and follow-up CT indicated completed resolution.

Accurate classification of gastric varices is essential in determining optimal management. The most widely used classification is that proposed originally by Sarin *et al*^[1]. Unlike esophageal varices, a high porto-systemic pressure (> 12 mmHg) does not cause gastric variceal bleeding, likely due to the high frequency of spontaneous gastro-renal shunts^[22]. In one study, fundal varices had a significantly higher bleeding incidence (78% and 55% for IGV1 and GOV2, respectively) than either GOV1 or IGV2 (10%)^[23]. In the present study, of 127 patients, GOV1 was seen in 56 (44.1%), GOV2 in 61 (48%), IGV1 in 8 (6.3%), and IGV2 in 2 (1.6%). No significant difference in 1-year rebleeding rate rates between GOV1 and GOV2 were detected ($P = 0.173$).

Billi *et al*^[24] conducted a 24 mo follow-up of 50 patients who underwent Histoacryl® injections for gastric varices and reported that acute hemostasis was achieved in 92% of patients. Also Fuster *et al*^[25] reported good results

from Histoacryl® use in pediatric patients. In this study, the success rate of acute hemostasis was 98.4%, comparable to or better than that reported elsewhere^[26,27].

The reported rebleeding rate after Histoacryl® injection for acute gastric variceal bleeding ranged from 22%-59%^[26,28]. This was reduced by improvement of treatment technique, use of prophylactic antibiotics and effective vasoactive agents, and so on. Also the higher the MELD score was the more rebleeding occurred^[29]. In our study the rebleeding rate was 22.8% ($n = 29$). This is consistent with other reports^[26,28]. Episodes of rebleeding from 1 d to 1 year occurred in 23 patients (18.1%). Twenty-two patients experienced successful hemostasis after rebleeding (73.9%).

In our study, rebleeding within 1 year was associated with the presence of HCC ($P = 0.000$) and Sarin classification (GOV2) ($P = 0.009$). However, no statistically significant difference in rebleeding rates among Child-Pugh scores A, B, and C ($P = 0.230$) or form of gastric varices ($P = 0.269$), sex ($P = 0.597$) and etiology ($P = 0.707$) was found. We assume that complete obliteration in GOV2 is not easy because GOV2 is wider than GOV1, and that was the reason for more frequent rebleeding in GOV2 than GOV1.

Use of β -blockers for the primary prevention of gastric variceal bleeding is not supported by significant data^[5]. Because the mortality rate of gastric variceal bleeding is high, it has been suggested that patients at high risk for bleeding should undergo primary prophylaxis of the gastric varix^[10,30]. Hashizume *et al*^[9] reported a number of risk factors for gastric variceal bleeding: red colored spots, larger nodular varix, and location in the fundus. According to Kim *et al*^[10] advanced Child-Pugh class, varices > 5 mm in size, and the presence of a red spot were associated with an increased risk for a first bleed. On the basis of these facts, 27 patients with high-risk gastric varices (large, fundus, RCS, Child C) underwent primary prophylaxis with endoscopic Histoacryl® injection. Four of these patients died of hepatic failure or HCC. The others had survived to the time of writing this report. Thus the 6 mo cumulative survival rate of these patients was 75%. No treatment-related complications were noted.

In conclusion, Histoacryl® injection therapy is effective for the treatment of gastric varices with high initial hemostasis and rare complications. On the basis of our analysis, rebleeding rates were linked to the presence of HCC or GOV2. Furthermore, Histoacryl® injection therapy is effective for prophylaxis of gastric varices which carry high risk of bleeding.

COMMENTS

Background

Gastric variceal bleeding tends to be more severe than esophageal variceal bleeding. Furthermore, acute bleeding was controlled, gastric varices have a high rate of rebleeding. Precisely what triggers a bleed from a gastric varix is not known. Also the effect of primary prophylaxis of gastric varices is not studied well.

Research frontiers

In this study, the authors demonstrate the long-term efficacy and safety of endoscopic obliteration with Histoacryl® for treatment of gastric variceal bleeding and prophylaxis.

Innovations and breakthroughs

The rate of initial hemostasis was 98.4% and recurrent bleeding within one year

occurred in 18.1% of patients. The rebleeding rate in patients with hepatocellular carcinoma (HCC) or GOV2 was higher than in those with other conditions. Median survival was 50 months (95% CI 30.5–69.5). Cumulative survival rates of the 127 patients at 6 months, 1, 3, and 5 years were 92.1%, 84.2%, 64.2%, and 45.3%, respectively. The 6-month cumulative survival rate of the 27 patients treated prophylactically was 75%.

Applications

Histoacryl® injection therapy is effective for the treatment of gastric varices with high initial hemostasis and rare complications. On the basis of our analysis, rebleeding rates were linked to the presence of HCC or GOV2. Furthermore, Histoacryl® injection therapy is effective for prophylaxis of gastric varices which carry high risk of bleeding.

Terminology

Histoacryl® (N-butyl-2-cyanoacrylate) is a watery solution, which polymerizes and hardens within 20 seconds in a physiological milieu and instantaneously upon contact with blood. This makes it ideal for obliterating vessels and controlling bleeding. It is necessary to dilute it with the oily contrast agent Lipiodol® is not only compatible with the tissue glue for dilution but also allows fluoroscopic monitoring of delivery of the substance.

Peer review

The authors reported their results of a retrospective study on patients who underwent endoscopic NBC injection for gastric varices. Important data including the success rate of initial haemostasis, rebleeding rate, long-term complications and prognostic predictors were reported. Though being limited by the retrospective nature of the study, the strength of the study was the relatively large number of patients with active bleeding.

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Clinicopathologic significance of HER-2/neu protein expression and gene amplification in gastric carcinoma

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1+ were measurable in 6.9%, 8.3% and 17.2% of the samples, respectively. No HER-2 was stained in 67.6% of the samples. FISH showed that *HER-2* gene was amplified in 18 samples, 10 HER-2 3+ samples, 5 HER-2 2+ samples, and 3 HER-2 1+ samples with IHC staining. HER-2 status was not correlated with the sex and age of patients, and tumor size, location or differentiation, but with the depth of invasion, TNM stage, lymph node and distant metastasis as well as histopathological classification of gastric cancer ($P < 0.05$).

CONCLUSION: All samples with IHC as HER-2 expression should be analyzed with FISH. Detection of *HER-2* gene amplification can assess the malignant biological behaviors and prognosis of gastric cancer.

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Key words: Gastric carcinoma; HER-2; Clinicopathologic significance; Immunohistochemistry; Fluorescence *in situ* hybridization

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Yan SY, Hu Y, Fan JG, Tao GQ, Lu YM, Cai X, Yu BH, Du YQ. Clinicopathologic significance of HER-2/neu protein expression and gene amplification in gastric carcinoma. *World J Gastroenterol* 2011; 17(11): 1501-1506 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i11/1501.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i11.1501>

Abstract

AIM: To study the HER-2/neu protein expression and gene amplification in gastric carcinoma and their relation.

METHODS: One hundred and forty-five formalin-fixed and paraffin-embedded tumor tissue samples from Chinese gastric carcinoma patients were studied with immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) methods. Clinicopathologic data about all patients were collected.

RESULTS: The levels of HER-2 3+, HER-2 2+ and HER2

INTRODUCTION

Gastric cancer is the second most common cause of cancer-related death worldwide. Surgery is the only curative procedure for localized gastric cancer, which is at the advanced stage when diagnosed in most cases. Currently

available agents are not very effective, resulting in a high recurrence rate, a low survival rate, and a poor prognosis of advanced gastric cancer patients. Thus, treatment of gastric cancer remains a challenge for physicians. New targeted therapies for advanced gastric carcinoma are needed, which may open up a new avenue for cancer treatment. Current targeted therapy for advanced gastric carcinoma depends on the evaluation of target gene status^[1-3].

HER-2/neu or CerbB-2 is a member of growing factors (EGFR, erbB-2, erbB-3 and erbB-4) in the HER family with intrinsic protein tyrosine kinase activity, and its increased activity is the assumed mechanism underlying cell transformation^[4]. It is an oncogene that regulates the biological functions such as cell proliferation, differentiation, motility, and apoptosis. It was reported that HER-2 is over-expressed in cancer of breast, lungs, salivary gland, ovary, colon, prostate and pancreas^[5,6]. HER-2 plays an important role in activation of HER-2 protein, and is over-expressed in 10%-38% of gastric cancer patients^[7]. However, the correlation between the expression of HER-2 protein and the prognosis of gastric cancer is still controversial. Different studies showed different HER-2 expression levels in gastric carcinoma^[8-10].

Detecting the HER-2 status in gastric carcinoma is a prerequisite of monoclonal antibody therapy for gastric cancer. In this study, immunohistochemistry (IHC) was used in detecting HER-2 oncoprotein and fluorescent *in situ* hybridization (FISH) was used as a follow-up test for ambiguous results.

MATERIALS AND METHODS

Materials

Paraffin-embedded tumor tissue blocks were provided by Cancer Hospital of Fudan University and Institute of Traditional Chinese Medicine of Jiangsu Province. One hundred and forty-five (95 males and 50 females) out of the 476 Chinese patients with gastric adenocarcinoma, admitted to Department of Surgery, Cancer Hospital of Fudan University, and Institute of Traditional Chinese Medicine of Jiangsu Province in 2006-2009 for surgery, were enrolled in this study. Tumor tissue blocks from patients with full clinical data (including diagnosis, age, sex, address, disease history, *etc.*) were cut into 20 slides. The average age of the patients was 60 years. Cancer was classified as stage I in 32 cases, stage II in 35 cases, stage III in 37 cases, and stage IV in 41 cases, respectively, according to the TNM Cancer Staging System of the American Joint Committee of Cancer. The study was approved by The Review Board of Fudan University Cancer Hospital and Institute of Traditional Chinese Medicine of Jiangsu Province. Informed consent was obtained from all patients.

Tumor tissue was cut into 4- μ m thick sections which were stained with hematoxylin and eosin for histopathological types, differentiation stage, IHC and FISH evaluation.

Immunohistochemical staining

Slides were deparaffinized, rehydrated, and heated in a microwave oven containing 0.01 mol/L citrate buffer (pH 6.0) for 5 min at 100°C for antigen retrieval, cooled for 20 min and washed with water and a buffer solution. Peroxidase was applied for 5 min and washed with a buffer solution for 2 \times 5 min. Primary antibody diluted at 1:200 was applied for 30 min, link and streptavidin were applied for 10 min, respectively, and then washed with a buffer solution for 2 \times 5 min. The bound antibody was visualized using a DAB-chromogen substrate. The sections were then counterstained with hematoxylin, covered with a cover-slip. Negative control was stained by omission of the primary antibody. Over-expression of HER-2 protein in paraffin-embedded invasive breast carcinoma tissue slides was used as a positive control.

A strong brown staining was located in cell membrane of malignant cells using this staining method. The DAKO Hercep Test™ Protocol System^[11] was used to grade the membrane staining. The staining was scored as negative (0) when no membrane was stained or when membrane was stained in less than 10% of tumor cells, weakly positive (1+) if focal membrane was stained in more than 10% of tumor cells, intermediately positive (2+) if complete membrane was weakly- moderately stained in more than 10% of tumor cells, and strongly positive (3+) if complete membrane was intensely stained in more than 10% of tumor cells. Scores 0 and 1 were considered negative, while scores 2 and 3 were considered positive.

FISH analysis

HER-2 gene was amplified with dual-color FISH using a Passvision HER-2 DNA probe kit (Vysis Inc. Downers Grove, IL, USA) according to its manufacturer's instructions. Briefly, hybridization buffer, DNA probe, and purified water were centrifuged, and heated to 73°C for 5 min in a water bath. Slides were immersed in a denaturing bath (70% formamide 2 \times SSC) for 5 min at 73°C, followed by dehydration in increasing ethanol concentrations, and then dried. The probe mixture was applied to each slide. The slides were placed in a 42°C incubator for 30 min, washed with 0.4 \times SSC/0.3% NP-40 for 2 min, air-dried in darkness, counterstained with 4',6-diamidino-2-phenylindole (DAPI), and covered with a cover-slip. HER-2/neu-spectrum orange probe contains a DNA sequence specific for the HER-2 human gene locus and hybridized to the region 17q11.2-q12 of human chromosomes. CEP17 (chromosome enumeration probe 17)/spectrum green probe containing alpha-satellite DNA that hybridizes to the D17Z1 locus (centromere region of chromosome 17) was used as a control. Nuclei were counterstained with DAPI. The slides were observed under a B \times 60 fluorescence microscope equipped with a digital camera (DP50; Olympus, Tokyo, Japan) and the images were captured on a Windows PC with the Viewfinder Lite software. A cell was considered to be amplified when a definite cluster or more than 10 signals for

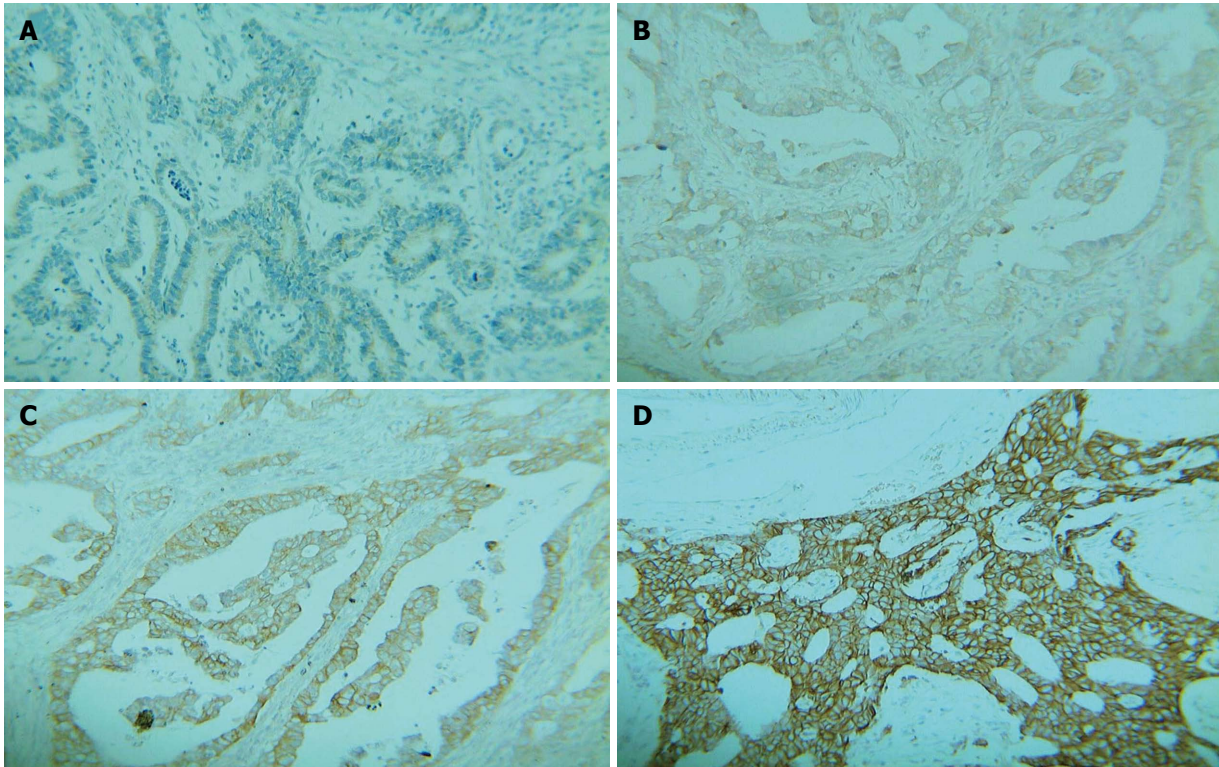


Figure 1 Immunohistochemistry staining of gastric carcinoma tissue samples showing negative HER-2 protein expression (A, B), positive HER-2 protein expression (C, D) (original magnification $\times 200$).

Table 1 Correlation between HER-2 protein expression and *HER-2* gene amplification

HER-2 FISH	Immunohistochemistry for HER-2				Total
	0 (<i>n</i> = 98)	1 (<i>n</i> = 25)	2 (<i>n</i> = 12)	3 (<i>n</i> = 10)	
Negative	98	22	7	0	127
Positive	0	3	5	10	18

FISH: Fluorescence in situ hybridization.

HER-2 were found. Known positive and negative cells were used as controls for each FISH. Gene amplification was scored when a minimum of 20 cancer cell nuclei exhibited a HER-2/CEP17 ratio ≥ 2 , or when a HER-2 signal cluster was observed.

Statistical analysis

Statistical analysis of data was performed by Student's *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

Expression of HER-2 protein in gastric carcinoma

HER-2 protein status in 145 gastric carcinoma tissue samples was determined with immunohistochemical staining (Figure 1). Of the 145 gastric carcinoma tissue samples, 98 (67.6%) were scored as 0, 25 (17.2%) as 1, 12 (8.3%) as 2, and 10 (6.9%) as 3. The positive rate was approximately 15.2% (22/145).

HER-2 gene amplification in gastric carcinomas

The HER-2/CEP17 ratio was determined using the rate of HER-2/neu signals and CEP17 signals in 20 nuclei. The total number of HER-2/neu signals was divided by the total number of CEP17 signals, with a ratio was ≥ 2 according to the indications given by the Abbott-Vysis Company. IHC showed complete membrane immunostaining (2+ and 3+). In addition, 123 samples negative for *HER-2* over-expression with IHC staining were also analyzed by FISH. *HER-2* gene was amplified in 18 out of the 145 gastric carcinoma tissue samples, including HER-2 3+ in 10 samples, HER2 2+ in 5 samples, and HER2 1+ in 3 samples with IHC staining (Table 1). The 10 tumors with strong complete membrane immunostaining (3+) exhibited *HER-2* gene amplification, accounting for 55.6% (10/18) of all HER-2 amplifications. The positive amplification rate of *HER-2* gene was about 12.4% (18/145) in all gastric carcinoma tissue samples (Figure 2).

Correlation between clinicopathologic findings and HER-2 status

The clinicopathological differences were observed in gastric carcinoma tissue samples with or without HER-2 protein expression or *HER-2* gene amplification. The HER-2 protein expression and *HER-2* gene amplification rates were 86.4 % (19/22) and 94.4 % (17/18) in intestinal type gastric carcinomas, respectively (*P* < 0.05). The HER-2 status was correlated with the depth of invasion, TNM stage, lymph node and distant metastasis (*P* < 0.05). However, no significant relation was found between clini-

Table 2 HER-2 protein expression and *HER-2* gene amplification in gastric carcinoma tissue samples

Clinicopathologic data	<i>n</i>	HER-2 (IHC)		<i>P</i> value	HER-2 (FISH)		<i>P</i> value
		Pos	%		Amp	%	
Sex							
Male	95	14	14.7	NS	10	10.5	NS
Female	50	8	16.0		8	16.0	
Age (yr)							
> 60	68	10	14.7	NS	7	10.3	NS
≤ 60	77	12	15.6		11	14.3	
Diameter of the tumor (cm)							
> 5	85	15	17.6	NS	12	14.1	NS
≤ 5	60	7	11.7		6	10.0	
Location							
Cardia or fundus of stomach	55	8	14.5	NS	7	12.7	NS
Body of stomach	39	5	12.8		4	10.3	
Sinus ventriculi/ostium pyloricum	51	9	17.6		7	13.7	
Differentiation							
Well	36	6	16.7	NS	5	13.9	NS
Moderately	44	5	11.4		4	9.1	
Poorly/undifferentiated	65	11	16.9		9	13.8	
Serosa invasion							
Positive	107	20	18.7	< 0.05	17	15.9	< 0.05
Negative	38	2	5.3		1	2.7	
Lymph node metastasis							
Positive	91	20	22.0	< 0.05	18	19.8	< 0.05
Negative	54	2	3.7		0	0.0	
Distant metastasis							
Positive	11	10	90.9	< 0.05	9	81.8	< 0.05
Negative	134	12	9.0		8	6.0	
TNM stage							
I + II	67	3	4.5	< 0.05	2	2.99	< 0.05
III + IV	78	19	24.4		16	20.5	
Histopathological classification(Lauren)							
Intestinal	86	19	22.1	< 0.05	17	19.8	< 0.05
Diffuse	37	2	5.4		0	0.0	
Non classified	22	1	4.5		1	4.5	

IHC: Immunohistochemistry; FISH: Fluorescence in situ hybridization; NS: Not significant.

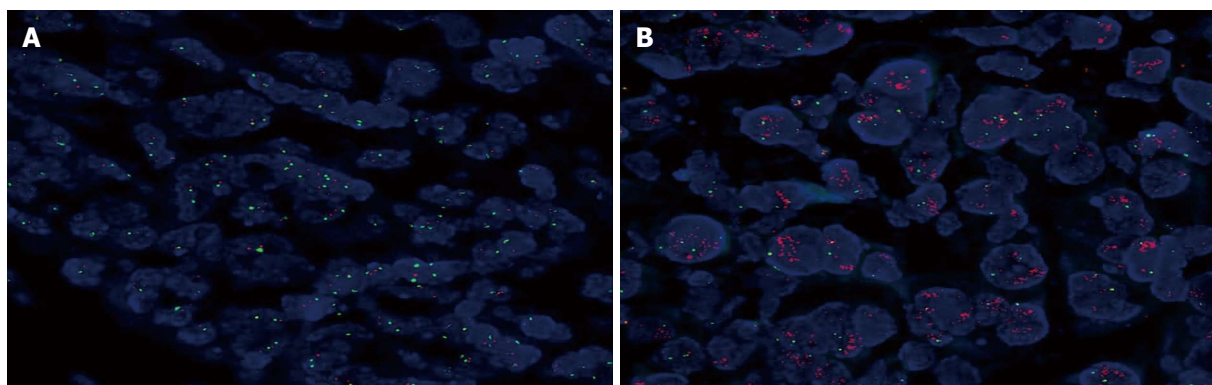


Figure 2 Fluorescence *in situ* hybridization targeting HER-2 in gastric carcinoma specimens showing intestinal type of carcinoma without *HER-2* gene amplification (A) and intestinal type of carcinoma with *HER-2* gene amplification (B) (original magnification × 1000).

copathologic variables (sex and age of the patients, and tumor diameter, differentiation, location) (Table 2).

DISCUSSION

Gastric cancer is the second most frequent cause of cancer-related death worldwide and the most common

cancer in Asian countries^[12]. Although various therapies for gastric carcinoma are available, such as gastrectomy with extensive lymphadenectomy and surgery combined with chemotherapy, the control of advanced stage gastric cancer is still a challenge for physicians. In recent years, molecular target therapy is a new treatment modality for gastric cancer and HER-2 has been identified as a poten-

tial therapeutic target. However, molecular target therapy for gastric cancer depends on the evaluation of the target gene status.

HER-2 amplification and over-expression play a key role in initiation, progression, and metastasis of some common cancers, including breast and gastric cancer. HER-2 status has been recognized as an important prognostic factor for cancer. The survival time of patients with breast cancer and positive HER-2 disease is significantly shorter than that of those with HER-2 negative tumors^[13-15]. Thus, detecting HER-2 status is of important significance in diagnosis of gastric cancer.

It was reported that the HER-2 over-expression rate in gastric carcinoma is 8.2%-34.0%^[2,16-18], whereas the concordance of FISH and IHC is 93.5% in diagnosis of gastric cancer^[9]. In the present study, the HER-2 gene amplification was evaluated using the FISH method and the HER-2 protein expression levels were compared. The positive rate of HER-2 gene amplification was 12.4% (18/145). Recent studies showed that the HER-2 gene amplification rate in gastric carcinoma is 8.2%-5%^[19,20], which is consistent with our findings (12.4%). HER-2 gene amplification is a golden criterion for target therapy with trastuzumab. In this study, the HER-2 gene was amplified in all samples with oncoprotein over-expression at 3+ level, but HER-2 gene was not amplified at 0 level in all samples, which is consistent with the reported data^[21], suggesting that target therapy is not necessary for gastric cancer patients with HER-2 protein expression but necessary for those with HER-2 protein over-expression at 3+ level. Although FISH shows a high sensitivity and specificity and remains a criterion for determining gene amplification status, IHC for HER-2 protein expression may be a good alternative when FISH cannot be performed.

In this study, the HER-2 protein expression and the HER-2 gene amplification rates were 86.4% (19/22) and 94.4% (17/18), respectively, in most intestinal types of gastric carcinoma ($P < 0.05$), and the HER-2 status was correlated with the depth of invasion, TNM stage, lymph node and distant metastasis of gastric cancer ($P < 0.05$), with no significant relation found between clinicopathologic variables (sex and age of patients, and tumor diameter, differentiation, and location), which is consistent with the reported findings^[22]. Furthermore, it has been shown that HER-2 status is only correlated with the histopathological classification of gastric cancer^[23]. It was also reported that HER-2 over-expression is correlated with well or moderately differentiated gastric cancer but not with pathologic stage of gastric cancer or the age and sex of gastric cancer patients^[24].

In conclusion, HER-2 status is correlated with the depth of invasion, TNM stage, lymph node and distant metastasis of gastric cancer. Detecting HER-2 status may contribute to the target therapy for gastric carcinoma using trastuzumab.

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COMMENTS

Background

HER-2 protein over-expression and gene amplification in gastric carcinoma are the prerequisite for monoclonal antibody therapy. Detecting HER-2 status in gastric carcinoma is very important in clinical practice. HER-2 protein expression and gene amplification were detected using immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) in this study.

Research frontiers

HER-2 is over-expressed in 10%-38% of gastric cancer patients. However, few studies are available on HER-2 status in gastric carcinoma in China.

Innovations and breakthroughs

In this study, HER-2 over-expression was detected with IHC and HER-2 gene amplification was found with FISH. FISH testing is unnecessary for patients with HER-2 protein expression at 0 or 3+ level and necessary for patients with HER-2 protein expression at 1+ or 2+ levels. HER-2 status was correlated with the depth of invasion, TNM stage, lymph node and distant metastasis of gastric cancer.

Applications

IHC may be used to screen the HER-2 status in gastric carcinoma patients. FISH testing may be necessary for patients with HER-2 protein expression at 1+ or 2+ levels. The screening method may determine whether target therapy is necessary for gastric carcinoma using trastuzumab.

Terminology

IHC and FISH are the abbreviation forms of IHC and FISH, respectively. HER-2 status contains negative or positive HER-2 protein expression, HER-2 gene amplification or no HER-2 gene amplification.

Peer review

The authors showed the expression of HER-2 in approximately one third of patients. Interestingly, they found that HER-2 was amplified not only in IHC +3 cases but also in some of IHC +2 and even +1 cases, suggesting that FISH should be performed in all cases with positive IHC and HER-2 expression is correlated with the key indicators for cancer progression, such as TNM stage and metastasis.

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Epigallocatechin gallate inhibits HBV DNA synthesis in a viral replication - inducible cell line

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Abstract

AIM: To analyze the antiviral mechanism of Epigallocatechin gallate (EGCG) against hepatitis B virus (HBV) replication.

METHODS: In this research, the HBV-replicating cell line HepG2.117 was used to investigate the antiviral mechanism of EGCG. Cytotoxicity of EGCG was analyzed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Hepatitis B virus e antigen (HBeAg) and hepatitis B virus surface antigen (HBsAg) in the supernatant were detected by enzyme-linked immunosorbent assay. Precore mRNA and pregenomic RNA (pgRNA) levels were determined by semi-quantitative reverse transcription polymerase chain reaction (PCR) assay. The effect of EGCG on HBV core promoter activity was measured by dual luciferase reporter assay. HBV covalently closed circular DNA and replicative intermediates of DNA were quantified by real-time PCR assay.

RESULTS: When HepG2.117 cells were grown in the presence of EGCG, the expression of HBeAg was suppressed, however, the expression of HBsAg was not affected. HBV precore mRNA level was also down-regulated by EGCG, while the transcription of precore mRNA was not impaired. The synthesis of both HBV covalently closed circular DNA and replicative intermediates of DNA were reduced by EGCG treatment to a similar extent, however, HBV pgRNA transcribed from chromosome-integrated HBV genome was not affected by EGCG treatment, indicating that EGCG targets only replicative intermediates of DNA synthesis.

CONCLUSION: In HepG2.117 cells, EGCG inhibits HBV replication by impairing HBV replicative intermediates of DNA synthesis and such inhibition results in reduced production of HBV covalently closed circular DNA.

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Key words: Covalently closed circular DNA; Epigallocatechin gallate; Hepatitis B virus e antigen; Hepatitis B virus; Precore mRNA; Replicative intermediates of DNA

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INTRODUCTION

Hepatitis B virus (HBV) infection remains a major world

health problem despite the availability of an effective vaccine. Approximately 350 million people are chronically infected with HBV worldwide^[1,2]. HBV possesses a 3.2 kb relaxed circular partially double-stranded DNA (RC-DNA) genome^[3]. After infection, this viral genome is released into cytoplasm and converted into covalently closed circular DNA (cccDNA) in the host cell nucleus. The cccDNA is the template for production of all viral RNAs. Among HBV RNAs, the pregenomic RNA (pgRNA) is packaged into new capsid and serves as the template for HBV DNA synthesis. The nucleocapsid acquires an envelope and then buds as a mature virion. Progeny nucleocapsids may redeliver their RC-DNA genomes to the nucleus for accumulation of a cccDNA pool^[4].

Currently, there is no reliable treatment that completely eliminates HBV infection in all chronic carriers. Several nucleos(t)ide analogues (lamivudine, adefovir, entecavir, and telbivudine) and interferon- α (IFN- α) have been approved as antiviral drugs against HBV infection, however, they have their own limitations, e.g. drug resistance and side effects^[5]. Several non-nucleoside natural products have also been reported to inhibit HBV replication using various mechanisms. For example, oxymatrine was shown to block HBV DNA synthesis and cccDNA formation^[6]. A helioxanthin derivative, 8-1, was demonstrated to diminish HBV promoter activities and thus suppress HBV gene expression and DNA synthesis^[7,8].

Epigallocatechin gallate (EGCG) is one of the most abundant polyphenols present in green tea^[9]. EGCG is well known for its anti-tumor activity while other pharmaceutical effects were discovered subsequently^[10-14]. Recently, it has also been reported that EGCG could inhibit HBV replication in a HBV stably replicating cell line - HepG2-N10^[15]. However, the mechanism by which EGCG inhibits HBV replication is unclear. In fact, it is difficult to dissect the detailed anti-HBV mechanism of EGCG using HepG2-N10 cells, because the processes from cccDNA to antigen expression are severely affected by transcription from integrated HBV DNA^[16].

In order to investigate the antiviral mechanism of EGCG against HBV in detail, we adopted a newly reported HepG2.117 cell line^[17]. HBV replication is controllable using doxycycline in this cell line. More importantly, due to the delicate design, HBV precore mRNA can only be transcribed from replicated HBV DNA but not the integrated HBV DNA. As a result, hepatitis B virus e antigen (HBeAg) can only be translated from precore mRNA that is solely produced from replicated HBV DNA. Therefore, the precore mRNA and HBeAg level represents HBV DNA synthesis. On the other hand, PreS1/S2 mRNAs, which are templates for the translation of hepatitis B virus surface antigen (HBsAg), are mainly transcribed from integrated HBV DNA rather than replicated HBV DNA in this cell line, thus, HBsAg has little correlation to HBV DNA synthesis level.

In this study, we investigated the inhibition of HBV replication by EGCG in HepG2.117 cells. Our results showed that HBV replicative intermediates of DNA (RI-

DNA) synthesis was significantly inhibited by EGCG, which resulted in less cccDNA production. In contrast, the production of HBV pgRNA, precore mRNA and the translation of HBeAg were not affected by EGCG.

MATERIALS AND METHODS

Cell line and cell culture

HepG2.117 (a gift from Dr. Dian-Xing Sun, University Hospital Freiburg, Germany) is an inducible HBV-replicating cell line^[17]. It contains a slight over-length HBV genome under the control of a tetracycline responsive CMV promoter. Transcription of HBV pgRNA is induced upon doxycycline removal from the culture medium, leading to capsid assembly and DNA synthesis. A small portion of the *de novo* synthesized RC-DNA is transported into the nucleus and converted to cccDNA which serves as a transcriptional template for the production of viral RNAs.

The HBeAg start codon and precore mRNA transcription initiation point are inexistent in chromosome integrated HBV genome in HepG2.117 cells^[17]. Therefore, HBeAg and precore mRNA can not be generated from the integrated genome. However, once HBV starts to replicate, a functional precore mRNA would be produced from cccDNA in this cell line, since the promoter and transcription initiation point would be restored in the process of cccDNA formation^[17]. Subsequently, HBeAg would be produced from this precore mRNA where HBeAg start codon is also restored.

HepG2.117 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 μ g/mL streptomycin (Sigma). When needed, doxycycline (Sigma) was routinely added at 1.5 μ g/mL to suppress HBV pgRNA transcription.

Plasmid construction

To generate pHBVCP-Luc reporter plasmid, a 442 bp fragment corresponding to the HBV core promoter region containing enhancer II was amplified from pCH9-3091 (an ayw subtype HBV over-length genomic plasmid, kindly provided by Professor Michael Nassal, University Hospital, Freiburg)^[18] by polymerase chain reaction (PCR) (forward primer: GGGGTACCCGC-GGGACGTCCTTTG; reverse primer: CCAAGCTTCAAGAGATGATTAGGCAG). This fragment was then inserted into the pGL3-basic vector (Promega, Madison, WI, USA), producing pHBVCP-Luc reporter construct.

Drug treatment and cytotoxicity assay

Three days after the removal of doxycycline, HepG2.117 cells were seeded into 24-well plates. Twenty-four hours later, cells were treated with fresh DMEM medium containing various concentrations of EGCG (Hubei Institute of Chemistry, Wuhan), or lamivudine (NIH AIDS Research and Reference Reagent Program, Rockville, MD, USA), respectively. The drug-containing media were

replaced each day for 3 d. The medium was then collected for antigen detection and attached cells were used for DNA and RNA analysis (see below) or EGCG toxicity analysis using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells cultured with DMEM medium without EGCG were used as a negative control.

Enzyme-linked immunosorbent assay detection of HBsAg and HBeAg

Collected media were subjected to $10\,000 \times g$ centrifugation to remove cellular debris. Secreted HBsAg and HBeAg were quantified by enzyme-linked immunosorbent assay (ELISA) kits (Kehua, Shanghai) following the manufacturer's instructions. All the data on HBsAg or HBeAg are presented as percentage of antigen level detected in the negative control samples.

Semi-quantitative reverse transcription polymerase chain reaction detection of HBV mRNA

Total cellular RNA was extracted with Trizol reagents (Invitrogen) and treated with RQ1 RNase-free DNase I (Promega) to digest residual DNA molecules. For the semi-quantification of precore mRNA and pgRNA, 1 μ g total cellular RNA was subjected to cDNA synthesis using MMLV reverse transcriptase (Promega) and random hexamer as a primer based on the manufacturer's instructions. The cDNAs were then measured by semi-quantitative PCR using Ex *Taq* (Takara, Dalian) and subsequently analyzed by agarose gel electrophoresis. The specific primers for semi-quantitative detection of precore mRNA were as follows: forward, 5'-TCTGCGCACCAG-CACCATG; reverse, 5'-TGCCTCGTCGTCTAACAA. The forward primer for pgRNA amplification was 5'-TC-GGGAAGCCTTAGAGTC, while the reverse primer was 5'-TGCCTCGTCGTCTAACAA. Primers used for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA amplification were as previously reported^[19].

Dual luciferase reporter assay detection of HBV core promoter activity

Three days after the removal of doxycycline, HepG2.117 cells were seeded into 24-well plates. Twenty-four hours later, the cells were transiently co-transfected with pH-BVCP-Luc reporter plasmid and normalizing plasmid pRL-TK with FuGENE-HD reagent according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Twelve hours after transfection, cells were subjected to a 3-d period of treatment with EGCG. HBV core promoter activity was determined by measuring luciferase activity using the Dual Luciferase Reporter Assay System (Promega).

Real-time PCR detection of HBV DNA

Extraction of cccDNA was carried out using a modified Hirt extraction procedure^[16,20]. Briefly, cells from one well in the 24-well plate were lysed in 300 μ L of 10 mmol/L Tris-HCl, 10 mmol/L EDTA and 0.7% SDS.

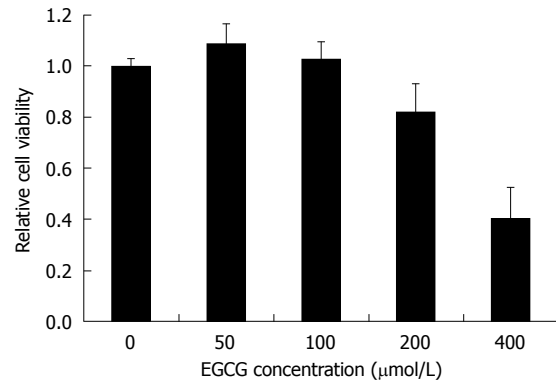


Figure 1 The cytotoxic effect of Epigallocatechin gallate on HepG2.117 cells. Twenty four hours after seeding, cells were incubated with medium containing different concentrations of Epigallocatechin gallate (EGCG) for 3 d, and cell viability was determined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability of the control well not treated with EGCG was determined and arbitrarily designated as 1. Error bars indicate standard deviation of 3 independent experiments.

After 30 min incubation at room temperature, the lysate was mixed with 80 μ L 5 mol/L NaCl and incubated at 4°C overnight. The lysate was clarified by centrifugation at $12\,000 \times g$ for 30 min at 4°C, and then extracted twice with phenol and once with phenol:chloroform. DNAs were precipitated with ethanol overnight at -20°C and dissolved in TE buffer, followed by further treatment of plasmid-safe DNase (Epicenter) to digest residual linear and open circular DNA molecules. When the reaction was finished, the plasmid-safe DNase was removed by phenol:chloroform extraction and the DNA was precipitated with ethanol at -20°C overnight and dissolved in TE buffer. Total cellular HBV DNA was extracted from the cell monolayer with the total DNA extraction kit (Takara, Dalian) according to the manufacturer's instructions.

A pair of primers (corresponding to HBV S ORF) introduced by Liu *et al.*^[21] was applied to quantify HBV DNA copies. In order to quantify cccDNA, extracted cccDNA samples were subjected to real-time PCR and the quantification was normalized by cell numbers. The total HBV DNA samples, which contained several types of HBV DNA forms (99% of them were HBV DNA replicative intermediates), were analyzed by real-time PCR and the quantification was normalized to the GAPDH DNA copies.

RESULTS

HBsAg expression level in HepG2.117 cells was down-regulated by EGCG

In order to choose an appropriate initial concentration of EGCG in the HBV inhibition assay, the MTT assay was used to determine the cytotoxicity of EGCG to HepG2.117 cells. As shown in Figure 1, the cytotoxic effects of EGCG were undetectable when the EGCG concentration was below 100 μ mol/L, while slight cytotoxicity (18% inhibition of cell viability) and significant cytotoxicity (60% inhibition of cell viability) were ob-

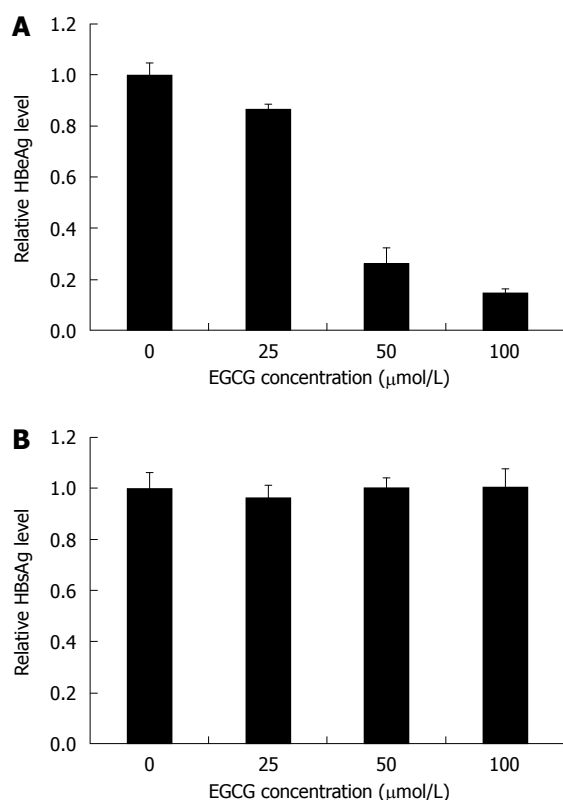


Figure 2 Epigallocatechin gallate inhibited hepatitis B virus e antigen expression level but not hepatitis B virus surface antigen expression level in the HepG2.117 cell line. Twenty four hours after seeding, cells were incubated with medium containing different concentrations of Epigallocatechin gallate (EGCG) for 3 d. The hepatitis B virus e antigen (HBeAg) and hepatitis B virus surface antigen (HBsAg) expression levels in the supernatants were measured using enzyme-linked immunosorbent assay kits for measuring HBeAg and HBsAg, respectively. The level of HBeAg (A) and HBsAg (B) from cells not treated with EGCG (the control) were arbitrarily designated as 1. The levels of HBeAg (A) and HBsAg (B) in EGCG-treated cells were normalized with the control and expressed as relative levels. Error bars indicate standard deviation of 3 independent experiments.

served when 200 and 400 μmol/L of EGCG were used, respectively. Therefore, the maximum non-toxic concentration was 100 μmol/L, and this was used as the highest concentration of EGCG in the HBV inhibition assay.

EGCG was reported to inhibit the expression of both HBeAg and HBsAg in HepG2-N10 cells^[15]. To investigate the antiviral mechanisms of EGCG against HBV in HepG2.117 cells, a cell-based assay was used. The levels of HBeAg and HBsAg in the supernatant of EGCG-treated HepG2.117 cells were measured by ELISA. As shown in Figure 2A, HBeAg expression was dose-dependently inhibited by EGCG with an IC₅₀ of 39.4 μmol/L. However, the expression level of HBsAg was largely unaffected (Figure 2B). These results indicated that EGCG could down-regulate HBeAg level but not HBsAg level in HepG2.117 cells.

HBV precore mRNA level was down-regulated in the presence of EGCG

Unlike several HBV DNA stably transfected cell lines (e.g. HepG2.2.15) whose HBeAg is mainly translated from the

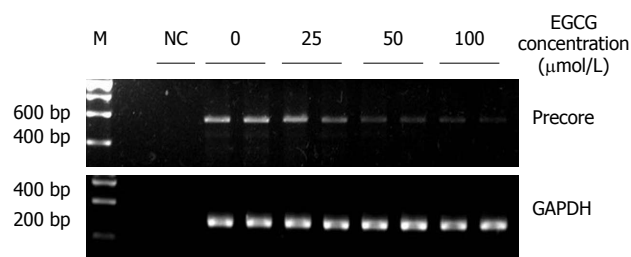


Figure 3 Epigallocatechin gallate inhibited the production of hepatitis B virus precore mRNA. Twenty four hours after seeding, HepG2.117 cells were treated with medium containing different concentration of Epigallocatechin gallate (EGCG) for 3 d. Total RNAs were prepared and were quantitated by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) with a pair of precore mRNA selective primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was semi-quantitatively RT-PCR amplified and used for normalization. NC: Total RNA sample was subjected to PCR without reverse transcriptase and used as the negative control.

long early RNA that is transcribed from the inserted exogenous promoter (e.g. CMV promoter), in the HepG2.117 cell line, HBeAg is exclusively translated from precore mRNA that is transcribed from cccDNA, akin to the actual HBV life cycle in HBV-infected hepatocytes. Since EGCG down-regulates HBeAg level but not HBsAg level in HepG2.117 cells, we determined whether the down-regulation of HBeAg by EGCG was due to inhibition of the transcription of precore mRNA.

Pcore mRNA and pgRNA share the same sequence, except that pcore mRNA is 35 nt longer at the 5' terminal^[16]. In order to distinguish pcore mRNA from pgRNA, a specific forward primer complementary to the 5' terminal of pcore mRNA was designed for the pcore mRNA specific reverse transcription PCR (RT-PCR) assay. Briefly, the 19 nt forward primer was designed to anneal to the 5' terminal of pcore RNA (nt 1800-1818) which is absent in pgRNA. The reverse primer was complementary to a sequence (nt 2345-2362) present in both pcore mRNA and in pgRNA. Thus, a 563 bp fragment could be amplified from pcore mRNA but not pgRNA.

After HepG2.117 cells were treated with EGCG for 3 d, total RNAs were extracted and subjected to semi-quantitative RT-PCR analysis using the pcore mRNA specific primers. As shown in Figure 3, the amount of 563 bp specific RT-PCR products amplified from pcore mRNA decreased in a dose-dependent manner with increasing concentrations of EGCG. The total RNA sample for RT-PCR was free of DNA contamination as this specific product did not appear in reactions without reverse transcription (Figure 3, NC lane). These results indicated that after EGCG treatment, pcore mRNA level was down-regulated in HepG2.117 cells, and the lowered pcore mRNA level accounted for the decreased production of HBeAg.

Transcriptional efficiency of HBV precore mRNA was not affected by EGCG

Pcore mRNA is transcribed from cccDNA and this process is controlled by HBV core promoter^[22]. To investigate

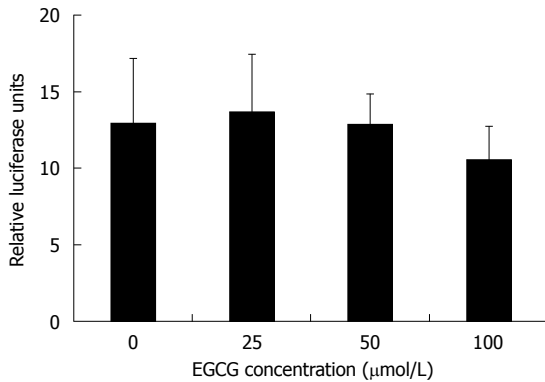


Figure 4 Epigallocatechin gallate does not affect hepatitis B virus core promoter activity. Twelve hours after cotransfection with pHBVCP-Luc and pRL-TK, HepG2.117 cells were treated with media containing different concentrations of Epigallocatechin gallate (EGCG) for 3 d, and were lysed for the dual luciferase reporter analysis. Core promoter activity was normalized with control TK promoter activity and presented as relative luciferase units. Error bars indicate standard deviation of 3 independent experiments.

whether EGCG can inhibit HBV core promoter activity, a reporter plasmid, pHBVCP-Luc, was constructed by inserting HBV core promoter before the firefly luciferase gene in the pGL3-basic vector and thus the measured firefly luciferase activity represented core promoter activity. Twelve hours after HepG2.117 cells were transfected with pHBVCP-Luc, different concentrations of EGCG were added and maintained for 3 d, and the cells were then subjected to luciferase reporter assay. As shown in Figure 4, HBV core promoter activity was not inhibited even in the presence of 100 μmol/L of EGCG, in which the expression of HBeAg was down-regulated by 85.5%. Thus, the down-regulation of precore mRNA level was not attributed to the inhibition of core promoter activity by EGCG.

The production of HBV cccDNA and RI-DNA was dose-dependently inhibited by EGCG

Since the precore mRNA in the HepG2.117 cell line was exclusively transcribed from HBV cccDNA, and EGCG did not suppress HBV precore mRNA transcription efficiency, we speculated that the down-regulation of precore mRNA by EGCG may be due to decreased HBV cccDNA formation.

To quantitatively analyze the amount of HBV cccDNA, EGCG-treated HepG2.117 cells were collected to obtain cccDNA, and real-time PCR analysis was used to detect cccDNA production. The results showed that EGCG could decrease cccDNA formation in a dose-dependent manner (Figure 5A). The inhibitory rates of cccDNA formation were 36.3%, 63% and 72.4%, following treatment with 25, 50 and 100 μmol/L of EGCG, respectively. As a control, the HBV polymerase inhibitor, lamivudine, also showed dose-dependent inhibition of HBV cccDNA production (Figure 5B).

HBV cccDNA is repaired from RC-DNA. RC-DNA and other forms of HBV DNA (double-stranded linear DNA and single-stranded DNA), which are collectively known as HBV replicative intermediates of DNA (RI-

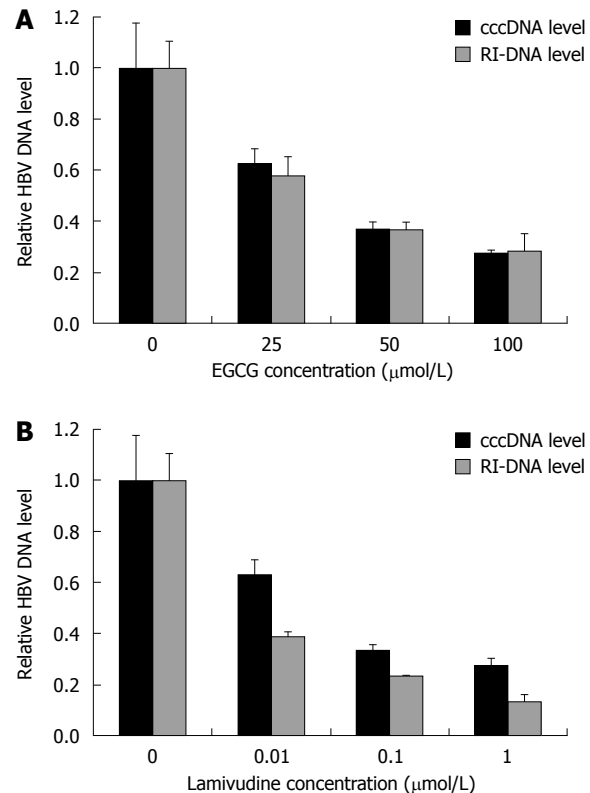


Figure 5 Epigallocatechin gallate down-regulates hepatitis B virus covalently closed circular DNA and replicative intermediates of DNA level.

A: Epigallocatechin gallate (EGCG) treatment was the same as described in Figure 1. Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) and total cellular HBV DNA were extracted and subjected to real-time polymerase chain reaction analysis; B: Lamivudine, the potent HBV DNA synthesis inhibitor, was used as a control inhibitor in this analysis. DNA level in the no treatment control was arbitrarily designated as 1. Error bars indicate standard deviation of 3 independent experiments. RI-DNA: Replicative intermediates of DNA.

DNA), represent HBV DNA synthesis efficiency. In HepG2.117 cells, RI-DNA molecules constitute 99% of HBV DNA molecules^[17]. Therefore, total HBV DNA level can reflect HBV RI-DNA level as well as HBV DNA synthesis level.

We suspected that impairment of cccDNA production was a result of less RI-DNA molecules produced when the cells were treated with EGCG. To verify this, total HBV DNA was extracted from EGCG-treated HepG2.117 cells and then subjected to real-time quantitative PCR analysis. As shown in Figure 5A, EGCG inhibited the level of RI-DNA dose-dependently, and the inhibitory rates were 42.2%, 63.4% and 71.8% following treatment with 25, 50 and 100 μmol/L of EGCG, respectively. The inhibitory rates were almost the same as that for cccDNA production, suggesting that the inhibition of cccDNA formation was mainly due to the decrease in HBV RI-DNA template.

Interestingly, it was observed that when treated with the same concentration of lamivudine, the decline in RI-DNA level was slightly stronger than the decline in cccDNA level (Figure 5B), e.g. 1 μmol/L lamivudine decreased RI-DNA production by 86.8% and only decreased cccDNA production by 72.4%. The reason for this could

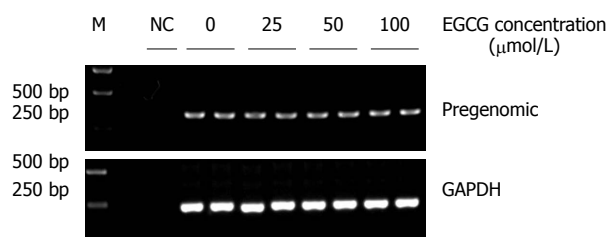


Figure 6 Epigallocatechin gallate did not inhibit hepatitis B virus pregenomic RNA transcription. When doxycycline was removed from the cell culture medium, different concentrations of Epigallocatechin gallate (EGCG) were added to the HepG2.117 cells. After 3 d of culture, total RNA were extracted from the treated cells and the pregenomic RNAs (pgRNAs) were analyzed by semi-quantitative reverse transcription polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were used for normalization. NC: Total RNA sample was subjected to polymerase chain reaction without reverse transcription.

be that part of the cccDNA can be repaired from a certain amount of pre-existing RC-DNA^[23,24]. The observed minimal difference in the inhibition of cccDNA and RI-DNA production implies that EGCG may partly inhibit cccDNA formation.

Transcription of pgRNA from integrated HBV genome was not affected by EGCG

HBV RI-DNA is synthesized from pgRNA and catalyzed by HBV polymerase. In the HepG2.117 cell line, pgRNA is transcribed from chromosome-integrated HBV DNA genome. Since RI-DNA level was down-regulated by EGCG in this cell line, we determined whether this down-regulation was actually caused by inhibition of RI-DNA synthesis rather than suppression of pgRNA transcription.

To analyze whether EGCG inhibited pgRNA transcription, doxycycline was removed from the culture medium, and EGCG was added at the same time. Three days later, total RNAs were extracted from HepG2.117 cells and pgRNAs were analyzed semi-quantitatively. As shown in Figure 6, EGCG treatment did not interfere with pgRNA production even when 100 $\mu\text{mol/L}$ of EGCG was applied. Therefore, the transcription of pgRNA from integrated HBV DNA genome was not affected by EGCG. Taken together, we conclude that the decrease in RI-DNA resulted from the inhibition of RI-DNA synthesis, rather than the down-regulation of pgRNA production.

DISCUSSION

In this study, we investigated the anti-HBV mechanism of EGCG in HepG2.117 cells, an inducible HBV-replicating cell line. Our results showed that EGCG down-regulated HBeAg level, precore mRNA level, cccDNA level and RI-DNA level to a similar extent, however, the production of HBV pgRNA was not affected. Therefore, the inhibition of HBV replication mainly occurred in HBV RI-DNA synthesis. When comparing the down-regulation of HBV cccDNA and RI-DNA production

mediated by EGCG and lamivudine, we speculated that EGCG may inhibit HBV cccDNA formation to a minor extent. Taken together, suppression of HBV DNA synthesis is the major anti-HBV mechanism of EGCG, while cccDNA formation might be slightly impaired in the presence of EGCG. Lowered RI-DNA level leads to decreased cccDNA formation, which in turn leads to a decline in precore mRNA level and HBeAg level. HBV precore mRNA transcription and HBeAg translation were unaffected by EGCG.

In a previous study, the anti-HBV activity of EGCG was reported to down-regulate extracellular HBsAg and HBeAg level, intracellular cccDNA level and RI-DNA level. That research was conducted in the HepG2-N10 cell line which was constructed by stable transfection of a 1.3-fold adw subtype HBV genome into HepG2 cells. We reasoned that EGCG could inhibit HBsAg production in HepG2-N10 but not in HepG2.117 cells, which may be due to the different HBV subtype (adw *vs* ayw) and DNA length (1.3-fold *vs* slightly more than 1.0-fold). The results obtained in HepG2-N10 cells were not adequate to elucidate the antiviral mechanism of EGCG against HBV replication, because all HBV mRNAs are mainly transcribed from integrated HBV genomic DNA in HepG2-N10, and thus the antigens (representing mRNA levels) can not truly reflect the levels of HBV RI-DNA synthesis, cccDNA formation and mRNA transcription in the cells.

On the other hand, in the HepG2.117 cell line, HBsAg could be produced from both integral HBV genome and cccDNA, however, cccDNA copy numbers constitute only 1% of the integrated HBV genome copy number. Thus, nearly all of the HBsAg produced from mRNA is transcribed from integrated HBV genome rather than from HBV cccDNA. Therefore, we speculate that the inability of EGCG to inhibit HBsAg production in the HepG2.117 cell line was attributed to the unchanged transcription level of HBsAg mRNA from integrated HBV DNA, even in the presence of EGCG. However, in actual infected hepatocytes, HBsAg can only be produced from cccDNA^[25,26], like HBeAg in the HepG2.117 cell line. Thus, we speculate that EGCG is able to inhibit HBsAg in HBV infection because cccDNA can be suppressed by EGCG treatment.

HBV DNA stably transfected cell lines are frequently used in cell-based assays for the discovery of antiviral drugs against HBV^[27,28]. In such cell lines, all HBV replicative intermediate DNA forms, cccDNA, nucleocapsid and infectious virion (Dane particle) can be detected, hence, it is generally accepted that the HBV life cycle is complete. However, all HBV RNAs and antigens can be produced from both chromosome-integrated HBV genomic DNA and replicated HBV genomic DNA. More importantly, the strong HBV DNA synthesis inhibitor, lamivudine, does not affect HBV RNA levels and antigen levels in such cell models^[29], indicating that HBV RNA transcription from integrated HBV DNA is much stronger than that from cccDNA. The amount of HBV RNAs and antigens in these cell lines do not reflect the

actual HBV DNA replication level. In contrast, in the HepG2.117 cell line, HBeAg and precore mRNA exclusively come from HBV cccDNA, consequently, the pathway from HBV cccDNA to precore mRNA and then HBeAg can be quantitatively analyzed. Although the authenticity of the HBeAg signal in antigen detection is controversial^[30], the precore mRNA is a solid and positive parameter in this cell system. In summary, the HepG2.117 cell line is a better cell model to analyze the anti-HBV mechanism of potential drugs against HBV replication.

Currently, researchers have focused mainly on the potential application of EGCG in treating cancers. EGCG is known to block each stage of carcinogenesis by modulating signal transduction pathways involved in cell proliferation, transformation, inflammation, apoptosis, metastasis and invasion^[31,32]. In this study, we investigated the anti-HBV mechanism of EGCG in detail and found that the suppression of HBV RI-DNA synthesis is the major anti-HBV mechanism of EGCG, while cccDNA formation might be slightly impaired in the presence of EGCG. Although the detailed replication mechanism of HBV remains unclear, it is estimated that multiple cellular factors are involved in HBV genome replication. We assume that EGCG may interact with certain cellular proteins and interfere with the function of the DNA replication machinery. As a result, HBV RI-DNA synthesis is impaired and HBV replication is inhibited. To fully understand the antiviral mechanism of EGCG against HBV and explore the potential use of EGCG as anti-HBV drug, further study needs to be done to determine how EGCG impairs HBV RI-DNA synthesis and which cellular factors are affected.

COMMENTS

Background

Hepatitis B virus (HBV) infection remains a major world health problem despite the availability of an effective vaccine. Currently, there is no reliable treatment that completely eliminates HBV infection in all chronic carriers. Epigallocatechin gallate (EGCG) is one of the most abundant polyphenols present in green tea. Recently, it has been reported that EGCG could inhibit HBV replication in a HBV stably replicating cell line. However, the mechanism by which EGCG inhibits HBV replication is unclear.

Research frontiers

Currently, there are very few cell lines which can be used to investigate HBV replication. HepG2.117 is a newly reported cell line in which HBV replication is controllable using doxycycline. Hepatitis B virus e antigen (HBeAg) can only be translated from precore mRNA solely produced from replicated HBV DNA. HBeAg level correlates well with HBV DNA synthesis, whereas hepatitis B virus surface antigen level does not. Hence, this cell line is more convenient when investigating the antiviral mechanisms of leading compounds against HBV.

Innovations and breakthroughs

Results from this report showed that HBV replicative intermediates of DNA synthesis was significantly inhibited by EGCG, which resulted in reduced covalently closed circular DNA (cccDNA) production. In contrast, the production of HBV pregenomic RNA and the translation of HBeAg were not affected by EGCG.

Applications

The finding that EGCG can inhibit HBV relaxed circular partially double-stranded DNA synthesis is important both when exploring the potential of new drugs against HBV and in understanding which targets might be valid in eliminating viral replication.

Peer review

The authors investigated the anti-viral mechanism of epigallocatechin gallate against HBV replication. It was observed that epigallocatechin gallate impairs replicative intermediate DNA synthesis, resulting in reduced production of cccDNA of the virus. The study is well conducted and the methodology was sound.

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Spontaneous resolution of multiple lymphangiomas of the colon: A case report

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Abstract

Lymphangioma of the colon is a relatively rare non-epithelial tumor and usually presents as a submucosal polypoid lesion. Many cases incidentally discovered are usually asymptomatic. However, they may present as abdominal pain or bleeding, and their resection is normally required. Lymphangioma itself is generally recognized as a benign tumor and no cases of malignant transformation have yet been reported, although its natural history is currently unknown. To the best of our knowledge, this study is the first to describe a case of spontaneous resolution in multiple colonic lymphangiomas without any specific treatment.

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Key words: Lymphangioma; Colon; Natural history

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INTRODUCTION

Lymphangioma is a benign tumor commonly found in children. The majority of lymphangiomas are considered to be lymph vessel anomalies. The condition involves several expanded lymphatics surrounded by benign endothelial cells and usually occurs in the head, neck and axillary regions, and rarely in the gastrointestinal tract^[1-3]. Recently, the number of case reports has been increasing with the increased use of endoscopic colon examination. Many lymphangiomas are asymptomatic and require no treatment, because the condition is considered absolutely benign. No cases, in which lymphangioma underwent malignant transformation, have been reported. However, some cases of lymphangioma accompany colon cancer^[2]. When symptoms such as bleeding, intussusceptions or protein losing enteropathy are present, resection of lymphangioma is necessary^[4-7]. Thus far, the natural history of colonic lymphangioma has remained unknown. Herein, we report the first case of spontaneous resolution of multiple colonic lymphangiomas during the follow-up.

CASE REPORT

A 54-year-old male patient suffered intermittently from

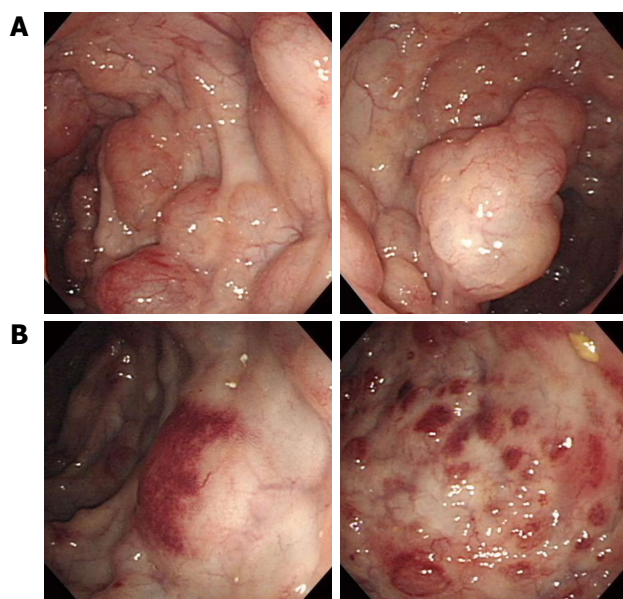


Figure 1 Initial colonoscopy showing multiple elevated or protruding lesions with semi-transparent and smooth surfaces (A), and petechiae on the surfaces of lesions in the sigmoid colon (B).

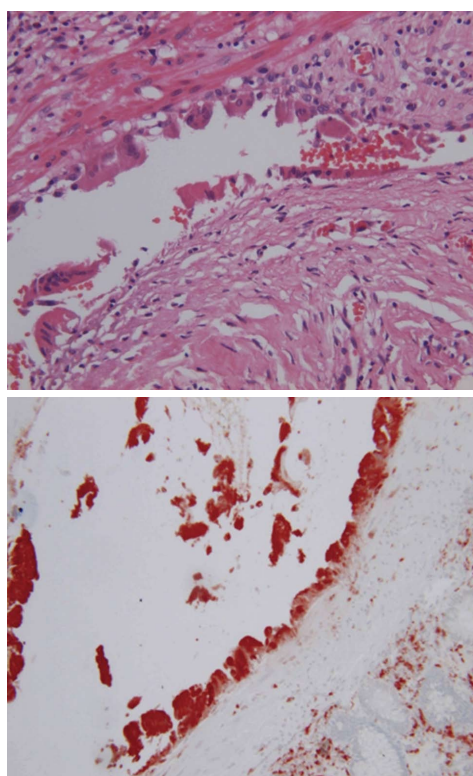


Figure 2 Initial histopathological examination showing a submucosal cystic structure lined with cuboidal endothelial cells for the CD68 histiocytic marker (bottom) and a few giant cells (upper, HE stain, × 200).

blood-tinged stool with no personal or familial history of any specific disease. Physical examination showed no remarkable abdominal abnormality. Laboratory test or chest and abdominal radiography displayed no specific findings. Initial colonoscopy revealed numerous elevated

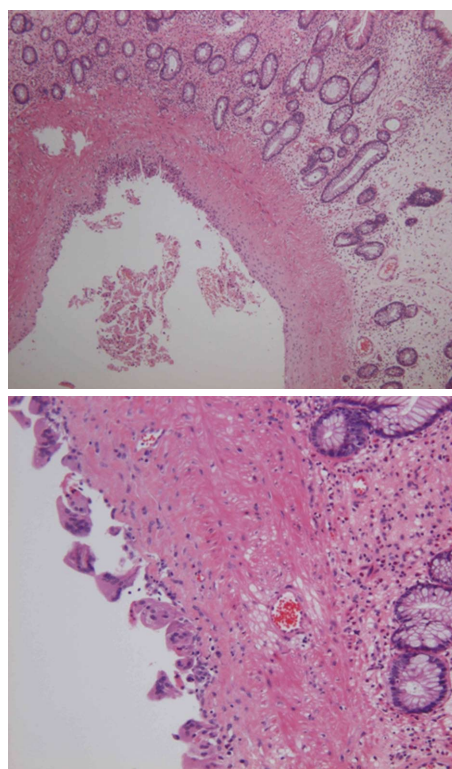


Figure 3 Histopathological examination 12 mo after initial examination showing cystically dilated lesion lined with endothelium (upper, HE stain, × 40) and focal multinucleated giant cells (bottom, HE stain, × 100).

lesions with smooth surfaces throughout the entire colon, which evidenced morphological changes as shown by endoscopy. In particular, the lesions on the left side of the colon evidenced surface petechiae (Figure 1). A diagnostic procedure and endoscopic mucosal resection were performed for the appropriate diagnosis and treatment of the lesions. Histopathological examination showed that the cystic lesion was lined with cuboid-shaped cells that were positive for CD68 histiocytic markers (Figure 2) but negative for CD34 and CK on immunohistochemical examination, the patient was thus diagnosed with colonic lymphangioma. Abdominal computed tomography showed no definite abnormalities, and the possibility of secondary events was excluded. The complaint of mild symptoms led to medical observation and use of stool softener.

After 12 mo, the patient visited our department for colonoscopy, which showed no more remarkable changes than the previous findings. Biopsy procedure was repeated, which demonstrated that the cystic lesion had no lining endothelium but was lined with focal multinucleated giant cells in the submucosa (Figure 3). After 24 mo, colonoscopy revealed complete resolution of colonic lymphangiomas without any specific treatment (Figure 4).

DISCUSSION

In most reported cases of colonic lymphangioma, the condition is solitary. Only a few cases of multiple lymph-

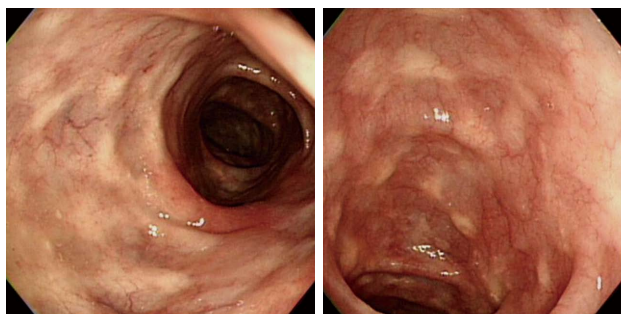


Figure 4 Follow-up colonoscopy 24 mo after initial examination showing multiple whitish scars throughout the entire colonic mucosa and resolved lymphangioma without specific therapy.

angiomas of the colon, the so-called “colonic lymphangiomatosis”, have been reported so far^[8,9]. Endoscopic findings of colonic lymphangioma are characterized by the presence of submucosal tumor properties covered with normal mucosa, round and soft surfaces, a wide base area, pink color and semi-transparency. Histological observations of lymph vascular dilation and outgrowth in the submucosal layer are important for its correct diagnosis in these cases. The condition is classified into capillary, cavernous, and cystic types^[10]. The cystic type, as in our case, is also the most frequently reported and characterized by dilated lymphatic vessels surrounded by flat endothelial cells between fat, fibrotic and lymphatic structures. To the best of our knowledge, the formation of lymphangioma impedes lymph flow for a prolonged period, resulting in lymph vessel expansion or further progression and is generally perceived as a developmental malformation or agenesis of lymphatic tissue. Other causes such as abdominal trauma, lymphatic obstruction, inflammatory processes, cancer, surgery, or radiation may result in secondary lymphangioma formation^[11-16]. In the case of lymphangioma appearing late in adulthood, the lesion may have developed secondarily from a local disturbance of the lymphatic circulation^[2]. Therefore, the clinicians should look out for the causative condition, particularly in adults. Abdomen computed tomography showed no co-morbid disease in our case.

Although a definitive diagnosis of colonic lymphangioma requires complete excision including the submucosal layer, unnecessary surgery should be avoided. Moreover, as in our case, it is quite difficult to perform a total colectomy to remove lesions involving the entire colon. The spontaneous resolution of cystic hygromas originating in the neck or mediastinum has been reported^[14,15]. Several investigators have suggested that increased pressure in the lymphatic system may overcome incomplete obstructions, which may explain why the lesions are spontaneously resolved. In contrast, spontaneous resolution presumably results from the establishment of alternative routes of lymphatic drainage in cases of septated lesions^[16-18]. Although this hypothesis has been applied to cystic hygroma of the neck or the spleen, it may match well in our case. The mild leakage induced by in-

creased pressure of the cyst may stimulate dendritic cells or macrophages in the stroma. In a prolonged state of lymphatic stagnation, CD68-positive cells, which can be dendritic cells or macrophages, might correspond to the cells equipped with phagosomes and lysosomes^[19]. These stimulated cells would evidence phagocytic activity on the lining cells, which continues until the lymphangioma is resolved.

In conclusion, conservative management coupled with close surveillance may be an effective treatment strategy for lymphangioma, and resection of lymphangioma should be reserved in mild symptomatic or asymptomatic patients, because of its benign nature and possible spontaneous resolution.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

March 21-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

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22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatria 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
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Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

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Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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Books

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- Electronic journal** (list all authors)
- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>
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- 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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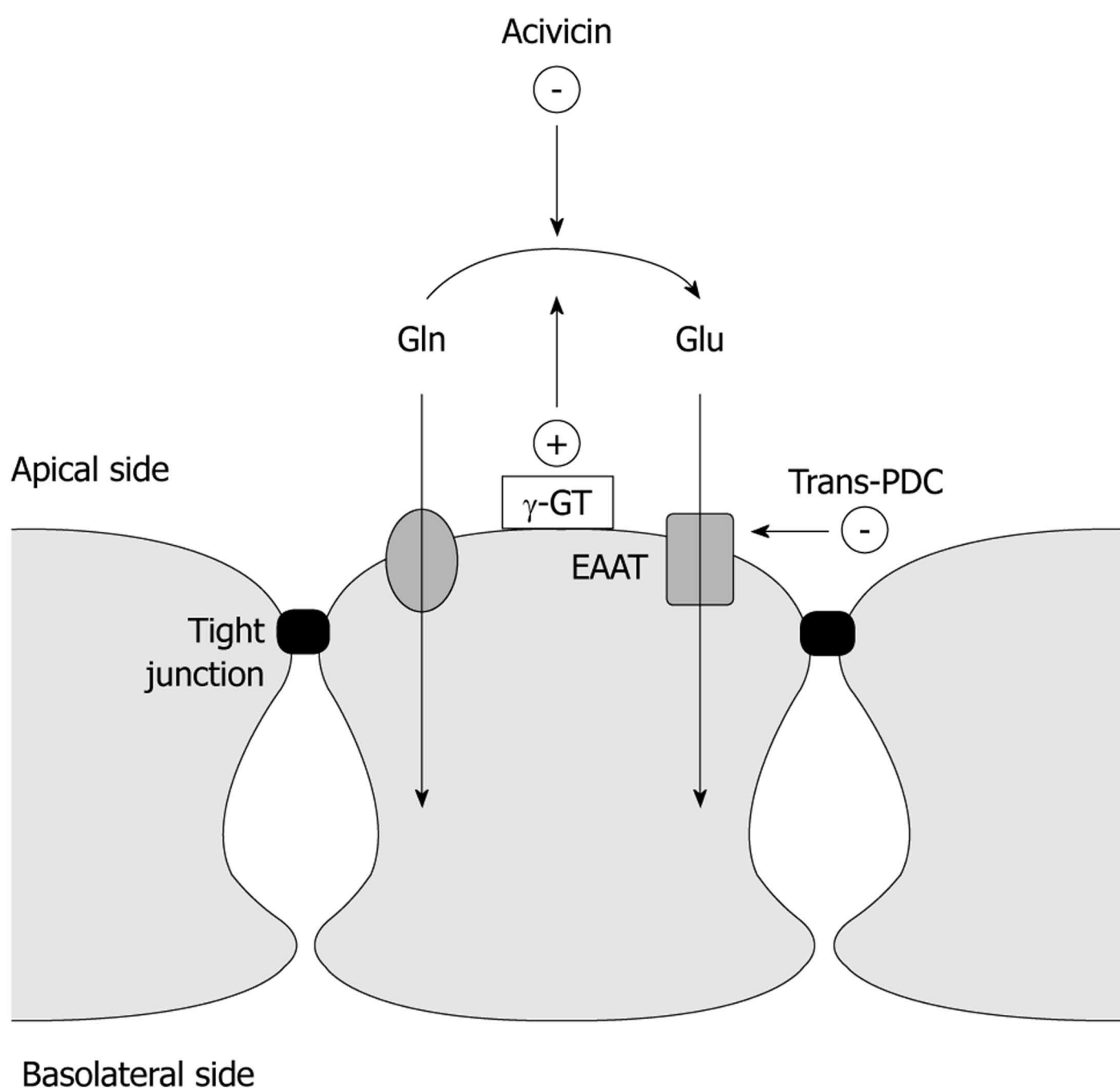
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**EDITORIAL**

- 1519 Potential beneficial effects of butyrate in intestinal and extraintestinal diseases
Berni Canani R, Di Costanzo M, Leone L, Pedata M, Meli R, Calignano A
- 1529 Occult hepatitis B virus infection: A complex entity with relevant clinical implications
Larrubia JR

TOPIC HIGHLIGHT

- 1531 Reactivation of hepatitis B virus infection after cytotoxic chemotherapy or immunosuppressive therapy
Manzano-Alonso ML, Castellano-Tortajada G
- 1538 Prevalence of occult hepatitis B virus infection
Gutiérrez-García ML, Fernández-Rodríguez CM, Lledo-Navarro JL, Buhigas-García I
- 1543 Pathogenesis of occult chronic hepatitis B virus infection
Aller de la Fuente R, Gutiérrez ML, García-Samaniego J, Fernández-Rodríguez C, Lledó JL, Castellano G
- 1549 Clinical significance of occult hepatitis B virus infection
Romero M, Madejón A, Fernández-Rodríguez C, García-Samaniego J
- 1553 Diagnostic strategy for occult hepatitis B virus infection
Ocana S, Casas ML, Buhigas I, Lledo JL
- 1558 Influence of occult hepatitis B virus infection in chronic hepatitis C outcomes
Fernandez-Rodríguez CM, Gutierrez ML, Lledó JL, Casas ML
- 1563 Management of occult hepatitis B virus infection: An update for the clinician
Lledó JL, Fernández C, Gutiérrez ML, Ocaña S

ORIGINAL ARTICLE

- 1569 Glutamate reduces experimental intestinal hyperpermeability and facilitates glutamine support of gut integrity
Vermeulen MAR, de Jong J, Vaessen MJ, van Leeuwen PAM, Houdijk APJ

1574 Omeprazole decreases magnesium transport across Caco-2 monolayers
Thongon N, Krishnamra N

1584 Over-starvation aggravates intestinal injury and promotes bacterial and endotoxin translocation under high-altitude hypoxic environment
Zhou QQ, Yang DZ, Luo YJ, Li SZ, Liu FY, Wang GS

1594 Simotang enhances gastrointestinal motility, motilin and cholecystokinin expression in chronically stressed mice
Cai GX, Liu BY, Yi J, Chen XM, Liu FL

BRIEF ARTICLE

1600 Epidemiological trends and geographic variation in hospital admissions for diverticulitis in the United States
Nguyen GC, Sam J, Anand N

1606 Is spleen circulation impaired in systemic sclerosis and what is the role of liver fibrosis?
Tarantino G, Spanò A, Loi G, Parisi A, Tarantino M, Brancaccio G, Gaeta GB, Riccio A

1614 Survivin isoforms and clinicopathological characteristics in colorectal adenocarcinomas using real-time qPCR
Pavlidou A, Dalamaga M, Kroupis C, Konstantoudakis G, Belimezi M, Athanasas G, Dimas K

1622 Pyogenic liver abscess: An audit of 10 years' experience
Pang TCY, Fung T, Samra J, Hugh TJ, Smith RC

1631 Trefoil factors: Tumor progression markers and mitogens *via* EGFR/MAPK activation in cholangiocarcinoma
Kosriwong K, Menheniott TR, Giraud AS, Jearanaikoon P, Sripa B, Limpaiiboon T

1642 Comparison between surgical outcomes of colorectal cancer in younger and elderly patients
Jin L, Inoue N, Sato N, Matsumoto S, Kanno H, Hashimoto Y, Tasaki K, Sato K, Sato S, Kaneko K

1649 Application of MPVR and TL-VR with 64-row MDCT in neonates with congenital EA and distal TEF
Wen Y, Peng Y, Zhai RY, Li YZ

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APPENDIX I Meetings
I-VI Instructions to authors

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World J Gastroenterol 2011; 17(12): 1569-1573
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Potential beneficial effects of butyrate in intestinal and extraintestinal diseases

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Abstract

The multiple beneficial effects on human health of the short-chain fatty acid butyrate, synthesized from non-absorbed carbohydrate by colonic microbiota, are well documented. At the intestinal level, butyrate plays a regulatory role on the transepithelial fluid transport, ameliorates mucosal inflammation and oxidative status, reinforces the epithelial defense barrier, and modulates visceral sensitivity and intestinal motility. In addition, a growing number of studies have stressed the role of butyrate in the prevention and inhibition of colorectal cancer. At the extraintestinal level, butyrate exerts potentially useful effects on many conditions, including hemoglobinopathies, genetic metabolic diseases, hypercholesterolemia, insulin resistance, and ischemic stroke. The mechanisms of action of butyrate are different; many of these are related to its potent regulatory effects on gene expression. These data suggest a wide spectrum of positive effects exerted by butyrate, with a high potential for a therapeutic use in human medicine.

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INTRODUCTION

The development of the intestinal ecosystem is crucial for many gastrointestinal functions and body health. The intestinal ecosystem essentially comprises the epithelium, immune cells, enteric neurons, intestinal microflora, and nutrients. The coordinate interplay between all these components is the object of intensive research efforts to design new strategies for many intestinal and extraintestinal diseases. In this context, short-chain fatty acids (SCFAs), produced by intestinal microflora, represent a clear example of the importance of the intestinal ecosystem. SCFAs are organic acids produced by intestinal microbial fermentation of mainly undigested dietary carbohydrates, specifically resistant starches and dietary fiber, but also in a minor part by dietary and endogenous proteins. SCFAs are 2-carbon to 5-carbon weak acids, including acetate (C2), propionate (C3), butyrate (C4), and valerate (C5). SCFAs are essentially produced in the colon. The ratio of SCFA concentrations in the colonic lumen is about 60% acetate, 25% propionate, and 15% butyrate. As a result of increasing concentrations of acidic fermentation products,

the luminal pH in the proximal colon is lower. This pH seems to boost the formation of butyrate, as mildly acidic pH values allow butyrate-producing bacteria to compete against Gram-negative carbohydrate-utilizing bacteria, such as *Bacteroides spp.*^[1]. The ability to produce butyrate is widely distributed among the Gram-positive anaerobic bacteria that inhabit the human colon. Butyrate-producing bacteria represent a functional group, rather than a coherent phylogenetic group. Numerically, two of the most important groups of butyrate producers appear to be *Faecalibacterium prausnitzii*, which belongs to the *Clostridium leptum* (or clostridial cluster IV) cluster, and *Eubacterium rectale*/*Roseburia spp.*, which belong to the *Clostridium coccoides* (or clostridial cluster XIVa) cluster of firmicute bacteria^[2]. Butyrate is the major energy source for colonocytes and is involved in the maintenance of colonic mucosal health^[3]. Recently several intestinal and extraintestinal effects of butyrate have been demonstrated (Figure 1 and Table 1). This review is focused on new evidence for possible applications of butyrate in human medicine.

EFFECTS OF BUTYRATE AT THE INTESTINAL LEVEL

Effects on transepithelial ion transport

Potentially, SCFAs are absorbed by each intestinal segment, as demonstrated in animal models and human volunteers. The colonocytes absorb butyrate and other SCFAs through different mechanisms of apical membrane SCFA uptake, including non-ionic diffusion, SCFA/HCO₃⁻ exchange, and active transport by SCFA transporters. The transport proteins involved are monocarboxylate transporter isoform 1 (MCT1), which is coupled to a transmembrane H⁺-gradient, and SLC5A8, which is Na⁺-coupled co-transporter^[3,4]. The absorption of these fatty acids has a significant impact on the absorption of NaCl and on the electrolyte balance generally^[5]. In particular, butyrate is able to exert a powerful pro-absorptive stimulus on intestinal NaCl transport and an anti-secretory effect towards Cl⁻ secretion. The powerful regulatory pro-absorptive/anti-secretory effects induced by butyrate on the transepithelial ion transport occurs through several mechanisms: (1) Stimulation of NaCl absorption by the action of two coupled transport systems on the intestinal brush border: Cl⁻/HCO₃⁻ and Na⁺/H⁺ and Cl⁻/butyrate and Na⁺/H⁺; and (2) inhibition of Cl⁻ secretion by blocking the activity of the cotransporter Na-K-2Cl (NKCC1) on the enterocyte basolateral membrane. *In vitro* studies have shown that butyrate has an inhibitory effect on Cl⁻ secretion induced by prostaglandin E₂, cholera toxin, and phosphocholine. This effect is due to reduced production of intracellular cAMP secondary to the expression and regulation of adenylate cyclase^[4]. Comparison studies showed that the pro-absorptive and anti-secretory effects of butyrate are significantly higher than those of all other SCFAs^[6]. Clinical studies in chil-

dren with acute diarrhea caused by *V. cholerae* showed a reduction in stool volume and a more rapid recovery in patients who received oral rehydration therapy in addition to resistant starch, a precursor of butyrate, in the diet^[7,8]. These results were confirmed in other forms of infectious diarrhea in children and in animal models studies^[9,10]. Moreover, butyrate therapy is beneficial in patients affected by Congenital Chloride Diarrhea (CLD)^[11,12]. This rare genetic disease is caused by mutations in the gene encoding the solute-linked carrier family 26-member A3 (SLC26A3) protein, which acts as a plasma membrane anion exchanger for Cl⁻ and HCO₃⁻^[13]. The mechanism underlying this therapeutic effect could be related, at least in part, to stimulation of the Cl⁻/butyrate exchanger activity^[11]. It is also possible that butyrate could reduce mistrafficking or misfolding of the SLC26A3 protein, as demonstrated for other molecules involved in transepithelial ion transport^[14]. Alternatively, butyrate may enhance gene expression: the *SLC26A3* gene contains a 290-bp region between residues -398 and -688 that is crucial for high-level transcriptional activation induced by butyrate. This may explain the variable response of patients affected by CLD to butyrate^[12]. In fact, depending on the patient's genotype, mutations in the above-mentioned regulatory regions of the *SLC26A3* gene could affect the gene transcription rate. It is also conceivable that other channels could be involved in the therapeutic effect of butyrate in CLD. SLC26A3, like other components of the SLC26 family, interacts with cystic fibrosis transmembrane conductance regulator (CFTR)^[15,16]. The interaction between CFTR and these components is mediated by binding of the regulatory domain of CFTR to the sulfate transporter and anti sigma factor antagonist (STAS) domain of SLC26. The interaction is enhanced by phosphorylation of the regulatory domain by protein kinase A^[17] and is modulated by PDZ-binding scaffold proteins. An important consequence of this interaction is that SLC26 anion exchange activity is enhanced when CFTR is activated by phosphorylation. Moreover, the two genes regulate each other: the overexpression of SLC26A3 or -A6 causes upregulation of CFTR and *vice versa*^[18]. In patch-clamp experiments, protein kinase A-stimulated CFTR channel activity was six-fold higher in HEK293 cells co-expressing both SCL26 exchanger and CFTR than in HEK293 cells expressing CFTR alone^[12,15,16,18]. Mutations may impair the interactions between channels and thus reduce the effect of butyrate therapy. Interestingly, it has been demonstrated that butyrate can act by different mechanisms in *in vitro* models of cystic fibrosis: it can increase the expression of the apical epithelial membrane of the CFTR, and it can act as a "chaperone-like" molecule, as shown in the ΔF508del CFTR cell line model^[19]. Similar mechanisms could occur in CLD. Lastly, Clausen *et al.*^[20] demonstrated that antibiotic-associated diarrhea was related to reduced fecal concentrations and production rates of butyrate. Their results suggest that

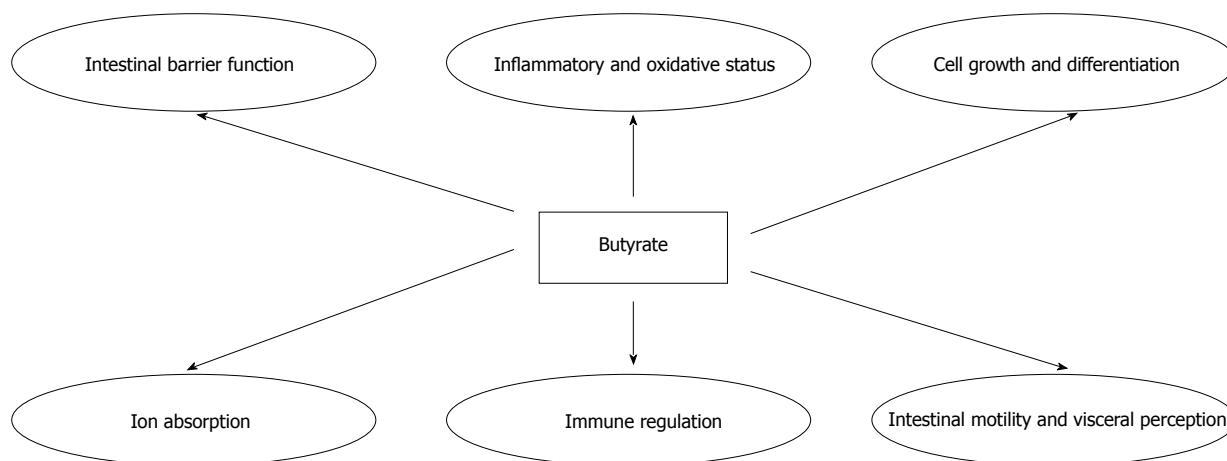


Figure 1 The multiple effects of butyrate at intestinal level.

Table 1 Main butyrate effects potentially useful in human medicine

Intestinal level	Extraintestinal level
Ion absorption	Insulin sensitivity
Cell proliferation	Cholesterol synthesis
Cell differentiation	Energy expenditure
Intestinal barrier function	Ammonia scavenger
Immune-regulation	Stimulation of β -oxidation of very long chain fatty acids and peroxisome proliferation
Oxidative stress	CFTR function
Intestinal motility	Neurogenesis
Visceral perception and rectal compliance	HbF production

CFTR: Cystic fibrosis transmembrane conductance regulator; HbF: Butyrate to increase fetal hemoglobin.

the antibiotic-associated diarrhea might be secondary to impaired colonic fermentation in otherwise disposed subjects, resulting in decreased butyrate and fluid absorption. In this case, the administration of butyrate could also alleviate the symptoms associated with antibiotic use.

Effects on cell growth and differentiation

Several epidemiological studies support the role of dietary fiber in the protection against colorectal cancer^[21-26]. Different mechanisms have been proposed for fiber's cancer preventive properties: reduction in transit time of the feces in the gut, which reduces exposure of the mucosa to luminal carcinogens; absorption of bile acids, biogenic amines, bacterial toxins, and production of butyrate. Most of the anticarcinogenic effects of butyrate are observed in *in vitro* carcinoma cell lines. In these models, addition of butyrate leads to inhibition of proliferation, induction of apoptosis, or differentiation of tumor cells^[27-30]. Butyrate's anticarcinogenic effects are in contrast with the effects of this compound in normal enterocytes. In fact, it has been shown that butyrate stimulates the physiological pattern of proliferation in the basal crypt in the colon,

whereas it reduces the number and the size of aberrant crypt focus, which are the earliest detectable neoplastic lesions in the colon^[31]. These contradictory patterns of butyrate represents the so called "butyrate paradox"^[27]. An important mechanism by which butyrate causes biological effects in colon carcinoma cells is the hyperacetylation of histones by inhibiting histone deacetylase (HDAC). This compensates for an imbalance of histone acetylation, which can lead to transcriptional dysregulation and silencing of genes that are involved in the control of cell cycle progression, differentiation, apoptosis and cancer development^[32,35]. In particular, in human colon cancer cell lines butyrate, acting as HDAC inhibitor, increases the p21 (*WAF1*) gene expression by selectively regulating the degree of acetylation of the gene-associated histones, and induces G1 cell cycle arrest^[36]. A novel contributory mechanism to the chemopreventive effect of butyrate is the downregulation of the key apoptotic and angiogenesis regulator Neuropilin-1 (NRP-1), which has been shown to promote tumor cell migration and survival in colon cancer in response to vascular endothelial growth factor (VEGF) binding^[37]. Several reports have shown that the apoptosis triggered by butyrate *in vitro* is associated with dysregulation of Bcl2 family proteins, especially upregulation of BAK and downregulation of BclxL^[38,39], rather than cellular damage. A study by Thangaraju *et al* suggests a novel mode of action of butyrate in the colon involving GPR109A, a G-protein-coupled receptor for nicotinate^[40,41], which recognizes butyrate with low affinity. This receptor is expressed in the normal colon on the lumen-facing apical membrane of colonic epithelial cells, but is silenced in colon cancer *via* DNA methylation. Thangaraju *et al*^[42] showed that inhibition of DNA methylation in colon cancer cells induces GPR109A expression and that activation of the receptor causes tumor cell-specific apoptosis. Butyrate is an inhibitor of HDAC, but apoptosis induced by activation of GPR109A with its ligands in colon cancer cells does not involve inhibition of histone deacetylation. The primary changes in this apoptotic pro-

cess include downregulation of Bcl-2, Bcl-xL, and cyclin D1 and upregulation of death receptor pathway. Moreover, a recent study suggested that the protective role of dietary fiber, and its breakdown product butyrate, against colorectal cancer could be determined by a modulation of canonical Wnt signaling, a pathway constitutively activated in the majority of colorectal cancers^[43]. Butyrate is recognized for its potential to act on secondary chemoprevention, by slowing growth and activating apoptosis in colon cancer cells^[44], but it can also act on primary chemoprevention. The mechanism proposed is the transcriptional upregulation of detoxifying enzymes, such as glutathione-S-transferases (GSTs). This modulation of genes may protect cells from genotoxic carcinogens, such as H₂O₂ and 4-hydroxynonenal (HNE)^[45,46].

Effects on inflammatory and oxidative status

Butyrate has a role as an anti-inflammatory agent, primarily *via* inhibition of nuclear factor κ B (NF- κ B) activation in human colonic epithelial cells^[47], which may result from the inhibition of HDAC. NF- κ B regulates many cellular genes involved in early immune inflammatory responses, including IL-1b, TNF- α , IL-2, IL-6, IL-8, IL-12, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), T cell receptor- α (TCR- α), and MHC class II molecules^[48-50]. The activity of NF- κ B is frequently dysregulated in colon cancer^[51,52] and in inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease (CD)^[53-55]. In CD patients, butyrate decreases pro-inflammatory cytokine expression *via* inhibition of NF- κ B activation and I κ B α degradation^[53]. The upregulation of peroxisome proliferator-activated receptor γ (PPAR γ) a nuclear receptor highly expressed in colonic epithelial cells, and the inhibition of IFN γ signaling, are another two of butyrate's anti-inflammatory effects^[56,57]. Butyrate can act on immune cells through specific G-protein-coupled receptors (GPRs) for SCFAs, GPR41 (or FFA3) and GPR43 (or FFA2), which are both expressed on immune cells, including polymorphonuclear cells, suggesting that butyrate might be involved in the activation of leucocytes^[58]. The possible immune-modulatory functions of SCFAs are highlighted by a recent study on GPR43 -/- mice. These mice exhibit aggravated inflammation, related to increased production of inflammatory mediators and increased immune cell recruitment^[59].

Most clinical studies analyzing the effects of butyrate on inflammatory status focused on UC patients. Hallert *et al.*^[60] instructed 22 patients with quiescent UC to add 60 g oat bran (corresponding to 20 g dietary fiber) to their daily diet. Four weeks of this treatment resulted in a significant increase of fecal butyrate concentration and in a significant improvement of abdominal symptoms. In a double blind, placebo-controlled multicenter trial, Vernia *et al.*^[61] treated 51 patients with active distal UC with rectal enemas containing either 5-aminosalicylic acid (5-ASA) or

5-ASA plus sodium butyrate (80 mmol/L, twice a day). The combined treatment with topical 5-ASA plus sodium butyrate significantly improved the disease activity score more than 5-ASA alone. These and other intervention studies^[62-64] suggested that the luminal administration of butyrate or stimulation of luminal butyrate production by the ingestion of dietary fiber results in an amelioration of the inflammation and symptoms in UC patients.

Numerous studies have reported that butyrate metabolism is impaired in intestinal inflamed mucosa of patients with IBD. Recent data show that butyrate deficiency results from the reduction of butyrate uptake by the inflamed mucosa through downregulation of MCT1. The concomitant induction of the glucose transporter GLUT1 suggests that inflammation could induce a metabolic switch from butyrate to glucose oxidation. Butyrate transport deficiency is expected to have clinical consequences. Particularly, the reduction of the intracellular availability of butyrate in colonocytes may decrease its protective effects toward cancer in IBD patients^[65].

Limited evidence from pre-clinical studies shows that oxidative stress in the colonic mucosa can be modulated by butyrate. Oxidative stress is involved in both inflammation^[66] and the process of initiation and progression of carcinogenesis^[67]. During oxidative stress there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanisms, leading to a cascade of reactions in which lipids, proteins, and/or DNA may get damaged. In healthy humans, it has been demonstrated that locally administered butyrate in physiological concentrations increased the antioxidant GSH and possibly decreased ROS production, as indicated by a decreased uric acid production^[68]. As the human colon is continuously exposed to a variety of toxic stimuli, enhanced butyrate production in the colon could result in an enhanced resistance against toxic stimuli, thus improving the barrier function. This might be relevant for the treatment of gastrointestinal disorders, such as post-infectious irritable bowel syndrome (IBS), microscopic colitis, IBD, and diversion colitis.

Effects on non-specific intestinal defense mechanisms

The main components of nonspecific intestinal barrier defense mechanisms are the mucous layer covering the epithelium, the production of antimicrobial peptides, and tight junctions, which protect the gastrointestinal mucosa against pathogens. Evidence suggests a role for butyrate in reinforcing the colonic defense barrier. Butyrate stimulates MUC2 mucin production in a human colonocytes cell line (LS174T). The increased expression of *MUC2* gene, and the induction of mucin synthesis, can affect the mucous layer leading to enhanced protection against luminal agents^[69,70].

Combined with other components of the innate immune system, antimicrobial peptides (AMPs) form the first line of defense against infections. The two major

classes of AMPs found in humans are defensins and cathelicidins. While the intestine expresses numerous defensins, LL-37 is the only cathelicidin-derived peptide expressed in humans. Several studies demonstrated an effect of butyrate on LL-37 gene expression and proposed that the molecular mechanism may be linked to an increase in histone acetylation and mitogen-activated protein (MAP) kinase signaling^[71-75]. The use of HDAC inhibitors, such as butyrate, to enhance the expression of the LL-37 gene may become a novel approach for strengthening innate immunity to treat or prevent intestinal infections.

Butyrate also regulates the colonic defense barrier through its effects on intestinal permeability, which depends on its concentration. At low concentrations, butyrate induces a concentration-dependent reversible decrease in permeability in intestinal cell line models^[76,77]. The effect of butyrate on the intestinal epithelial permeability involves the assembly of tight junctions *via* AMP-activated protein kinase (AMPK)^[78].

Effects on visceral perception and intestinal motility

Little is known about the environmental and nutritional regulation of the enteric nervous system (ENS), which controls gastrointestinal motility. Butyrate regulates colonic mucosa homeostasis and can modulate neuronal excitability. Soret *et al.*^[79] investigated the effects of butyrate on the ENS and colonic motility, and showed, *in vivo* and *in vitro*, that butyrate significantly increased the proportion of choline acetyltransferase (ChAT), but not nitric oxide synthase (nNOS) immunoreactive myenteric neurons. Butyrate increases the cholinergic-mediated colonic circular muscle contractile response *ex vivo*. The authors suggest that butyrate might be used, along with nutritional approaches, to treat various gastrointestinal motility disorders associated with inhibition of colonic transit.

A recent study by Van Houten *et al.*^[80] shows that intraluminal administration of a physiologically relevant dose (50 to 100 mmol/L) of butyrate into the distal colon increases compliance and decreases pain, urge, and discomfort measured with a rectal barostat procedure in healthy subjects. This study suggests a potential beneficial effect of butyrate in disorders that are associated with visceral hypersensitivity, such as IBS and infantile colics, and provides a basis for future trials with dietary modulation resulting in intracolonic butyrate production in both healthy and IBS subjects. The decrease in visceral perception induced by butyrate treatment could be due to an increased 5-HT release, as previously suggested by others^[81]. Another possible mechanism by which butyrate could affect visceral perception is the previous reported inhibition of histone deacetylase. In fact, Chen *et al.*^[82] showed that these inhibitors induce microglial apoptosis and attenuate inflammation-induced neurotoxicity in rats, which may affect visceral perception. Butyrate has been reported to induce enhancement of colonic motility *via* the release of 5-HT^[83]. In functional studies, butyrate and propionate induced phasic and tonic contractions in rat

colonic circular muscle. The dose-dependent contractile effect occurred only when SCFAs were applied on the mucosal side and disappeared in mucosal free preparations, suggesting the presence of sensory mechanisms near the epithelium^[84,85].

EFFECTS AT THE EXTRAINTESTINAL LEVEL

Hemoglobinopathies

Clinical trials in patients with sickle cell disease and β -thalassemia confirmed the ability of butyrate to increase fetal hemoglobin (HbF) production^[86-89]. Butyrate is an inducer of HbF through an epigenetic regulation of fetal globin gene expression *via* HDAC inhibition, resulting in global histone hyperacetylation, including nucleosomes at the γ -globin promoters^[90]. Other experiments have shown that butyrate can cause a rapid increase in the association of γ -globin mRNA with ribosomes^[91]. Other authors have demonstrated activation of p38 mitogen-activated protein kinases (MAPK) and cyclic nucleotide signaling pathways in association with butyrate induction of HbF^[92]. Taken together, these studies suggest that global histone hyperacetylation induced by HDAC inhibition is not the unique mechanism underlying butyrate stimulation of HbF.

Genetic metabolic diseases

Sodium phenylbutyrate 4 (4-PBA) was approved by the Food and Drug Administration (FDA) for use in patients with urea cycle enzyme deficiency, in which it acts as a scavenger of ammonia. Indeed, 4-PBA is oxidized to phenylacetate, which binds to glutamine and determines the urinary excretion. In patients with ornithine transcarbamylase deficiency, the use of 4-PBA allows for better metabolic control and increased intake of natural protein in the diet^[93].

The possible use of butyrate in the treatment of X-linked Adrenoleukodystrophy (X-ALD), a disorder of peroxisomes characterized by altered metabolism and accumulation of very long chain fatty acids, has also been studied. Sodium phenylbutyrate 4 induces, *in vitro* on fibroblasts from patients with X-ALD and *in vivo* in X-ALD knockout mice, an increase in β -oxidation of very long chain fatty acids and peroxisome proliferation^[94].

Hypercholesterolemia

Under normal lipidemic conditions, the liver is the most important site of cholesterol biosynthesis, followed by the intestine. Biosynthesis in the liver and intestine account for about 15% and 10%, respectively, of the total amount of cholesterol biosynthesis each day^[95,96]. In hypercholesterolemia, when cholesterol biosynthesis is suppressed in most organs by fasting, the intestine becomes the major site of cholesterol biosynthesis, and its contribution can increase up to 50%. Importantly, recent evidence shows that the global effect of butyrate is to downregulate the

Table 2 Possible therapeutic indications of butyrate in gastroenterology

Functions	Therapeutic indications	Potential applications
Regulation of fluid and electrolyte uptake	Acute gastroenteritis ^[9,10] Cholera ^[7,8] Congenital chloride diarrhea ^[11,12]	Irritable bowel syndrome ^[80] Aspecific chronic diarrhea ^[4] Traveler's diarrhea ^[4] Antibiotic associated diarrhea ^[4,20] Chronic secretory diarrhea ^[4] Cystic fibrosis ^[14,19] Mucosal atrophy in malnutrition ^[101]
Effects on proliferation and differentiation of epithelial intestinal cells	Acute gastroenteritis ^[9,10] IBD ^[53-65]	Mucosal atrophy in total parenteral nutrition ^[101,102] Mucosal atrophy in radiotherapy or chemotherapy ^[101] Short bowel syndrome and intestinal failure ^[103] Prevention of colorectal cancer ^[46]
Anti-inflammatory effect	IBD ^[53-65]	Intestinal polyposis ^[104] Pouchitis ^[105] Allergic colitis ^[106]

IBD: Inflammatory bowel disease.

expression of nine key genes involved in intestinal cholesterol biosynthesis, potentially inhibiting this pathway^[97].

Obesity and insulin resistance

Dietary supplementation with butyrate can prevent and treat diet-induced obesity and insulin resistance in mouse models. After a 5-wk treatment with butyrate, obese mice lost 10.2% of their original body weight. Consistent with the change in body weight, fat content was reduced by 10%. Furthermore, fasting glucose was reduced by 30%, insulin resistance was reduced by 50%, and intraperitoneal insulin tolerance was improved significantly by butyrate. The mechanism of butyrate action is related to promotion of energy expenditure and induction of mitochondrial function. Stimulation of peroxisome proliferator-activated receptor (PPAR) coactivator (PGC-1 α) activity has been suggested as the molecular mechanism of butyrate. Activation of AMPK and inhibition of histone deacetylases may contribute to the PGC-1 α regulation. These data suggest that butyrate may have potential application in the prevention and treatment of metabolic syndrome in humans^[98].

Ischemic stroke

Cerebral ischemia enhances neurogenesis in neurogenic and non-neurogenic regions of the ischemic brain of adult animal models. A recent study demonstrated that post-insult treatment with sodium butyrate stimulated the incorporation of bromo-2'-deoxyuridine (BrdU) in the ischemic brain of rats subjected to permanent cerebral ischemia. Butyrate treatment also increased the number of cells expressing polysialic acid-neural cell adhesion molecule, nestin, glial fibrillary acidic protein, phospho-cAMP response element-binding protein (CREB), and brain-derived neurotrophic factor (BDNF) in various brain regions after cerebral ischemia^[99]. Furthermore, extensive co-localization of BrdU and polysialic acid-neural cell adhesion molecule was observed in multiple regions after ischemia, and butyrate treatment upregulated protein levels of BDNF, phospho-CREB, and glial fibrillary acidic protein. Intraventricular injection of K252a, a tyrosine kinase B receptor antago-

nist, markedly reduced the long-lasting behavioral benefits of butyrate, inhibiting cell proliferation, nestin expression, and CREB activation^[99]. Together, these results suggest that butyrate-induced cell proliferation, migration, and differentiation require BDNF-tyrosine kinase B signaling and may contribute to long-term beneficial effects of butyrate after ischemic injury.

ISSUES RELATED TO THE CLINICAL USE OF BUTYRATE

Data from literature and clinical experience of several research groups show a wide spectrum of possibilities for potential therapeutic use of butyrate by oral administration without having serious adverse events (Table 2). Some butyrate-based products are marketed, but their spread is still very limited and greatly understaffed in view of the wide spectrum of possible indications, especially in chronic diseases, where it is possible to predict a lasting use of the compound. The main problem is of the availability of formulations of butyrate that can be easily administered orally, in particular for pediatric patients, and to the extremely poor palatability of the products available on the market. The unpleasant taste and odor make oral administration of butyrate extremely difficult, especially in children. Thus, new formulations of butyrate with a better palatability, which can be easily administered orally, are needed. Another possible solution could be the modulation of intestinal microflora by probiotics. Probiotics are live and viable microorganisms, which, if given in adequate amounts, confer a beneficial effect to the host. Probiotic microorganisms generate small molecular metabolic byproducts, referred to as "postbiotics", which exert beneficial regulatory influence on host biological functions, including butyrate^[100].

CONCLUSION

The SCFA butyrate, a main end product of microbial fermentation of dietary fibers in the human intestine,

plays an important role in the maintenance of intestinal homeostasis and overall health status. The effects exerted by butyrate are multiple and involve several distinct mechanisms of action. Its well-known epigenetic mechanism, through the inhibition of HDACs, results in the regulation of gene expression and in the control of cell fate. At the intestinal level, butyrate exerts multiple effects such as the prevention and inhibition of colonic carcinogenesis, the improvement of inflammation, oxidative status, epithelial defense barrier, and the modulation of visceral sensitivity and intestinal motility. At the extraintestinal level, potential fields of application for butyrate seem to be the treatment of sickle cell disease, β -thalassemia, cystic fibrosis, urea cycle enzyme deficiency, X-linked adrenoleukodystrophy, hypercholesterolemia, obesity, insulin resistance, and ischemic stroke.

In conclusion, a growing number of studies have revealed new mechanisms and effects of butyrate with a wide range of potential clinical applications from the intestinal tract to peripheral tissues. However, more clinical studies to elucidate the role of butyrate in health and diseases and new solutions for easier administration are needed.

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Occult hepatitis B virus infection: A complex entity with relevant clinical implications

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entity in cryptogenetic liver diseases, hepatitis C virus/HIV infected patients and immunosuppressed individuals. It is also necessary to increase our knowledge in this fascinating field to define better strategies to diagnose and treat this infection.

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Abstract

Occult hepatitis B virus (HBV) infection is a world-wide entity, following the geographical distribution of detectable hepatitis B. This entity is defined as the persistence of viral genomes in the liver tissue and in some instances also in the serum, associated to negative HBV surface antigen serology. The molecular basis of the occult infection is related to the life cycle of HBV, which produces a covalently closed circular DNA that persists in the cell nuclei as an episome, and serves as a template for gene transcription. The mechanism responsible for the HBsAg negative status in occult HBV carriers is a strong suppression of viral replication, probably due to the host's immune response, co-infection with other infectious agents and epigenetic factors. There is emerging evidence of the potential clinical relevance of occult HBV infection, since this could be involved in occult HBV transmission through orthotopic liver transplant and blood transfusion, reactivation of HBV infection during immunosuppression, impairing chronic liver disease outcome and acting as a risk factor for hepatocellular carcinoma. Therefore it is important to bear in mind this

EDITORIAL

The persistence of hepatitis B virus (HBV) in hepatitis B surface antigen (HBsAg) negative individuals is termed occult HBV infection (OBI)^[1]. Since the early 80's it was suspected that hepatitis B virus could persist in the host despite not being detectable. In the last decade after the appearance of highly sensitive molecular biology techniques the presence of HBV in the liver and serum of HBsAg negative individuals has been shown^[2]. OBI-infected subjects maintained HBV infection due to the peculiar HBV life cycle which is characterised by the production of covalently closed circular DNA that serves as a template for gene transcription^[3]. In occult HBV carriers the viral replication from this episome is strongly suppressed and several factors could contribute to this situation^[4]. A high HBV-specific immunological pressure could contribute to the development of occult HBV infection and this could

explain why in immunosuppressive condition an HBV infection reactivation is observed. Another hypothetical factor associated with OBI is the co-infection with other pathogens. HCV infection is strongly associated with HBV occult infection and moreover it has been shown that HCV core protein is able to inhibit *in vitro* HBV replication. Finally, there is some evidence that epigenetic factors could contribute to reduce HBV replication efficiency through the regulation of the HBV transcriptional program. The prevalence of this entity is difficult to assess due to the difference in the technical procedures and the tissues studied. The distribution is similar to the HBVAg positive HBV infection being more prevalent in certain groups such as HCV and HIV populations^[5,6]. Anyway epidemiological data are still scarce and more studies in the general population and cryptogenic hepatitis are necessary^[7]. From the clinical point of view this entity is extremely relevant because it could be the cause of liver disease in different scenarios not well described currently. Carriers of occult HBV infection may be a source of HBV transmission in the case of blood transfusion^[8]. Moreover, HBV occult infection can be also transmitted during orthotopic liver transplantation from anti hepatitis B core positive individuals as well as from HBV seronegative cases^[9]. Another important clinical manifestation of HBV occult infection is its reactivation of the infection during immunosuppression. After the restoration of the immune system after finishing immunosuppressive treatment it is possible that acute hepatitis may occur. This is important in haematological malignancies, hematopoietic stem cell transplantation and organ transplantation^[10]. Clinicians should be aware of these clinical events to prevent this situation with the appropriate prophylaxis. Moreover, hepatologists should not think that this infection in immunocompetent individuals is an inoffensive condition. The long-lasting persistence of the virus in the liver may provoke a mild but continuous inflammation which could have clinical implications in cases of previous liver damage. Different reports suggest that HBV occult infection could be responsible for the acceleration of chronic HCV progression and interfere with treatment response^[5]. More studies are needed to clarify this important issue. Finally HBV occult infection could be related to the development of hepatocellular carcinoma among HBsAg negative chronic hepatitis patients^[11]. This could be through the induction of chronic inflammation and by means of HBV DNA integration into the host's genome. In summary, it is clear that a large amount of information has been produced in recent years about this topic but the data are still too incomplete to describe properly the clinical impact of this infection and more bio-medical research is still needed in this fascinating field. In this issue of *World Journal of Gastroenterology* the current knowledge on the HBV occult infection field is updated, addressing all the topics^[12-18] with a practical point of view to make this "Topic Highlight" interesting and useful to most clinicians.

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Reactivation of hepatitis B virus infection after cytotoxic chemotherapy or immunosuppressive therapy

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that caused by chemotherapy. Baseline HBV serology is recommended for all patients receiving chemotherapy and immunosuppressive drugs, and HBsAg positive patients should receive anti-HBV prophylaxis to decrease virus reactivation and death rates.

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Key words: Hepatitis B; Immune response; Immunosuppression; Reactivation

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Abstract

Reactivation of hepatitis B is defined as the recurrence or an abrupt rise in hepatitis B virus (HBV) replication, often accompanied by an increase in serum transaminase levels, and both events occurring in a patient with a previous inactive hepatitis B infection. This reactivation can occur in situations in which the ratio of HBV replication and immune response is altered. It can happen during the treatment of hemato-oncological malignancies with chemotherapy and in immunosuppression of autoimmune diseases. Clinical manifestations of hepatitis B reactivation are variable and can range from asymptomatic to acute hepatitis, which are sometimes serious and result in acute liver failure with risk of death, and usually occur in the periods between cycles or at the end of chemotherapy. Immunosuppressive drugs such as corticosteroids or azathioprine can induce HBV reactivation in patients carrying hepatitis B virus surface antigen (HBsAg) or anti-HBc, but much less frequently than chemotherapy treatments. The tumor necrosis factor α inhibitors infliximab, etanercept and adalimumab may cause reactivation of hepatitis B, and the overall frequency with infliximab may be similar (50%-66%) to

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SITUATIONS ASSOCIATED WITH REACTIVATION OF HEPATITIS B

Hepatitis B is a major health problem worldwide with a prevalence that varies according to geographic area. This prevalence is changing due to the growing phenomenon of immigration. There are four distinct dynamic phases of chronic infection with hepatitis B virus (HBV) and this process is unidirectional, depending on the interaction between the virus itself, hepatocytes and the host immune system^[1]. Because of this interaction, situations that lead to immunosuppression in patients with chronic HBV infection may alter the natural history of this infection and give rise to the phenomenon of reactivation.

Reactivation of hepatitis B (HBV reactivation) is defined as the recurrence or abrupt rise in HBV replication by at least an increase in serum HBV DNA levels of 1 log₁₀, often accompanied by an increase in transaminase levels (at least three times the baseline). Both events occur in a patient with a previous inactive HBV infection, i.e. either an inactive carrier state or a patient with resolved hepatitis^[2]. HBV reactivation can occur in situations in which HBV replication increases or the immune response decreases. This can happen spontaneously, generally when the virus mutates and the immune system needs time to rebuild the immune response. It also appears when the virus replicates again abruptly and becomes resistant to a drug or when antiviral medications have been withdrawn. In patients with coinfection by the human immunodeficiency virus (HIV) this happens when progressive immunodeficiency lowers specific T cells response against HBV. A major cause of HBV reactivation is solid organ transplantation. Finally, the most common causes and the focus of this report, are chemotherapy (CMT), used in the treatment of onco-hematological diseases, and immunosuppressive drugs used in the treatment of autoimmune diseases (Table 1)^[2]. The risk of HBV reactivation depends on factors such as the state of HBV: the higher the level of viral replication, the higher the risk of reactivation. This risk is much lower in patients with resolved infection. Another factor that influences HBV reactivation is the type of disease: the risk is higher in patients with lymphoma than in those with solid tumors. This can be attributed to the fact that hematological disease itself induces a greater degree of immunosuppression or that CMT is stronger in cases of hematological malignancies^[3]. All types of drugs used in CMT have been involved in HBV reactivation, from the classic cytostatics to monoclonal antibodies and steroids, as they all have immunosuppressive activity (Table 2). HBV reactivation associated with CMT occurs frequently. In one of the first series, published in 1991, reactivation in 48% of hepatitis B virus surface antigen (HBsAg) carriers and in 4% of resolved hepatitis B patients were observed^[4]. In patients with reactivation, 53% showed icteric hepatitis and 20% died. In a meta-analysis carried out in 2008, which included 14 studies of HBsAg carriers, an average reactivation rate of 33% (with a wide range of 24%-88%) was observed, in addition to hepatitis in 33% and death in 7% of cases^[5]. Therefore, HBV reactivation does not occur in all HBsAg carriers undergoing CMT. Multivariate analysis identified various risk factors: male gender, young age, high HBV replication at baseline (HBV-DNA > 20 000 IU/mL or hepatitis B virus e antigen positive), CMT regimens including corticosteroids or rituximab, and bone marrow transplantation (BMT). In patients with the same hematologic disease and undergoing the same CMT treatment, the addition of corticosteroids significantly increased the HBV reactivation risk from 38% to 72%, and the number of cases of hepatitis and severe hepatitis^[6]; in contrast, patients treated with steroids had an improvement in treatment outcome in terms of remission and survival, endpoints which need

to be clarified in larger series. Rituximab is a HBV reactivation risk factor even greater than corticosteroids: in a series of patients with lymphoma treated with CMT, the only significant difference between the reactivation group with resolved hepatitis and the group without reactivation was treatment with rituximab alone or associated with steroids^[7]. Rituximab is a monoclonal anti-B lymphocyte that causes apoptosis, and the B cell plays a key role in the multiple immune response against HBV: besides neutralizing antibodies, it is an antigen presenting cell and enhances the cytotoxic response of CD8 T lymphocytes. Therefore, its destruction decreases dramatically favoring the immune response of HBV replication. This also explains why rituximab can cause fulminant hepatitis and death in 50% of patients with resolved hepatitis B in the case of seroreversion^[8].

BMT is a major risk factor. Reactivation was reported almost universally among patients with positive HBsAg, and reverse seroconversion from resolved hepatitis B is twice as common in these patients as in patients without BMT. High doses of CMT with or without radiation are necessary to destroy the recipient's bone marrow before infusion of donor bone marrow, followed by an initial intense and prolonged immunosuppression followed by maintenance immunosuppression^[2].

CLINICAL MANIFESTATIONS OF HEPATITIS B REACTIVATION

HBV reactivation is associated with a wide range of clinical manifestations, from asymptomatic to acute hepatitis that can be serious and cause liver failure which can be life-threatening. In some patients, HBV reactivation has a subclinical onset and resolves spontaneously. In other patients it progresses to a persistent infection that can not be detected. At the time of diagnosis there is already advanced liver disease^[2]. HBV reactivation often manifests in periods between cycles of CMT/immunosuppressive treatment or at the end of therapy. Following immunosuppression caused by CMT/immunosuppressive treatment, a period of time is necessary for immune reconstitution and subsequent attack on the liver^[9]. Therefore, in HBsAg carriers the time between CMT treatment and HBV reactivation detection is very variable: from 1-36 mo, but usually ranges between 1 and 4 mo. Although not all patients go through the following stages, HBV reactivation usually has three phases: (1) replication, in which serum HBV-DNA levels increase; and (2) hepatitis, which appears 2-3 wk after HBV-DNA levels increase and is characterized by an elevation of transaminases and, sometimes, symptoms such as fatigue, malaise and jaundice. In some cases, hepatitis can be fulminant (hepatic encephalopathy, coagulopathy, *etc.*) and if the patient does not die of fulminant hepatitis, the infection moves to the third phase or recovery. In this phase, HBV-DNA and transaminase levels return to their previous state, usually by suspending CMT. In patients

Table 1 Onco-hematological diseases and reactivation of hepatitis B

Hematologic diseases	Hematological tumors
Non-hodgkin lymphoma	Breast cancer
Hodgkin lymphoma	Lung cancer
Chronic lymphocytic leukemia	Hepatocellular carcinoma
Chronic myeloid leukemia	Nasopharyngeal cancer
Acute myeloid leukemia	Other cancers
Acute lymphoblastic leukemia	
Multiple myeloma	
Waldenstrom macroglobulinemia	
Plasmacytoma	
Aplastic anemia	
Myelodysplastic syndrome	
Bone marrow transplantation	

Table 2 Chemotherapy and reactivation of hepatitis B

Classes	Drugs
Alkylating agents	Cyclophosphamide, chlorambucil, cisplatin
Alkaloids	Vincristine, vinblastine
Antibiotics	Doxorubicin, epirubicin, daunorubicin, bleomycin, mitomycin C, actinomycin D
Antimetabolites	Cytarabine, fluorouracil, gemcitabine, mercaptopurine, methotrexate, thioguanine
Monoclonal antibodies	Rituximab (anti-CD20), alemtuzumab (anti-CD52)
Corticosteroids	Dexamethasone, methylprednisolone, prednisone
Others	Folinic acid, colaspase, docetaxel, etoposide, fludarabine, interferon, procarbazine

with resolved hepatitis, HBV reactivation usually begins later than 4 mo, but has the same clinical features. Sometimes reactivation only occurs with HBV-DNA elevation, without increased transaminases. Other times the patient does not move into the full recovery phase, but has elevated HBV DNA levels, although there is no significant immune reconstitution or liver damage. This is common in patients undergoing organ transplantation and treatment with immunosuppressive drugs. Finally in some cases, the hepatitis phase persists and a chronic hepatitis is established^[10].

TREATMENT AND PREVENTION OF HEPATITIS B REACTIVATION

The first step following HBV reactivation is to suspend CMT. However, this can affect the efficacy of this treatment.

Interferon- α is the classic treatment of chronic hepatitis B, but should not be used in cases of HBV reactivation, because its immunomodulatory action can cause a serious hepatitis outbreak that added to HBV reactivation can have serious consequences.

Lamivudine is the most frequently used nucleoside analog in the treatment of HBV reactivation and has been available since 1999. This drug is capable of inhibiting HBV replication and can reverse HBV reactivation.

However, treatment with lamivudine can cause mortality in patients with HBV reactivation, which ranges between 13% and 80% with an average of about 36%^[2]. Therefore, at least one third of patients die from HBV reactivation despite treatment with lamivudine. This could be due to its lower antiviral potency (less than that of other nucleoside/nucleotide analogs such as telbivudine, entecavir and tenofovir), its high rate of resistance (up to 67% at 4 years in immunocompetent individuals), and possibly because HBV reactivation is less effective when it is already in progress. These facts led to the conclusion that in such patients it would probably be best to administer preventive treatment for HBV reactivation with the aim of inhibiting the replication of HBV, as this would hinder the development of hepatitis and thus mortality.

Prophylaxis in patients treated with chemotherapy

In eight retrospective case studies of patients with positive HBsAg treated with CMT, HBV reactivation occurred in 2.4% of those receiving prophylaxis with lamivudine compared with 56% who did not receive prophylaxis. Similarly, in four prospective case studies with historical controls, HBV reactivation occurred in 4% of those receiving prophylaxis with lamivudine compared with 28% who did not receive prophylaxis^[8]. In a prospective controlled clinical trial, prophylaxis with lamivudine for 2 wk before starting CMT and for six wk thereafter was compared to treatment with lamivudine when there was already HBV reactivation. HBV reactivation occurred in 53% of patients who received no prophylaxis, however, none of those who received prophylaxis showed evidence of HBV reactivation; hepatitis occurred in 47% of those without prophylaxis and 7% died. None of the patients who received prophylaxis developed hepatitis or died^[11]. A similar trial also compared lamivudine prophylaxis 2 wk prior to CMT until 2 mo after CMT with lamivudine treatment for HBV reactivation. Again there were significant differences in favor of prophylaxis, although this did not prevent reactivation in 12% of cases and hepatitis in 8%, attributable to the average antiviral potency of lamivudine and the possibility of the development of resistance. Importantly, following discontinuation of prophylaxis an increase in the number of patients with hepatitis reactivation, including death, occurred indicating that prophylaxis should be maintained beyond 2 mo after completion of CMT^[12]. In another study, prophylaxis was continued for 3 mo after CMT. After 26 mo of follow-up, hepatitis B was reactivated in 24% of patients, and reached 40% at 40 mo, being more common in those patients with high HBV replication at baseline^[13]. A meta-analysis of 14 studies also found significant differences in favor of prophylaxis with lamivudine compared with untreated controls, although again prophylaxis with lamivudine did not eliminate all risk of HBV reactivation^[5]. Finally, in another systematic review with a meta-analysis that included 21 studies, significantly lower numbers of reactivation, hepatitis and death were found in the group receiving prophylaxis with lamivudine^[14]. In summary, the

findings from these 8 retrospective case series, 4 prospective case series with historical controls, two controlled trials published to date and 2 meta-analyses showed that prophylactic lamivudine significantly reduces HBV reactivation. In addition, prophylaxis should last approximately 3 mo after completion of CMT, especially in patients with high HBV replication at baseline. There are no studies on the time prophylaxis should be extended and recommendations by experts range from 6-12 mo post-CMT treatment. These recommendations have even been extended to patients with risk factors such as high basal HBV-DNA, use of rituximab or BMT. All consensus and clinical practice guidelines^[15-17] recommend baseline screening for HBV (HBsAg, anti-HBc and anti-HBs) in all patients who are scheduled to receive CMT. In seronegative patients, the possibility of anti-HBV vaccination should be assessed. HBsAg positive patients should receive anti-HBV prophylaxis: patients who undergo CMT for less than 1 year may be treated with lamivudine (100 mg/d) from 1-2 wk before and 6-12 mo after. If CMT continues over 1 year and, especially, if there is high HBV replication, more potent drugs with less resistance such as telbivudine, tenofovir or entecavir should be evaluated. Patients with resolved hepatitis may be carriers of hidden HBV. Thus, HBV reactivation prophylaxis in patients assessed for BMT or subjected to aggressive and prolonged immunosuppressive therapy, such as patients undergoing solid organ transplantation should be considered^[4]. The appearance of HBsAg and HBV DNA in up to 50% of patients with anti-HBc undergoing BMT have been reported. The serial determination of anti-HBs in the serum of these bone marrow recipients has shown a steady decline to undetectable levels by 1-3 years after transplantation. With the loss of anti-HBs (anti-HBc), HBV DNA increases and HBsAg reappears. Some of these patients with HBs seroreversion do not develop clinical hepatitis, but among those who have HBs seroreversion, severe infection is rare. HBsAg seroreversion occurs late in patients with BMT, and therefore, in these cases long-term antiviral prophylaxis is recommended^[18,19]. Resolved hepatitis B patients with hematologic malignancies who receive CMT may also develop HBV reactivation, especially those with only positive anti-HBc (HBV reactivation in 25%). Risk factors are: negative anti-HBs or serum HBV DNA levels often < 100 IU/mL, treatment with more than one chemotherapeutic agent or with rituximab and BMT. In 20%-40% of cases, HBV reactivation may be fatal and prophylaxis with lamivudine again does not prevent reactivation in all cases^[20,21].

Prophylaxis in patients receiving biological treatments

Classical immunosuppressive drugs (corticosteroids, azathioprine, methotrexate) have been used for many years in multiple autoimmune diseases and organ transplantation. These drugs can induce HBV reactivation in patients carrying HBsAg or anti-HBc, but much less frequently than CMT. Therefore, reported cases of HBV reactivation are isolated^[2].

Table 3 Infliximab treatment and cases of hepatitis B reactivation

17 reports (2003-2009) (n = 21)	n
Michel <i>et al</i> ^[26] , 2003	1
Ostuni <i>et al</i> ^[27] , 2003	1
Oniankitan <i>et al</i> ^[28] , 2004	1
Esteve <i>et al</i> ^[29] , 2004	3
Wendling <i>et al</i> ^[30] , 2005	1
Ueno <i>et al</i> ^[31] , 2005	1
Anelli <i>et al</i> ^[32] , 2005	1
Millonig <i>et al</i> ^[33] , 2006	1
Roux <i>et al</i> ^[34] , 2006	1
Calabrese <i>et al</i> ^[35] , 2006	1
Colbert <i>et al</i> ^[36] , 2007	1
Madonia <i>et al</i> ^[37] , 2007	1
Sakellariou <i>et al</i> ^[38] , 2007	2
Ojio <i>et al</i> ^[39] , 2008	1
Chung <i>et al</i> ^[40] , 2009	1
Conde-Taboada <i>et al</i> ^[41] , 2009	1
Wendling <i>et al</i> ^[42] , 2009	1

The new immunosuppressants are called biological therapies, because these drugs block the action of biological products involved in the immune-inflammatory pathogenesis of many diseases. There are four main types: anti-inflammatory cytokines, anti-lymphocyte, anti-leukocyte adhesion and migration and anti-immunoglobulin^[22]. Within the first group of anti-cytokines there are tumor necrosis factor (TNF) α inhibitors, such as infliximab, etanercept and adalimumab. All three are used in the treatment of various rheumatic diseases and inflammatory bowel disease^[23], since the cytokine TNF α is dominant in these diseases and its inhibition improves the disease. However, the inhibition of TNF α can reactivate hepatitis B^[24], as TNF α is also important in the immune pathogenesis of hepatitis B, it takes part in cytolytic immunodepletion by CD8 cytotoxic lymphocytes, and is involved in noncytolytic immunodepletion with other cytokines produced by CD4 lymphocytes suppressing HBV replication and, ultimately, increases all immunocompetent cells. Therefore, administration of anti-TNF α inhibits the anti-HBV immune response, thus favoring HBV replication and the expression of a large amount of hepatitis B virus core antigen (HBcAg) in infected hepatocytes. When the administration of anti-TNF α is suspended, inhibition of the immune response is stopped and thus, there is immune reconstitution which attacks the large number of hepatocytes expressing HBcAg in their membranes. This results in an outbreak of hepatitis^[25].

Seventeen publications in the past 6 years have reported 21 patients who experienced reactivation of hepatitis B when treated with infliximab (Table 3). Infliximab was used to treat Crohn's disease in 8 cases, rheumatoid arthritis in 6 patients, ankylosing spondylitis in 5 cases, Still's disease in 1 case and psoriasis in 1 case. HBV infection at baseline was the inactive carrier state in 15 cases, chronic active hepatitis in 4 cases, occult HBV in 1 case and delta virus co-infection in 1 case. None of the six

patients who received lamivudine prophylaxis had HBV reactivation. This was not the case in the remaining 15 patients who did not receive prophylaxis and were distributed as follows: in 8 cases (53%), reactivation was treated with lamivudine and the outcome was good, 3 cases (20%) had fatal fulminant hepatitis, in another 3 cases (20%) withdrawal of infliximab was followed by regression of the alterations, and in one case (7%) evolution was spontaneously favorable. It should be noted that the usual pattern of treatment with infliximab in these publications was three doses at week 0, 2 and 6 followed by maintenance treatment every 8 wk. It is, therefore, a long-term treatment and resistance to lamivudine can appear, particularly in patients with high HBV replication. In such cases, it is preferable to use drugs with lower rates of resistance such as tenofovir or entecavir.

In the last 3 years, 7 patients who had HBV reactivation with etanercept have been reported. All had rheumatic disease and 5 patients at baseline were in the inactive carrier state of HBV, one patient had chronic active hepatitis and the other case had hidden hepatitis B infection. None of the three patients receiving prophylaxis with lamivudine showed reactivation of hepatitis B. Of the four patients without prophylaxis, 3 responded favorably to treatment with lamivudine and 1 responded without treatment. These results suggest that etanercept could lead to a revival milder than that with infliximab, but it is difficult to determine because it was described as a case of reactivation of resolved hepatitis, which generally is interpreted as a result of increased immunosuppression^[43].

To our knowledge, there are only two case reports of HBsAg-positive patients treated with adalimumab, which accounted for three patients with rheumatoid arthritis and an inactive carrier state of HBV^[44,45]. In one case, lamivudine prophylaxis was administered and there was no HBV reactivation. Of the other two cases, one had an increase in HBV DNA, which remained stable for 2 years without associated hepatitis, despite continuing with adalimumab; the other case did not show any evidence of reactivation. It is difficult to draw conclusions from so few data, except to say that prophylaxis prevents HBV reactivation.

Both etanercept and adalimumab probably cause less HBV reactivation than infliximab. In a series of 103 patients with rheumatic diseases treated with anti-TNF α , 8 cases were inactive HBsAg carriers and 2 cases were treated with infliximab, 4 cases with etanercept and 2 cases with adalimumab HBV reactivation occurred in one of these 8 patients and this patient was one of the two treated with infliximab^[46]. Thus, the frequency of reactivation with infliximab may be 50%, similar to that observed with cancer treatments. However, there is evidence that this frequency may be even higher. In another series of 80 cases of Crohn's disease treated with infliximab, 3 patients were HBsAg positive: one of them received prophylaxis with lamivudine and demonstrated no evidence of HBV reactivation, but the other two cases

who received no prophylaxis demonstrated evidence of HBV reactivation, i.e. the frequency of HBV reactivation was 66%^[47].

CONCLUSION

In HBsAg carriers who undergo CMT, the risk of HBV reactivation is high: 30%-50%. The risk is greater in patients with high HBV replication at baseline, and in patients receiving CMT regimens which include corticosteroids or rituximab, or individuals who undergo a BMT. These patients should be screened for HBV at baseline: HBsAg, anti-HBc and anti-HBs. Anti-HBV vaccination should be assessed for seronegative patients.

Anti-HBV prophylaxis is indicated in patients with positive HBsAg, to decrease reactivation rates, hepatitis and death. Although there is a lack of information, prophylaxis should begin 1-2 wk prior to CMT and should be maintained until 6-12 mo after treatment. A longer treatment period may be necessary in patients with factors that promote reactivation.

Most studies on prophylaxis have been conducted with lamivudine. This drug should be used in patients with low HBV DNA levels at baseline who will receive treatment for less than 1 year. In other cases, more potent drugs with less risk of resistance such as tenofovir or entecavir should be used.

Positive anti-HBc patients may have an occult HBV infection that can be reactivated by CMT. There is insufficient evidence to recommend routine prophylaxis, but treatment is recommended for patients with risk factors and, in other cases, follow-up and early treatment should be recommended in case of reactivation.

The TNF α inhibitors infliximab, etanercept and adalimumab may cause reactivation of HBV. The overall frequency seems less than that caused by CMT, but the frequency with infliximab may be similar (50%-66%). Prophylaxis prevents HBV reactivation in patients treated with infliximab. There are insufficient data to advise routine prophylaxis with etanercept and adalimumab, but until such data are available it seems prudent to administer prophylaxis.

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Prevalence of occult hepatitis B virus infection

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Abstract

Occult hepatitis B virus (HBV) infection (OBI) is characterized by the persistence of HBV DNA in the liver tissue in individuals negative for the HBV surface antigen. The prevalence of OBI is quite variable depending on the level of endemic disease in different parts of the world, the different assays utilized in the studies, and the different populations studied. Many studies have been carried out on OBI prevalence in different areas of the world and categories of individuals. The studies show that OBI prevalence seems to be higher among subjects at high risk for HBV infection and with liver disease than among individuals at low risk of infection and without liver disease.

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Key words: Occult hepatitis B virus infection; Hepatitis B virus DNA; Anti-HBc; Hepatitis B virus seronegative; Hepatitis C; Cryptogenetic hepatitis; Dialysis; Human immunodeficiency virus; Blood donors

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is characterised by persistence of HBV surface antigen (HBsAg) and presence of HBV DNA in serum.

Occult HBV infection (OBI) is the persistence of viral genome in the liver tissue in individuals negative for HBsAg. OBI is defined by the presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) in patients with serological markers of previous infection (anti-HBc and/or anti-HBs positive) or in patients without serological markers (anti-HBc and/or anti-HBs negative). The prevalence of OBI is quite variable depending on the level of endemic disease in different parts of the world, the different assays utilized in the studies, and the different populations studied^[1]. The populations in which prevalence of OBI has been investigated are: patients with liver disease (HCV infected patients and patients with cryptogenetic liver diseases), patients at high risk of parenteral-transmitted infection (intravenous drug addicts, hemophiliacs), patients on hemodialysis, human immunodeficiency virus (HIV) infected patients and apparently healthy individuals (blood donors, general population)^[1]. The purpose of this review is to provide comprehensive information on overall OBI prevalence as well as in patients with different chronic liver diseases.

HCV INFECTED PATIENTS

As HBV and HCV share many of the same transmission

routes, and infection with both viruses is common, the high prevalence of OBI in patients with hepatitis C is not unexpected. HCV infected patients have the highest prevalence of OBI^[2,3]. Cacciola *et al*^[2] published the first study of prevalence of OBI in patients with chronic hepatitis C; in this study HBV sequences were found in liver tissue from 66 of the 200 (33%) HCV infected patients and in 7 of the 50 (14%) HCV negative patients, 46 of the 66 patients were anti-HBc positive and 20 of the 66 were anti-HBc negative. They also found very low levels of viremia and the prevalence of OBI was particularly high among patients with anti-HBV antibodies although OBI was also detected in patients who were negative for all HBV serum markers^[2]. The study of Cacciola *et al*^[2] also demonstrated that OBI was significantly correlated with cirrhosis among HCV infected patients; 22 of the 66 patients (33%) with HCV infection and OBI had cirrhosis as compared with 26 of the 134 (19%) with HCV infection and no OBI, suggesting that OBI can accelerate the evolution to cirrhosis in HCV infected patients. Bréchet *et al*^[4] reviewed all the studies published in anti-VHC patients using PCR on serum and liver and the conclusion was that about 20%-30% and 40%-50% of serum and livers respectively showed HBV DNA positivity.

CRYPTOGENETIC LIVER DISEASE

In patients with cryptogenetic liver disease there is less available information than in HCV patients but the prevalence is thought to range from 19% to 31%^[5,6]. Chemin *et al*^[6] studied 50 patients with chronic hepatitis non-A non-E and reported a high prevalence of low-grade HBV infection. HBV DNA was detected by PCR in serum in a high proportion of cases (15/50; 30%); in all cases HBV DNA detection in serum was further confirmed on liver biopsies. 11 of the 15 (73%) patients who were HBV DNA positive were found to be anti-HBc positive, and all the patients had 10⁴ or less HBV DNA copies per mL. Among the positive HBV DNA patients 8/15 (53%) had severe fibrosis and cirrhosis and none of the patients had steatosis, so low-grade HBV infection was associated with more severe liver disease. The histopathological follow-up showed that some patients progressed to cirrhosis^[5]. Berasain *et al*^[5] investigated 1075 patients with chronic liver disease and in 109 (10%) the aetiology could not be defined by clinical, biochemical and serological data. In these cases liver biopsy was reviewed, then the histopathological findings and implication of hepatitis viruses B and C was investigated in cryptogenetic liver disease. HBV DNA and HCV RNA were determined in serum by PCR. HBV DNA and HCV RNA were detected in the serum of 18% and 8% patients with cryptogenetic and noncryptogenetic liver diseases respectively. Liver biopsies showed non specific changes or non-alcoholic steatohepatitis (NASH) in 48% and chronic hepatitis or cirrhosis in 52%. The proportion of cases with detectable HBV DNA or HCV RNA was 14% in the first group, 30% in the group with

chronic hepatitis and 61% in the group with cirrhosis, so occult viral infection was found in a high proportion of patients with chronic hepatitis or cirrhosis and in a low percentage of patients with NASH or non-specific changes. Two patients with cryptogenetic cirrhosis underwent liver transplantation, and in these 2 cases HBV DNA was detected in the explanted liver^[6].

DIALYSIS

Hemodialysis patients are at high risk of acquiring parenterally transmitted infections, not only because of the large number of received blood transfusions, and the invasive procedures that they undergo, but also because of their immunosuppressed state. Several reports have been published about prevalence in haemodialysis patients ranging from 0% to 36%. Most of these studies show that OBI is usually associated with low levels of HBV and have investigated the presence of OBI in the context of chronic HCV infection^[7-13]. These studies demonstrated HBV DNA by PCR in serum samples; there were no studies demonstrating HBV DNA in liver extracts because of the lack of available liver tissue in the setting of haemodialysis. The studies of Fabrizi *et al*^[9] and Minuk *et al*^[10] show that conventional serological features of HBV DNA positive subjects do not distinguish these individuals from the remainder of the dialysis patient population; therefore, routine serological testing is not able to identify the occult infection in this population. Several studies have demonstrated that the prevalence of OBI is not associated with the presence of anti-VHC antibodies in hemodialysis patients^[14-16]. Recently, one study has shown an OBI prevalence of 9.8% in continuous ambulatory peritoneal dialysis (CAPD)^[17].

HIV INFECTED PATIENTS

OBI in HIV infected patients may be viewed as the result of opportunistic reactivation of HBV due to cellular immune deficiency, as reflected by the decreased CD4 counts in HIV infection. The prevalence of OBI in HIV infected patients remains controversial and the available data are widely divergent. Published studies report a prevalence between 0% to 89%^[18-26]. The cause of these variations is the same as in HIV-negative patients: level of endemic disease in different parts of the world, the different assays utilized in the studies, and the different populations studied. HIV patients with OBI have significantly lower CD4 counts and high plasma HIV RNA loads^[20,27]. The risk factors, the clinical significance and the effect of highly active antiretroviral therapy (HAART) are unknown. Recently, Cohen Stuart *et al*^[28] analyzed the prevalence of OBI in 191 HIV and anti-HBc positive before HAART and also during the immune reconstitution phase that follows initiation of HAART. Anti-HBs was positive in 128/191 (67%), and negative in 45/191 (24%). Plasma HBV DNA was detected in 9/191 corresponding to a prevalence of 4.7%. In the isolated anti-HBc group

the prevalence was 11.1%, whereas in those anti-HBs positive the prevalence was 3.1%; this difference was not significant. The study demonstrated the absence of hepatic flares after start of HAART and showed that HBV DNA remained undetectable in all patients after starting HAART. Therefore OBI has no clinical impact when immune reconstitution is achieved with HAART containing at least one HBV inhibiting compound.

BLOOD DONORS

Despite continuous technical improvement in blood donation screening, hepatitis B infection remains a major risk of transfusion-transmitted viral infection. Reduction of HBV residual risk is achieved by developing more sensitive HBsAg tests, by adopting anti-HBc screening if appropriate and implementing HBV nucleic acid test (NAT).

The prevalence of OBI among HBsAg negative blood donors is quite variable depending on the level of endemic disease and on the assays employed in routine serological or NAT screening. Screening of anti-HBc is feasible in non-endemic areas, but would cost an unnecessary loss of blood donations in endemic areas (where nearly 90% of adults are positive for both anti-HBc and anti-HBs due to past exposure to HBV).

Hollinger^[29] provide an excellent summary of the prevalence of serological markers in HBsAg negative blood donors in different regions of the world. The studies of prevalence in North America reveal that HBV DNA was detected in 0.1%-1.05% of those who were HBsAg negative and anti-HBc-positive (with or without anti-HBs) and that HBV DNA was detected in 2.03%-2.8% in the anti-HBc only category (no anti-HBs)^[30-34]. The studies of prevalence in Europe reveals that HBV DNA was detected in 0%-1.59% of those who were HBsAg negative and anti-HBc-positive (with or without anti-HBs) and HBV DNA was not detected in patients who were anti-HBc only^[35-38]. In the study of Allain *et al.*^[35] no occult hepatitis B was detected in any of the samples because the level of sensitivity was only approximately 1300 copies/mL. The studies of prevalence in the Middle East and Asia revealed that HBV DNA was detected in 1.09%-3% of those who were HBsAg negative and anti-HBc-positive (with or without anti-HBs) and that HBV DNA was detected in 8.1% in the anti-HBc only category (no anti-HBs)^[39-41].

GENERAL POPULATION

There are few studies about OBI prevalence in the general population. Minuk *et al.*^[42] detected a prevalence of OBI in 18% of those with serological evidence of previous HBV infection and in 8% of HBV seronegative individuals. Kim *et al.*^[43] found HBV DNA in 16% of Korean healthy subjects with normal transaminase values and who were HBV/HCV negative. Hui *et al.*^[44] detected occult HBV genomes in 15% of healthy hematopoietic stem cell donors from Hong-Kong. Raimondo *et al.*^[45] investigated the prevalence of OBI in subjects free from liver disease

through the analysis of liver DNA extracts by performing four different in-house nested-PCR amplification assays. HBV DNA sequences were detected in liver tissues from 16 of the 98 cases examined (16.3%). DNA was detected in 10 of the 16 (62.5%) anti-HBc positive cases vs 6 of the 82 (7.3%) HBV marker negative cases, so OBI status was strongly related with the anti-HBV antibody positive status.

CONCLUSION

Although studies on OBI prevalence have been extensive, the precise prevalence of this clinical entity remains very difficult to define for several reasons. These studies show that OBI prevalence seems to be higher among subjects at high risk of HBV infection and with liver disease than among individuals at low risk of infection and without liver disease. In general about 20% of OBI individuals are negative for all serological markers, and 80% are positive for serological markers of previous infection. Most studies show that OBI is usually associated with low levels of HBV DNA. The importance of this entity is that OBI may have significant impact in several clinical contexts. It might favour the progression of liver fibrosis and the development of hepatocellular carcinoma in patients with additional causes of liver damage; OBI may become reactivated when an immunosuppressive status occurs; and it may be transmitted through blood transfusion and organ transplantation. While awaiting for more sensitive methods for blood HBV DNA measurement, anti-HBc should be recommended in patients undergoing chemotherapy or immunosuppressive treatments as well as all organ donors.

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Pathogenesis of occult chronic hepatitis B virus infection

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the viral DNA, is due in most cases to the strong suppression of viral replication and gene expression that characterizes this "occult" HBV infection; although the mechanisms responsible for suppression of HBV are not well understood. The majority of OBI cases are secondary to overt HBV infection and represent a residual low viremia level suppressed by a strong immune response together with histological derangements which occurred during acute or chronic HBV infection. Much evidence suggests that it can favour the progression of liver fibrosis and the development of hepatocellular carcinoma.

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Abstract

Occult hepatitis B infection (OBI) is characterized by hepatitis B virus (HBV) DNA in serum in the absence of hepatitis B surface antigen (HBsAg) presenting HBsAg-negative and anti-HBc positive serological patterns. Occult HBV status is associated in some cases with mutant viruses undetectable by HBsAg assays; but more frequently it is due to a strong suppression of viral replication and gene expression. OBI is an entity with world-wide diffusion. The failure to detect HBsAg, despite the persistence of

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem. It is estimated that about 350 000 000 individuals are infected by HBV; infection can induce a wide spectrum of clinical forms, ranging from a healthy carrier state to cirrhosis and hepatocellular carcinoma^[1]. HBV infection is usually diagnosed when circulating hepatitis B sur-

face antigen (HBsAg) is detected. However, the availability of highly sensitive molecular biology techniques has also allowed the identification of HBV infection in HBsAg-negative individuals, with or without circulating antibodies to HBsAg (anti-HBs) and/or hepatitis B core antigen (anti-HBc)^[2-5]. Furthermore, it is estimated that as much as one third of the world population have been exposed to HBV^[6]. Many of these individuals may unknowingly carry the virus. The natural history of chronic HBV infection is highly heterogeneous as many host, virus and environmental factors play an integrated role in affecting rates of disease progression and long term clinical outcomes.

CHRONIC HBV INFECTION AND ITS VIROLOGICAL PHASES

The natural course of chronic infection includes four phases based on the virus-host interaction: immune tolerance, immune clearance, low or non-replication, reactivation and HBsAg negative (occult) HBV^[6-8].

Immune tolerance phase

This phase is characterized by presence of hepatitis B e antigen (HBeAg), high serum levels of HBV DNA, normal or minimally elevated serum alanine aminotransferase (ALT) and normal liver or only minimal histological activity. The immunotolerant phase may persist for 10-30 years in perinatally infected subjects, whereas it is generally short-lived or absent in childhood or adult-acquired HBV infection.

Immune clearance phase

This initiates when immune tolerance to the virus is lost and the immune response starts to kill infected hepatocytes. This phase is characterized by fluctuating, but progressively decreasing, HBV DNA levels, high levels of ALT and hepatic necroinflammation. Serum HBV DNA levels are > 20000 IU/mL (10^5 copies/mL) in the phase of HBeAg positive chronic hepatitis and may remain elevated or drop rapidly at the time of anti-HBe seroconversion.

Inactive carrier state

An important outcome of the active immune response is seroconversion from HBeAg to anti-HBe, with transition to the third phase of inactive HBsAg carrier state, characterized by HBeAg negativity and anti-HBe positivity, low levels of HBV DNA < 2000 IU/mL (10^4 copies/mL), normal ALT and inactive liver histology.

Reactivation phase

Some inactive HBsAg carriers may develop HBV reactivation while remaining anti-HBe positive. This phase is characterized by HBeAg negativity with anti-HBe positivity, HBV DNA levels > 2000 - 20000 IU/mL (10^4 - 10^5 copies/mL), high ALT and moderate or severe necroinflammation with variable amounts of fibrosis on liver biopsy (HBeAg negative chronic hepatitis).

HBs negative (occult) HBV

A final phase of chronic HBV infection may eventually follow in some long term carriers, with loss of HBsAg but persistence of HBV DNA in liver. These cases usually do not have active liver disease but show the histological consequences of previous damage with variable amounts of residual fibrosis. Immunosuppression, however, may cause HBV reactivation and sometime severe exacerbation of liver disease with HBsAg, HBV DNA, and even HBeAg rebound.

DEFINITION OF HBsAg LOSS

In "inactive carriers" the HBsAg may become undetectable which is defined as HBsAg loss. A loss of HBsAg is a sign for deep and usually durable suppression of HBV DNA replication and usually announces a stable status of recovery, even though other complications caused by pre-existing liver cirrhosis or fibrosis or an elevated risk for development of hepatocellular carcinoma can still lead to severe complications^[9,10]. However, HBsAg may reappear and replication may reincrease, especially if the HBsAg loss was observed during a period of antiviral therapy. The spontaneous HBsAg loss in anti-HBe-positive asymptomatic HBsAg carriers has long been recognized as a very rare event with increasing probability during long term follow up. Accordingly, a cumulative rate of HBsAg seroclearance of 8.1% was described after the first 10 years of infection. Chu *et al.*^[11] observed that the cumulative probability of HBsAg seroclearance increased to 24.9% after 20 years and to 44.7% after 25 years of HBV infection. The authors also pointed out that age at infection, sustained remission of liver disease and male sex were factors independently associated with HBsAg seroclearance.

ETIOPATHOGENESIS OF OCCULT HEPATITIS B INFECTION

Occult HBV is found more frequently in patients with serologic evidence of HBV infection (anti-hepatitis B core antibody positive) than in core antibody-negative individuals^[12,13]. Occult HBV is found in a significant proportion of patients with chronic hepatitis due to hepatitis C virus, with HBV DNA detectable in up to 30% of serum samples and 50% of liver biopsies^[12]. Occult HBV infection is defined as the existence of HBV DNA in the serum, cells of the lymphatic (immune) system, and/or hepatic tissue in the absence of serum HBsAg. Most frequently, occult HBV infection follows resolution of acute hepatitis and continues indefinitely after clearance of HBsAg and biochemical improvement in liver function^[13-17]. Recent estimates suggest that up to 20% of individuals with occult HBV carriage evidenced by HBV DNA detection could be nonreactive for anti-HBc or any other serological indicator of exposure to HBV^[15]. Although viruses with replication deficits could theoretically explain occult HBV, the finding of cccDNA, RNA transcripts, and pregenomic replicative RNA intermediates in a large proportion of pa-

tients suggests that most occult infections are due to low-level replication of wild-type virus^[19,20]. In addition, the transmissibility of acute HBV *via* liver transplant or blood product transfusion from donors with occult infection^[21-23] provides evidence against the presence of defective viruses. Occult HBV may also result from mutations in HBsAg coding or transcription control regions that alter antigenicity or expression levels^[24-26]. Such mutant viruses have been reported as the sole circulating strain in up to 40% of patients with occult HBV^[27-30]. Occult hepatitis B infection (OBI) has been detected in the following clinical situations: (1) chronic hepatitis unrelated to HVC, atypical alcoholic hepatitis and hepatocellular carcinoma (HCC); (2) viral reactivation following immunosuppression; and (3) transmission through transplantation, transfusion or experimental transmission to chimpanzees^[31]. Low level HBV DNA has been detected in liver tissue of patients with HCC^[32] and in serum of blood donors and their recipients. Occult silent or serologically negative HBV infection was reported for the first time more than 20 years ago in the context of blood transfusion *via* the transmission of HBV by a donor positive for anti-HBc as the only marker of HBV infection^[33]. Schematically, the available data suggest that (a) the host's immune response; (b) coinfection with others infectious agents; and (c) epigenic factors may play important roles in inducing the occult status.

VIROLOGICAL ASPECTS

The molecular basis of occult infection is strictly related to the peculiar life cycle of the HBV. A fundamental step is the conversion of the ~3 kb relaxed circular genome into a covalently closed-circular DNA (cccDNA), a long lived HBV replicative intermediate that persists in the cell nuclei as a stable chromatinized episome and that serves as a template for gene transcription^[34,35]. The stability and long-term persistence of viral cccDNA molecules, together with the long half-life of hepatocytes, imply that HBV infection, once it has occurred, may possibly continue for life^[36]. Reduced HBV viraemia may result from extrahepatic HBV replication, such as in peripheral blood mononuclear cells or from coinfection with others viruses, especially with VHC, which down regulate HBV replication^[31].

Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen

Several recent reports have shown that HBV could be frequently transmitted to recipients from donors who have antibodies to hepatitis B core antigen (anti-HBc) through liver transplantation. Marusawa *et al.*^[19] provide the molecular evidence of latent HBV infection accompanied with ongoing viral replication in the liver tissue of anti-HBc-positive healthy individuals. They have demonstrated that HBV DNA was detectable in 13 of 14 healthy donors who were positive for both anti-HBc and antibodies to hepatitis B surface antigen (anti-

HBs), but in none of 3 who were positive for anti-HBs alone. The detected HBV genomes from these subjects included cccDNA and pregenomic RNA, the replication intermediate of HBV. Notably, 5 of 7 cases tested were predominantly infected with wild type HBV strains without any mutations in the precore and core promoter regions under the presence of circulating antibody to hepatitis B e antigen, but we can not draw conclusions because of the small number of patients.

Therefore the majority of healthy individuals positive for anti-HBc, which had been assumed to denote a past history of transient HBV infection, were latently infected with the episomal form of HBV accompanied by ongoing viral replication and few nucleotide mutations in the precore and core regions.

Why are the occult HBV carriers HBsAg negative despite the presence in their liver of episomal, free HBV genomes?

This is an unresolved question. Some of these individuals are infected by viral variants either producing an antigenically modified HBV S protein undetectable by the available HBsAg assays^[37], or carrying mutations capable of inhibiting S gene expression and/or viral replication^[38,39]. The occult infection appears to be mostly due to a strong suppression of viral replication and gene expression affecting viruses whose genetic variability is comparable to that of HBV strains from individuals with "overt" chronic HBV infection^[40].

EXPERIMENTAL OCCULT HBV INFECTION

Recent studies^[41] on this silent form of hepadnavirus carriage in an experimental woodchuck hepatitis virus (WHV) infection, which is considered to be the closest natural model of HBV disease, revealed that the life-long occult persistence of traces of pathogenic virus is an invariable consequence of recovery after hepadnaviral invasion and that this state always co-exists with a steady low-rate virus replication in both the liver and the lymphatic system. This experimental model has been validated during the last 2 decades as one of the most valuable tools in reach on hepatitis B^[42,43]. Importantly, this serologically concealed infection can be accompanied by development of hepatocellular carcinoma in convalescent animals and is transmissible from mothers to offspring as an asymptomatic, indefinitely long infection which involves the lymphatic system but not always the liver^[44]. Furthermore, it became apparent that the detection of anti-WHc in the absence of other serological indicators of infection is a reliable indicator of occult WHV persistence^[45]. This state, which is similar in strength to HBV-specific cytotoxic T cell (CTL) and T helper lymphocyte responses detectable years after resolution of acute hepatitis B^[46,47], is likely a consequence of sustained stimulation of the immune system with a viral protein produced during low-level virion assembly^[15]. The high degree of compatibility between WHV and HBV infections and the data from studies on otherwise healthy anti-HBc-positive individuals suggest

that the occurrence of isolated anti-HBc could also be of value in identifying occult HBV persistence. The virus recovered from woodchucks with OBI remains infectious. It was observed that WHV harvested from PBMC isolated during OBI and *ex vivo* stimulated with lipopolysaccharide (LPS) induced classical acute WHV hepatitis in virus-naïve animals^[45]. In the case of hepadnaviruses, studies on occult WHV infection point to a direct link between virus persistence and its lymphotropism. They also reveal that infection of the lymphatic system could be a natural and unavoidable consequence of hepadnavirus invasion and that, under certain circumstances, such as a low virus dose, lymphoid cells can be the only temporary or permanent site of virus propagation. Another important fact established through analysis of the WHV model is that a state of occult virus persistence is a normal and ultimate aftermath of WHV infection, which is symptomatic, followed by recovery, or serologically silent from the start. A generalized concept is proposed for the possible progression pathways and outcomes of hepadnaviral infection in relation to variable virus and/or variation of virus in the host. Because of significant virological and pathobiological similarities between HBV and WHV, and close analogies in the patterns of progression and outcomes of the induced liver disease, this concept might also be applicable to human HBV infection^[14].

RELATIONSHIP OF OCCULT HEPADNAVIRAL INFECTION AND THE HOST'S IMMUNE SYSTEM

The host's immune-surveillance probably has a critical role in the development of occult HBV infection, as suggested by at least two arguments: (1) there is evidence showing that a vigorous memory T-cell response against HBV antigens is still present many years after clinical recovery from acute B hepatitis, probably because the long-lasting persistence of the occult infection produces a minute amount of antigens able to maintain an efficient antiviral T-cell response^[46,47]; and (2) all the conditions inducing immunosuppression may provoke the reactivation of the occult HBV infection with the reappearance of the typical serological profile of the productive infection^[48,49]. During occult infection a balance between the virus and the host's immune system is established, and as well as the cytotoxic T lymphocytes, the cytokines synthesized in the liver might also exert a control on HBV replication^[16]. On the other hand, it also is conceivable that this extrahepatic pattern of virus expression is a consequence of another property of the virus that, at low doses, displays its natural predisposition for invading lymphoid cells. In this regard, of note are our findings documenting the existence of the cell-binding site in the preS1 domain of the WHV envelope which mediates a strictly host and cell-type-specific recognition. Synthetic analogues of the site have considerably greater ability to interact with woodchuck lymphoid cells than with hepatocytes^[50]. Different molecular forms of HBV

DNA, including replicative intermediates and cccDNA, and viral RNA have been detected in lymphoid cells of patients with clinically evident hepatitis B^[51,52], although discrimination between synthesized *de novo* viral nucleic acids and those potentially adhered to the cell surface has not always been made. Also, traces of HBV antigens have been found on peripheral blood mononuclear cells isolated from actively infected individuals^[53]. The observation that cultured peripheral blood mononuclear cells from WHsAg-positive animals produced infectious virus strongly argued that lymphoid cells can support the complete replication cycle of WHV^[54].

TRANSMISSIBILITY OF MATERNAL OCCULT HEPADNAVIRAL INFECTION TO NEWBORNS

Vertical transmission of viral hepatitis is considered to be a cause of many perinatal viral infections, and it is postulated that a specific variant can influence virus tropism and outcome of infection in the newborn.

Since mother-to-child transmission is one of the main routes for HBV dissemination, it was important to determine whether hepadnavirus persistently carried as an occult infection can be passed from mothers to their newborns. To investigate this possibility, Michalak *et al.*^[44] have examined woodchuck offspring born in captivity and they found not only that such mothers transmit WHV to newborns but also that the induced infection is asymptomatic and progresses for years after birth in the absence of serum WHsAg, anti-WHs and, in contrast to convalescent adults, anti-WHc-antibodies. Results of these experiments revealed that under certain natural conditions long-term persistence of hepadnavirus can be maintained exclusively at an extrahepatic location. Thus, there were newborns that did not have any detectable WHV DNA in sequential liver biopsies, yet the viral genomes were expressed in livers of other offspring from the same litter. There also were offspring that initially had lymphoid-restricted patterns of WHV infection, yet at 19 mo after birth became liver WHV DNA reactive^[55].

Albeit maternal transmission of hepadnavirus from mother to offspring might differ in some aspects in humans and woodchucks, the essential pathobiological similarity between HBV and WHV suggests that HBV could also be passed from apparently healthy mothers convalescent from hepatitis B to their babies. Children born to these mothers may persistently carry traces of infectious virus and, possibly, have an increased long-term risk for the development of disorders of the lymphatic system and, in some cases, the liver.

CONCLUSION

Occult HBV infection is defined as HBV DNA detection in serum or in the liver of HBsAg-negative patients with or without serologic markers of previous viral

exposure. With the introduction of highly sensitive diagnostic tests for viral proteins the paradigms of HBV infection were challenged. Accumulated evidence indicates that occult HBV can be both a source of virus contamination in blood and organ donations, as well as the reservoir from which full blown hepatitis can arise. The oncogenic potency of occult HBV persistence becomes progressively evident. Certain co-morbidities support occult HBV infection as co-infection with hepatitis C virus or human immunodeficiency virus. Persistence of OBI could involve different mechanisms: mutation or integration of the viral sequence which may alter HBsAg expression, decrease of HBV replication, or hindrance of HBsAg through circulating immune complex. OBI has important clinical implications: any case reports indicate that immunosuppression caused by chemotherapy^[56], immunomodulatory agents^[57], or immune deficiencies, such as HIV infection^[58] or hematological malignancies^[59], can reactivate occult infection. However most patients with hepatitis B who recover from the infection do not experience any problems during their lives.

In the case of hepadnavirus, studies on occult WHV infection point to a direct link between virus persistence and its lymphotropism. Infection of the lymphatic system could be a natural and unavoidable consequence of hepadnavirus invasion and that, under certain circumstances, such as low virus dose, lymphoid cells can be the only temporary or permanent site of virus propagation. Occult HBV may also result from mutations in HBsAg coding or transcription control regions that alter antigenicity or expression levels^[19,25,26]. In addition, the transmissibility of acute HBV *via* liver transplant or blood product transfusion from donors with occult infection^[5,22,23] provides evidence against the presence of defective viruses.

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Juan-Ramón Larrubia, PhD, Series Editor

Clinical significance of occult hepatitis B virus infection

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Abstract

Occult hepatitis B virus (HBV) infection (OBI) is defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in serum) for individuals testing HBV surface antigen negative. Until recently, the clinical effect of OBI was unclear on the progression of liver disease; on the development of hepatocellular carcinoma; and on the risk for reactivation or transmission of HBV infection. Several studies suggest a high prevalence of OBI among patients with cryptogenic chronic liver disease, but its role in the progression to cirrhosis remains unclear. Although OBI has been well documented in human immunodeficiency virus (HIV)-positive patients, especially among those coinfecting with hepatitis C virus, further studies are needed to determine its current clinical impact in HIV setting.

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Key words: Occult hepatitis B virus infection; Liver dis-

ease; Cryptogenic cirrosis; Hepatitis B virus coinfection; Human immunodeficiency virus

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CLINICAL SIGNIFICANCE OF OCCULT HEPATITIS B VIRUS INFECTION

Occult hepatitis B virus (HBV) infection (OBI) was redefined by international experts, meeting in Italy in 2008, as the presence of HBV DNA in the liver (with or without detectable HBV DNA in serum) for individuals testing HBV surface antigen (HBsAg) negative^[1]. These experts also introduced a cut-off value for HBV DNA of < 200 IU/mL. A serum concentration greater than 200 IU/mL should be interpreted as an infection caused by escape mutants, not an OBI. Nevertheless, other experts prefer the traditional definition of OBI: the presence of HBV DNA in blood or liver tissues in patients negative for HBsAg, with or without any HBV antibodies.

Patients with OBI were further classified as either seronegative, when both antiHBs and antiHBc are negative, or seropositive when antiHBc is present^[2]. These patients are also found to be positive for antiHBcs plus antiHBs or only positive for antiHBc but without antiHBs. For both of these latter patients, the HBV viral load is greater^[3]. Moreover, viral replication seems to be controlled by different mechanisms: for antiHBc positive patients by a T-cell response of protective memory; whereas antiHBc negative individuals have no HBV-specific T-cell expan-

sion, suggesting that low level infection is insufficient for protective memory to mature.

There are scant data for the molecular mechanisms that explain the lack of detection of HBsAg in the presence of HBV DNA. One possible explanation is defective transcription control of both the HBV-polymerase and HBsAg coding regions that can lead to a significant decrease of the viral replication levels and the circulating HBsAg titres. The presence of HBsAg/anti-HBs immune-complex, especially in those patients with low titres of surface antigen may interfere in the detection of circulating HBsAg.

Until recently, the clinical effect of OBI was unclear in the following contexts: for its influence on the progression of liver disease; on the development of hepatocellular carcinoma; and on the risk for reactivation or transmission of HBV infection^[4].

OBI IN OTHERWISE HEALTHY INDIVIDUALS

For patients with OBI but who are otherwise healthy, the key issues are HBV prognosis and transmission. From the 5 blood donors with OBI and the 55 recipients of their blood studied by Gerlich *et al*^[5], 22 probable cases of HBV transmission were identified, but these patients remained healthy. Nevertheless, fulminant hepatitis B was found in 3 of these blood recipients that were immunosuppressed. Although transmission is possible, this risk appears to be negligible when concurrent antiHBs is present (> 100 mIU/mL)^[6]. In addition, HBV transmission was not found by Satake *et al*^[7] for OBI blood donors that were antiHBs positive, whereas 27% of antiHBs negative donors transmitted HBV infection. These results were confirmed by other studies^[8,9]. While HBV transmission by OBI donors is possible transmission risk is negligible when antiHBs titres are present (even at low levels).

After transplantation, organs, particularly livers from antiHBc positive donors, may transmit HBV infection. Cholongitas *et al*^[10] reviewed 39 studies that included 903 recipients of antiHBc positive donors. *De novo* HBV infection developed in 19% of HBsAg-negative recipients, but was less frequent for antiHBc and antiHBs positive recipients than for HBV naïve recipients. Adequate prophylaxis with lamivudine or specific gammaglobulin, or both in combination, reduced *de novo* infections in these two groups.

A second issue is patients with OBI developing complications. Mild necrosis and inflammation can be present in liver biopsies of humans after acute hepatitis and in woodchucks infected by woodchuck hepatitis virus (WHV). This mild inflammation probably has no repercussion if the patient has no other cause of liver disease^[1]. For 18 patients with OBI who underwent liver transient elastometry^[11], the median liver stiffness was 4.2 kPa (healthy people 4.6 kPa), showing absence of fibrosis for them. Additionally, no significant mortality was found caused by hepatocellular carcinoma in patients developing

unapparent hepatitis B^[6]. Therefore, immune individuals with only OBI but no other concomitant liver disease usually show no clinical evidence of hepatic damage.

OBI AND CRYPTOGENIC CHRONIC LIVER DISEASE

For a cohort of 159 patients with cryptogenic chronic liver disease, the prevalence of OBI investigated by Fang *et al*^[12] was 28% for serum HBV DNA positivity, but for antiHBc positive individuals the prevalence reached 100%. Similar results for patients with cryptogenic cirrhosis were reported by Chan *et al*^[13]: 32% had OBI, most of them with antiHBc and/or antiHBs. By contrast, Kaviani *et al*^[14] only found OBI in 1.9% of patients with cryptogenic hepatitis. These results, however, may be explained by both the variation in methods for the measurement of HBV DNA and differences in endemicity for the geographic areas studied. In the paper by Kaviani *et al*^[14], OBI was found in patients with cryptogenic hepatitis by qualitative polymerase chain reaction (PCR) with a sensitivity of 150×10^3 copies/L. Therefore, it is very important to use the most sensitive tools to measure HBV viral load, such as “real-time” PCR methods (lower detection limit 10-15 IU/mL) in order to define OBI. Yet, it is nevertheless unclear if OBI was the main cause of this liver disease and its role in the progression to cirrhosis.

OBI AND DIALYSIS PATIENTS

In a study including 366 patients from six dialysis units in Central Greece, an OBI prevalence of 0.9% was recently reported by Mina *et al*^[15]: 15 patients were HBV DNA positive, 12 had overt HBV infection (one HBeAg positive, 10 antiHBe positive and 1 antiHBc plus antiHBs positive) and OBI was diagnosed in 3 patients (two had no serological markers of HBV infection and one was antiHBc and antiHBs positive). For both groups the HBV DNA levels were low. Additionally, there was no association with HCV infection. In the setting of continuous ambulatory peritoneal dialysis and hemodialysis (71 patients in each group), another study^[16] reported a higher prevalence of OBI than the Greek study (17% in peritoneal dialysis and 10% in hemodialysis). Similarly, HBV viral loads were low and HCV positivity was not a contributing factor to OBI. These low HBV DNA titres may be explained by both the transfer of HBV DNA from serum to the dialysate compartment and also the destruction of the HBV genome during the haemodialysis session^[15].

OBI AND HUMAN IMMUNODEFICIENCY VIRUS-INFECTED PATIENTS

Human immunodeficiency virus (HIV) infection modifies the natural history of hepatitis B disease. Coinfected patients have higher HBV DNA levels and are more likely to have accelerated loss of protective anti-HBs and an increased risk for liver morbidity and mortality. The in-

creasing survival of patients with HIV-HBV treated with HAART allows a longer time for liver cirrhosis to develop, with some patients experiencing accelerated progression to clinically significant liver disease.

The prevalence of OBI for patients with HIV has been estimated by several studies with controversial results^[17-20]: from a Brazilian group's^[19] estimate of 5% to one in India^[18] of nearly 25%. The Indian group described the presence of detectable HBV DNA in 21% of antiHBc positive patients. Additionally, HIV patients are at a higher risk for HCV coinfection^[18]. Morsica *et al*^[17] reported that OBI is more frequent in HCV-coinfected patients. However, other studies^[18,19] did not confirm these results.

Because most HIV-infected patients currently on HAART receive drugs with anti-HBV activity, recognition of OBI in these patients may be difficult. Although reactivation of HBV infection in HIV-positive/HBsAg-negative is rare, it would be possible after withdrawal of antivirals against HBV, such as lamivudine or tenofovir^[21]. Recently, Bloquel *et al*^[20] described reactivation for two HIV patients with anti-HBc after withdrawal of HAART with anti-HBV activity. Consequently, it would be very important to know the HBV virological status of these patients before starting antiretroviral therapies in order to avoid reactivations if treatment is stopped. Further studies are needed to determine the current clinical impact of OBI for HIV setting.

OBI AND HCV INFECTED PATIENTS: REACTIVATION AFTER IMMUNOSUPPRESSION THERAPY

These issues will be reviewed in other chapters. Although the presence of OBI in HCV infected patients has been well established, its significance is currently under investigation. Some studies have shown higher fibrosis stages in patients with OBI compared with individuals without it. Nevertheless, others have failed to show this finding. Central questions include the implication of OBIs on hepatic carcinogenesis and their effect on response to HCV treatment.

Patients positive for HBsAg have a high risk of reactivation of hepatitis B after cytotoxic or immunosuppressive therapy. In this regard, OBI patients under immunosuppressant conditions may show a reactivation of viral replication when immunological reconstitution is achieved with a consequently CTL-mediated hepatocyte injury^[1]. Therefore, reactivation more often appears after end of treatment. Hui *et al*^[22] studied 244 patients who received rituximab plus steroids. All were HBsAg-negative and 8 patients developed HBV reactivation. Thus, for these patients starting antivirals early, close monitoring of HBV DNA is mandatory before the occurrence of *de novo* hepatitis.

CONCLUSION

Although the clinical significance of OBI remains unclear,

the more recent data suggest an important role in reactivation of hepatitis B in immunosuppressed patient (especially after treatment with cytotoxic drugs and biologic therapies) and in HBV transmission in liver transplantation, particularly livers from antiHBc positive donors. Other issues such as cryptogenic liver disease, or the relevance in "healthy" patients should be clarified with more studies.

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Diagnostic strategy for occult hepatitis B virus infection

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perhaps, to identify the target population for screening OBI. Viremia is very low or undetectable in occult HBV infection, even when the most sensitive methods are used, and the detection of the viral DNA reservoir in hepatocytes would provide the best evaluation of occult HBV prevalence in a defined set of patients. However, this diagnostic approach is obviously unsuitable: blood detection of occult hepatitis B requires assays of the highest sensitivity and specificity with a lower limit of detection < 10 IU/mL for HBV DNA and < 0.1 ng/mL for HBsAg.

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Key words: Occult hepatitis B virus infection; Hepatitis B surface antigen; Hepatitis B virus DNA; Anti-HBc

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Abstract

In 2008, the European Association for the study of the liver (EASL) defined occult hepatitis B virus infection (OBI) as the "presence of hepatitis B virus (HBV) DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing hepatitis B surface antigen (HBsAg) negative by currently available assays". Several aspects of occult HBV infection are still poorly understood, including the definition itself and a standardized approach for laboratory-based detection, which is the purpose of this review. The clinical significance of OBI has not yet been established; however, in terms of public health, the clinical importance arises from the risk of HBV transmission. Consequently, it is important to detect high-risk groups for occult HBV infection to prevent transmission. The main issue is,

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INTRODUCTION

According to European Association for the study of the liver (EASL), about one third of the world's population have serological evidence of past or present hepatitis B virus (HBV) infection, and more than 350 million people may be affected by chronic HBV infection^[1]. In addition, chronic HBV infection is the worldwide primary cause of cirrhosis and hepatic cellular carcinoma, and it is among

the top ten causes of death^[2]. The clinical evolution of HBV is variable, ranging from mild liver disease to fulminate hepatitis, cirrhosis, or hepatic cellular carcinoma (HCC). In some individuals, in whom the HBV infection persists, serological markers can identify different clinical states of viral persistence^[2,3].

Chronic hepatitis B

Patients with hepatitis B surface antigen (HBsAg) detectable for six months or more are defined as having chronic hepatitis B. Usually these patients have elevated serum liver enzymes, high levels of HBV DNA, and high risk of transmission, both related to the positivity of the hepatitis B e antigen (HBeAg). They also have the highest risk of cirrhosis and HCC^[2]. In some patients, HBeAg is undetectable, in spite of persistent replication of the virus.

In these patients, the virus has mutations that prevent expression of the “e” protein. The mutations are located in the basal core promoter (BCP) region (A1762T and G1764A) and in the precore (PC) region (G1896A) of HBV genome^[4]. These variants are more common in Mediterranean countries and Asia. The appearance of hepatitis B e antibody (anti-HBe) does not necessarily indicate clinical improvement. The HBV DNA levels in these subjects tend to be lower^[5].

“Healthy” carrier

These patients are characterised by a positive HBsAg that persists more than six months, but with normal liver enzymes values. They are negative for HBeAg, and are associated with low or undetectable serum HBV DNA and low risk for progression to cirrhosis or HCC^[2].

Occult hepatitis B infection

Owing to modern molecular analysis we know the viral genome of HBV can persist indefinitely in previously infected HBsAg-negative subjects^[5]. This persistence occurs by conversion to a covalently closed circular HBV DNA (ccc) DNA in the hepatocyte, which then binds to proteins, forming a mini chromosome. This cccDNA is the molecular basis of occult hepatitis B infection because of its stability and long-lasting persistence in the nuclei of hepatocytes^[6].

SEROLOGICAL PATTERN OF OCCULT HEPATITIS B INFECTION

The antibodies produced by the host and proteins released from the virus provide us with valuable information. Within the group of occult hepatitis B infection (OBI) patients, it is possible to observe differences based on the results from serological markers.

Seropositive subjects

These are OBI subjects with anti-HBc and/or positive anti-HBs in which serum HBsAg is not detected because of the resolution of acute hepatitis B (after a few months of HBsAg carriage) or after years of chronic

HBsAg positive infection^[1]. Thirty-five percent of patients with OBI have positive anti-HBs and forty-two percent of have positive anti-HBc^[2]. The HBV DNA detection rate is higher in individuals who are positive anti-HBc but negative for anti-HBs. When patients give a positive result for both antibodies, they have intermediate HBV DNA levels^[3]. One explanation for this serological pattern is that positive anti-HBc patients with chronic HBV infection clear HBsAg to an undetectable level, with or without anti-HBs: this pattern is associated with older age and anti-HBe^[5].

Seronegative OBI

Patients who are not positive for anti-HBc and anti-HBs represent twenty-two percent of OBI patients^[2]. They have very low levels of HBV DNA^[7]. This pattern of antibodies may appear from the beginning of the infection when patients have not yet developed positive hepatitis B specific antibody (“primary OBI”) or because of clearance of the hepatitis B specific antibodies^[1]. Therefore, this pattern should always be kept in mind, because almost anybody can be a potential carrier of occult B hepatitis.

Moreover, there are cases termed as “False” OBI. They are carriers of mutations in HBsAg (in the S gene) that are not recognized by some routine detection assays. In these cases, the DNA result resembles other cases of HBV, because they are usually positive for HBsAg^[1].

MARKERS FOR SCREEN OBI PATIENTS

HBsAg

OBI diagnosis is based upon detection of HBV DNA when HBsAg is absent. It is very important to define the optimal methodology to test this marker to prevent false positive results, depending on HBsAg assay sensitivity.

The quantification of HBsAg presents several problems associated with the virus, the host, or the test kits. To correctly test HBsAg, International Standard samples with known quantities of HBsAg are required. Thus, using World Health Organization (WHO) International Standard for HBsAg (00/588) with a potency of 33 IU/vial, it is possible to know that 1 international unit (IU) is equivalent to 5.6 Abbott ng, 1.9 French ng, and 0.43 PEI units or ng (confirming that ng values applied to other standards are not equivalent and some of the values have changed over time)^[7].

The correlation between the HBsAg and the number of HBV particles is a key point, because this marker is used with a wide range of HBV particles in blood, depending on the infection state. One ng of HBsAg protein is equivalent to approximately 2×10^8 22 nm subviral particles and to approximately 5×10^7 HBV particles (assuming the virus particles have a four times larger surface). Different assays for testing HBsAg have variable detection limits, from 0.04 to 0.62 ng/mL. At a detection limit of 0.04 ng/mL, there must be around 2 million particles in the blood for a positive result. The presence of excess

Table 1 Serological markers and hepatitis B virus DNA in different states of persistence of hepatitis B virus^[2,4]

	HBV DNA	HBsAg	Total anti-HBc	anti-HBs	HBsAg	anti-Hbe
Chronic hepatitis B	+++	+	+	-	+	-
Chronic hepatitis B with variants pre-core	++	+	+	-	-	+/-
Healthy carrier	< 10 ⁶ IU/mL	+	+	-	-	+
OBI seropositive	< 1000 IU/mL	-	+	+/-	+/-	+/-
OBI seronegative	< 1000 IU/mL	-	-	-	-	-

HBV DNA data are copies/mL. -: Negative, the marker is not present in the serum of the patient; +/-: The marker can be present in the serum of the patient; +: Positive, the marker is present in the serum of the patient; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; anti-HBs: Hepatitis B surface antibody; HBeAg: Hepatitis B e antigen; anti-Hbe: Hepatitis B e antibody; OBI: Occult hepatitis B virus infection; IU: International unit.

subviral particles can improve detection.

A level of 2 million HBV genomes/mL (quantity estimated at the time of HBsAg seroconversion) is considered to exist in a ratio of 1:1000 for the amount of HBsAg in the virus to that in subviral particles^[8]. However, this is not always true. Minegishi *et al*^[9] found six HBsAg seroconverters among 76 prism-negative blood donations with > 10⁴ genomes/mL, generating a ratio of 1: < 200. Gerlich *et al*^[8] found a blood donor in the incubation phase with a ratio of 1:100, which became 1:500 during the chronic phase.

Accordingly, the ratio is variable, from 1:100 in patients with OBI (HBsAg negative with high levels of HBV DNA) to 1:100000 or more when HBsAg is detected in association with low concentrations of HBV DNA. Most HBsAg commercial assays are able to detect all genotypes and subtypes of the wild-type virus, but some of them may miss mutations in the S region^[9,10]. Usually, wild-type virus is the dominant species detected at the beginning of the infection; however, mutations can increasingly appear because of the lack of viral proofreading exonuclease activity.

Mutations in the S gene cause changes in the amino acids of the “a” region, which is very important for inducing immunity, being a target of anti-HBs^[7,10]. Immunological pressure may cause a decrease of HBsAg, but might favour the selection of HBsAg mutants. Thus, the presence of anti-HBs and the clearance of AgHBs do not necessarily reflect viral clearance.

In this apparent “resolved” infection, cytotoxic T cells are responsible for controlling the replication (but absolutely eliminating it). The role of Anti-HBs would be in controlling traces of circulating virus, although there is a risk of selecting mutants. This process may underlie seropositive OBI, and it is very important in patients under immunosuppressive therapy, in patients with liver disease, or in cases of blood donors, because of the risk for transmitting the virus^[8]. Mutants have also been detected in vaccinated patients, in patients who have been treated with hepatitis B immunoglobulin, and in patients with chronic infection^[11]. As a consequence, all patients with a serological pattern consistent with possible OBI should be investigated to rule out HBsAg mutants. HBsAg should be tested with an alternative method that can detect the most common mutants. Quantitative HBV DNA testing should also be considered^[11].

HBV DNA

The gold standard for OBI diagnosis is the study of extracted DNA (from liver or blood). For this purpose, a very sensitive and specific assay is required. The experts meeting in Taormina^[1] recommended assays with detection limits of less than 10 copies of HBV DNA per reaction. Current technologies used for DNA detection are: nested-PCR, real-time PCR, and transcription based mediated amplification (TMA). Using these assays, it is possible to decrease the lower detection limit (< 5 IU/mL of HBV DNA). This is particularly important in OBI, because the HBV DNA levels vary from < 10 to 425 copies/mL. However, the false negative and positive rates are around the cut-off level due to the Poisson distribution of the virions and blank specimens^[7].

According to Taormina Group recommendations, primers must be specific for different HBV genomic regions and be complementary to highly conserved (genotype shared) nucleotide sequences^[1]. Usually, the genes amplified by PCR are S and X; the former has been found to be the most sensitive in serum, and the second has been described as the most sensitive in the liver^[2]. The use of very sensitive methods, such as PCR, increases the risk of false positive results, due to contamination or to different amplicons within the target. To resolve these problems, inclusions of appropriate controls in each assay run, as well as sequencing the amplicons, are recommended. In the case of serum samples, it is recommended DNA from is extracted from at least 1 mL of serum^[1,2].

Differences can be observed because of the different amounts of material used in the assay. To reduce this problem, it is possible to quantify the DNA in comparison with, or normalized to, a host cell gene (such as beta-globin)^[1]. DNA detection from a liver biopsy would be the best option because of the persistence of viral genomes in hepatocytes. For this procedure, frozen samples are preferred to formalin fixed tissues^[1,7].

Usually, when a blood sample is positive for HBV DNA, the liver sample is too; however, HBV DNA from the liver can be detected even when HBV cannot be detected in serum. Patients with undetectable HBV DNA in the liver have also undetectable levels of HBV DNA in peripheral blood^[2]. However, there are no standardized assays and liver specimens are not routinely obtained^[7]. Commercial assays use serum or plasma to de-

termine the presence and the amount of HBV DNA. In some studies using the woodchuck model, occult WHV was shown to persist in peripheral blood mononuclear cells (PBMC)^[13,14]. Samples for studying HBV DNA should be collected and stored in appropriate conditions, and the risk of cross-contamination should be avoided^[1]. However, which is the best DNA extraction method to apply in the OBI diagnosis remains unclear^[2].

To reduce variability and risk of contamination, reagents that are ready to use or that use automatic systems to extract DNA have been proposed. Nine years ago twenty-two laboratories participated in an international collaborative study to establish a WHO international standard (97/746) for HBV DNA nucleic acid amplification techniques (NAT)^[15]. A subtype adw2 genotype A isolate was used. Based on this study, one IU of the standard is equivalent to 6.31-6.42 genomic equivalents (geg) if a PCR assay is used. Therefore, the results of the DNA for HBV must be expressed as IU/mL, but accurate conversion factors depend on the chemistry used for HBV DNA quantification, and range from 5.26 to 7.3 copies/IU. Despite these attempts, there is still variability in the results; therefore, using one assay should be used to monitor any particular patients or group of patients^[7]. In case of blood banks the NAT is used to screen for Hepatitis C virus (HCV), Human Immunodeficiency Virus 1 (HIV-1), and HBV. Plasma pooling is often used because of high cost issues, which introduces a dilution factor and decreases the sensitivity of the assay. This is critical in the case of OBI, because the levels of HBV DNA are very low. Moreover, the use of plasma pooling can be aggravated by using triplex assays to detect the three viral genomes at the same time. González *et al*^[16], using blood donors in Madrid, found that donors in the window period and donors with OBI were not uncommon. Furthermore, they were detected at a higher frequency using individual nucleic acid testing (NAT) than with minipool NAT blood.

Other assays to detect a genome use a probe labelled with a different dye to permit the identification of the different viruses. However, a positive result from this type of screen needs to be confirmed by other tests^[17].

Anti-core antibody

This is the first antibody to appear, even preceding HBsAg, and targets the nucleocapsid of HBV. The anti-core antibody can induce anti-HBc responses without T-cell activation. This antibody can be found in almost every patient with a previous contact with HBV, even in HBV carriers without other responses. This serological pattern is called "anti-HBc alone", and might reflect an occult HBV infection. Anti-HBc is present in the different phases of hepatitis, including recovery, and may persists longer than anti-HBs or anti-HBe; however, it is not protective. Anti-HBc IgM may help in the diagnosis of the acute phase. Moreover, this IgM can be positive during flares^[3].

In some patients, anti-HBc cannot be detected at any phase of HBV infection because of a defective host immunological response (such as in HIV coinfection or organ transplantation) or virus infection by variants of

HBV^[3,8]. Although anti-HBc is not an ideal marker, the Taormina group recommended its use as a surrogate marker whenever an HBV DNA test is not available to identify potential seropositive OBI individuals such as in cases of blood, tissue or organ donation, or in cases of patients undergoing immunosuppressive therapy^[1].

In addition, anti-HBc determination is useful in OBI diagnosis, even when HBV DNA is available, because of the possibility of intermittent viremia^[3]. In such cases, not all anti-HBc positive individuals are positive for HBV DNA, and anti-HBc tests might provide false-positive results^[1]. Furthermore, the absence of this antibody does not exclude OBI (seronegative OBI). If this marker is used in combination with HBV DNA, the prevalence of HBV infection in the area should be considered, because when prevalence of anti-HBc is higher than a 50% of the donor population, a positive result is unhelpful^[17].

Anti-surface antibody

This is the last antibody to appear (about three months after acute phase), and it is able to neutralize the virus. In vaccinated subjects it is the only positive marker.

This antibody can be used with anti-HBc to study the serological status of patients with a probable OBI.

TARGET POPULATIONS FOR INVESTIGATING THE PRESENCE OF OBI

Investigation of possible OBI should be done in case of blood and solid organ donors, because of the risk of transmission to others^[7]. The study and monitoring of reactivation in case of serologic markers of past HBV infection must also be done in patients undergoing immunosuppressant therapy, because of the risk of reactivation after the therapy.

Reactivation risk can be explained if immunosuppression permits viral replication, represses the function of immune cells, and after the treatment, the response of the immune system is exaggerated, leading to cellular injury. The main factors for reactivation are positive HBsAg, grade of immunosuppressant, liver disease, primary malignancy, and toxicity of these drugs. Among the group of patients treated with immune suppressors, there are patients with autoimmune liver diseases. In this group Georgiadou *et al*^[18] studied the prevalence of OBI in patients treated with immunosuppressants, and they concluded that there was a significantly higher proportion of OBI cases among these patients compared to blood donors. Interestingly, under immunosuppression, these patients did not seem to deteriorate during the follow-up^[18,19].

Patients undergoing haemodialysis must also be studied for OBI because of the risk of reactivation due to immunosuppression and the risk of infection^[7].

On the other hand, patients with chronic hepatitis C, patients affected by a Hepatic cellular Carcinoma, and patients with cryptogenetic liver disease, must be investigated for OBI because of its possible influence on the development of these diseases^[7].

In case of pregnant women, Kwon *et al.*^[20] studied the prevalence of HBV DNA in 202 healthy pregnant women. They concluded that the vertical transmission of OBI through the cord blood does not represent a clinical problem because of the low HBV DNA level in the mother's blood, although they acknowledged that more studies are needed.

CONCLUSION

The study of occult HBV infection involves serological and molecular assays. The serology should include, firstly, AgHBs. This test must be done using the most sensitive method, because, depending on this result, the hepatitis B infection can be further classified. With a negative HBsAg result, HBV DNA should be studied only in cases previously exposed, to rule out OBI. Moreover, serological tests should include anti-HBc and anti-HBs.

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Influence of occult hepatitis B virus infection in chronic hepatitis C outcomes

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INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) share common routes of transmission, which explains the high prevalence of occult HBV infection reported in patients with chronic hepatitis C^[1-5]. While there is persuasive evidence suggesting that HBV-HCV co-infection accelerates the liver disease progression and increases the risk of developing hepatocellular carcinoma (HCC)^[6] the effect of occult hepatitis B infection (OBI) on the natural history of chronic hepatitis C infection remains elusive. Despite its potential clinical importance, knowledge on the effect of OBI on chronically HCV infected subjects is limited as HBV-DNA detection may require liver tissue and liver biopsies are not routinely performed in the majority of patients. In addition, most studies addressing this issue are cross-sectional or have included small size cohorts or heterogeneous populations. Furthermore, the use of different methods with variable sensitivity for HBV-DNA determination in serum, PBMCs and liver may explain the discrepant results on the effect of OBI on chronic hepatitis C^[1-3,7-12]. The purpose of this review is to critically examine the current evidence for a potential effect of OBI on HCV chronic infection. Specifically, we will review possible mechanisms of viral interaction, the

Abstract

Persistence of hepatitis B virus-DNA in the sera, peripheral blood mononuclear cells or in the liver of hepatitis B surface antigen (HBsAg)-negative patients with or without serological markers of previous exposure (antibodies to HBsAg and/or to HB-core antigen) defines the entity called occult hepatitis B infection (OBI). Co-infection with hepatitis B and hepatitis C viruses is frequent in highly endemic areas. While this co-infection increases the risk of liver disease progression, development of cirrhosis and hepatocellular carcinoma and also increases the rate of therapeutic failure to interferon-based treatments than either virus alone, a potentially negative effect of OBI on clinical outcomes and of therapeutic response to current antiviral regimes of patients with chronic hepatitis C remains inconclusive.

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potential effect on liver histology, on clinical outcomes such as the risk of developing HCC or disease decompensation in these patients.

LITERATURE SEARCH

Electronic searches of the National Library of Medicine's (PubMed and OVID Technologies), EMBASE (OVID Technologies), Current Contents (Institute for Scientific Information) and manual of selected specialty journals were made to select all relevant literature. The key words "Occult hepatitis B virus AND hepatitis C virus", "Impact of occult hepatitis B virus on chronic hepatitis C", were used. All articles were identified by a search from June 1999 to May 2010. Eligibility and exclusion criteria were previously specified. Case reports and human immunodeficiency virus co-infection articles were excluded while case-series, cross sectional, retrospective and prospective studies of occult hepatitis B and chronic hepatitis C were included.

DO HEPATITIS B AND HEPATITIS C VIRUSES INTERACT IN THE HOST?

Some *in vitro* studies have shown that the HCV "core" protein suppresses HBV replication^[13-15]. However, these results have not been confirmed by more recent studies which have demonstrated little or null interaction between HCV and HBV in a Huh7 cells culture^[16,17]. Nonetheless, *in vitro* experiments cannot be extrapolated to the host viral infection scenario as a host active immunological and cytokine response to the human infection is lacking in *ex vivo* experiments. This immunological response may determine both the liver damage and the clinical outcome. In the clinical setting, Jardi *et al*^[18] found that HCV displayed strong inhibitory action in the reciprocal viral inhibition seen in HBV/HCV coinfecting individuals. An inhibition of HCV replication by HBV-DNA was also observed in hepatitis B surface antigen (HBsAg)-negative Austrian patients^[19]. However, Alberti *et al*^[20] studied 30 patients with symptomatic acute hepatitis and markers of active HBV and HCV coinfection; all patients underwent long-term follow-up and their chronic infection rates were similar to those patients with single HBV and HCV infection. Nevertheless, the risk of fulminant/subfulminant hepatitis is increased in cases of acute HCV superinfection in chronic hepatitis B^[21-23] and causes a higher cumulative risk of cirrhosis and HCC than HDV superinfection does^[24].

OBI AND CHRONIC HEPATITIS C: EFFECT ON HISTOLOGY AND CLINICAL OUTCOMES

Cacciola *et al*^[2] found that patients with chronic hepatitis C and OBI more frequently had cirrhosis than patients with chronic hepatitis C alone. Likewise, Mrani *et al*^[10] found

that 47 of a cohort of 203 HCV positive French patients (23%) had occult HBV infection with a low HBV load (10^2 - 10^4 copies/mL). The serum HCV-RNA titer, the liver inflammatory activity and the stage of fibrosis were significantly higher in HBV-DNA positive than in HBV-DNA negative patients. However, these findings have not been confirmed by other studies. Sagnelli *et al*^[7] found occult HBV infection by using PCR as defined by two different positive results of HBV-DNA in plasma, peripheral blood mononuclear cells (PBMCs) and liver compartments in 37 of 89 patients with biopsy proven chronic hepatitis C (41.6%) and found no association between occult HBV infection and the degree of liver necro-inflammation and fibrosis. Fabris *et al*^[12] studied a cohort of 51 HBsAg-negative patients with chronic hepatitis C, and studied liver fibrosis progression by using paired liver biopsies. HBV-DNA was found by nested PCR in 1.9% of sera and 29.4% of liver tissue samples. The authors found no significant differences in mean serum aminotransferase values, baseline HCV viral load, HCV genotypes, or grading and staging in patients with or without HBV-DNA. Hui *et al*^[25] retrospectively compared fibrosis progression and progression to severe fibrosis (fibrosis stage 3 or 4) in 74 HCV patients with at least two consecutive biopsies, and found occult HBV infection in 31 (41.9%). Patients with occult HBV co-infection did not progress more than patients without occult HBV infection. Kannangai *et al*^[26] reported liver flares that were associated with serum HBV-DNA detection in a small group of patients with OBI and hepatitis C; the authors proposed that flares might be the pathogenetic mechanism underlying liver disease progression in patients with OBI and chronic hepatitis C^[19]. By contrast, no effect on liver biochemistry was observed in other studies^[27,28]. In summary, results of the combined effect of OBI and chronic hepatitis C on liver disease progression have yielded controversial results and no firm conclusion can be reached on this issue.

EFFECT OF OBI ON THE RISK FOR DEVELOPMENT OF HCC IN CHRONIC HEPATITIS C

Pollicino *et al*^[29] found a significant association between OBI and HCC, and provided persuasive evidence that OBI maintains several of the oncogenic mechanisms of HBV such as the capacity to be integrated in the host's genome and production of transforming proteins. Therefore, it is conceivable that OBI might increase the risk for developing HCC in patients with chronic hepatitis C in the same way as HBV infection does. Adachi *et al*^[11] found that positive HBcAb, which indicates a previous HBV infection, but not positive HBV-DNA patients, was associated with an increased risk for developing HCC. Independent risk factors for development of HCC were male gender, α -fetoprotein ≥ 20 ng/mL, serum ALT ≥ 80 IU/L and the presence of anti-HBc. Likewise, Ikeda *et al*^[30] prospec-

Table 1 Studies assessing the effect of occult hepatitis B infection on liver histology, clinical outcomes and effect on the sustained virological response rate in patients with chronic hepatitis C

Author and references	Type of study	Population of HCV infected	OBI	Method of HBV-DNA detection	Geographic area	Effect on histology and/or clinical outcomes	Effect on CHC SVR
Cacciola <i>et al</i> ^[2]	Cross-sectional	<i>n</i> = 200	33.0%	Nested PCR	Italy	Increased cirrhosis	Less sustained virological response rate
Sagnelli <i>et al</i> ^[7]	Cross-sectional	<i>n</i> = 89	41.6%	PCR	Italy	No effect on histology	Not reported
Chen <i>et al</i> ^[9]	Cross-sectional	<i>n</i> = 126	4.8%	bDNA assay	Taiwan	No effect on histology	No effect on sustained virological response
Mrani <i>et al</i> ^[10]	Cross-sectional	<i>n</i> = 203	23.0%	Real-time PCR	France	Increased proportion of patients with inflammatory activity and liver fibrosis	Less sustained virological response rate
Adachi <i>et al</i> ^[11]	Longitudinal F-U	<i>n</i> = 123	11.4%	Real-time PCR	Japan	Increased risk of HCC in patients with HBcAb (+) but not in patients with DNA-HBV +	Not reported
Fabris <i>et al</i> ^[12]	Cross-sectional	<i>n</i> = 51	1.9% of HBV-DNA in sera and 29.4% in liver	Nested PCR	Italy	No effect on aminotransferases, HCV-RNA titre or liver histology	No effect on sustained virological response
Hui <i>et al</i> ^[25]	Retrospective	<i>n</i> = 74	41.9%	Real-time PCR	USA	No effect on fibrosis progression	Not reported
Kannangai <i>et al</i> ^[26]	Cross-sectional	<i>n</i> = 15	12% IgM HBc	Real-time PCR	USA	Increased proportion of flares in patients with OBI	Not reported
Shetty <i>et al</i> ^[31]	Prospective	<i>n</i> = 50	50% in explant livers and 29.4% in serum	Real-time PCR	USA	Increased prevalence of HCC	Not reported
Ikedo <i>et al</i> ^[30]	Multicenter prospective-observational	<i>n</i> = 872 F-U 846	46.3% HBcAb (+)	DNA probe assay	Japan	Increased risk of HCC in HbcAb (+)	Less sustained virological response rate
Matsuoka <i>et al</i> ^[28]	Prospective	<i>n</i> = 468	43.6% in serum	Nested-PCR	Japan	Increased inflammation and increased risk of HCC	Not reported
Tamori <i>et al</i> ^[32]	Retrospective	<i>n</i> = 16 and a control group; <i>n</i> = 50	50% in liver	Nested-PCR in liver	Japan	Increased rate of OBI in chronic hepatitis C patients with SVR who subsequently developed HCC	Not reported
Hasegawa <i>et al</i> ^[35]	Retrospective	<i>n</i> = 140	7.9%	Real-time PCR	Japan	No effect on HCC risk	No effect on sustained virological response
Levast <i>et al</i> ^[36]	Retrospective	<i>n</i> = 140	0% in sera 4.4% in liver tissue	Real-time PCR	France	No effect on histology	No effect on sustained virological response

OBI: Occult hepatitis B infection; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PCR: Polymerase chain reaction; SVR: Sustained virological response; CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; F-U: Follow up.

tively studied a large multicenter cohort of patients with chronic HCV infection and occult HBV infection (negative results for HBsAg and HBV-DNA but positive for anti-HBc on serologic testing). Patients with HCV-related cirrhosis and positive anti-HBc were at higher risk for HCC. Anti-HBc positivity was associated with increased risk for HCC, even in patients with a prior virological response to interferon therapy. Shetty *et al*^[31] prospectively examined the rate of HCC in 44 explanted livers from patients with HCV-associated cirrhosis and found that those patients with occult HBV infection had a significantly higher rate of explant-proven HCC (59%) compared to patients without OBI (36%); OR: 3.1 (2.1-5.4). In another large prospective study, Matsuoka *et al*^[28] investigated the influence of occult HBV infection on the histopathological features and clinical outcomes of 468 HBsAg-negative patients with chronic hepatitis C. These authors determined the HBV-DNA in serum and the hepatitis B core (HBc) parti-

cles in hepatocytes by immunohistochemistry and electron microscopy. The authors found a significant increase in the degree of inflammatory cell infiltration, higher irregular regeneration of hepatocytes and a higher probability of developing HCC in patients with OBI. Tamori *et al*^[32] found that patients with chronic hepatitis C who achieved sustained virological response and developed HCC had a higher rate of OBI than a control group of 50 patients with chronic hepatitis C without OBI. Miura *et al*^[33] found that occult HBV infection, high ALT levels (≥ 80 IU/L) and the staging of liver fibrosis after interferon (IFN) therapy were important independent factors affecting the appearance of HCC. By contrast, Toyoda *et al*^[34] found that Circulating low-level HBV does not appear to play an important role in hepatocarcinogenesis in HBsAg-negative HCC. Overall, these results suggest that OBI may increase the likelihood of developing HCC in patients with chronic hepatitis C.

DOES OCCULT HBV INFECTION IMPAIR SUSTAINED ANTIVIRAL RESPONSE RATE IN CHRONIC HEPATITIS C INFECTED PATIENTS?

Cacciola *et al*^[2] found that the sustained virological response (SVR) rate to alfa IFN monotherapy was lower in patients with chronic hepatitis C and OBI. By contrast, Fabris *et al*^[12] studied twenty-five patients who were treated with alfa IFN and ribavirin and followed for at least 18 mo; there was no significant difference in the SVR among patients with and without OBI. Mrani *et al*^[10] reported that sustained response to IFN and Ribavirin was achieved in 11 (28%) of 40 HBV-DNA positive cases with chronic hepatitis C, compared with 65 (45%) of the 144 HBV-DNA negative cases ($P < 0.05$). Hasegawa *et al*^[35] analyzed 140 HCV patients without HBsAg and found that 7.9% of the cohort patients were positive for serum HBV-DNA; 4 of these 11 patients achieved SVR with IFN compared with 39 of 129 without HBV-DNA (NS). However this small group of patients precluded drawing firm conclusions regarding the SVR. Levast *et al*^[36] retrospectively studied a cohort of 140 HCV patients in France and found no effect on the SVR. Overall, these results do not support the concept that OBI impairs SVR in patients with chronic hepatitis C. Table 1 summarizes the main results analyzing the effect of OBI on liver damage, on clinical outcomes, risk of developing HCC and on response to antiviral treatment in patients with chronic hepatitis C.

CONCLUSION

Prospective studies using standardized laboratory techniques and well-designed large prospective studies with homogeneous cohorts and uniform selection criteria of patients are needed to elucidate the effect of OBI on individuals with chronic hepatitis C. Currently available data do not support a conclusive role of OBI in accelerating liver disease progression in patients with chronic hepatitis C or a potential negative effect of OBI on the SVR in patients with chronic hepatitis C. However, populations studied were small and heterogeneous and most of them included patients prior to the current standard of treatment, i.e. peginterferon-alfa plus ribavirin. By contrast, most studies including those with a longitudinal design that incorporated large cohorts strongly suggest that the risk of HCC is increased in OBI/HCV co-infection.

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Management of occult hepatitis B virus infection: An update for the clinician

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Abstract

Occult hepatitis B virus (HBV) infection (OBI) is defined by the presence of HBV DNA in the liver tissue of individuals who test negative for hepatitis B surface antigen (HBsAg). Patients who have recovered from acute hepatitis B can carry HBV genomes for a long time and show histological patterns of mild necro-inflammation, even fibrosis, years after the resolution of acute hepatitis, without showing any clinical or biochemical evidence of liver disease. At least in conditions of immunocompetence, OBI is inoffensive itself, but when other relevant causes of liver damage are present it might make the course of the liver disease worse. The risk of HBV transmission through transfusion is related to blood donations negative for HBsAg that have been collected during the pre-seroconversion period or during chronic OBI. Use of HBV nucleic acid amplification testing and multivalent anti-HBs antibodies in the HBsAg assays is recommended for detection of true and false OBI, respectively. It is not known if prior hepatitis B immunization with an optimal anti-HBs response in cases of HBV transmission through organ transplantation can effectively modulate or abort the infection. Use of anti-

viral agents as prophylaxis in patients with serological evidence of past HBV infection prevents reactivation of OBI after transplantation in most cases. Reactivation of OBI has been observed in other conditions that cause immunosuppression, in which antiviral therapy could be delayed until the HBV DNA or HBsAg becomes detectable. OBI might contribute to the progression of liver fibrosis and hepatocellular carcinoma development in patients with chronic liver disease.

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Key words: Occult hepatitis B; Management; Blood transfusion; Organ transplantation; Virus reactivation; Chronic liver disease; Hepatocellular carcinoma

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INTRODUCTION

Occult hepatitis B virus (HBV) infection (OBI) is defined by the presence of HBV DNA in the liver tissue of individuals who test negative for hepatitis B surface antigen (HBsAg), by currently available assays, regardless of the detection of HBV DNA in the serum. When detectable, the level of HBV DNA in the serum is usually very low (< 200 IU/mL). Depending on the HBV antibodies detected, OBI may be seropositive [anti-hepatitis B core (HBc)

and/or anti-HBs positive] or seronegative (anti-HBc and anti-HBs negative) (> 20 %) [1].

The molecular basis of OBI is linked to the intrahepatic persistence of covalently closed circular DNA and to strong suppression of viral replication activity and gene expression. Host immune response, co-infection with other infectious agents, and epigenetic factors probably play a relevant role in HBV inhibition. This suppression of HBV activity is responsible for the very low or undetectable levels of serum HBV DNA in OBI cases [2,3]. If serum HBV-DNA levels are similar to those detected in serologically evident HBV infection, it should be considered as false OBI, usually due to infection by HBV variants with mutations in the S gene. These variants produce a modified HbsAg that is not recognized by commercially available detection assays [1]. OBI is more prevalent among subjects at high risk for HBV infection and with liver disease [1].

The gold standard for diagnosis of OBI is the analysis of HBV-DNA extracts from the liver and blood samples. As liver samples are only available in a minority of cases, the most common diagnosis of OBI is based on the analysis of serum samples [1]. In all cases, it is recommended to use a highly sensitive and specific test, like HBV nucleic acid amplification testing (NAT), a PCR technique with detection limits of < 10 copies HBV DNA per reaction. Only if this highly sensitive HBV-DNA testing is not possible, should anti-HBc be used to identify potential seropositive OBI cases [1].

OBI is a complex entity that comprises many conditions and different situations. The evidence of its potential relevance is the reason for the growing interest in this topic. The purpose of this review is to synthesize the current evidence regarding OBI management, with special emphasis on strategies for prevention of OBI transmission in different scenarios (Table 1).

OBI AFTER ACUTE HEPATITIS

Patients who have recovered from acute hepatitis B might carry HBV genomes for several years without showing any clinical or biochemical evidence of liver disease [4-6]. The question is if patients with this disorder are at risk for transmitting infection to others or for progression of their disease.

In a woodchuck model of OBI, maternal-fetal transmission has been observed from mothers with occult infection. Low levels of virus were detected in peripheral blood mononuclear cells and liver of newborns that had recovered from hepatitis, and this was associated with liver injury that occasionally led to hepatocellular carcinoma (HCC) development [4,7-9].

In immunocompetent humans who have developed anti-HBc and anti-HBs following acute hepatitis B, no transmission of HBV has ever been demonstrated in blood donations [10]. The persistence of virus-specific cytotoxic T lymphocytes as a consequence of stimulation by viral replication and gene expression in this population is necessary to control HBV infection and maintain the long-term persistence of anti-HBc and anti-HBs in these pa-

Table 1 Scenarios in which occult hepatitis B virus infection is of clinical importance

After acute hepatitis B
Blood donation
Organ transplantation
Immunosuppression
Cryptogenic chronic liver disease
Hepatocellular carcinoma development

tients [9]. Mild necro-inflammation, even fibrosis, has been observed in patients who have recovered from acute hepatitis B for several years after resolution of hepatitis [11,12].

With regard to the management of these patients, we know that, at least in conditions of immunocompetence, OBI is innocuous in itself, but when other relevant causes of liver disease are present, mild liver damage produced by the occult virus might contribute to making the course of the liver disease worse [2,13].

OBI AND BLOOD DONATIONS

HBsAg-negative blood donations that contain HBV DNA are considered infectious and might transmit HBV that usually induces typical type B hepatitis in recipients [14]. Nowadays, OBI is the major cause of post-transfusion hepatitis B in western countries [15] and in countries like India and Taiwan where the incidence of this problem is considerable [16,17]. Although post-transfusion hepatitis B is rare in western countries [15], the risk of transmission of HBV by transfusion is probably higher than for hepatitis C virus or human immunodeficiency virus (HIV) [18].

The risk of HBV transmission through transfusion is related to blood donations that have been collected during the so-called pre-seroconversion period or during chronic OBI [10]. The risk of transmission is high with blood that lacks anti-HBs, but it might not reach 100%. There are several possible explanations as to why not all recipients of HBV-DNA-positive, HBsAg-negative blood develop hepatitis [9]: (1) vaccination or prior disease in recipients can induce immunity to HBV; (2) concurrent infusion of anti-HBs in another blood component; (3) presence of immune complexes; (4) inocula below the minimum infectious dose of HBV; (5) presence of defective or replication-incompetent virions; and (6) viral interference from another pathogen. The risk of transmission is insignificant when anti-HBs is present in the blood, regardless of anti-HBc status [19,20]. The concentration of anti-HBs which makes transfusion safer is a matter of debate. However, caution is recommended when immunodeficient patients receive anti-HBc-positive, anti-HBs-positive donations. This is important if we consider that almost 50% of transfused blood in Western Europe is given to immunodeficient patients [20].

With regard to the management of these patients, the use of multivalent anti-HBs antibodies in the HBsAg assays is strongly recommended for detection of false OBI [1]. In cases of pre-seroconversion period donation, HBsAg or anti-HBc screening cannot detect OBI, and we

must use HBV-DNA NAT^[10]. NAT detects potentially infectious blood units before donation and consequently reduces the risk of transmitting HBV through blood transfusion. In HBV-endemic regions of the world, where a universal hepatitis B vaccination program is not available, NAT has higher potential benefit for reducing this risk. However, in low-prevalence countries, the availability of highly sensitive and specific HBsAg and anti-HBc assays limits the benefit of NAT^[9,10,21].

OBI AND ORGAN TRANSPLANTATION

Grafts from donors who are HBsAg-negative and anti-HBc-positive might transmit HBV to recipients after organ transplantation; particularly in the case of orthotopic liver transplantation (OLT), and especially if the recipient is negative for all HBV serum markers, because of the presence of viral strains in the hepatocytes, which can be reactivated during immunosuppression^[22-24]. In OLT, this occurs in 17%-90% of cases^[24]. Transmission of occult infection from HBV-seronegative (anti-HBs negative/anti-HBc-negative) individuals is uncertain. There is no evidence of this theoretical possibility, which is probably underestimated by a regular allocation to *de novo* HBV infection after transplantation^[1].

The risk of occult HBV transmission is very low after kidney, heart or bone marrow transplantation^[25,26]. Reactivation of OBI is possible in liver transplant recipients with a serological profile of past exposure to hepatitis B (anti-HBc positive), as a consequence of immunosuppression after transplantation^[27]. Hepatitis B infection usually has a benign course and is often less severe following solid organ transplantation obtained from anti-HBc positive donors when compared to hepatitis B that develops as a result of recurrent disease^[22,28].

With regard to the management of these patients, it is not known if prior hepatitis B immunization with an optimal anti-HBs response can modulate or abort the infection^[9]. Prophylaxis with antiviral agents prevents reactivation of OBI in most of these cases^[24].

REACTIVATION OF OBI

The risk of HBV reactivation is well documented in HBsAg-positive patients who receive chemotherapy and/or with hemato-oncologic diseases, and there is consensus that these patients require prophylaxis with an antiviral agent^[29,30]. However, the risk of HBV reactivation in OBI is less defined^[31-33]. The state of strong suppression of viral replication and gene expression activity by the host immune system in OBI patients might be discontinued, which leads to the development of a classical hepatitis B that often has a severe clinical course^[2]. This situation has been observed in several conditions including HIV infection^[34,35], hematological malignancies^[29], patients undergoing chemotherapy^[36,37], transplantation (bone marrow, liver, or kidney)^[38-40], and treatment with potent immunosuppressive drugs like rituximab (anti-CD20), alemtuzumab (anti-CD52) or infliximab (anti-tumor necrosis factor)^[41-43].

Various mechanisms are involved in HBV reactivation^[9]: (1) immunosuppression with cytotoxic agents can enhance HBV replication and lead to direct hepatic damage; (2) cytotoxic/immunosuppressive agents can suppress T-cell function and/or deplete B cells; and (3) suppressed immunological response leads to widespread HBV infection of hepatocytes. Once recovery is achieved following withdrawal of cytotoxic agents and immune surveillance is reconstituted, a rebound in cytotoxic-T-cell response is induced that leads to the development of cellular injury and hepatitis.

The clinical significance of HBV reactivation in HIV-positive patients is uncertain^[44-46]. Severe HBV reactivation has been reported after withdrawal of antiretrovirals that are active against HBV^[35].

Graft reinfection and reactivation of OBI is possible in liver transplant recipients with a serological profile of past exposure to hepatitis B (anti-HBc positive)^[27,47]. OBI patients with cirrhosis need close monitoring because the mortality rate following reactivation approaches 5%-40%^[9].

All patients who receive chemotherapy and immunotherapy should be tested for HBV serology and/or viremia before starting therapy, especially if they are positive for antibody to viral antigens, and monitored for several months or years after stopping treatment^[2,29]. Early identification of virological reactivation is essential to start antiviral therapy and prevent the occurrence of hepatitis B, which can be very dangerous in these patients^[2,32,48].

Use of antiviral agents as prophylaxis against HBV in HBsAg-positive patients who are undergoing cytotoxic chemotherapy is a standard strategy^[9,30,49]. However, for patients with OBI and those who are serologically HBV-DNA-negative but anti-HBc-positive, current data are insufficient to recommend routine prophylaxis and antiviral therapy could be delayed until the HBV DNA becomes detectable^[9,49-51].

For those with OBI, especially in the absence of anti-HBs, a prudent therapeutic approach is to initiate HBV antiviral therapy (lamivudine, telbivudine, adefovir, entecavir or tenofovir) prior to chemotherapy. This should be continued for ≥ 6 mo after stopping immunosuppressive treatment. If long-term treatment (> 12 mo) is predicted, then adefovir, entecavir or tenofovir should be chosen, and if a more rapid response is needed, then entecavir or tenofovir could be considered. Antiviral therapy is usually unsuccessful if started after alanine aminotransferase becomes elevated^[9].

For those patients who are HBV-DNA-negative and anti-HBc-positive, the following approach could be considered based on the kinetics of reactivation^[9,32]: (1) monitor at 4-wk intervals with HBV-DNA NAT (low limit of detection < 10 IU/mL) and begin antiviral therapy when the result is > 30 IU/mL; or (2) monitor at 4-wk intervals with a highly sensitive HBsAg assay (low limit of detection < 0.1 ng/mL) and begin antiviral therapy when the test becomes positive. Further studies are needed to clarify the clinical usefulness, safety and cost-effectiveness of these strategies in OBI. In HIV-positive patients, the risk

of HBV sero-reversion is low; therefore, it does not justify any prophylaxis.

OBI AND CHRONIC LIVER DISEASE

OBI has been detected in patients with cryptogenic chronic liver disease^[52-54] and could be associated with progression of liver fibrosis and cirrhosis development in these patients^[52,55,56]. HBV-infected patients might present with progressive reduction of viral replication and serum HBsAg levels. HBsAg might disappear over time, despite the presence of severe liver injury that has been provoked by overt hepatitis B, and then maintained once the occult HBV status has been established^[2,57].

It has been reported that close monitoring of serum HBV-DNA levels and liver-enzyme levels could be useful in the management of patients with OBI and cryptogenic liver disease in two respects^[58]: (1) to predict the risk of cirrhosis or HCC; and (2) to decide on the possibility of antiviral treatment to prevent HBV reactivation or transmission in the case of transplantation. However, the role of OBI in accelerating the development of cirrhosis is still unresolved. Prospective studies using well-defined selection criteria of patients and standardized laboratory techniques are needed^[1,56,59].

OBI AND HCC DEVELOPMENT

Many epidemiological and molecular studies have indicated that OBI is a potential risk factor for HCC development. OBI seems to maintain HBV oncogenic mechanisms such as the capacity to be integrated in the host genome, and production of transforming proteins^[1,52,59-68]. This pro-oncogenic role is not only the consequence of the integration of viral DNA into the host genome. Other factors might contribute^[2]: (1) persistence of replicating virus might induce mild liver necro-inflammation that continues for life; (2) occult strains usually persist as free genomes, and maintain the capacity to transcribe and replicate^[68-70]; and (3) OBI might contribute to progression towards cirrhosis, which is the most important risk factor for HCC development. However, further molecular pathogenesis studies and prospective molecular epidemiological studies are needed to reach the conclusion that OBI plays a major role in hepatocellular transformation. Until then, it is premature to recommend testing all HBsAg-negative patients with HCC for OBI^[62].

CONCLUSION

OBI is a complex entity that comprises many conditions and different situations. Patients who have recovered from acute hepatitis B can carry HBV genomes for a long time, and the virus might aggravate the course of their liver disease, when other causes of liver damage are present. Use of HBV-DNA NAT and multivalent anti-HBs antibodies in the HBsAg assays is recommended for detection of true and false OBI, respectively, and to minimize the risk of HBV transmission through transfusion. It is not known if

prior hepatitis B immunization with an optimal anti-HBs response can effectively modulate or abort the infection in the case of HBV transmission through organ transplantation. In patients with serological evidence of past infection with hepatitis B, prophylaxis with antiviral agents prevents reactivation of hepatitis B after transplantation in most cases. Reactivation of OBI has been observed in several other conditions that cause immunosuppression in which antiviral therapy could be delayed until HBV DNA or HBsAg becomes detectable. OBI might contribute to the progression of liver fibrosis and HCC development in patients with chronic liver disease. However, further studies are needed to clarify the clinical usefulness, safety and cost-effectiveness of strategies for management of OBI.

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Glutamate reduces experimental intestinal hyperpermeability and facilitates glutamine support of gut integrity

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Abstract

AIM: To assess whether glutamate plays a similar role to glutamine in preserving gut wall integrity.

METHODS: The effects of glutamine and glutamate on induced hyperpermeability in intestinal cell lines were studied. Paracellular hyperpermeability was induced in Caco2.BBE and HT-29CL.19A cell lines by adding phorbol-12,13-dibutyrate (PDB) apically, after which the effects of glutamine and glutamate on horseradish peroxidase (HRP) diffusion were studied. An inhibitor of glutamate transport (L-trans-pyrrolidine-2,4-dicarboxylic acid: trans-PDC) and an irreversible blocker (acivicin) of the extracellular glutamine to glutamate converting enzyme, γ -glutamyltransferase, were used.

RESULTS: Apical to basolateral HRP flux increased significantly compared to controls not exposed to PDB ($n = 30$, $P < 0.001$). Glutamine application reduced hyperpermeability by 19% and 39% in the respective cell lines. Glutamate application reduced hyperpermeability by 30% and 20%, respectively. Incubation of HT29CL.19A cells with acivicin and subsequent PDB and glutamine addition increased permeability levels. Incubation of Caco2.BBE cells with trans-PDC followed by PDB and glutamate addition also resulted in high permeability levels.

CONCLUSION: Apical glutamate -similar to glutamine- can decrease induced paracellular hyperpermeability. Extracellular conversion of glutamine to glutamate and subsequent uptake of glutamate could be a pivotal step in the mechanism underlying the protective effect of glutamine.

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Key words: Apical; Basolateral; Flux; Glutamate; Glutamine; Gut protection; Gut wall integrity; Intestine; Permeability

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INTRODUCTION

Intestinal hyperpermeability, whether cause or effect, seems

to be related to the occurrence of sepsis, bacteraemia, and multiple organ failure^[1,2].

Managing this change in gut physiology might contribute to substantial health improvement. The semi-essential amino acid, glutamine, is thought to improve clinical outcome in these situations. It has been ascribed several properties that are supportive of intestinal cell function and relevant to cell survival^[3]. Additionally, plasma and muscle glutamine concentrations drop dramatically in critically ill patients^[4-6]. *In vivo* experiments, however, have not yet provided definitive evidence to support the claim that glutamine supplementation has a beneficial effect on gut permeability^[7]. In contrast, *in vitro* experiments do show a positive influence of glutamine. Kouznetsova *et al*^[8] induced hyperpermeability in the intestinal HT-29CL19A cell line and found that glutamine significantly reduced this increased permeability. Furthermore, Le Bacquer *et al*^[9] demonstrated that glutamine helps to preserve adequate paracellular permeability levels in nutritionally deprived intestinal Caco-2 cells. The precise mechanisms underlying these findings remain to be clarified. Glutamate might play a pivotal role in the effects of glutamine therapy considering the metabolic fate of glutamine: it is mostly converted to glutamate, either intra- or extracellularly^[10]. Welbourne *et al*^[11] provide support for this theory by demonstrating that blocking the extracellular glutamine to glutamate converting enzyme γ -glutamyltransferase (γ -GT) and blocking glutamate uptake, both increase paracellular permeability in the proximal tubule-like LLC-PK₁-F⁺ cells.

The aim of this study was to assess whether glutamate might play a similar role in the intestine (Figure 1). To do so, an experimental model that allows differentiation between the effects of glutamine and glutamate on induced hyperpermeability in intestinal cell lines was used.

MATERIALS AND METHODS

Study design

We created an experimental set-up using two intestinal cell lines: Caco2.BBE and HT-29CL19A (both human colon adenocarcinoma derived cell lines). In culture, both cell lines exhibit polarity and apical brush-border membranes, similar to *in vivo* structure^[12,13]. Cells were therefore placed in a bicameral system to simulate a physiological situation in which they are exposed to distinct apical and basolateral compartments, and thereby allowing permeability experiments.

Paracellular hyperpermeability was induced by adding phorbol-12,13-dibutyrate (PDB) to the apical compartment after which the effects of glutamine and glutamate on horseradish peroxidase (HRP) diffusion were studied. To differentiate between the effect of glutamine and glutamate on permeability, an inhibitor of glutamate transport (L-trans-pyrrolidine-2,4-dicarboxylic acid: trans-PDC) and an irreversible blocker (acivicin) of the extracellular glutamine to glutamate converting enzyme, γ -GT, were used.

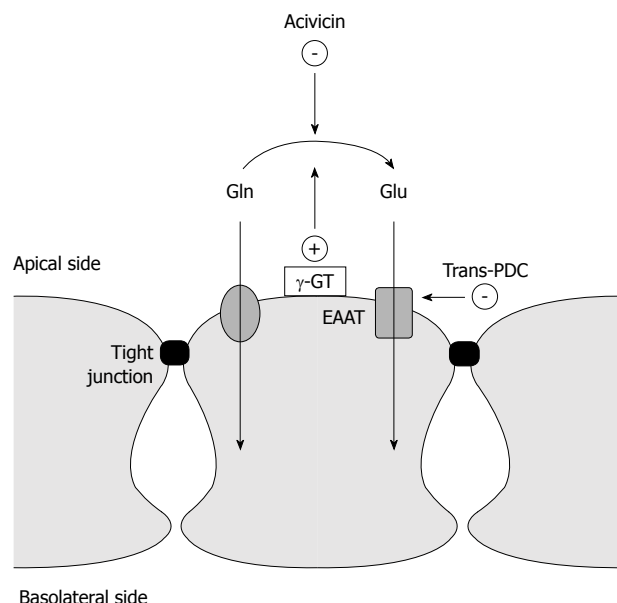


Figure 1 Model illustrating intestinal cell lineage with tight junctions and EAAT transporter. The extracellular enzymatic conversion of glutamine to glutamate by γ -glutamyltransferase (γ -GT) is shown on the apical side. The experimental design of the study using the γ -GT blocking enzyme, acivicin, and the blocker of the glutamate transporter, EAAT L-trans-pyrrolidine-2,4-dicarboxylic, is included in the figure.

Cell culture

The HT29CL19A cell line, passage number 14-35 and the Caco2.BBE cell line, passage number 34-62, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The medium contained penicillin 40 mg/L, ampicillin 8 mg/L and streptomycin 9 mg/L. The cells were seeded in 12 cm² culture flasks which were placed in an incubator with a humidified atmosphere of 5% CO₂ and 95% O₂. The cells were subcultured on transparent filters (12 mm diameter; Falcon, Micronic, Lelystad, The Netherlands) for 14 (HT29CL19A) and 21 (Caco2.BBE) days to form confluent monolayers. The medium contained glutamine (2 mmol/L) and was replaced every other day. During the last 2 d before the experiments, the cells were cultured in glutamine-free medium.

Flux experiments

The culture medium was discarded after cell cultivation and filters were rinsed with Ringer's solution (containing 117.5 mmol/L NaCl, 5.7 mmol/L KCl, 25 mmol/L NaHCO₃, 1.2 mmol/L NaH₂PO₄, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄ and 27.8 mmol/L mannitol, kept at pH 7.4). Filters were placed in a bicameral system with 300 μ L of Ringer's solution added to the apical chamber and 700 μ L to the basolateral chamber. The bicameral setup with filters was then placed in an incubator with humidified gas (5% CO₂, 95% O₂) where a temperature of 37°C was maintained. After an equilibration period of 30 min, HRP (type IV; Sigma Chemical Co., St Louis, MO, USA) dissolved in Ringer's solution was added apically to reach a final concentration of 10⁻⁵ mol/L. For the next 4 h, basolateral samples of 5 μ L were taken, in

triplicate, each hour and replaced by oxygenated Ringer's solution. The appearance of HRP in these samples was measured enzymatically. To this end, samples were mixed with 180 μ L citrate buffer (0.1 mol/L citrate + 0.1 mol/L citric acid at pH 5.5) containing 3.6 μ L bovine serum albumin (BSA 20 μ L/mL). Three samples of 25 μ L were taken from the resulting mixture and were added to 200 μ L substrate. Substrate was prepared by adding 340 μ L TMB stock (3.3-5.5 tetramethylbenzidine) (6 mg/mL in H₂O) and 200 μ L 0.3% H₂O₂ to 20 mL citrate buffer.

Samples were then incubated for 30 min at normal room temperature, after which the positive samples were blue in colour. The reaction was stopped by adding 50 μ L HCl (2 mol/L). The samples were read at 450 nm by a spectrophotometer. Data were recorded using Microplate Manager 5 Software, Bio-Rad Laboratories Ltd., UK.

Experiments with PKC-mediated hyperpermeability were conducted by simultaneously adding 1 μ mol/L of PDB and HRP to the apical chamber. The effects of L-glutamine (0.6 mmol/L) and L-glutamate (0.6 mmol/L) were (separately) studied by apical application with simultaneous PDB and HRP application. Acivicin experiments were conducted by incubating the cells with 10 μ L of the following solution: 1.7 mg acivicin, dissolved in 50 μ L HCl (2.0 mol/L) and 50 μ L DES (buffering the medium). Inhibition of glutamate transporters EAAT 1-5 was achieved by pre-incubating the cells with 1 mmol/L trans-PDC.

Chemicals

Falcon filters were obtained from Micronic (Lelystad, The Netherlands), penicillin/streptomycin from Boehringer Mannheim (Almere, The Netherlands) and ampicillin from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). All other cell culture materials were obtained from Gibco (Breda, the Netherlands). Chemicals used for the Ringer's solution were obtained from Merck (Merck Nederland BV, Amsterdam). PDB, L-glutamate, L-glutamine and trans-PDC were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands).

Statistical analysis

Statistical analyses of differences between groups were performed by one way ANOVA and the Tukey-Kramer test. A *P*-value < 0.05 was considered significant. HRP flux results are presented graphically as percentages of total flux. Total flux is defined by the HRP + PDB groups, which therefore represent the 100% mark. Graphpad Prism 3.03 for Windows® (GraphPad Software Inc., California, USA) was used for analyses and graphical output.

RESULTS

Hyperpermeability was successfully induced by PDB stimulation: apical to basolateral HRP flux increased significantly in the HT29Cl.19A and the Caco2.BBE cell line (with a maximum after 4 h) compared to controls not exposed to PDB ($n = 30$, $P < 0.001$). Cells in the PDB group defined the 100% mark, and all values were

composed of triplicate measurements per group per experiment and were repeated 3-11 times.

In HT29Cl.19A cells, glutamine application reduced hyperpermeability by 45% ($n = 11$, $P < 0.001$) (Figure 2A). In the Caco2.BBE cell line, glutamine application reduced hyperpermeability by 30% ($n = 3$, $P < 0.05$) (Figure 2B).

Glutamate application reduced hyperpermeability by 25% in the HT29Cl.19A cell line ($n = 3$, $P < 0.01$) (Figure 2C) and by 25% in the Caco2.BBE cell line ($n = 4$, $P < 0.001$) (Figure 2D).

Incubation of HT29CL.19A cells with acivicin and subsequent PDB and glutamine addition resulted in high permeability levels which were not significantly different from the PDB group ($n = 11$, Figure 2A).

Incubation of Caco2.BBE cells with trans-PDC and subsequent PDB and glutamate addition also resulted in high permeability levels, once again not significantly different from the PDB group ($n = 4$, Figure 2D).

Control experiments revealed that acivicin and trans-PDC did not alter HRP permeability (results not shown).

DISCUSSION

We found that both glutamine and glutamate can reduce an induced form of hyperpermeability in human colon derived cell lines. The effect of glutamine could be nullified by blocking the extracellular converting enzyme, γ -GT, whereas the effect of glutamate could be nullified by blocking the glutamate transporters EAAT 1-5.

These results lead to two suggestions: firstly, the conversion of glutamine to glutamate is essential for its beneficial effect on permeability. Secondly, transport of glutamate into the cell is essential for the beneficial effect of glutamate on permeability.

Because the effect of trans-PDC on the protective action of glutamine was not studied, and similarly, the effect of acivicin on the protective action of glutamine was not studied, further research will be necessary to confirm these suggestions. Not all of the experiments were performed with both cell lines due to inherent differences between the two cell lineages. The HT29Cl.19A cell line proved unstable during later experiments compared to the Caco2.BBE cell line. Additionally, the Caco2.BBE cell line has been shown to possess EAATs^[14], making it the favourable cell line for trans-PDC related experiments. Moreover, these inconsistencies might account for the observed differences in the reduction of induced hyperpermeability between the cell lines^[15]. However, our study design was not focussed or powered on cell line comparison.

Glutamine and glutamate seem to reduce this hyperpermeability by acting on the paracellular permeability (tight junction) as opposed to transcellular permeability (endocytosis). PDB, induces a Protein Kinase C (PKC)-mediated hyperpermeability. This signal transduction pathway is also activated by clinically relevant mediators, including lipopolysaccharides^[16]. PKC is thought to regulate tight junction (TJ) permeability *via* tightening and loosening of the cell's perijunctional actomyosin ring

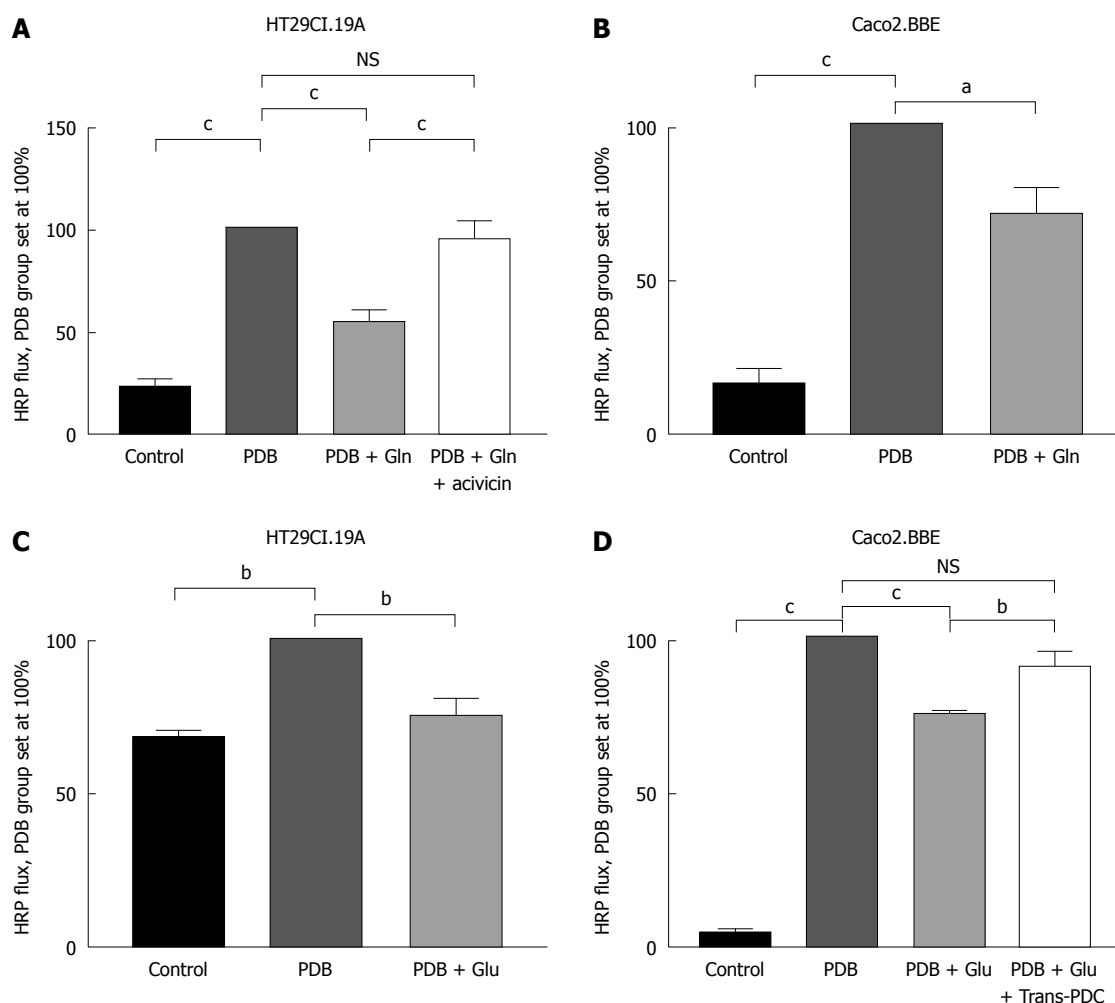


Figure 2 Effects of glutamine and glutamate on phorbol-12,13-dibutyrate-induced permeability in the two intestinal cell lines. A: In the HT29Cl.19A cell line, glutamine addition resulted in a 45% decrease in permeability. Acivicin nullified this effect, $n = 6$; B: In the Caco2.BBE cell line, glutamine addition resulted in a 30% decrease in permeability, $n = 3$; C: In the HT29Cl.19A cell line, glutamate addition resulted in a 25% decrease in permeability, $n = 3$; D: In the Caco2.BBE cell line, glutamate addition resulted in a 25% decrease in permeability. Trans-PDC nullified this effect, $n = 4$. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.01$.

(PAMR)^[17-19]. Furthermore, rinsing the PDB from the cells restored permeability levels to control values, indicating that the effect of PDB is not due to cell destruction (results not shown). In such, PDB addition creates a paracellular hyperpermeability which can be monitored by HRP diffusion from apical to basolateral compartments.

HRP needs to remain enzymatically active to be measured. Approximately 97% of the HRP that reaches the basolateral compartment *via* the transcellular pathway is degraded^[20,21] and loses its enzyme activity. The detection of enzymatically active HRP in this study therefore verified that we measured paracellular permeability.

Our results suggest that glutamine needs transamination to glutamate to exert its effect. In a broader scope, it would be interesting to quantify the transamination by glutaminase intracellularly. However, since blocking γ -GT extracellularly immediately showed a decrease in the effect of glutamine, the extracellular conversion seems important independent of intracellular mechanisms.

The protective effect of glutamine on gut mucosa is often thought to result from cell proliferation and at-

tenuation of apoptosis^[22]. Our study indicates that this is probably not the sole reason. HRP flux was inhibited within the four hour window of this study. Enterocyte proliferation, however, takes more than 4 h, thus cell proliferation can not (completely) explain the observed favourable effect. To exclude indirect effects of glutamine and glutamate metabolism, the measurement of metabolites by HPLC could pinpoint such effects.

Proliferation and maintaining the integrity of enterocytes requires an adequate supply of glutamine. Hence, plasma levels are normally maintained around 0.6 mmol/L^[23,24]. This physiological concentration was therefore used in the present study. For easier comparison the glutamate concentration was also set at 0.6 mmol/L, even though its physiological concentration approaches 24-80 μ mol/L^[23,24].

The 0.6 mmol/L of glutamine and glutamate were applied to the apical chamber. *In vivo*, however, luminal concentrations of glutamine and glutamate commonly exceed 0.6 mmol/L after protein-rich meals^[25]. It is, therefore, interesting to see that this concentration can already elicit advantageous effects. Future studies comparing differ-

ent concentrations of glutamine and glutamate should be performed to optimally quantify dosage effects. To allow a comparison with catabolic patients, it would also be interesting to detect a minimum dose of glutamine and glutamate which still elicits a protective effect on hyperpermeability.

In summary, we have shown that apical glutamate-similar to glutamine can decrease an induced paracellular hyperpermeability in two human colon derived cell lines. Because of the nature of the permeability inducing agent, PDB, glutamine and glutamate probably exert their effect through interaction with tight junctions. Furthermore, the extracellular conversion of glutamine to glutamate and the subsequent uptake of glutamate could be a pivotal step in the mechanism underlying the protective effect of glutamine. Yet, to certify this mechanism, the focus should be on different concentrations of apically applied glutamine and glutamate in different cell lines or in co-cultured cell lines, in parallel with research on intracellular conversion.

COMMENTS

Background

Intestinal hyperpermeability seems to be related to the occurrence of sepsis, bacteraemia, and multiple organ failure. The semi-essential amino acid, glutamine, is thought to improve clinical outcome in these situations. Glutamate might play a pivotal role in the effects of glutamine therapy considering the metabolic fate of glutamine: it is mostly converted to glutamate.

Research frontiers

The authors found that both glutamine and glutamate can reduce an induced form of hyperpermeability in colon cell lines.

Innovations and breakthroughs

Glutamine and glutamate seem to reduce this hyperpermeability by acting on paracellular permeability as opposed to transcellular permeability.

Applications

These results suggest further research on glutamate in feeding.

Peer review

The paper by Vermeulen *et al* describes experiments demonstrating that glutamate has a similar protective effect on intestinal hyperpermeability as glutamine. The used two different cell lines and appropriate inhibitors to show that extracellular conversion of glutamine to glutamate and subsequent uptake of glutamate could be a pivotal step in the mechanism underlying the protective action of glutamine. This is the first report showing the protective effect of glutamate itself.

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Omeprazole decreases magnesium transport across Caco-2 monolayers

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Abstract

AIM: To elucidate the effect and underlying mechanisms of omeprazole action on Mg^{2+} transport across the intestinal epithelium.

METHODS: Caco-2 monolayers were cultured in various dose omeprazole-containing media for 14 or 21 d before being inserted into a modified Ussing chamber apparatus to investigate the bi-directional Mg^{2+} transport and electrical parameters. Paracellular permeability of the monolayer was also observed by the dilution potential technique and a cation permeability study. An Arrhenius plot was performed to elucidate the activation energy of passive Mg^{2+} transport across the Caco-2 monolayers.

RESULTS: Both apical to basolateral and basolateral to apical passive Mg^{2+} fluxes of omeprazole-treated epithelium were decreased in a dose- and time-dependent manner. Omeprazole also decreased the paracellular cation selectivity and changed the paracellular selective permeability profile of Caco-2 epithelium to Li^+ , Na^+ , K^+ ,

Rb^+ , and Cs^+ from series VII to series VI of the Eisenman sequence. The Arrhenius plot revealed the higher activation energy for passive Mg^{2+} transport in omeprazole-treated epithelium than that of control epithelium, indicating that omeprazole affected the paracellular channel of Caco-2 epithelium in such a way that Mg^{2+} movement was impeded.

CONCLUSION: Omeprazole decreased paracellular cation permeability and increased the activation energy for passive Mg^{2+} transport of Caco-2 monolayers that led to the suppression of passive Mg^{2+} absorption.

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Key words: Magnesium; Paracellular; Proton pump inhibitor; Transepithelial; Tight junction

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INTRODUCTION

Magnesium plays an important role in numerous biological functions. Mg^{2+} deficiency is associated with several diseases, e.g. Alzheimer's disease^[1], osteoporosis^[2], and hypertension^[3]. Therefore, its plasma level is tightly regulated within a narrow range (0.7-1.1 mmol/L) by intestinal absorption and renal excretion^[4]. In human intestine, fractional Mg^{2+} absorption varies from 11% to 65% depending on the amount of Mg^{2+} intake^[5]. Intestinal epithelium absorbs Mg^{2+} via both saturable transcellular and non-saturable paracellular pathways. Transcellular Mg^{2+} transport is an

active process that requires the activity of transient receptor potential melastatin 6 (TRPM6) and the basolateral Na⁺/Mg²⁺ exchanger^[6,7]. On the other hand, paracellular Mg²⁺ transport is a passive mechanism and is implicated in about 90% of intestinal Mg²⁺ absorption^[7]. The paracellular Mg²⁺ transport process is modulated by the tight junction proteins, i.e. Claudin-16 and Claudin-19^[8].

Omeprazole is a common therapeutic tool for acid-peptic disorders. Its active sulphenamide form selectively and covalently interacts with the H⁺/K⁺-ATPase, particularly the extracellular cysteine 813, leading to potent inhibition of H⁺/K⁺-ATPase activity^[9]. Previous reports demonstrated that prolonged omeprazole administration led to hypomagnesemia and hypomagnesuria in humans^[10,11]. Withdrawal of omeprazole and intravenous Mg²⁺ replacement, but not high dose oral Mg²⁺ supplement, could normalize the plasma and urinary Mg²⁺ levels^[10,12]. Renal Mg²⁺ handling was normal in patients with severe hypomagnesemia associated with long-term use of omeprazole^[12-14]. This body of evidence suggested an inhibitory effect of omeprazole on intestinal Mg²⁺ absorption. However, the direct action of omeprazole on intestinal Mg²⁺ transport is still elusive. The present study, therefore, aimed to elucidate the effect of omeprazole as well as obtain information regarding possible mechanisms of omeprazole action on Mg²⁺ transport across the intestinal epithelium. This study employed a monolayer of Caco-2 cells which is a suitable *in vitro* model for studying intestinal transport of divalent cations, e.g. Ca²⁺^[15] and Mg²⁺^[16].

MATERIALS AND METHODS

Cell culture

Caco-2 cells (ATCC No. HTB-37) were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS-Gold) (PAA Laboratories GmbH, Pasching, Austria), 1% L-glutamine (Gibco, Grand Island, NY, USA), 1% non-essential amino acid (Sigma, St. Louis, MO, USA), and 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY, USA) and maintained at a humidified atmosphere containing 5% CO₂ at 37°C. The Caco-2 monolayers were developed by seeding cells (5.0 × 10⁵ cells/cm²) onto permeable Snapwell[™] inserts (12-mm diameter and 0.4-μm pore size polyester filter) (Corning, Corning, NY, USA). In the omeprazole-treated group, Caco-2 monolayers were grown in 200, 400, 600, 800, or 1000 ng/mL omeprazole (Calbiochem, San Diego, CA, USA) containing culture media. The culture medium was changed three times a week. On day 14 or 21 after seeding, the Snapwell was inserted into a modified Ussing chamber (1.13 cm² exposed area).

Measurement of Mg²⁺ flux

In the Ussing chamber, the monolayer was equilibrated for 20 min in bathing solution at 37°C, pH 7.4, and osmolarity of 290-293 mmol/kg H₂O^[17]. To avoid the unstirred water layer and to maintain pH at 7.4, the bathing solution in each hemi-chamber was continuously gassed with humidified 5% CO₂ in 95% O₂. After equilibration, the api-

cal or basolateral bathing solution was replaced with 2.5, 5, 10, 20, 40, or 80 mmol/L MgCl₂-containing bathing solution, while the contralateral side was replaced with MgCl₂-free bathing solution. At 1 and 2 h, 500 μL solution was collected from the side that contained MgCl₂-free bathing solution and Mg²⁺ concentration was measured. Mg²⁺ flux (nmol/h per cm²) was calculated using Equation (Eq. 1):

$$\text{Mg}^{2+} \text{ flux} = C_{\text{Mg}} / (t \times S) \quad (1)$$

Where C_{Mg} is Mg²⁺ concentration (nmol/L); t is time (h); and S is transport surface area (cm²).

To elucidate the involvement of solvent drag-induced mechanism on Mg²⁺ transport, 100 μmol/L phlorizin (Fluka Chemie AG, Buchs, Switzerland) and 100 μmol/L phloretin (Calbiochem, San Diego, CA, USA) were added to the apical and basolateral solution, respectively. Mg²⁺ transport was also observed at different temperatures (15, 25, or 35°C) and the results were presented as an Arrhenius plot^[18] (Eq. 2):

$$\ln(P_{\text{Mg}}) = (-E_a)/(RT) + \ln(E) \quad (2)$$

Where $\ln(P_{\text{Mg}})$ is the natural logarithm of Mg²⁺ permeability (cm/s); E_a is activation energy (kJ/mol); R is gas constant; T is absolute temperature (273+°C), E is pre-exponential factor. The temperature coefficient Q_{10} was determined as previously described^[19].

Measurement of Mg²⁺ concentration

The concentration of Mg²⁺ was determined by Xylidyl Blue (Sigma, St. Louis, MO, USA) colorimetric assay, modified from the method of Tang and Goodenough^[20]. In brief, the sample solutions were spun at 1000 × g for 10 min and a 200 μL sample of the upper solution was collected. An aliquot was added to 100 μL water, gently mixed, and then 200 μL of 1.25 mmol/L EGTA was added to the assay tube. After mixing well, 500 μL of Xylidyl Blue solution (pH 10.5) was added to the assay tube. After 5 min of incubation at room temperature, the assay solution was subjected to colorimetric analysis using a spectrophotometer at 520 nm (model UV-2550; Shimadzu, Kyoto, Japan).

Measurement of epithelial electrical parameters

Trans-epithelium resistance (TER), potential difference (PD), and short-circuit current (I_{sc}) were determined as previously described^[21]. These electrical parameters were recorded after 20 min equilibration at 30 min intervals throughout the 2 h of Mg²⁺ flux study.

Ion permeability measurement

Absolute permeabilities of Na⁺ (P_{Na}) and Cl⁻ (P_{Cl}), as well as the relative permeability of Na⁺ to Cl⁻ ($P_{\text{Na}}/P_{\text{Cl}}$), of Caco-2 monolayers were obtained by the dilution potential technique as previously described^[21]. The absolute permeability of group I alkaline metals (Li⁺, K⁺, Rb⁺, and Cs⁺), i.e. P_{Li} , P_{K} , P_{Rb} , and P_{Cs} was determined as previously described^[21] using the same calculation as that used to obtain P_{Na} .

The Mg²⁺ permeability (P_{Mg}) of Caco-2 monolayers was calculated using Eq. 3:

$$P_{\text{Mg}} = \text{Mg}^{2+} \text{ flux} / \Delta C_{\text{Mg}} \quad (3)$$

Table 1 Kinetic data of $Mg_{B \rightarrow A}$ transport

	V_m (nmol/h per cm^2)	K_m (mmol/L)	m ($\times 10^{-3}$ cm/h)
14 d			
Control	57.22 \pm 8.41	5.62 \pm 1.83	2.18 \pm 0.11
Omeprazole treated (ng/mL)			
200	58.12 \pm 6.19	4.55 \pm 1.23	1.80 \pm 0.08 ^b
400	62.82 \pm 9.64	5.83 \pm 2.22	1.54 \pm 0.12 ^b
600	57.01 \pm 7.49	4.48 \pm 1.44	1.28 \pm 0.09 ^b
800	59.35 \pm 7.40	4.71 \pm 1.40	0.83 \pm 0.09 ^b
1000	58.84 \pm 7.52	5.84 \pm 1.59	0.52 \pm 0.10 ^b
21 d			
Control	55.82 \pm 8.02	5.09 \pm 1.89	2.17 \pm 0.10
Omeprazole treated (ng/mL)			
200	57.81 \pm 10.41	7.48 \pm 2.24	1.26 \pm 0.12 ^{b,d}
400	53.47 \pm 7.59	5.10 \pm 1.87	1.18 \pm 0.10 ^{b,d}
600	53.12 \pm 7.53	4.42 \pm 1.68	0.96 \pm 0.09 ^{b,d}
800	57.65 \pm 6.76	5.97 \pm 1.51	0.53 \pm 0.08 ^{b,d}
1000	57.99 \pm 7.72	6.09 \pm 1.48	0.49 \pm 0.07 ^b

^b $P < 0.001$ vs the age-matched control group, ^d $P < 0.001$ vs the concentration-matched 14 d-omeprazole-treated groups.

Where ΔC_{Mg} is the concentration difference of Mg^{2+} between the apical and basolateral solutions.

Mg^{2+} transport kinetic analysis

To estimate the kinetic values of the saturable active and non-saturable passive Mg^{2+} transport, the rate of apical to basolateral Mg^{2+} transport ($Mg_{A \rightarrow B}$ transport) was fitted to a modified Michaelis-Menten kinetic plus linear component as shown in Eq. 4:

$$Mg_{A \rightarrow B} \text{ transport} = (V_m \times C_{Mg}) / (K_m + C_{Mg}) + mC_{Mg} \quad (4)$$

Where V_m is the maximal rate of saturable $Mg_{A \rightarrow B}$ transport; K_m is the rate constant of saturable $Mg_{A \rightarrow B}$ transport; m is the rate constant for non-saturable $Mg_{A \rightarrow B}$ transport; and C_{Mg} as mentioned above. This study was performed using a nonlinear regression program of GraphPad Prism version 5.0 for Window (GraphPad Software Inc., San Diego, CA, USA).

Statistical analysis

Results were expressed as means \pm SE. Two sets of data were compared using the unpaired Student's *t*-test. One-way analysis of variance (ANOVA) with Dunnett's posttest was employed for multiple sets of data. The level of significance was $P < 0.05$. Linear regression and slope analysis were performed to obtain the basolateral to apical Mg^{2+} transport ($Mg_{B \rightarrow A}$ transport)- Mg concentration relationship. The curve of P_{Mg} - Δ magnesium relationship was obtained using one phase exponential decay equation. All data were analyzed by GraphPad Prism (GraphPad Software Inc.).

RESULTS

Omeprazole decreased $Mg_{A \rightarrow B}$ transport and P_{Mg} in both a dose- and time-dependent manner

As demonstrated in Figure 1, the $Mg_{A \rightarrow B}$ transport vs Mg^{2+} concentration plots of Caco-2 monolayers were curvi-

Table 2 Electrical parameters of Caco-2 monolayers

	n	PD (mV)	I_{sc} (mA/cm ²)	TER ($\Omega \cdot cm^2$)
14 d				
Control	9	0.99 \pm 0.12	3.09 \pm 0.36	322.19 \pm 6.37
Omeprazole treated (ng/mL)				
200	9	1.03 \pm 0.54	2.52 \pm 0.39	413.64 \pm 12.95
400	9	0.98 \pm 0.14	2.24 \pm 0.29	433.23 \pm 17.66
600	9	1.08 \pm 0.15	2.29 \pm 0.31	470.35 \pm 23.87 ^d
800	9	1.09 \pm 0.18	2.30 \pm 0.41	483.22 \pm 20.20 ^d
1000	9	1.20 \pm 0.11	2.41 \pm 0.20	502.88 \pm 30.99 ^d
21 d				
Control	9	1.00 \pm 0.15	3.18 \pm 0.47	314.05 \pm 4.64
Omeprazole treated (ng/mL)				
200	9	1.26 \pm 0.20	2.48 \pm 0.32	485.09 \pm 24.36 ^b
400	9	1.13 \pm 0.19	2.20 \pm 0.32	502.19 \pm 27.47 ^d
600	9	1.06 \pm 0.13	2.21 \pm 0.34	500.33 \pm 32.97 ^d
800	9	0.99 \pm 0.19	1.97 \pm 0.31	481.64 \pm 25.48 ^d
1000	9	1.07 \pm 0.18	2.06 \pm 0.30	500.84 \pm 26.61 ^d

^b $P < 0.01$, ^d $P < 0.001$ vs the age-matched control group.

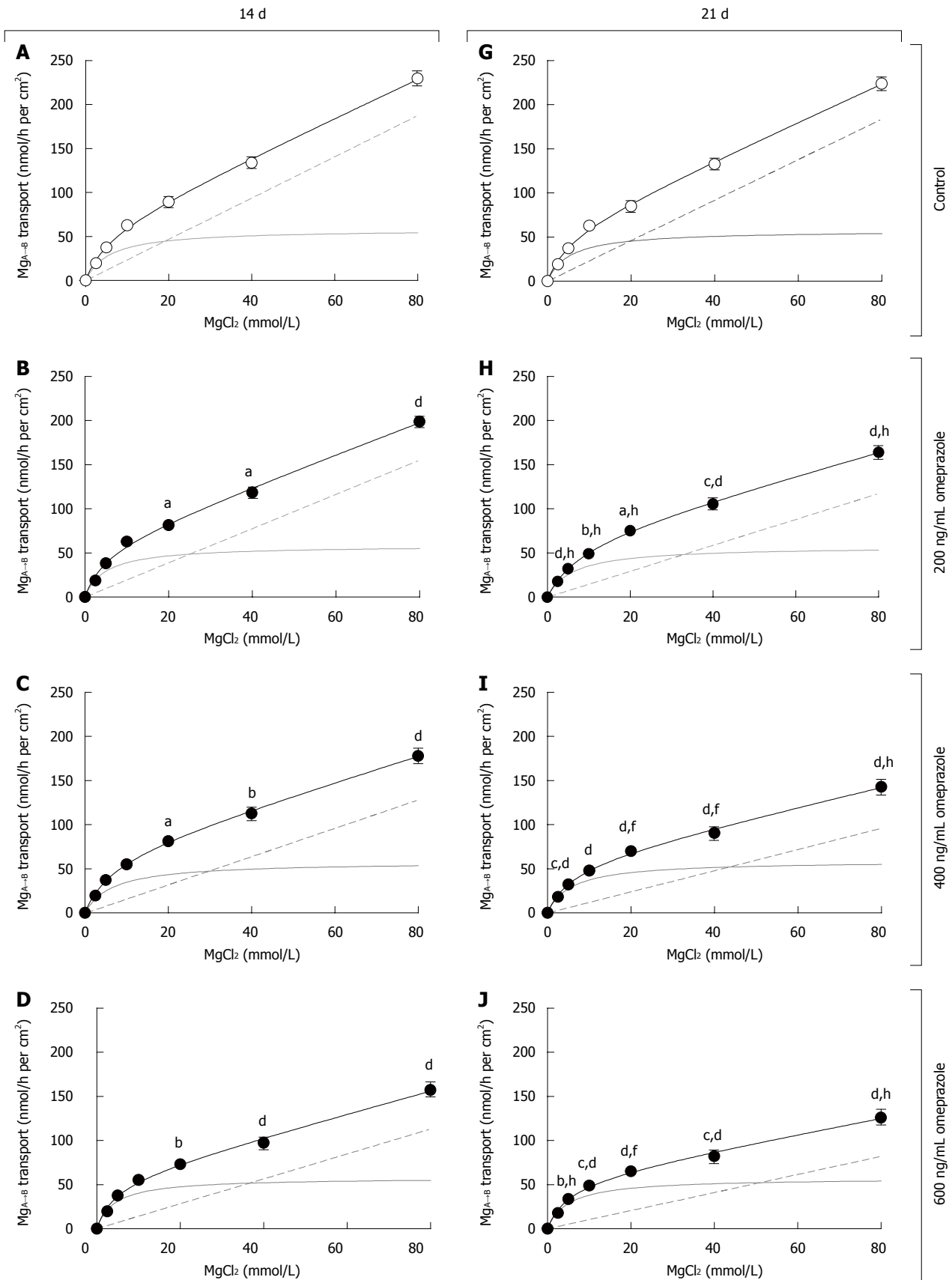
linear similar to that reported in humans^[5]. After 14 d in the omeprazole-treated groups, $Mg_{A \rightarrow B}$ transport was inhibited when compared with its corresponding untreated group (Figures 1A-F). The level of inhibition progressively increased with higher concentrations of omeprazole. Omeprazole selectively decreased non-saturable $Mg_{A \rightarrow B}$ transport, but not the saturated component, as clearly demonstrated by the lower rate constant for non-saturable $Mg_{A \rightarrow B}$ transport (Table 1). For 21 d omeprazole-treated groups, the results were similar to those of the 14 d omeprazole-treatment (Figure 1G-L, Table 1). When the same omeprazole concentration was considered, 21 d-treated groups showed a significantly lower $Mg_{A \rightarrow B}$ transport than the 14 d-treated groups (Figure 1, Table 1). Therefore, omeprazole decreased $Mg_{A \rightarrow B}$ transport in a dose- and time-dependent manner. According to the $Mg_{A \rightarrow B}$ transport, omeprazole also decreased the apical to basolateral P_{Mg} in a dose- and time-dependent mechanism (Figure 2). Moreover, omeprazole significantly increased TER, but not PD or I_{sc} , of Caco-2 monolayers (Table 2), indicating the lower paracellular permeability to ion transport.

Omeprazole decreased $Mg_{B \rightarrow A}$ transport

Since the $Mg_{B \rightarrow A}$ transport occurred solely through the paracellular pathway, the $Mg_{B \rightarrow A}$ transport vs Mg^{2+} concentration plot was linear (Figure 3A). Omeprazole significantly decreased the slope of the $Mg_{B \rightarrow A}$ transport- Mg^{2+} concentration plot. The slope progressively decreased with increased concentration of omeprazole (Figure 3A). In addition, omeprazole significantly suppressed the basolateral to apical P_{Mg} in a dose-dependent manner (Figure 3B). The collective results clearly showed that omeprazole suppressed paracellular passive Mg^{2+} transport across Caco-2 monolayers.

Omeprazole decreased paracellular cation selectivity

Similar to previous reports^[21,22], Caco-2 monolayers



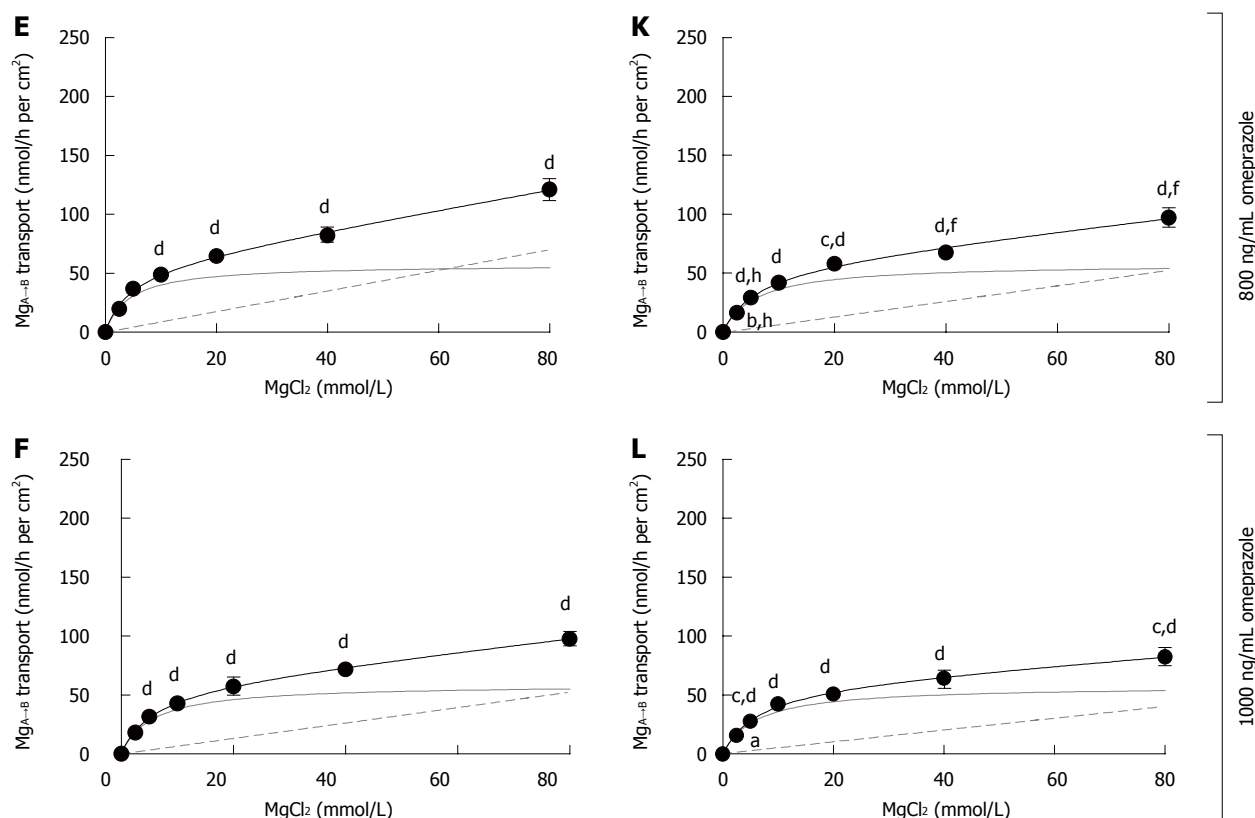


Figure 1 Mg_{A-B} transport across Caco-2 monolayers. For Mg_{A-B} transport of 14 d monolayers, A: Control; B: 200 ng/mL omeprazole-treated; C: 400 ng/mL omeprazole-treated; D: 600 ng/mL omeprazole-treated; E: 800 ng/mL omeprazole-treated; and F: 1000 ng/mL omeprazole-treated monolayers. For Mg_{A-B} transport of 21 d monolayers, G: control; H: 200 ng/mL omeprazole-treated; I: 400 ng/mL omeprazole-treated; J: 600 ng/mL omeprazole-treated; K: 800 ng/mL omeprazole-treated and L: 1000 ng/mL omeprazole-treated monolayers. Light solid lines represent the saturable component. Dashed lines represent the non-saturable component. ^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ vs the age-matched control group, ^c $P < 0.05$, ⁱ $P < 0.01$, ^h $P < 0.001$ vs the concentration-matched 14 d omeprazole-treated groups. For each data point, $n = 9$.

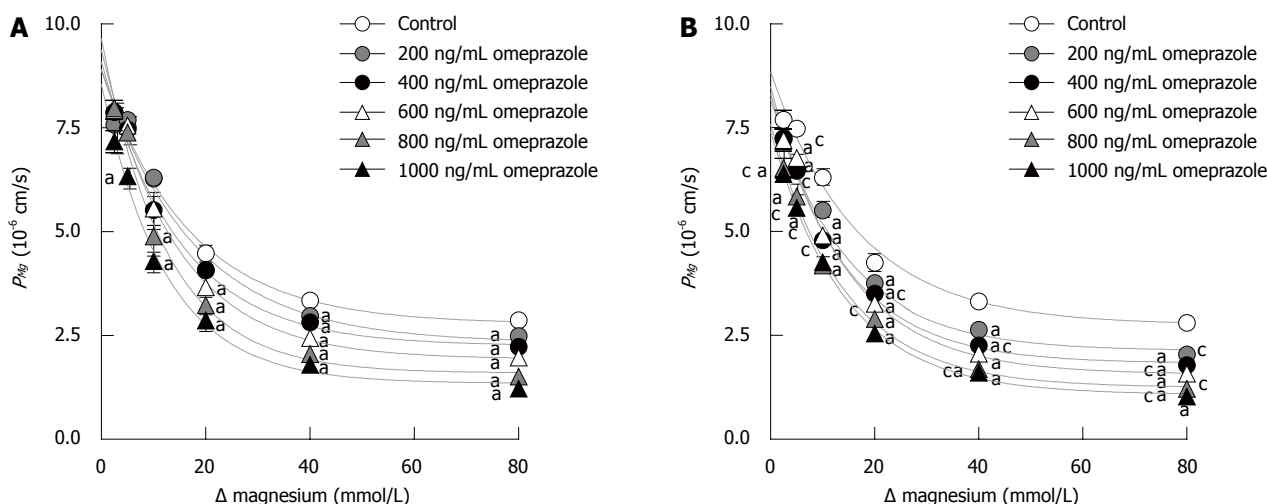


Figure 2 Apical to basolateral P_{Mg} . A: P_{Mg} of 14 d control and various dose omeprazole-treated monolayers; B: 21 d control and various dose omeprazole-treated monolayers. ^a $P < 0.05$ vs the age-matched control group, ^c $P < 0.05$ vs the concentration-matched 14 d omeprazole-treated group. For each data point, $n = 9$.

showed high P_{Na}/P_{Cl} (3.79 ± 0.15 in 14 d monolayers; 3.96 ± 0.22 in 21 d monolayers) from the higher P_{Na} (8.28 ± 0.20 in 14 d monolayers; 8.11 ± 0.25 in 21 d monolayers) than P_{Cl} (2.08 ± 0.09 in 14 d monolayers; 2.07 ± 0.11 in 21 d monolayers) (Figure 4A-C). Therefore, the Caco-2 monolayer was a cation selective epithelium. In 14 d- as well as

21 d-omeprazole-treated groups, omeprazole significantly suppressed P_{Na}/P_{Cl} and P_{Na} but enhanced P_{Cl} in a dose-dependent manner (Figure 4A-C), indicating that omeprazole decreased cation selectivity of Caco-2 monolayers.

Moreover, the present study also examined the paracellular permeability to monovalent cations, i.e. Li^+ , Na^+ ,

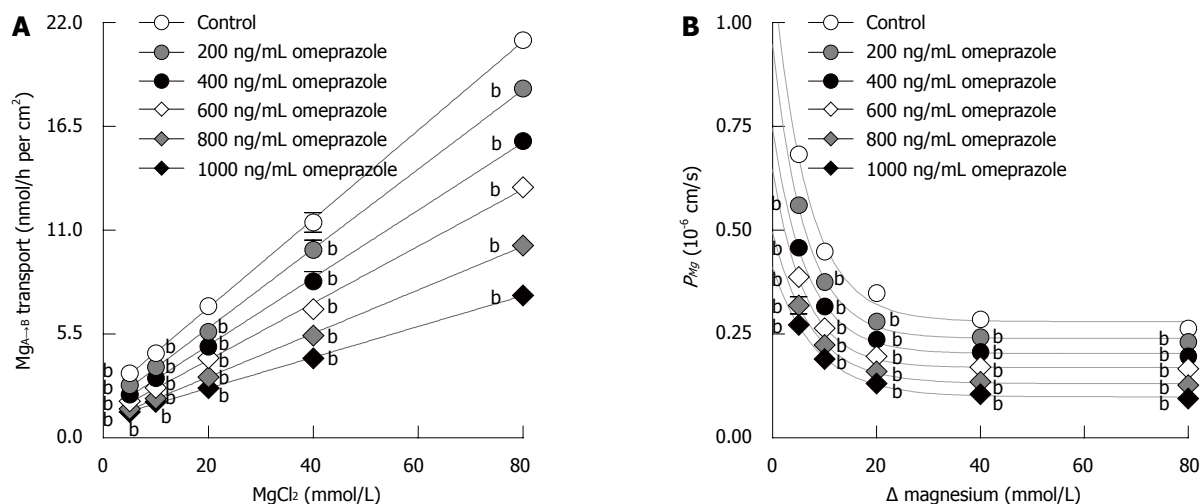


Figure 3 $Mg_{B \rightarrow A}$ transport and basolateral to apical P_{Mg} . A: $Mg_{B \rightarrow A}$ transport; B: Basolateral to apical P_{Mg} of 14 d control and various dose omeprazole-treated monolayers. $^bP < 0.01$ vs the control group. For each data point, $n = 6$.

K^+ , Rb^+ , and Cs^+ . In control conditions, Caco-2 monolayers showed the following selective sequence: P_{Na} (8.62 ± 0.18) $>$ P_K (7.99 ± 0.19) $>$ P_{Rb} (5.84 ± 0.08) $>$ P_{Cs} (4.82 ± 0.07) $>$ P_{Li} (3.98 ± 0.12) (Figure 4D). Interestingly, 14 d-omeprazole (600 ng/mL)-exposed monolayers showed a different permeability sequence as follows: $P_K > P_{Na} > P_{Rb} > P_{Cs} > P_{Li}$ (Figure 4D). In addition, omeprazole also inhibited Caco-2 permeability to all of these monovalent cations in a dose-dependent manner.

In a parallel study, TER was simultaneously recorded when the monolayers were exposed to group I alkaline metals containing solution. In control conditions, Caco-2 monolayers showed the highest conductance (lowest TER) to Na^+ (Figure 4E). The TER-Pauling radii relationship showed a V-shaped profile. Omeprazole-treated Caco-2 monolayers showed the lowest TER when the primary ion was K^+ (Figure 4E). Omeprazole also changed the TER-Pauling radii graph to a U-shape relationship and increased TER in all groups.

Omeprazole inhibited paracellular Mg^{2+} transport

Theoretically, ions can move transversely across Caco-2 monolayer *via* four transport mechanisms, i.e. solvent drag-induced active, voltage dependent active, transcellular active, and paracellular passive transport. Therefore, the present experiment aimed to identify the relative involvement of each mechanism in Mg^{2+} transport across Caco-2 monolayers. Inhibitors of solvent drag-induced ion transport (phlorizin and phloretin) had no effect on $Mg_{A \rightarrow B}$ transport (40 mmol/L Mg^{2+} concentration gradient) in both control and omeprazole-treated monolayers (Figure 5D). In another set of experiments, Caco-2 monolayers received continuous application of I_{SC} , simultaneously with the Mg^{2+} flux study, to nullify trans-epithelial PD and to abolish voltage dependent Mg^{2+} transport. The $Mg_{A \rightarrow B}$ transport in both control and omeprazole-treated monolayers were unaffected by I_{SC} (Figure 5A). The results indicated that solvent drag-induced and voltage dependent $Mg_{A \rightarrow B}$ transport were negligible.

Since transcellular Mg^{2+} transport required apical Mg^{2+} influx, inhibition of Mg^{2+} influx should abolish Mg^{2+} transport. When 20 μ mol/L ruthenium red (RR), a TRPM6 inhibitor^[23], was added to the apical solution, a linear relationship between $Mg_{A \rightarrow B}$ transport and Mg^{2+} concentration was observed (Figure 5B). The rate constant for non-saturable $Mg_{A \rightarrow B}$ transport of control monolayers (2.18 ± 0.13) was not different from the slope of $Mg_{A \rightarrow B}$ transport (2.09 ± 0.06) of RR-treated control monolayers. In parallel experiments, 14 d-600 ng/mL omeprazole-treated monolayers were bathed in bathing solution with or without 20 μ mol/L RR (Figure 5C). Similar to control conditions, RR inhibited the saturable component, but not the non-saturable component, of $Mg_{A \rightarrow B}$ transport in omeprazole-treated monolayers. In RR-treated monolayers, the 14 d-omeprazole-treated group showed a less steep slope when compared with that of the control group (1.29 ± 0.04 vs 2.09 ± 0.06 , $P < 0.001$, Figure 5B and C). Therefore, omeprazole suppressed the non-saturable passive Mg^{2+} transport across Caco-2 monolayers.

Temperature dependent Mg^{2+} permeability

To elucidate the temperature dependent Mg^{2+} transport, Caco-2 monolayers were bathed in 40 mmol/L $MgCl_2$ containing apical solution, while the basolateral solution had no $MgCl_2$. As shown by the Arrhenius plot (Figure 5E), the $\ln(P_{Mg})$ decreased in lower temperatures. The control monolayers showed E_a of 14.28 ± 1.19 kJ/mol and Q_{10} of 1.22 ± 0.04 . Fourteen days of 600 ng/mL omeprazole exposure significantly suppressed Mg^{2+} transport and increased E_a (19.24 ± 1.98 kJ/mol, $P < 0.05$), but not Q_{10} (1.31 ± 0.05), of Caco-2 monolayers.

DISCUSSION

The present study demonstrated the effect of omeprazole on Mg^{2+} transport across Caco-2 intestinal epithelium. Omeprazole-treated monolayers showed a dose- and time-dependent decrease in Mg^{2+} transport and P_{Mg} (Figures 1-3).

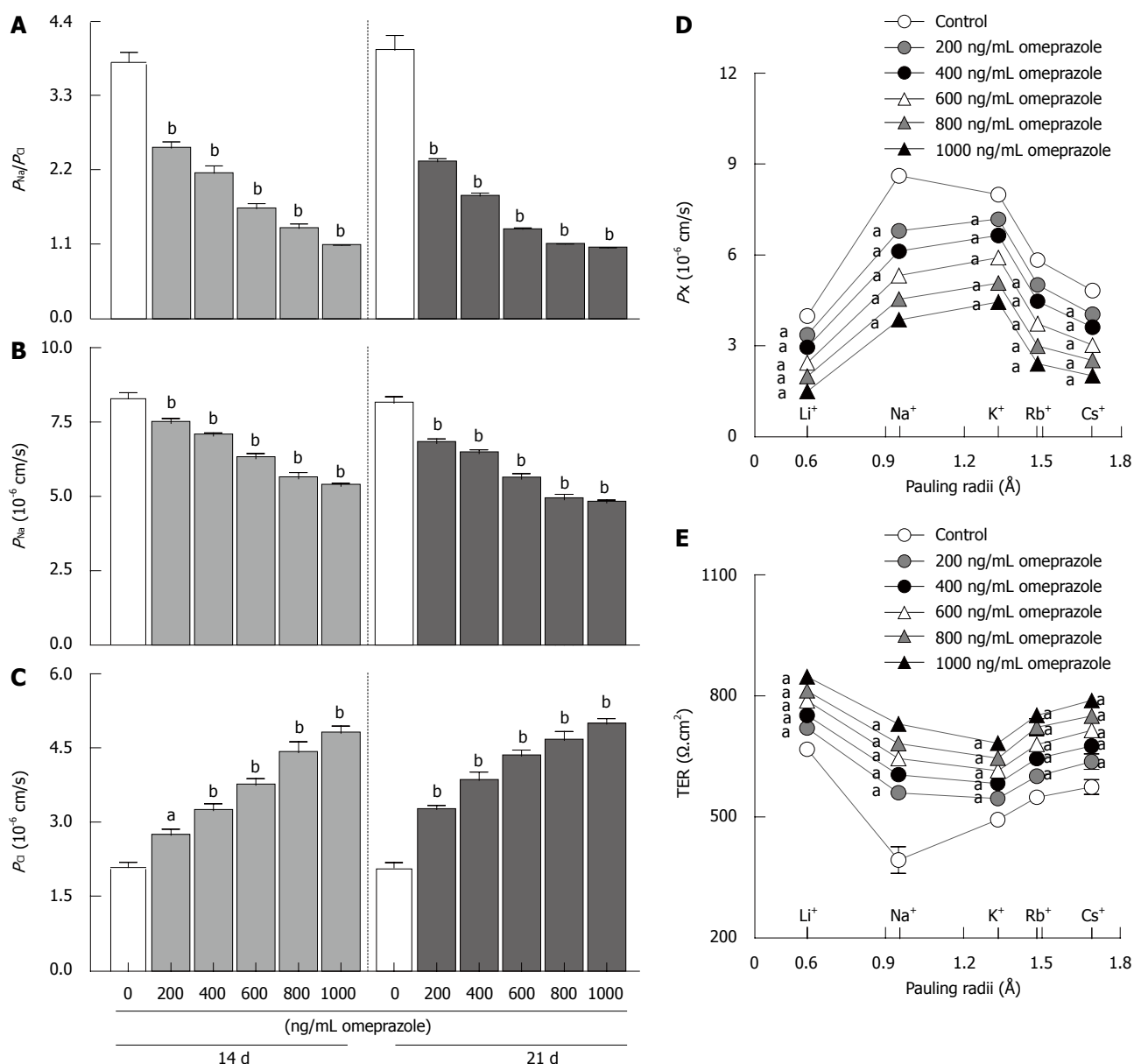


Figure 4 Paracellular charge selectivity and selective permeability profile. A: $P_{\text{Na}}/P_{\text{Ca}}$; B: P_{Na} ; C: P_{Ca} of 14 and 21 d control and various dose omeprazole-treated monolayers; D: Absolute alkaline metal ions (Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+) permeability; E: Trans-epithelium resistance (TER) of 14 d control and 600 ng/mL omeprazole-treated monolayers. ^a $P < 0.05$, ^b $P < 0.01$ vs the age matched control group. For each data point, $n = 6$.

Omeprazole selectively inhibited the non-saturable passive component, but not the saturable active component, of transepithelial Mg^{2+} transport (Table 1 and Figure 5). The paracellular cation selectivity of the monolayers was also reduced after prolonged exposure to omeprazole (Figure 4). Results of the Arrhenius plot (Figure 5) showed the higher E_a in the omeprazole-treated group, indicating impediment of the paracellular channel to Mg^{2+} movement.

In humans, intestinal Mg^{2+} absorption *vs* Mg^{2+} intake exhibited a curvilinear relationship^[5] from the combination of saturable active and non-saturable passive absorption. Moreover, lower intestinal passive Mg^{2+} absorption as compared with passive Ca^{2+} absorption was also demonstrated^[5,24]. Similarly, in Caco-2 monolayers, a plot of $\text{Mg}_{\text{A} \rightarrow \text{B}}$ transport (representing Mg^{2+} absorption) against Mg^{2+} concentration (in apical solution) was also curvilinear

(Figure 1) and $\text{Mg}_{\text{A} \rightarrow \text{B}}$ transport was lower than the apical to basolateral Ca^{2+} transport^[21]. Therefore, the Caco-2 monolayer was a suitable *in vitro* model of intestinal Mg^{2+} absorption^[16].

Several case reports demonstrated severe hypomagnesemia associated with prolonged omeprazole usage^[10-14], suggesting that intestinal Mg^{2+} absorption, but not renal Mg^{2+} handling, was defective. On the other hand, short-term omeprazole administration had no effect on intestinal Mg^{2+} absorption^[25] because its bioavailability was low and its half-life was short^[9,26]. Therefore, the later development of hypomagnesemia was probably associated with the depletion of Mg^{2+} store in the human body^[13]. The present study demonstrated an inhibitory effect of omeprazole on Mg^{2+} fluxes across 14 and 21 d-omeprazole-treated Caco-2 monolayers, suggesting that the intes-

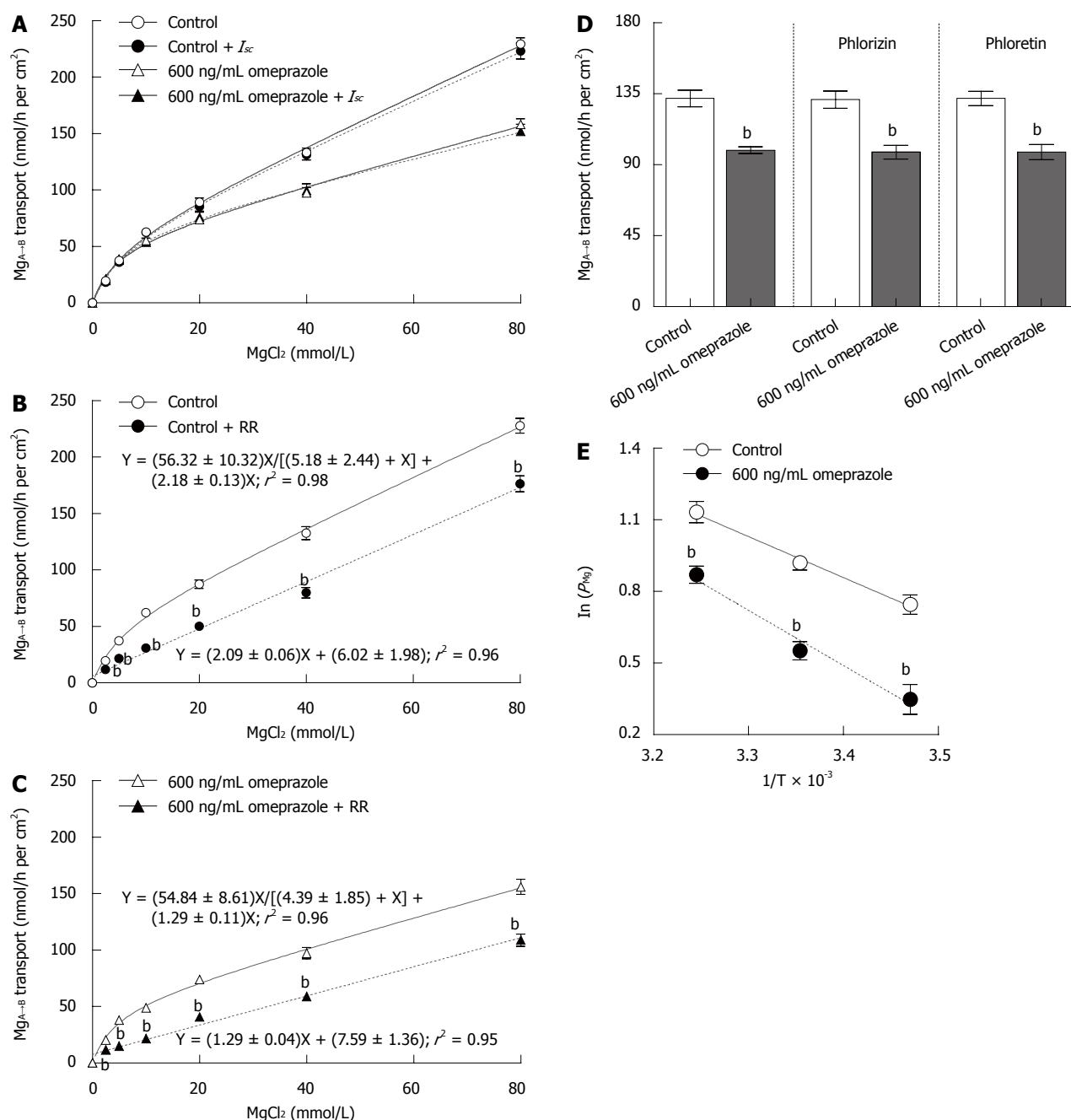


Figure 5 Mechanism of Mg_{A-B} transport. A: Mg_{A-B} transport 14 d control and 600 ng/mL omeprazole-treated monolayers with or without I_{sc} ; B and C: 20 μ mol/L ruthenium red (RR); D: 100 μ mol/L phlorizin or 100 μ mol/L phloretin. For each data point, $n = 9$ in A; $n = 6$ in B and C. E, Arrhenius plot of 14 d control and 600 ng/mL omeprazole-treated monolayers. ^b $P < 0.01$ vs the control group. For each data point, $n = 6$.

tinal Mg^{2+} flux defect could not be responsible for later development of hypomagnesemia in omeprazole use.

There are two transport mechanisms for Mg^{2+} absorption, i.e. transcellular active and paracellular passive transport, across the intestinal epithelium^[7]. Previous reports suggested that omeprazole inhibited active intestinal Mg^{2+} absorption and TRPM6 activity because high dose oral Mg^{2+} supplement partially^[13] and totally^[14] resolved hypomagnesemia in prolonged omeprazole use. On the other hand, other reports showed different results i.e. high dose oral Mg^{2+} supplement, but not intravenous Mg^{2+} replacement and withdrawal of omeprazole, failed to normalize plasma and urinary Mg^{2+} levels^[10,12]. The later evidence

indicated that omeprazole inhibited passive Mg^{2+} absorption, which agreed with the present findings. In the present study, omeprazole inhibited the non-saturable passive, but not saturable active, Mg^{2+} transport across Caco-2 monolayers (Table 1). In addition, the role of transcellular active Mg^{2+} transport was examined using the TRPM6 inhibitor RR. Inhibition of TRPM6 in Caco-2 cells^[27] abolished the saturable active Mg^{2+} transport and revealed the inhibition of non-saturable passive Mg^{2+} transport in omeprazole-treated monolayers (Figure 5B and C). Therefore, the paracellular passive Mg^{2+} absorption defect should be recognized in omeprazole usage.

Consistent with previous findings that the paracellular

passive transport of cations, such as Na^+ , Cs^+ , H^+ , Ca^{2+} , and Mg^{2+} , was a temperature variance mechanism^[18,20], the present Arrhenius plot (Figure 5E) showed the temperature-dependent Mg^{2+} transport. Since the temperature coefficient Q_{10} of passive ion diffusion through the open ion channel ranged from 1.2 to 1.4^[28] and the paracellular pore of the tight junction behaved as the channel^[20], therefore, the Q_{10} of control (1.22) and omeprazole treated (1.31) monolayers indicated that Mg^{2+} mainly moved through the paracellular channels of Caco-2 epithelium. The paracellular passive H^+ transport occurred *via* the claudin-8 channel of MDCK II epithelium^[18]. The paracellular claudin-8 channel was found to impede H^+ transport by increasing the E_a ^[18]. Therefore, the higher E_a of omeprazole-treated Caco-2 epithelium suggested that the paracellular channel of Caco-2 epithelium impeded Mg^{2+} transport. In addition, the higher TER (Table 2) also indicated lower paracellular permeability. The present study supported a previous report by Hou *et al.*^[29], who demonstrated that the epithelium with higher TER showed lower passive Mg^{2+} transport.

The paracellular transport of Mg^{2+} was regulated by the paracellular charge selectivity, i.e. cation selectivity, of the tight junction^[29,30]. Caco-2 epithelium was a cation selective epithelium (Figure 4A-C)^[21,22] that favored the transport of cations through the paracellular pathway. Similar to a previous report^[21], the paracellular selective permeability profile of Caco-2 monolayers to monovalent cations was $Na^+ > K^+ > Rb^+ > Cs^+ > Li^+$ (Figure 4D) which was classified as series VII of the Eisenman sequence^[31]. Series VII indicated the presence of moderate negative electrical field strength in the paracellular channel of Caco-2 epithelium. However, omeprazole changed the selective permeability profile to series VI of the Eisenman sequence ($K^+ > Na^+ > Rb^+ > Cs^+ > Li^+$)^[31]. Since series VI was characterized by lower negative electrical field strength than that of series VII^[31], the paracellular cation selectivity was decreased when the monolayers were exposed to omeprazole (Figure 4A-C). Hou *et al.*^[29] also demonstrated lower paracellular Mg^{2+} transport due to lower paracellular cation selectivity of the epithelium. Thereby, omeprazole-induced suppression of paracellular cation selectivity led to the inhibition of paracellular Mg^{2+} transport across Caco-2 epithelium.

The present study demonstrated the inhibitory effect of omeprazole on passive Mg^{2+} transport which was consistent with previous reports^[29,30,32-34]. The paracellular passive Mg^{2+} transport was mainly mediated by claudins at the tight junction^[29,30], the distribution of which could be affected by the change in extracellular fluid pH. Inhibition of H^+/K^+ -ATPase activity in Caco-2 cells^[35] by omeprazole might decrease the extracellular H^+ concentration, which in turn increased the sensitivity of the extracellular calcium sensing receptor (CaSR)^[32,33], which was expressed in Caco-2 cells^[36,37]. Ikari *et al.*^[34] clearly demonstrated that the activation of CaSR led to the translocation of claudin-16 from the tight junction into the cell, thus inhibiting paracellular Mg^{2+} transport. Therefore, omeprazole-inhibited passive Mg^{2+} transport appeared to involve the CaSR-tight junction-dependent mechanism.

In conclusion, omeprazole inhibited paracellular passive Mg^{2+} transport across Caco-2 epithelium in a dose- and time-dependent fashion. The inhibition of passive Mg^{2+} transport was due to the decrease in paracellular cation selectivity. The results from the present study provided evidence for the regulation of intestinal Mg^{2+} absorption. However, the underlying mechanism of omeprazole inhibiting passive Mg^{2+} transport requires further study.

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COMMENTS

Background

Previously, it was widely believed that intestinal Mg^{2+} transport in humans depended on the amount of Mg^{2+} intake and was not tightly regulated by any hormones. Omeprazole, a common therapeutic drug for acid-peptic disorders, has been found to have effects on Mg^{2+} metabolism.

Research frontiers

Several previous reports have demonstrated an association between severe hypomagnesemia and prolonged omeprazole usage in humans. Those patients had normal renal Mg^{2+} handling, suggesting that a defect in intestinal Mg^{2+} absorption may be responsible for hypomagnesemia. However, the direct action of omeprazole on intestinal Mg^{2+} absorption is unknown. In this manuscript, an inhibitory effect of omeprazole on intestinal passive Mg^{2+} absorption is demonstrated.

Innovations and breakthroughs

In this manuscript, the authors reported a direct inhibitory action of prolonged omeprazole treatment on paracellular passive Mg^{2+} absorption across the intestinal epithelium. This finding provides an explanation on how prolonged usage of omeprazole could lead to hypomagnesemia.

Applications

Acid-peptic disorders, e.g. gastro-oesophageal reflux disease, erosive oesophagitis, heartburn, and Barrett's disease, are chronic diseases that require prolonged omeprazole administration. Therefore, plasma Mg^{2+} assessment should help prevent hypomagnesemia in these patients.

Terminology

The paracellular charge selectivity is a property of epithelium that is selectively permeable to specific charged molecules, e.g. ions. This property is regulated by proteins of the tight junction, i.e. claudins. Alterations in claudin expression in the tight junction directly affect the charge selectivity and the paracellular ion transport across the epithelium.

Peer review

This is an interesting paper investigating the inhibitory action of omeprazole on magnesium transepithelial transport at intestinal level. The study is well-done, the rationale is clear, the experimental design correct, and the results shown convincingly support the conclusions drawn.

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Over-starvation aggravates intestinal injury and promotes bacterial and endotoxin translocation under high-altitude hypoxic environment

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Abstract

AIM: To study whether over-starvation aggravates intestinal mucosal injury and promotes bacterial and endotoxin translocation in a high-altitude hypoxic environment.

METHODS: Sprague-Dawley rats were exposed to hy-

pobaric hypoxia at a simulated altitude of 7000 m for 72 h. Lanthanum nitrate was used as a tracer to detect intestinal injury. Epithelial apoptosis was observed with terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Serum levels of diamino oxidase (DAO), malondialdehyde (MDA), glutamine (Gln), superoxide dismutase (SOD) and endotoxin were measured in intestinal mucosa. Bacterial translocation was detected in blood culture and intestinal homogenates. In addition, rats were given Gln intragastrically to observe its protective effect on intestinal injury.

RESULTS: Apoptotic epithelial cells, exfoliated villi and inflammatory cells in intestine were increased with edema in the lamina propria accompanying effusion of red blood cells. Lanthanum particles were found in the intercellular space and intracellular compartment. Bacterial translocation to mesenteric lymph nodes (MLN) and spleen was evident. The serum endotoxin, DAO and MDA levels were significantly higher while the serum SOD, DAO and Gln levels were lower in intestine ($P < 0.05$). The bacterial translocation number was lower in the high altitude hypoxic group than in the high altitude starvation group (0.47 ± 0.83 vs 2.38 ± 1.45 , $P < 0.05$). The bacterial translocation was found in each organ, especially in MLN and spleen but not in peripheral blood. The bacterial and endotoxin translocations were both markedly improved in rats after treatment with Gln.

CONCLUSION: High-altitude hypoxia and starvation cause severe intestinal mucosal injury and increase bacterial and endotoxin translocation, which can be treated with Gln.

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Key words: High altitude; Hypoxia; Starvation; Intestinal mucosal injury; Bacterial translocation; Endotoxin; Glutamine

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INTRODUCTION

Multiple organs can be damaged by rapid ascent to an altitude above 3000 m. High altitude cerebral edema (HACE) and high altitude pulmonary edema (HAPE) are the clinical manifestations of acute mountain sickness (AMS)^[1-3]. AMS is a threat to those who live at or ascend to a high altitude. Our previous study showed that multiple organ dysfunction syndrome (MODS) can occur if HAPE and HACE are not treated timely and properly, and the condition of such patients can deteriorate, thus complicating their treatment^[4]. However, the mechanism underlying acute severe mountain sickness (ASMS) accompanying MODS is still poorly understood.

The gastrointestinal tract is an important organ, and its primary function is to digest and absorb nutrients. However, in addition to nutrient absorption, the gastrointestinal tract functions as a barrier to prevent the translocation of intraluminal bacteria and endotoxin to systemic organs and tissues. It has been confirmed that intestinal mucosal injury caused by a variety of factors may decrease the intestinal barrier function, thus leading to the translocation of intraluminal bacteria and endotoxin to systemic organs and tissues, which is a principal cause of systemic inflammatory response syndrome (SIRS), MODS and multiple organ failure^[5-7]. However, little is known about the relation between intestinal barrier dysfunction and ASMS accompanying MODS, and whether the intestinal barrier dysfunction caused by hypobaric hypoxia promotes bacterial translocation and spread of endotoxin. In addition, the degree of dysfunction caused by hypobaric hypoxia and the role of acute intestinal barrier dysfunction in the occurrence and development of ASMS are still unclear.

To explore the detrimental effect of hypobaric hypoxia on intestinal barrier function and the role of acute intestinal barrier dysfunction in the occurrence and development of ASMS, we examined the microstructure and ultra-structure of intestinal mucosa from rats exposed to hypobaric hypoxia, under light and electron microscopes. The activity of serum and intestinal diamine oxidase (DAO), malondialdehyde (MDA), superoxide dismutase (SOD) and NO, as well as glutamine (Gln) was assayed. In addition, rats were given Gln intragastrically to explore its protective effect on intestinal mucosa. The results of

this study may provide the functional and morphological information about the effect of ASMS on the spread of endotoxin and bacterial translocation, as well as important information about the pathogenesis, prevention and treatment of MODS caused by hypobaric hypoxia.

MATERIALS AND METHODS

Instruments

Instruments used in the present study were animal decompression chamber (Guizhou Aviation Industry, China), electronic balance (Shanghai Balance, China), ultralow freezer (Heraneus, Germany), microplate reader (Biotek, USA), spectrophotometer (Changsha Persee, China), endotoxin detection system (Tianjin Wireless Electronics, China), scanning electron microscope (Hitachi, Japan) and transmission electron microscope (Philips, Netherlands).

Reagents

Reagents used in this study include compound glutamine granules (Heilongjiang Aolida, China), horseradish peroxidase (Shanghai Guoyuan Biotech, China), 3,3'-dimethoxybenzidine (Sigma, USA), cadaverine dihydrochloride (Sigma, USA), lysine (Sigma, USA), protein kinase (Amresco, USA), levamisole (Sigma, USA), sodium dimethyl arsenite (Shanghai Genebase, China), limulus test kit (Zhanjiang Bokang, China), control standard endotoxin (Zhanjiang Bokang, China), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche, Switzerland), MDA kit (Nanjing Jiancheng, China), NO kit (Nanjing Jiancheng, China), SOD kit (Nanjing Jiancheng, China), glutamine kit (Nanjing Jiancheng, China) and lanthanum nitrate (Chongqi Boyi, China).

Animals and grouping

A total of 40 male Sprague-Dawley rats weighing 200 ± 20 g were purchased from Animal Center of Third Military Medical University (Chongqing, China) and housed in dedicated cages. The rats were randomly divided into normal control group (C), hypobaric hypoxia group (H), hypobaric hypoxia plus starvation group (HH), and hypobaric hypoxia plus Gln group (HG), 10 rats in each group. All procedures were performed in accordance with the Animal Care Guidelines of Third Military Medical University, conforming to the Health Guide for the Care and Use of Laboratory Animals of Third Military Medical University. This study was approved by the Ethics Committee of Third Military Medical University.

Experimental regimen

Rats in the control group were housed under a normal atmospheric pressure and weighed daily, with free access to food. Rats in groups H, HH and HG were exposed to a simulated altitude of 7000 m for 72 h in an animal decompression chamber, to established rat model of hypoxia exposure. Rats in group H had free access to food and water and weighed daily, rats in group HH were fasted with free access to water, and rats in group HG had free access to food and water in addition to intragastric Gln (0.5 g/d per

100 g body weight) at 09:00, am, daily. Gln was prepared in warm water at a dilution of 1:4. The animals were taken out of the chamber for feeding, medication, weighing and cleaning for 30 min, and returned to the chamber for continuous hypoxia exposure.

Three days after exposed to hypobaric hypoxia, the rats were sacrificed by decapitation with 5 mL blood collected into a pyrogen-free tube. The blood was centrifuged at 2500 r/min at -4°C, and serum was stored at -20°C before use. Additionally, the heart, liver, spleen, lungs and mesenteric lymph nodes (MLN) were removed from rats, weighed, and subjected to bacterial culture. About 5 cm of ileum, approximately 5 cm from the ileocecal junction, was obtained for light and electron microscopy. About 3 cm of intestine was put into a 3-fold volume of PBS (0.1 mol/L, pH 7.2), homogenized and centrifuged at 1000 r/min for 30 min. Then, the supernatant was collected.

General observations

The spontaneous activity, mental state, eating status and weight of rats were observed.

Light microscopy

About 2 cm of intestine at 5 cm from the ileocecal valve was obtained and cut open longitudinally. The intestine was washed with normal saline, fixed in 10% formaldehyde at 4°C for 24 h, rinsed with PBS, embedded in paraffin and consecutively cut into 4-μm thick sections which were stained with hematoxylin and eosin (HE). The structure of intestinal mucosal epithelium was observed and the thickness of mucosa was measured^[8]. The height and area of 15 randomly selected intestinal villi were measured and averaged^[9] according to the following equation: $\text{Area} = 2\pi rh$, where r represents the radius of villus and h is the height of villus. The number of villi was counted in each field of vision.

Assay of apoptotic intestinal mucosal epithelial cells

About 2 cm of intestine at 5 cm from the ileocecal valve was obtained and cut open longitudinally. The intestine was washed with normal saline, fixed in 10% formaldehyde at 4°C for 24 h, rinsed with PBS, embedded in paraffin, and consecutively cut into 4-μm thick sections which were mounted onto 0.05% lysine-treated slides. The slides were dried at room temperature for 20 min, dry-heated at 60°C for 30 min and stored at room temperature before use. The sections were stained with TUNEL as previously described^[10]. The cells with blue nuclei were considered positive cells. Two slides of each sample were selected, and 4 randomly selected fields were used to calculate the apoptotic intestinal mucosal epithelial cells at a magnification $\times 400$. The counts were averaged. The apoptotic index (AI) was calculated as follows: number of TUNEL positive cells/number of total cells^[11].

Observation of intestinal mucosa ultra-structure by scanning electron microscopy

Part of the ileum was obtained, rinsed with cold normal

saline, and cut into 2 mm \times 2 mm sections which were fixed in 2.5% glutaraldehyde and 10% osmium, dehydrated in sucrose solution containing PBS. Subsequently, the cells were labeled with gold and observed under a scanning electron microscope^[12]. The arrangement of microvilli in intestinal mucosa and organelles in columnar epithelial cells were observed. Special attention was paid to the deformed and exfoliated villi and the intercellular space between epithelia.

Observation of intestinal mucosa ultra-structure by transmission electron microscopy

Jejunal tissue was cut into 0.7 cm \times 0.7 cm sections and adherent feces were removed from the colon mucosa with saline. The sections were immediately placed into a 4°C fixative (4 g paraformaldehyde, 20 mL glutaraldehyde, 100 mL 0.2 mol/L phosphate buffer solution, and 80 mL distilled water, pH 7.4) for 2 h, and then cut into 1 mm \times 1 mm \times 1 mm pieces. The specimens were washed three times (10 min each) with 10% sucrose phosphate buffer, fixed in 1% osmium tetroxide at 4°C for 1 h, dehydrated, embedded in resin, then cut into ultra-thin sections which were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy and photographed. The tight junctions between intestinal mucosal epithelia, arrangement of microvilli, integrity of columnar epithelial cells, organelles and nuclei, and structure of glands in lamina propria were observed.

Detection of intestinal mucosal injury

Two rats in each group were anesthetized through intraperitoneal administration of 1% sodium pentobarbital (1.0 mL/100 g body weight). Transcardial perfusion was performed with a mixture containing 3% glutaraldehyde, 4% paraformaldehyde and 2% lanthanum nitrate. The mixture was prepared with 0.1 mol/L sodium dimethyl arsenite in PBS. Part of the intestine was obtained and fixed in the same mixture followed by 1% osmium. The tissues were rinsed with 0.1 mol/L sodium dimethyl arsenite solution, and slides were prepared as indicated for transmission electron microscopy^[13].

Detection of bacterial translocation

The blood, heart, liver, spleen, lungs and MLN were independently placed into a 9-fold volume of normal saline, and the mixture was homogenized. Then, 0.5 mL homogenates was loaded onto the MacConkey solid medium to culture bacteria for 24 h, and the bacteria were analyzed biochemically.

Measurement of serum endotoxin level

The serum endotoxin level was measured by limulus test as previously described^[14]. The serum was prepared with warm water, and 0.1 mL serum was mixed with 0.9 mL processing solution and incubated at 70°C for 15 min. Subsequently, 0.2 mL serum was added into the enzyme reaction solution and reacted in the endotoxin detection

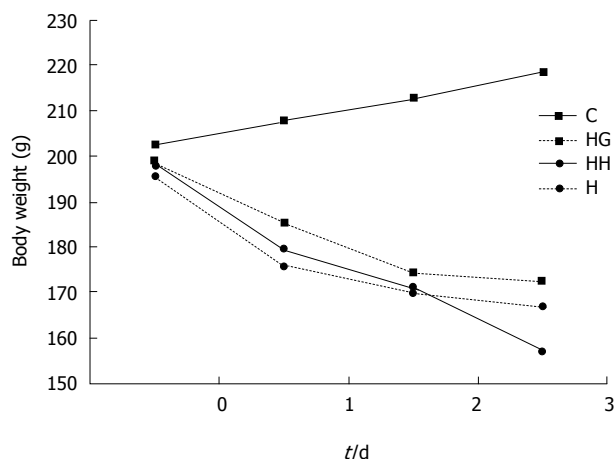


Figure 1 Body weight changes in rats of different groups. The body weight of rats in group C increased stably, and reduced body weight was observed in all rats exposed to hypobaric hypoxia. Decreased body weight was most evident in group HH, followed by groups H and HG. C: Control group; H: Hypobaric hypoxia group; HH: Hypobaric hypoxia plus starvation group; HG: Hypobaric hypoxia plus Gln group.

system EDS-99 for 1 h. The serum endotoxin level was automatically outputted from the system.

Measurement of serum DAO, SOD, MDA, NO, Gln, DAO and Gln levels in intestine

About 0.5 mL serum was mixed with a solution containing 0.1 mol/L PBS (3 mL, pH 7.2), horseradish peroxidase (4 g, 0.1 mL), 3,3'-dimethoxybenzidine (500 g, 0.1 mL) and cadaverine dihydrochloride (175 g, 0.1 mL) and incubated at 37°C for 30 min. The solution was replaced by PBS as a blank control. The optical density (OD) was detected at 436 nm, and the DAO content was measured. The standard curve was delineated with DAO^[15]. About 0.5 mL intestinal homogenates was used to measure the DAO content with the same method. Approximately 0.1 mL serum was used to detect the SOD activity and measure the NO and MDA content as previously described^[16]. The SOD and NO contents were measured using the nitrate reductase activity, the MDA content was measured using the thiobarbituric acid method, and the OD values of Gln were calculated using the Gln synthetic enzymatic method. In addition, the content of proteins in homogenates was measured with Coomassie brilliant blue, and the OD value of Gln was obtained and the Gln content was measured.

Statistical analysis

Statistical analysis was performed using the SPSS version 13.0, and quantitative data were presented as mean \pm SD. One-way ANOVA was used for comparison between groups. Qualitative data were expressed as percentages, and *t* test was used for comparison of means between groups. *P* < 0.05 was considered statistically significant.

RESULTS

General information

No rats died during the experiment. The activity of rats

Table 1 Morphological manifestations of intestinal mucosa in different groups (mean \pm SD)

Group	Rats (n)	Height of villi	Thickness of mucosa	Villous area (m ²)
C	10	242 \pm 19	380 \pm 4	15324 \pm 1027
H	10	228 \pm 23 ^a	296 \pm 7 ^b	13450 \pm 999 ^b
HH	10	200 \pm 24 ^b	314 \pm 7 ^b	11341 \pm 997 ^b
HG	10	215 \pm 23 ^c	370 \pm 9 ^d	12901 \pm 989 ^d

^a*P* < 0.05, ^b*P* < 0.01 vs group C; ^c*P* < 0.05, ^d*P* < 0.01 vs group H. C: Control group; H: Hypobaric hypoxia group; HH: Hypobaric hypoxia plus starvation group; HG: Hypobaric hypoxia plus Gln group.

was markedly lower in groups H, HH and HG with a poor mental state than in control group. The food-intake was significantly higher in group C than in group H (50 g/d vs 23 g/d, *P* < 0.05). The food intake was slight decreased in group HG (38 g/d). The body weight in group C increased stably, and decreased most evidently in group HH, followed by groups H and HG (Figure 1).

Light microscopy

The intestinal mucosa was smooth with intact epithelia and ordered arrangement of villi in group C with no defects detected in villi (Figure 2A). The intestinal mucosa was exfoliated and the villi became thinner in group H. In addition, the number of mucosal villi was reduced, and the villi were irregular and disorganized (Figure 2B). In group HH, atrophic and thinned of villi accompanying a loose and disordered arrangement were observed with edema and infiltration of inflammatory cells in the mastoid lamina of villi, lodged and exfoliated villi with loss of goblet cells and effusion of red blood cells around the capillaries (Figure 2C). In group HG, the intestinal mucosal villi were relatively intact with ordered arrangement with alleviated edema in the mastoid lamina of villi accompanying a few infiltrated inflammatory cells (Figure 2D). The height of intestinal villi and the thickness of mucosa accompanying a decreased villous area were lower in groups H and HH than group C (*P* < 0.05). The height and area of intestinal villi and the thickness of mucosa were significantly higher in group HG than in group H (*P* < 0.05, Table 1).

Scanning electron microscopy

Orderly intestinal villi with a smooth surface and fullness were observed in group C (Figure 3A). The epithelia were atrophic with disordered arrangement of the villi and widened villous spaces in group H (Figure 3B). Villous atrophy and disordered villi with widened villous spaces and exfoliated microvilli were observed in group HH (Figure 3C). The intestinal mucosa was almost intact with orderly villi with few lodged villi, less effusion of red blood cells and no disc-shaped cells and cellulose in group HG (Figure 3D).

Observation by transmission electron microscopy with nitric acid lanthanum tagging

Orderly mucosal villi and integrated tight junctions as

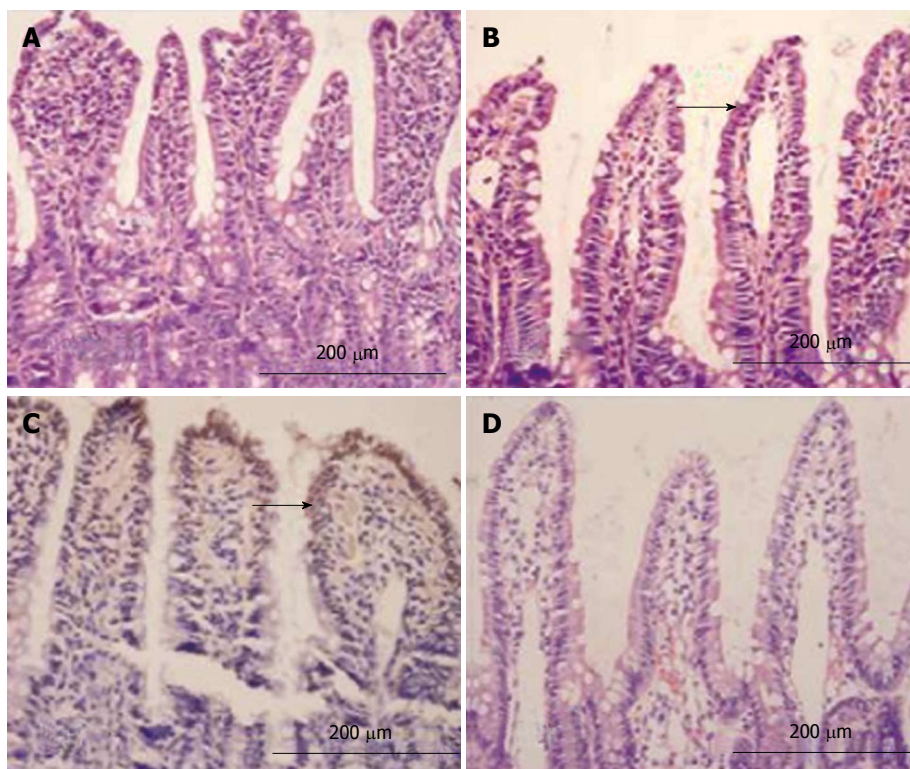


Figure 2 Light microscopy. Smooth intestinal mucosa with intact epithelia and ordered villi in group C (A), exfoliated and incomplete intestinal mucosa with thickened mucosa and reduced villi accompanying irregular morphology and disorganized villous epithelia in group H (B), atrophic and thinned villi accompanying a loose and disordered arrangement as well as edema and infiltration of inflammatory cells in mastoid lamina of villi and lodged and exfoliated villi with loss of goblet cells and red blood cell effusion around the capillaries in group HH (C), relatively intact intestinal mucosal villi with ordered arrangement and alleviated edema in mastoid lamina of villi accompanying a few infiltrated inflammatory cells in group HG (D) (HE, $\times 200$).

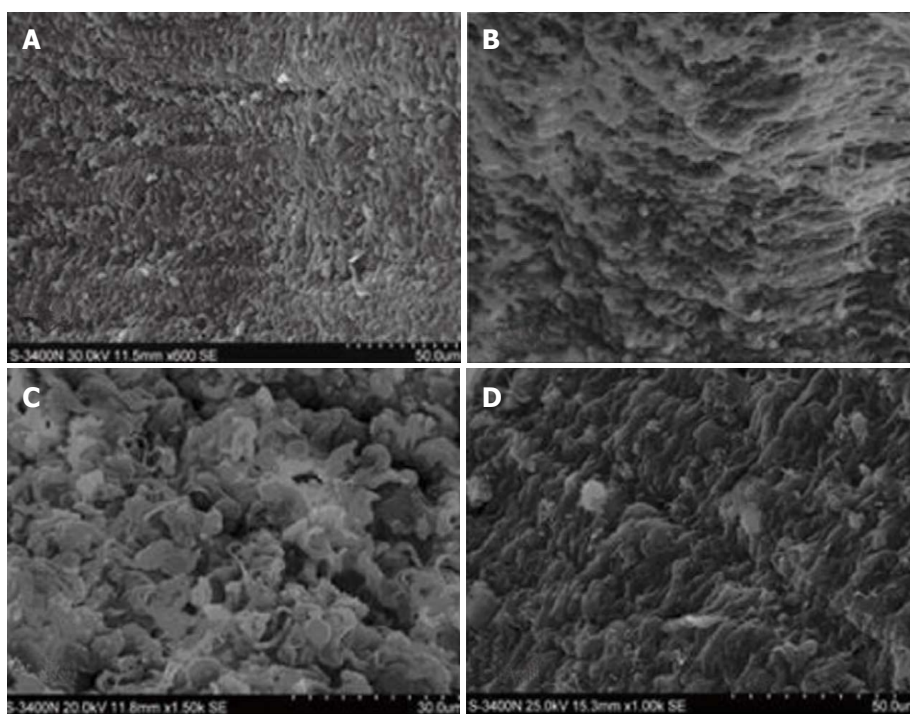


Figure 3 Scanning electron microscopy. A smooth surface of intestinal mucosa with a clear structure as well as complete and orderly villi in group C (A) ($\times 6900$), atrophic epithelial structure of intestinal mucosa and lodging villi accompanying a rough surface with disordered villi and widened villous spaces in group H (B) ($\times 6900$), severely injured intestinal mucosa as well as disc-shaped cells and cellulose in mucosal defects along with evident atrophy and disordered villi with widened villous spaces and exfoliated microvilli in group HH (C) ($\times 11500$), almost intact intestinal mucosa with orderly villi and less effusion but no disc-shaped cells and cellulose in group HG (D) ($\times 11500$).

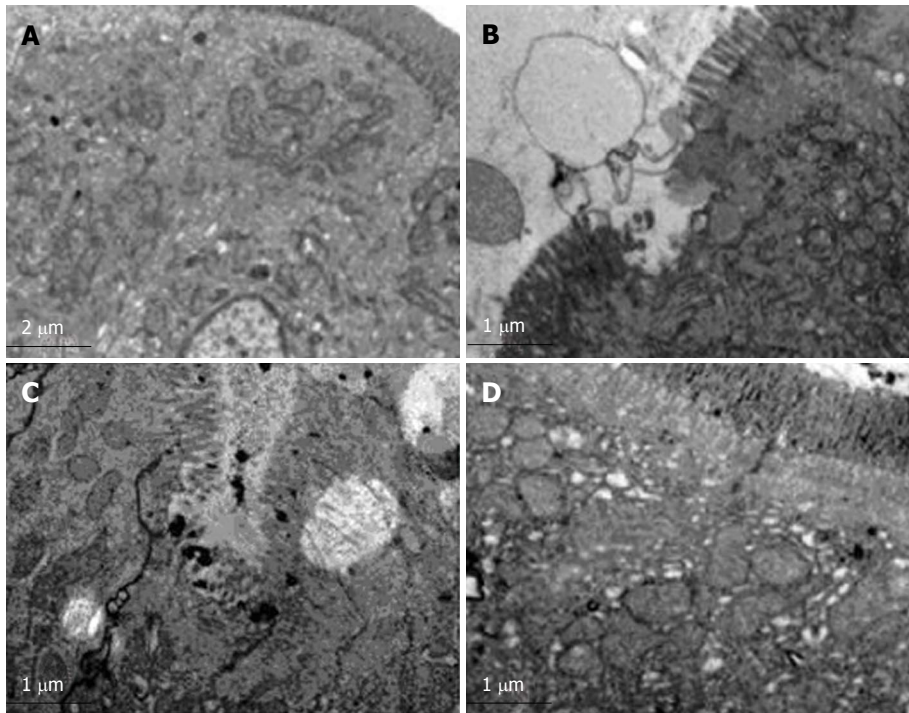


Figure 4 Transmission electron microscopy with nitric acid lanthanum tagging. Orderly mucosal villi and integrated tight junctions as well as intact organelles with regular nuclei in group C (A), exfoliated and incomplete microvilli accompanying widened intercellular spaces as well as swollen endoplasmic reticulum and mitochondria and a small number of lanthanum granules in tissue spaces in group H (B), dilated Golgi complex with irregular nuclei and edge aggregation in chromatin as well as lanthanum granules in the tight junction gap and cells in group HH (C), mildly deformed microvilli and swollen mitochondria in lamina propria accompanying a small number of lanthanum granules confined to vessels and epithelial surface in group HG (D) ($\times 8900$).

Table 2 Bacteria translocation in different organs (mean \pm SD)

Group	Rats (<i>n</i>)	Heart	Liver	Spleen	Lung	Lymph node	Blood	Bacteria translocation
C	10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H	10	0.00	0.00	1.5 \pm 0.85	0.00	1.3 \pm 0.95	0.00	0.47 \pm 0.83 ^a
HH	10	2.5 \pm 0.85	2.9 \pm 1.21	3.1 \pm 1.21	2.6 \pm 1.07	3.2 \pm 1.03	0.00	2.45 \pm 1.36 ^a
HG	10	0.00	0.00	0.7 \pm 0.48	0.00	0.6 \pm 0.52	0.00	0.22 \pm 0.42 ^c

^a $P < 0.05$ vs group C, ^c $P < 0.05$ vs groups H and HH. C: Control group; H: Hypobaric hypoxia group; HH: Hypobaric hypoxia plus starvation group; HG: Hypobaric hypoxia plus Gln group.

well as intact organelles with regular nuclei were observed with no edge aggregation in chromatin and no lanthanum granules in tissue space and cells in group C (Figure 4A). The microvilli were exfoliated and incomplete with widened cellular spaces, swollen endoplasmic reticulum and mitochondria as well as a small number of lanthanum granules in group H (Figure 4B). The Golgi complex was dilated with irregular nuclei with edge aggregation in chromatin and a large number of lanthanum granules in the tight junction gap and cells in group HH (Figure 4C). Mildly deformed microvilli and gland proliferation in the lamina propria and a small number of lanthanum granules were confined to vessels and epithelial surface in group HG (Figure 4D). The number of blue particles was 3.5 ± 1.5 unit/cell/gap in group C, 17.5 ± 2.5 unit/cell/gap in group H, 36 ± 2.7 unit/cell/gap in group HH, and 12 ± 2.1 unit/cell/gap in group HG, respectively.

Detection of bacterial translocation

Negative bacterial cultures were obtained in group C. Bacterial translocation occurred in MLN and spleen of group H and group HH but not in peripheral blood ($P < 0.05$). The translocation organ number of bacteria was the greatest in group HH. The incidence of bacterial translocation was markedly lower in group HG than in group H ($P < 0.05$, Table 2).

Serum DAO, Gln and intestinal homogenate levels in different groups

The serum DAO level was higher in groups H and HH than in group C ($P < 0.05$), and lower in group HG than in groups H and HH ($P < 0.05$). The serum Gln level was lower in groups H and HH than in group C ($P < 0.05$), and higher in group HG than in groups H and HH ($P < 0.05$, Table 3). The intestinal DAO and Gln levels were lower in

Table 3 Serum diamino oxidase and glutamine levels in different groups (mean \pm SD)

Group	Rats (n)	DAO (kU/L)	Gln (mmol/L)
C	10	0.861 \pm 0.359	3.083 \pm 0.186
H	10	3.533 \pm 0.584 ^b	1.472 \pm 0.079 ^b
HH	10	4.991 \pm 0.813 ^b	1.075 \pm 0.150 ^b
HG	10	1.810 \pm 0.450 ^a	1.951 \pm 0.070 ^a

^a*P* < 0.05 *vs* groups H and HH; ^b*P* < 0.01 *vs* group C. DAO: Diamino oxidase; Gln: Glutamine; C: Control group; H: Hypobaric hypoxia group; HH: Hypobaric hypoxia plus starvation group; HG: Hypobaric hypoxia plus Gln group.

Table 4 Intestinal diamino oxidase and glutamine levels in different groups (mean \pm SD)

Group	Rats (n)	DAO (kU/L)	Gln (mmol/L)
C	10	0.516 \pm 0.062	3.083 \pm 0.186
H	10	0.325 \pm 0.053 ^b	1.472 \pm 0.079 ^b
HH	10	0.271 \pm 0.042 ^b	1.075 \pm 0.150 ^b
HG	10	0.431 \pm 0.049 ^a	1.951 \pm 0.070 ^a

^a*P* < 0.05 *vs* groups H and HH; ^b*P* < 0.01 *vs* group C. DAO: Diamino oxidase; Gln: Glutamine; C: Control group; H: Hypobaric hypoxia group; HH: Hypobaric hypoxia plus starvation group; HG: Hypobaric hypoxia plus Gln group.

groups H and HH than in group C (*P* < 0.05) and higher in group HG than in groups H and HH (*P* < 0.05, Table 4).

Serum endotoxin, SOD, MDA, NO and AI levels in different groups

The serum MDA and endotoxin levels were higher in groups H and HH than in group C (*P* < 0.05) and lower in group HG than in groups H and HH (*P* < 0.05, Table 5). The serum SOD and NO levels were lower in groups H and HH than in group C (*P* < 0.05) and higher in group HG than in groups H and HH (*P* < 0.05, Table 5).

DISCUSSION

The normal intestinal barrier function depends on the intact intestinal mechanical, biological, immunological and chemical barriers. The mechanical barrier is the most important. Complete intestinal mucosal epithelium is the dominant component of the mechanical barrier, and the integrity of mucosal epithelium plays a critical role in protection against the spread of endotoxin and bacterial translocation^[17]. The tight junction between adjacent cells, composed of proteins with various functions, can be found between villous and duct epithelia, and plays a crucial role in preventing molecules and ions from passing between cells^[18,19].

Gastrointestinal motility is another component of the intestinal mechanical barrier. The swing of intestinal villi reduces the adhesion of pathogens to mucosal epithelia. Furthermore, intestinal peristalsis pushes the food residue to the distal end and reduces the stay time of bacteria in intestinal mucosa and the chance of bacteria reaching epithelia through the mucous layer, which re-

Table 5 Serum malondialdehyde, nitric oxide, superoxide dismutase and endotoxin levels in different groups (mean \pm SD)

Group	Rats (n)	endotoxin (kEU/L)	MDA (mol/L)	SOD (kU/L)	NO (mol/L)
C	10	0.032 \pm 0.003	6.332 \pm 0.649	146.659 \pm 3.554	31.097 \pm 1.491
H	10	0.277 \pm 0.053 ^b	9.732 \pm 0.675 ^b	66.550 \pm 6.144 ^b	23.397 \pm 1.909 ^b
HH	10	0.582 \pm 0.061 ^b	13.157 \pm 0.848 ^b	46.851 \pm 3.183 ^b	19.586 \pm 1.203 ^b
HG	10	0.113 \pm 0.015 ^a	7.183 \pm 0.497 ^a	119.682 \pm 5.481 ^a	27.584 \pm 1.168 ^a

^a*P* < 0.05 *vs* groups H and HH; ^b*P* < 0.01 *vs* group C. MDA: Malondialdehyde; SOD: Superoxide dismutase; C: Control group; H: Hypobaric hypoxia group; HH: Hypobaric hypoxia plus starvation group; HG: Hypobaric hypoxia plus Gln group; NO: Nitric oxide.

sults in intestinal self-cleaning. The normal flora in intestine forms a biological layer with multiple levels, which comprises the immunological barrier of intestine with no specific immune functions^[20].

The balance between bacterial location, amount and type is critical for the maintenance of intestinal homeostasis. Numerous environmental changes may lead to an imbalance between humans and bacteria, and between different types of bacteria, thus resulting in injury to the intestinal biological barrier^[21]. The immunological barrier is composed of secretory IgA, which is secreted by plasma cells in the lamina propria and lymphoid tissues in intestine. A few Paneth cells consume necrotic cells and secrete several immune substances, which function as an immunological barrier^[22]. Additionally, gastric acid, bile, lysozymes, mucopolysaccharide and proteolytic enzymes form a gastrointestinal chemical barrier that exerts bactericidal effects. Normally, a viscoelastic layer on intestinal mucosa constitutes the chemical barrier, with no specific immunological function. The mucus secreted by goblet cells mainly consists of mucin and its main function is to lubricate the intestinal mucosa, thus protecting the mucosa against mechanical and chemical injury. In addition, the non-specific and specific adhesions between oligosaccharides in mucin and cells interfere with the colonization of opportunistic pathogens.

It has been shown that a variety of factors cause intestinal barrier functional injury, and that hypobaric hypoxia may directly damage mucosal epithelia^[23]. As a result of energy deficiency, the swing of mucosal villi is also compromised, accompanying suppressed intestinal peristalsis, which enhances intestinal absorption. Furthermore, hypoxia may damage aerobic metabolism and increase glycolysis, resulting in intracellular acidosis. Subsequently, the mucosal permeability increases, leading to intestinal barrier functional injury. Intestinal mucosal injury, cytolysis of goblet cells, and reduced amount of mucus caused by hypobaric hypoxia may attenuate the ability of intestinal mucosa to combat gastric acid and pepsin. At the same time, the gastrointestinal vagus nerve is in an excitatory state, which increases the secretion of gastrin, and the gastric acid and pepsin deteriorate the intestinal injury. Hypobaric hypoxia may also reduce secretion of secretory IgG, which compromises the specific immune function of intestine. Moreover, the expressed adhesion molecules of white blood cells and endothelia enhance

the phagocytosis of neutrophils, which release several proteolytic enzymes, thus resulting in intestinal mucosal injury^[24,25]. There is evidence that hypobaric hypoxia can reduce bile secretion and cause disorderliness of enterohepatic circulation, leading to gastrointestinal dysfunction and overpopulation of intestinal bacteria, and further intestinal biological barrier damage. The damaged biological barrier, together with the injured mechanical barrier and increased mucosal permeability, increases the possibility of overproduced bacteria and endotoxin entering parenteral organs through injured mucosa, thus resulting in the spread of endotoxin and bacterial translocation^[26], which is also the basic cause of SIRS.

In the present study, after the rats were exposed to a simulated altitude of 7000 m for 72 h, their food intake was significantly reduced, accompanying weight loss. Light microscopy showed that the intestinal mucosa was exfoliated, and the height of mucosa decreased. The number of villi was reduced, along with their height, accompanying an irregular morphology. The epithelia had different sizes and disordered arrangement. The number of goblet cells was decreased, and a few of them showed signs of degeneration. Electron microscopy revealed atrophic and thinned intestinal villi and disordered epithelia. The villi were incomplete and exfoliated, accompanying widened intercellular spaces. Swollen endoplasmic reticulum and mitochondria and dilated Golgi complex were observed with irregular nuclei and edge aggregation of chromatin. In addition, lanthanum granules were found in intercellular spaces, basement membrane, tissue spaces, and intracellular compartment. At the same time, TUNEL staining showed that the number of apoptotic epithelial cells was significantly elevated after the rats were exposed to hypobaric hypoxia. Under a hypobaric hypoxia environment, starvation might markedly enhance the mucosal injury that leads to exfoliation, atrophy and decreased height of mucosal villi. Vacuolar degeneration was noted in a few epithelia with effusion of red blood cells around capillaries, accompanying infiltration of inflammatory cells. The intestinal mucosal injury was dramatically improved after treatment with Gln. These findings demonstrate that acute hypobaric hypoxia can severely damage intestinal mucosa, thus resulting in intestinal barrier functional injury. In this study, Gln exerted its protective effects on the injured intestinal mucosa to a certain extent.

Damage to the intestinal barrier function may increase mucosal permeability, which leads to bacterial translocation and SIRS. Therefore, detection of bacterial translocation and spread of endotoxin, as well as measurement of some parameters (DAO, MDA, SOD, NO and Gln) related to intestinal function and systemic inflammatory reaction, may directly reflect the intestinal barrier function. The spread of endotoxin and bacterial translocation represent an increased mucosal permeability, which occurs after intestinal mucosal injury. The activity of DAO in mucosal villi may reflect the structure and function of intestine^[27,28]. When mucosal cells are injured and necrotized, DAO is released into the blood or enters the intestinal tract together with necrotic mucosal cells, thus increasing the serum and intestinal tract DAO level and decreasing the DAO levels in intestinal mucosa.

The activity of SOD represents its ability to scavenge free radicals. MDA is the end product of lipid oxidation and indirectly represents lipid peroxidation. NO is an antioxidant. When the intestinal mucosa is damaged, the activity of SOD decreases, accompanying decreased NO and increased MDA contents. It was reported that the serum Gln level is decreased in intestinal mucosa of patients with some critical illnesses. In the present study, the activity of serum DAO and MDA and endotoxin levels were markedly higher in rats exposed to hypobaric hypoxia than in those not exposed to hypobaric hypoxia. However, the activity of DAO and the content of Gln, and the serum NO and Gln levels in intestinal mucosa were significantly lower in rats exposed to hypobaric hypoxia than in those not exposed to hypobaric hypoxia. These changes were more evident in rats after exposed to hypobaric hypoxia and starvation. The serum activity of DAO, MDA and endotoxin was dramatically decreased after treatment with Gln. Moreover, the activity of DAO in intestinal mucosa and the serum NO and Gln levels in intestine were markedly increased. These results suggest that Gln, as an intestinal nutrient, can confer protective effects against intestinal mucosal injury caused by hypobaric hypoxia.

The results of this study show that hypobaric hypoxia can severely injure the intestinal barrier function and increase the intestinal mucosal permeability. In addition, the release of factors involved in SIRS was enhanced accompanying reduced production of protective factors after exposed to hypobaric hypoxia. At the same time, the ability of hypobaric hypoxia to combat lipid peroxidation was reduced. These changes finally resulted in the spread of endotoxin and bacterial translocation. The spread of endotoxin and bacterial translocation, on one hand, activates Kupffer cells in the liver, resulting in the release of numerous cytokines, and on the other hand, leads to endotoxemia, which activates monocytes, macrophages, T and B lymphocytes, and promotes the release of a large number of cytokines, thus resulting in a cytokine cascade^[24]. Additionally, the release of numerous inflammatory mediators, including metabolites of arachidonic acid (prostaglandin E₂, prostacyclin, NO, platelet activating factor, leukotriene and bradykinin), may induce SIRS^[29]. SIRS further promotes the release of inflammatory mediators, which result in inflammatory-mediator-related cascade effects, exacerbation of intestinal mucosal injury^[30], and suppression of intestinal immune function, thus leading to aggravation of bacterial translocation and spread of endotoxin, which are the basis of the later stage of SIRS^[31]. Therefore, the intestinal tract is not only the target organ of SIRS but also the initiator of SIRS^[32], which forms a vicious cycle that results in an endogenous and uncontrollable systemic inflammatory reaction, deterioration of tissue, organ injury, and finally, MODS. Therefore, the damaged intestinal barrier function resulting from hypobaric hypoxia may be one of the important causes of high-altitude MODS.

Both high-altitude hypoxia and starvation may cause severe intestinal barrier function injury, and increased bacterial and endotoxin translocation, but high-altitude

starvation causes more severe intestinal mucosal injury, and bacterial and endotoxin translocation than simple hypoxic exposure. High-altitude over-starvation can aggravate intestinal mucosal injury and promote bacterial and endotoxin translocation, which can be markedly alleviated after intragastric administration of Gln.

COMMENTS

Background

Rapid access to 3000 m above sea level can lead to body function change, and even acute severe mountain sickness (ASMS), which can be life-threatening. High altitude pulmonary edema and high altitude cerebral edema are normal in ASMS. If they are not treated effectively, many people can develop multiple organ dysfunction syndrome (MODS). However, the mechanism for complication of ASMS by MODS is still unclear. It has been demonstrated that gastrointestinal mucosal barrier dysfunction plays an important role in translocation of intestinal bacteria and endotoxin, systemic inflammatory response syndrome (SIRS), and MODS. However, whether high altitude hypoxia can cause gastrointestinal mucosal barrier dysfunction promoting bacterial and endotoxin translocation is currently unknown.

Research frontiers

Studies have shown that high altitude hypoxia can directly cause pathological damage to the intestinal mucosa, and increase intestinal permeability. High altitude hypoxia can reduce secretion of IgG from the gastrointestinal mucosa, decrease the mucosal immune barrier, reduce bile secretion, cause enterohepatic circulation disorders, and destroy the intestinal biological barrier. Intestinal barrier damage can increase intestinal permeability, which results in bacterial translocation and occurrence of SIRS and MODS. Therefore, observation of intestinal translocation of bacteria and endotoxins can indirectly reflect intestinal mucosal barrier function.

Innovations and breakthroughs

In this study, the authors found that high altitude hypoxia altered intestinal barrier function, and increased permeability and bacterial translocation. High altitude hypoxia complicated by excessive hunger can increase damage to the intestinal barrier function and translocation of intestinal bacteria and endotoxins, and induce high altitude MODS. Glutamine has a protective effect on gastrointestinal mucosal injury in the hypoxic environment, reduces intestinal bacterial and endotoxin translocation, and promotes repair of intestinal injury.

Applications

This study has high clinical significance and practical value. First, it reminds people to improve monitoring of gastrointestinal mucosal injury in ASMS. Second, when gastrointestinal mucosal injury is found, glutamine should be administered early.

Terminology

Intestinal mucosal barrier function: It included mechanical barriers, biological barrier, immune barriers and chemical barriers. Mechanical barrier is complete gastrointestinal mucosa to prevent bacterial translocation; biological barrier is the normal intestinal bacteria group in the intestine to form a multi-level special biological layer, a non-specific immune intestinal biological barrier; immune barrier is the intestinal immune barrier lamina propria plasma cells by the secretion of secretory immunoglobulin A (SIgA) and together constitute the gut-associated lymphoid tissue; chemical barrier is the gastrointestinal tract such as gastric acid, bile, lysozyme, mucopolysaccharide and proteolytic enzymes have a certain material form the bactericidal effect of the chemical barrier. Bacterial and endotoxin translocation: Intestinal bacteria and endotoxin from the intestine into other organs or blood when the intestinal mucosal barrier is broken, the body can be an "intestinal" sepsis-like. Systemic inflammatory response syndrome (SIRS): When subject was suffered a variety of damage, and show a high metabolic response. As the body in a high metabolic state, it can increase oxygen consumption; on the other hand, metabolic hyperactivity can enhance the body break down protein, negative nitrogen balance; sugar enhanced anaerobic glycolysis, lactate accumulation, acidosis, eventually leading to tissue failure. Multiple organ dysfunction syndrome: MODS is defined as severe trauma, infection and shock, the original organ dysfunction in patients with no more than two successive system and organ dysfunction.

Peer review

This is a study of the effects of hypobaric hypoxia on intestinal integrity in rats

($n = 40$). Animals exposed to hypobaric hypoxia for 72 h demonstrated histological damage to the small intestine, translocation of lanthanum particles, and increased serum levels of DAO, MDA and endotoxin. This was accompanied by increased translocation of bacteria into lymph nodes and the spleen. Concomitant treatment of rats with glucosamine reduced the severity of intestinal injury.

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Simotang enhances gastrointestinal motility, motilin and cholecystokinin expression in chronically stressed mice

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Abstract

AIM: To investigate the effect of Simotang (Decoction of Four Powered Drugs) on gastrointestinal motility, motilin and cholecystokinin expression in chronically stressed mice.

METHODS: Forty mice were randomly divided into control group, stress group (model group), mosapride group and Simotang group, 10 in each group. A variety of unpredictable stimulations were used to induce chronic stress in mice. Then, the mice were treated with distilled water, mosapride or Simotang for 7 d. Gastric emptying and intestinal propulsion function were detected. Serum level of motilin was measured by enzyme-linked immunosorbent assay. Expression of cholecystokinin (CCK) in intestine, spinal cord and brain of mice was detected by immunohistochemistry and semi-quantitative reverse transcription polymerase chain reaction, respectively.

RESULTS: Simotang improved the gastric emptying

and intestinal propulsion in chronically stressed mice. Furthermore, the serum motilin level was significantly higher and the expression levels of CCK-positive cells and genes were significantly lower in intestine, spinal cord and brain of Simotang group than in those of model group ($P < 0.05$). No significant difference was found in serum motilin level and expression levels of CCK-positive cells and genes between the mosapride and Simotang groups.

CONCLUSION: Simotang enhances the gastrointestinal motility in chronically stressed mice by regulating the serum motilin level and the expression of cholecystokinin.

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Key words: Simotang; Chronic stress; Motilin; Cholecystokinin; Gastrointestinal motility

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INTRODUCTION

Functional dyspepsia (FD), a common functional gastrointestinal disorder, is steadily becoming a public health problem. Its prevalence in the United States is 15%^[1], 11% in the general Italian population^[2], and 12%-25% in China^[3].

The pathogenesis of FD remains unknown, but is likely to involve many factors. In recent years, gastrointestinal motor dysfunction has been considered as the

main pathogenesis of FD^[4]. Gastrointestinal motility is modulated by many hormones and brain-gut peptides. Motilin, a polypeptide hormone containing 22 amino acids, is secreted by endocrine M cells and regulates gastrointestinal motility by increasing the migrating myoelectric complex^[5,6]. cholecystokinin (CCK), a brain-gut peptide, is widely distributed in the gastrointestinal tract and central/peripheral nervous system, and regulates the gastrointestinal motility and food intake^[7,8].

Simotang (Decoction of Four Powered Drugs) is a classical formula that has been used in treatment of gastrointestinal disorders for hundreds of years^[9] and approved as an oral liquid drug by the Chinese National Food and Drug Administration in the 1980s. It has been shown that Simotang can affect the gastrointestinal motility in cold-restraint stressed mice^[10]. Some monomer constituents, such as arecoline^[11] and hesperidin^[12] detected in Simotang, can affect gastrointestinal function. However, the mechanism of Simotang underlying gastrointestinal motility is still unknown. In the present study, we investigated the effect of Simotang on gastrointestinal motility in chronically stressed mice and measured the serum motilin levels, expression of CCK-positive cells and genes in intestine, spinal cord and brain of chronically stressed mice.

MATERIALS AND METHODS

Drugs

Simotang, composed of *Fructus aurantii*, *Radix linderae*, *Radix Aucklandiae*, and *Semen arecae* (specification: 10 mL/division), was purchased from Hunan Hansen Pharmaceutical Company, Ltd (Yiyang, Hunan Province, China). Mosapride citrate was purchased from Chengdu Kanghong Pharmaceutical Company, Ltd (Chengdu, Sichuan Province, China), and dissolved in distilled water to a final concentration of 0.5 mg/mL.

Animals and drug administration

Forty male adult ICR mice weighing 15–22 g were provided by Hunan Slac Jingda Laboratory Animal Company, Ltd (Changsha, China) and randomly divided into control group, stress group (model group), mosapride group and Simotang group. Mice in mosapride and Simotang groups were fed with mosapride citrate (30 mg/kg) or Simotang (1.2 g/kg) consecutively for 7 d from day 21 after stress. Mice in control and model groups received the same volume of distilled water. All animals were housed in a 12 h light and dark cycle (7 Am), in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Mouse model of chronic stress

Mice in model, mosapride and Simotang groups were exposed to chronic stress. A mouse model of chronic stress was induced as previously reported^[13]. In brief, mice were randomly exposed to different stressors daily from day 1 to day 21 as follows: high-speed agitation for 1 min, food deprivation for 48 h, water deprivation for 24 h, tail pinching (2 cm apart from the end of the tail) for 1 min, hung

upside-down for 5 min, heat stimulation at 45°C for 5 min, day and night reversal, and cold stimulation at 4°C for 5 min. Each stressor was repeated twice during the 21 d stress procedure.

Detection of gastric emptying and intestinal propulsion

Gastrointestinal motility was evaluated by testing gastric emptying and intestinal propulsion. Six days after drug or water administration, mice were fasted for 24 h and then administered the last drug or water. One hour later, each mouse administered 0.4 mL semi-solid paste. Twenty minutes later, the mice were sacrificed with 10% chloral hydrate (10.0 mL/kg), and the rate of semi-solid paste in the stomach and the rate of semisolid paste propulsion in the small intestine were measured. The gastric emptying and intestinal propulsion rates in 20 min were calculated according to the following equations^[14]: gastric emptying rate (%) = (semi-solid paste quality-gastric residue quality)/semi-solid paste quality, and intestinal propulsion rate (%) = advanced length of black semi-solid paste/total length of the small intestine × 100%.

Tissue preparation

Intestine, spinal cord and brain were rapidly removed from the mice and immediately fixed with 4% paraformaldehyde for immunohistochemistry. Specimens were cut into 15-μm thick sections with a cryostat and mounted onto silane-coated slides, and dried at 37°C for 60 min. The sections were stored at -70°C. Samples were quickly removed from an environment with a low temperature and immediately stored in liquid nitrogen for RT-PCR.

Measurement of serum motilin level

Serum was collected from the heart of mice after anesthesia, and serum motilin level was measured by ELISA according to its manufacturer's protocol (R&D, USA).

Immunohistochemistry

The sections were incubated with 10 μL normal goat serum at room temperature for 10 min, and treated with rat anti-CCK diluted at 1/100 (Boster Biological Technology, Wuhan, China) at room temperature for 60 min to detect CCK-positive cells in intestine, spinal cord and brain of the mice. The sections were then incubated with biotinylated secondary broad IgG antibody (Zymed Laboratories, CA, USA) for 30 min, reacted with streptavidin peroxidase and aminoethyl carbazole (Zymed Laboratories CA, USA), counterstained with aminoethyl carbazole and observed under an Olympus BX51 microscope (Olympus, Tokyo, Japan). CCK-positive cells were counted under a light microscope (200 × magnification) using the Olympus MicroImage 4.0 software (BX51, Olympus). Ten 200 × microscopic fields per sample were randomly selected from each group, and the cell counts per mm² from 10 fields were averaged.

RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen,

Table 1 Effect of Simotang on gastric emptying and intestinal propulsion rates in chronically stressed mice (mean \pm SD)

Groups	n	Gastric emptying rate (%)	Small intestine advancing rate (%)
Control group	10	48.4 \pm 6.3	54.3 \pm 4.5
Model group	10	62.7 \pm 8.5 ^b	38.2 \pm 3.8 ^b
Simotang group	10	51.6 \pm 7.4 ^d	50.2 \pm 4.7 ^d
Mosapride group	10	52.5 \pm 7.2 ^d	51.5 \pm 4.2 ^d

^b*P* < 0.01 vs control group, ^d*P* < 0.01 vs model group.

CA, USA) according to its manufacturer's instructions. RNA concentration was measured by 1.5% agarose gel electrophoresis. RT-PCR was performed using a TaKaRa RNA PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) and the sequences of primers used are 5'-TCC-GTGCTTCTGCTAATA-3' for CCK forward primer and 5'-CAGCCATCACTGTCTTCC-3' (242 bp) for reverse primer, 5'-AGGGAAATCGTCGTGGAC-3' for β -actin forward primer and 5'-TGGAAGGTGGACAGT-GAGG-3' (443 bp) for reverse primer. The PCR products were electrophoresed on 1.5% agarose gels and analyzed using the GIS-1000 digital gel image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China). Expression levels of CCK mRNA were normalized to those of β -actin mRNA. The experiments were performed at least in triplicate.

Statistical analysis

Data are expressed as mean \pm SD. Statistical analysis was performed by one-way analysis of variance (ANOVA). *P* < 0.05 was considered statistically significant.

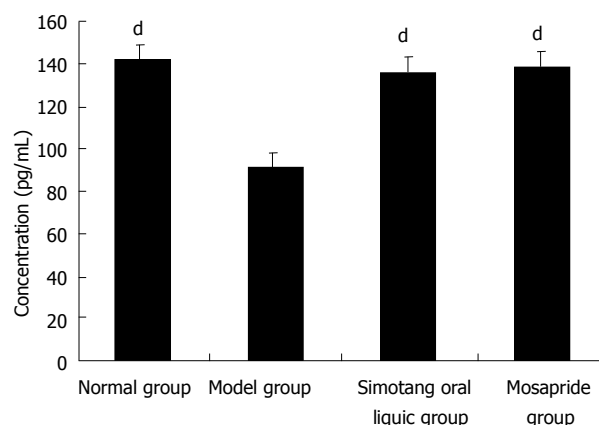
RESULTS

Gastric emptying and intestinal propulsion rates for chronically stressed mice after treatment with Simotang

Whether Simotang affects gastrointestinal motility in chronically stressed mice was observed by evaluating their gastric emptying and intestinal propulsion rates. The gastric emptying and intestinal propulsion rates were significantly lower in model group than in control group (*P* < 0.01, Table 1), suggesting that chronic stress leads to gastrointestinal motility disorder in chronically stressed mice. The gastric emptying and intestinal propulsion rates were significantly higher in Simotang or mosapride group than in model group (*P* < 0.01, Table 1).

Serum motilin level in chronically stressed mice after treatment with Simotang

To determine the potential mechanism of Simotang underlying gastrointestinal motility, the serum motilin levels were measured in chronically stressed mice, showing that the serum motilin levels were significantly lower in model group than in control group and significantly higher after treatment with Simotang or mosapride (*P* < 0.01, Figure 1).

**Figure 1** Effect of Simotang on serum motilin level in chronically stressed mice. Values are expressed as mean \pm SD, *n* = 10/group. ^d*P* < 0.01 vs model group.

Expression level of CCK-positive cells in small intestine, spinal cord and brain of chronically stressed mice after treatment with Simotang

The expression level of CCK-positive cells in control group is shown in Figure 2A-C. The expression level of CCK-positive cell protein was significantly higher in model group than in control group (*P* < 0.01, Figure 2D-F) and remarkably lower in Simotang group (Figure 2G-I) and mosapride group (Figure 2J-L) than in model group (*P* < 0.01, Figure 3).

Expression level of CCK mRNA in spinal cord and brain of chronically stressed mice after treatment with Simotang

The expression level of CCK mRNA was significantly higher in model group than in control group and significantly lower in Simotang and mosapride groups than in model group after treatment with Simotang (*P* < 0.01, Figure 4).

DISCUSSION

FD, a meal-related and pain-predominant symptom in the absence of organic disease according to the Rome III criteria^[15], is considered hazardous to health because of its high prevalence and recurrence rates. Although its etiology and pathogenesis have not been clearly identified, a wide variety of pathogenetic mechanisms are involved. For example, gastrointestinal motor abnormality is thought to be an important mechanism underlying FD^[16]. It has been reported that 20%-40% of FD patients have delayed gastric emptying and altered duodenojejunal motility^[17,18]. With the development of neural gastroenterology in recent years, it has been shown that gastrointestinal motility is modulated by the central nervous system, autonomic nervous system and enteric nervous system (brain-gut axis) through hormones or brain-gut peptides, and that external stimuli can affect the motility or sensation of the gastrointestinal tract through the brain-gut axis^[19]. Psychological factors are considered another important etiology of FD,

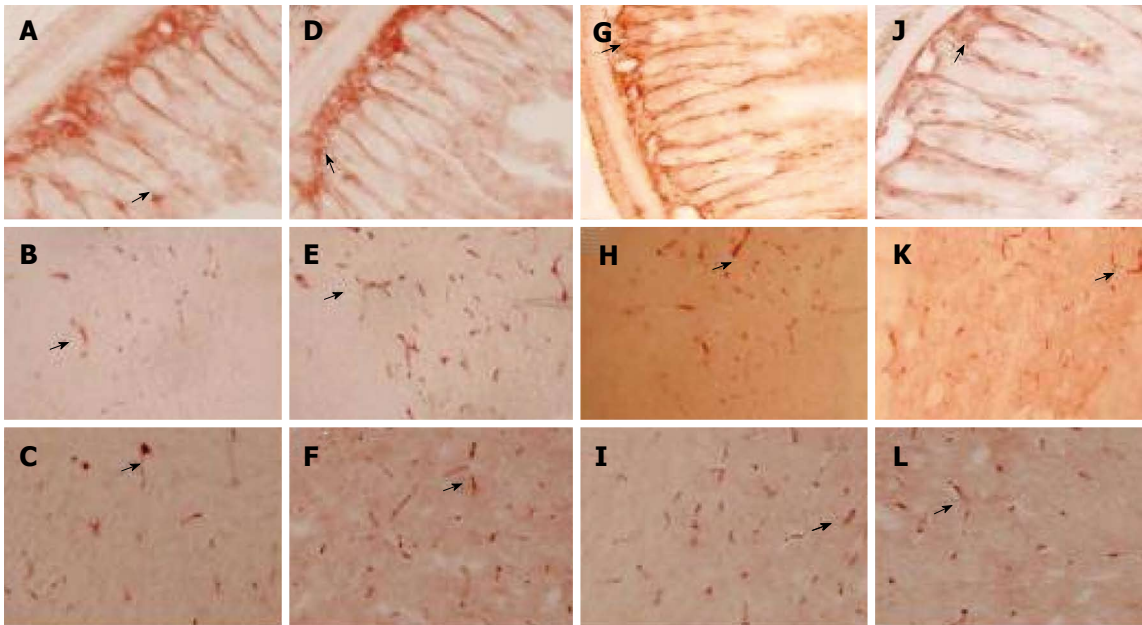


Figure 2 Effect of Simotang on expression of cholecystokinin-positive cells in the small intestine, spinal cord and brain of chronically stressed mice with immunostaining with cholecystokinin in small intestine (A, D, G and J), spinal cord (B, E, H and K) and brain cortex (C, F, I and L) in control group (A-C), model group (E, F), Simotang group (G-I) and mosapride group (J-L). Magnification $\times 200$.

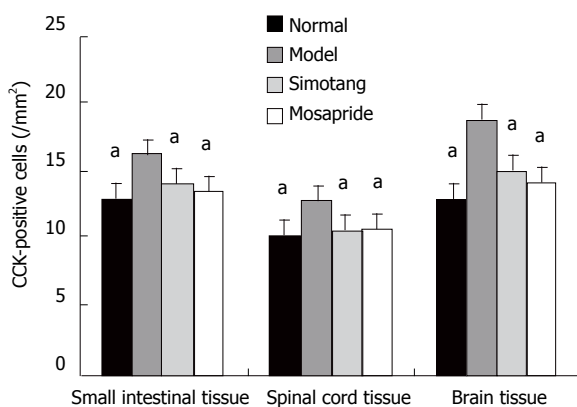


Figure 3 Effect of Simotang on the number of cholecystokinin-positive cells in the small intestine, spinal cord and brain of chronically stressed mice (mean \pm SD, $n = 5$ /group). ^a $P < 0.05$ vs model group.

and FD is currently considered to be a biopsychosocial disorder^[20]. The proportion of psychosocial factors, such as depression and anxiety, is higher in FD patients than in healthy people, and psychosocial factors also affect gastric motility^[21]. Stressed animals are often used as a model of FD. In the present study, the gastrointestinal motility was lower in chronically stressed mice than in unstressed control mice, which is consistent with the reported findings^[22].

Motilin, a hormone secreted by endocrine cells in the duodenal mucosa, interacts directly with its receptor, induces smooth muscle contraction and improves peristalsis in the small intestine^[23,24]. Its concentration is closely related with gastric emptying^[25]. Although the role of motilin in the pathogenesis of FD has not yet been determined, the plasma motilin concentration is lower in FD patients than in healthy people^[6]. FD patients have a similar proximal gastric motor response to motilin as healthy volunteers^[24]. In

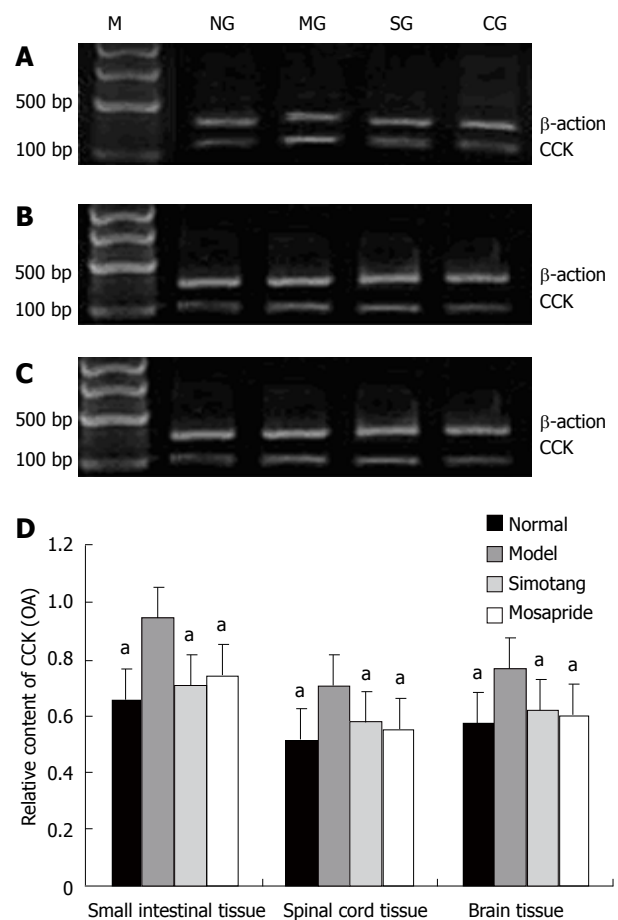


Figure 4 Effect of simotang on cholecystokinin gene expression in the small intestine (A), spinal cord (B), brain (C) of chronically stressed mice, and relative optical density of cholecystokinin RNA in small intestine, spinal cord and brain of chronically stressed mice (D). Values are expressed as mean \pm SD ($n = 5$ /group). M: Marker; NG: Control group; MG: Model group; SG: Simotang group; CG: Mosapride group. ^a $P < 0.05$ vs model group.

the present study, the serum motilin level was significantly lower in stressed mice than in unstressed control mice and significantly higher after treatment with Simotang.

CCK is not only a gastrointestinal hormone, but also a neurotransmitter which is widely distributed in both the enteric and central nervous systems^[7,8]. As an established brain-gut peptide, CCK transfers signals between the gut and central nervous system, plays an important role in regulation of gastrointestinal function, and is linked to the etiology of stress-related anxiety disorders. CCK not only inhibits gastric motility and emptying^[26], but also is related to food intake and satiety^[27]. It has been shown that the CCK levels are higher in FD patients than in controls^[28]. Exogenous CCK can suppress the appetite and gastric emptying in healthy volunteers^[29,30]. In the present study, the CCK protein and mRNA levels were increased not only in the small intestine but also in the spinal cord and brain of chronically stressed mice, and the CCK expression level was higher in the brain than in the small intestine and spinal cord of chronically stressed mice. Furthermore, the CCK expression level was higher in the small intestine, spinal cord and brain of chronically stressed mice after treatment with Simotang.

In conclusion, Simotang affects the gastrointestinal motility in chronically stressed mice by regulating the serum motilin level and expression of CCK, which provides a pharmacological basis for its clinical application in treatment of functional gastrointestinal disorders.

COMMENTS

Background

Simotang has been used in treatment of gastrointestinal disorders for hundreds of years but its mechanism is unknown. Dysfunction of the brain-gut axis is thought to be involved in the pathogenesis of functional dyspepsia.

Research frontiers

As one of the brain-gut peptides, cholecystokinin (CCK) not only affects gastric motility and emptying, but also plays an important role in the pathogenesis of Functional dyspepsia (FD). However, how CCK is regulated by Simotang still remains unknown. In the current study, the authors demonstrated that chronic stress could inhibit gastric emptying and intestinal propulsion by investigating the mechanism of Simotang underlying gastrointestinal motility.

Innovations and breakthroughs

The results of this study show that Simotang can improve gastric emptying and intestinal propulsion in chronically stressed mice by regulating the serum motilin level and expression of cholecystokinin in the small intestine, spinal cord and brain of chronically stressed mice.

Applications

By understanding the mechanism of Simotang underlying FD and providing a pharmacological basis for Simotang, it can be used in treatment of functional gastrointestinal disorders.

Peer review

The authors investigated the effect of Simotang on the expression of motilin and cholecystokinin, showing that Simotang can improve gastric emptying and intestinal propulsion in chronically stressed mice by regulating the expression of motilin and cholecystokinin, thus providing a pharmacological basis for the clinical application of Simotang in treatment of functional gastrointestinal disorders.

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Epidemiological trends and geographic variation in hospital admissions for diverticulitis in the United States

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Abstract

AIM: To characterize the increasing incidence and geographic variation of acute diverticulitis.

METHODS: Using the nationwide inpatient sample (NIS) we identified a cohort who had been admitted with diverticulitis between 1998 and 2005. We calculated age-, sex-, and region-specific rates of hospitalizations for diverticulitis over time.

RESULTS: The age-adjusted hospitalization rate for diverticulitis increased from 61.8 per 100 000 to 75.5 per 100 000 between 1998 and 2005, and increased similarly in both sexes. Diverticulitis-associated admissions were male-predominant in those younger than age 45 years but were female-predominant thereafter. Admission rates increased the most among those < 45 years, while remaining unchanged for those ≥ 65 years. By 2005, the majority of hospitalized patients were < 65 years. Age-adjusted rates of diverticulitis-associated

hospitalizations were lower in the West (50.4/100 000) compared to the Northeast (77.7/100 000), South (73.9/100 000), and Midwest (71.0/100 000).

CONCLUSION: Diverticulitis-associated hospitalizations have steeply risen, especially in young adults. These epidemiological trends vary by geographic region and warrant further investigation into potential dietary and environmental etiologies.

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Key words: Diverticulitis; Geographic variation; Hospitalization; Young adults

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INTRODUCTION

Diverticular disease of the colon is among the most prevalent conditions in Western society and is among the leading conditions for outpatient visits and hospitalizations^[1,2]. The prevalence of diverticular disease increases with age, occurring in less than 10% of those who are younger than 40 years and being as high as 66% in those older than 80 years^[3]. Between 10%-25% of individuals with colonic diverticula will develop diverticulitis^[4], of which a quarter can develop life threatening complications such as obstruction, perforation and intraperitoneal abscess formation^[5].

There is significant geographic variation in the preva-

lence of diverticular disease, occurring much less commonly among Asians compared to western populations^[4]. This observation has led to the theory that low-fibre diets may contribute to the development of colonic diverticula^[6]. Additionally, there is emerging evidence that obesity and body mass index may also be predisposing factors^[7,8].

Though diverticular disease is generally thought to be a disease of older adults, there are increasingly common reports of diverticulitis in individuals younger than 50 years^[9]. Based on single-centre reports, these cases were often male-predominant^[10] accompanied by a more aggressive disease course^[8,11]. Using the nationwide inpatient sample (NIS), we sought to ascertain nationwide trends in hospitalizations for diverticulitis particularly among cases of younger onset and to evaluate for geographic variations in hospitalization rates within the US.

MATERIALS AND METHODS

Data source

All data were extracted from the NIS between 1998 and 2005. The NIS is maintained as part of the Healthcare cost and utilization project (HCUP) sponsored by the Agency for healthcare research and quality (AHRQ). These databases reflect a 20% stratified sample of non-federal, acute-care hospitals in the United States. The sampling frame includes community and general hospitals and academic medical centers comprising approximately 90% of all hospital discharges in the United States. Hospitals were grouped into 60 strata based on five hospital characteristics: US census region, location (urban versus rural), teaching versus non-teaching status, ownership (non-federal private or public), and bed-size (tertiles). Each data entry included a unique identifier, demographic variables (defined as age, gender, and race/ethnicity, median income for ZIP code), discharge disposition, primary and secondary diagnoses (up to 15), primary and secondary procedures (up to 15), primary insurance payers, total hospital charges, and length of stay. NIS data concurs with the National hospital discharge Survey, supporting data reliability^[12].

Eligibility criteria

Our analysis included all hospital discharges between the years of 1998 and 2005 that were admitted with a primary or secondary diagnosis of diverticulitis as identified by Clinical Modification of the international classification of diseases, 9th revision (ICD-9-CM) codes (562.11, 562.13) and with a length of stay greater than 1 d.

Statistical analysis

Data were analyzed using the Stata 10.0 SE software package (Stata Corp LP, College Station, Texas). Analyses took into account the stratified two-stage cluster design using stata's SVY (survey data) commands and incorporating individual discharge-level weights. Weighting functions using these hospital and discharge weights were applied to the 20% NIS sample to estimate the total number hospitalizations for diverticulitis throughout the US stratified.

Table 1 Demographics of admissions for acute colonic diverticulitis

	Geographic region (%)			
	Northeast	Midwest	South	West
Age group				
15-24 yr	< 1	< 1	< 1	< 1
25-44 yr	17	15	15	16
45-64 yr	38	35	36	37
≥ 65 yr	44	50	49	46
Sex				
Male	42	40	39	43
Female	58	60	61	57
Race ¹				
Non-hispanic white	86	93	80	76
Black	6	5	9	4
Hispanic	6	< 1	9	15
Other	2	2	2	3

¹Based on admissions with race data which was missing in 25%.

The primary outcome was rate of hospitalization for diverticulitis in the US population. We used the US resident population census from 1998 to 2005 published by the US Census Bureau as the denominator for rate calculations. We calculated age-adjusted rates with the direct standardization method using the US standard population from 2000. We also calculated age-specific and sex-specific rates of hospitalization for diverticulitis and then calculated the percent increase for each year of the study relative to the baseline rate in 1998 within each age group. These analyses were repeated stratified by the US geographic regions: the Northeast, West, South, and Midwest.

Ethical considerations

The analysis of the Nationwide inpatient sample uses completely unidentified data with no risk of loss of confidentiality and an initial expedited review by the Institutional Review Board of the Johns Hopkins Medical Institutions deemed it exempt from further ethical review.

RESULTS

Demographics of diverticulitis admissions

There were 323 097 hospital admissions for acute diverticulitis in the NIS database between 1998 and 2005. The demographic characteristics of the study population are shown in Table 1 stratified by geographic region. There was a greater proportion of diverticulitis admissions in patients who were 65 and older in the Midwest (50%) and the South (49%) compared to the Northeast (44%) and the West (46%). There was a greater percentage of minority Blacks and Hispanics in the South and the West (Table 1).

Epidemiological trends

The rate of hospitalization increased with age in both males and females as shown in Figure 1. Diverticulitis admissions were male-predominant in those younger than 44 years with male-to-female ratio that is as high as 2.8 among those between those 25 and 34 years. For those

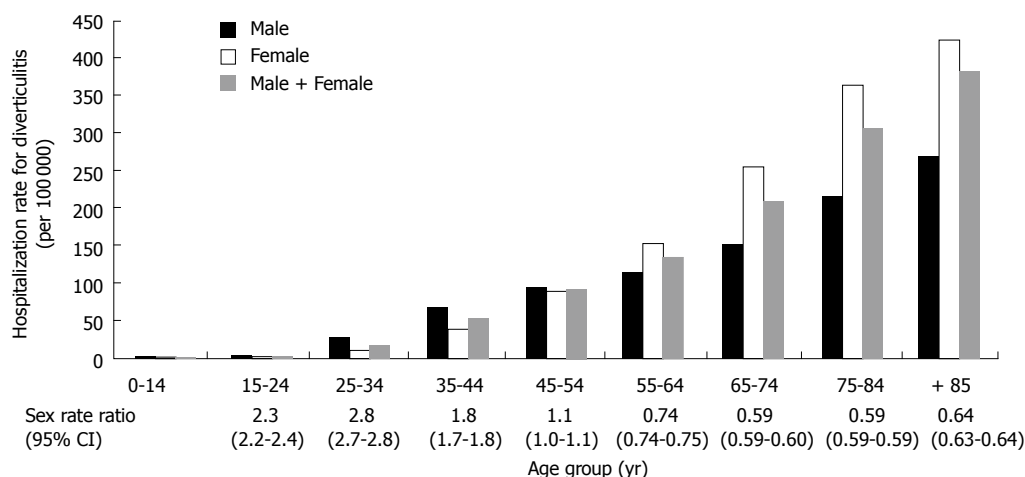


Figure 1 Age-specific hospitalization rates for diverticulitis stratified by sex in the US. Males are shown in black, females in white, and both sexes in gray. Male-to-female ratios with 95% confidence intervals (95% CI) are shown below each age subgroup.

Table 2 Age- and sex-specific time trends in hospitalization rates for colonic diverticulitis

	Calendar year hospitalization rates for diverticulitis (per 100 000)							
	1998	1999	2000	2001	2002	2003	2004	2005
15-24 yr								
M	1.4	1.7	1.8	2.2	2.6	3.2	3.4	3.2
F	0.5	0.6	0.9	1.1	1.3	1.3	1.4	1.6
B	1.0	1.1	1.3	1.6	1.9	2.3	2.5	2.4
25-44 yr								
M	33.4	38.5	44.1	47.3	54.4	55.0	55.7	57.6
F	16.6	18.9	23.3	24.2	28.1	28.5	30.8	31.7
B	24.7	28.7	33.7	35.7	41.2	41.9	43.4	44.8
45-64 yr								
M	84.4	90.1	98.9	97.9	107	109	108	117
F	99.7	99.3	110	114	125	122	126	132
B	92.3	94.9	105	106	116	116	117	125
65+ yr								
M	181	178	180	189	187	187	182	191
F	322	298	317	329	331	331	323	330
B	264	249	261	271	272	271	265	272

M: Males; F: Females; B: Both sexes.

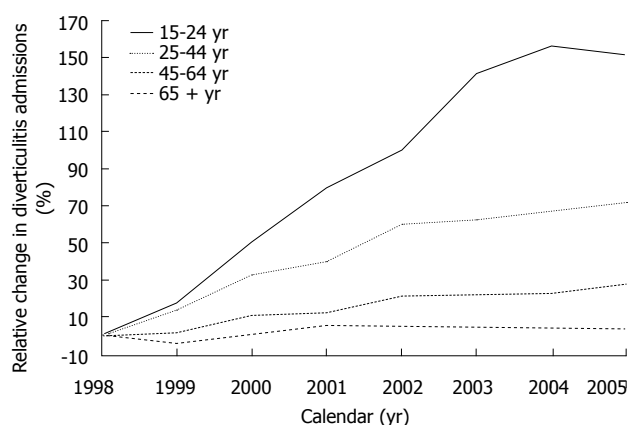


Figure 2 Relative increase in hospitalization rates for diverticulitis in the US between 1998 and 2005, compared to the reference year (1998) stratified by age group.

between 45 and 54 years of age, hospitalizations for diverticulitis were similar between sexes, while after age 54 years, it became increasingly female-predominant.

Table 2 shows the age- and sex-specific rates of hospitalization for acute diverticulitis during the 8 year study period. Age-adjusted diverticulitis-associated hospitalizations increased in both males (52.8 to 59.2 per 100 000) and females (67.3 to 79.6 per 100 000) and across all age groups. The overall age-adjusted hospitalization rate for diverticulitis increased from 61.8 per 100 000 to 75.5 per 100 000 during the study period. Figure 2 depicts the relative percentage increase in hospitalization rate for each age group relative to the baseline rate in 1998. This relative increase was inversely proportional with age, being sharpest in those between 15-24 years (150% overall increase), and gradually less with a 70% increase in those aged 25-44 years, 30% increase in those 45-64 years, and approaching 8% in those

Table 3 Age- and region-specific time trends in rates of hospitalization for colonic diverticulitis

	Calendar year rates of hospitalization for diverticulitis (per 100000)							
	1998	1999	2000	2001	2002	2003	2004	2005
15-24 yr								
N	1.4	1.8	1.6	1.8	2.2	2.7	2.9	2.9
M	0.6	0.9	1.2	1.7	1.9	2.1	2.1	2.7
S	1.0	0.9	1.2	1.8	2.1	2.4	2.6	2.3
W	0.9	1.2	1.6	1.2	1.4	1.9	2.2	2.0
25-44 yr								
N	33.5	39.6	43.4	44.3	55.0	54.2	58.3	65.3
M	24.8	27.4	31.4	37.2	41.9	42.5	44.6	48.2
S	24.9	28.5	33.8	36.7	41.6	43.1	43.3	43.6
W	17.0	20.1	26.3	24.7	27.2	28.2	29.5	26.6
45-64 yr								
N	106	114	125	126	138	137	147	155
M	85	86	102	108	119	123	115	130
S	105	107	111	113	122	122	119	128
W	66.8	67.3	78.2	73.3	83.1	81.4	89.7	87.6
65+ yr								
N	245	256	275	260	283	276	267	291
M	271	254	265	298	299	299	285	296
S	304	277	284	302	288	292	290	286
W	206	184	200	199	200	198	196	203

N: Northeast; M: Midwest; S: South; W: West.

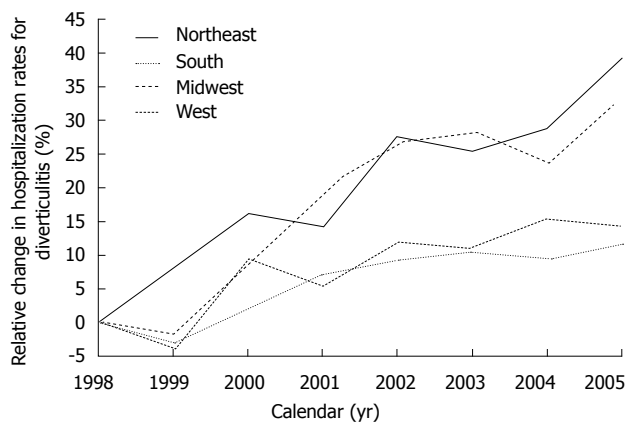


Figure 3 Relative increase in hospitalization rates for diverticulitis in the US between 1998 and 2005, compared to the reference year (1998) stratified by geographic region.

aged 65 years and older. In 1998, the proportion of admissions for diverticulitis in patients who were older than 65 years was 55%. Over the 8 year study period, that percentage decreased to 44%, with the majority of patients being under the age of 65 years by 2005. Those who were under the age of 45 years comprised 12.7% of all admissions for diverticulitis in 1998 and increased to 16.3% in 2005.

Geographic variation in diverticulitis admissions

Table 3 shows age-specific and region-specific rates of admissions for diverticulitis. There was significant geographic variation in overall and age-specific growth rate of hospitalizations for acute diverticulitis. The overall age-adjusted rates of diverticulitis-related hospitalizations were 77.7 per 100000 for the Northeast, 71.0 per 100000

for the Midwest, 73.9 per 100000 for the South, and 50.4 per 100000 for the West. The age-adjusted incidence rate ratio for diverticulitis-associated admission in the West compared to the Northeast was 0.60 (95% CI, 0.59-0.60). There was significant variation in the region-specific proportional change in hospitalization rates relative to the baseline rate in 1998, as shown in Figure 3. The sharpest increases were observed in the Northeast (64.8 to 90.2 per 100000; overall 39%) and the Midwest (60.5 to 74.8 per 100000; overall 33%). The trends were more moderate in the South (69.9 to 76.5 per 100000, overall 11%) and in the West (46.7 to 53.3 per 100000, overall 14%).

There was also regional variation in rising incidence of hospitalizations for diverticulitis among those younger than age 45 years. Rates increased the most in the Northeast (from 24.2 to 44.7 per 100000, overall 85%) and Midwest (from 17.2 to 32.6 per 100000, overall 90%). The rise in hospitalizations in patients younger than 45 years was more moderate in the South (from 17.5 to 29.9 per 100000, overall 71%) and was lowest in the West (from 12.1 to 18.4 per 100000, overall 53%).

DISCUSSION

Our nationwide analysis has demonstrated geographic variations in the burden of diverticulitis and underscores rapidly increasing rates of diverticulitis-associated hospitalizations among individuals younger than age 45 years. These findings have implications for understanding the underlying etiology of diverticulitis as well as for the timely diagnosis of this condition in younger individuals. The rising epidemiological trends and geographic variations in diverticulitis-associated admissions, particularly

among younger individuals, may correlate with observed temporal changes and regional differences in diet and obesity in America.

Diverticular disease is an age-related disorder of the large bowel affecting greater than half of the population over the age of 65 years^[13]. Current evidence suggests that dietary deficiency (of fibre), colonic pressure, motility changes and colonic structural alterations may collectively contribute to diverticula formation^[4]. Parallel epidemiological trends of decreasing dietary fibre and increasing diverticular disease have led to a hypothesized role of fibre deficiency in the pathogenesis of diverticular disease^[6,14]. A large prospective study of 43 888 US male health care professionals^[15] found that a decreased intake of insoluble dietary fibre, specifically fruits and vegetables, was associated with increased symptomatic diverticular disease. Similarly, obesity has also been linked to higher incidence of diverticulitis and diverticular bleeding^[7,8]. It is hypothesized that adipose tissue secretes numerous cytokines that are known to participate in local and generalized inflammation and may play a role in the development of diverticulitis^[16].

Our study showed that the overall rates of acute diverticulitis hospitalizations increased in the US in the last decade, which has been previously reported in the US^[9] as well as in the UK^[17] and Finland^[18]. An epidemiological study of adults aged 40–74 years has also shown a rise in obesity, decreased physical activity, and decreased fibre intake which may all contribute to the increasing incidence of diverticular disease^[19]. Similarly, a decline in fibre intake among children and 3-fold rise in childhood obesity in the US over the last 3 decades may also partially explain the sharp rise in admissions for diverticulitis among younger age groups^[20,21].

The higher prevalence of obesity in the South and Midwest may correlate with our findings of higher rates of diverticulitis admissions in those regions compared to the West. Data from the Center for Disease Control showed that in 1991, the Midwest and the South had higher obesity rates compared to the Northeast and West, and this difference had persisted in the ensuing decade 2000^[22]. Data from NHANES-III also showed that BMI was greater in the South and Midwest compared to the other regions^[23]. Childhood obesity has been similarly shown to be most prevalent in the South ($\geq 18\%$) while being least prevalent in the West (11.4%)^[20].

Regional variation in diet may also contribute to geographic differences in diverticulitis admissions. Based on self-reported data, residents from the South consumed more fatty acids and the least amount of fibre, while those from the West consumed higher amounts of fibre than other regions^[23]. One could hypothesize that there may be a protective association between higher fibre intake in the West and corresponding relatively lower age-adjusted rates of diverticulitis admissions. However, the roles of dietary fibre and obesity in geographic variations in diverticulitis remain speculative, and do not explain high rates of diverticulitis admissions in the Northeast. Thus, other environmental and health systems-based factors may be involved. Racial and ethnic differences in risk of diverticulitis may

also contribute to geographic variations in hospital admissions for diverticulitis, particularly in the West, where there is a higher composition of Hispanics and Asians. There is evidence that these ethnic groups may be at lower risk for diverticulitis^[24,25]. Unfortunately, because nearly 25% of racial and ethnic data is missing in the NIS database, we were not able to readily determine race- and ethnic-specific rates in diverticulitis admissions.

Our current study has several limitations inherent to administrative data analyses. The NIS data set does not contain personal identifiers, which does not allow linkage to medical records in order to validate ICD-9 codes for diverticulitis. However, we would not expect the degree of errors in administrative coding to be different with respect to time or age. Thus, this type of non-differential misclassification usually leads to conservative estimates of temporal trends. Additionally, this study evaluated only rates of hospitalization, and would not have included milder cases of diverticulitis managed in an outpatient setting. Furthermore, this is a cross-sectional study and we are unable to longitudinally follow patients after discharge to assess long-term mortality and morbidity such as recurrent diverticulitis or surgery.

Despite these limitations, this nationally representative analysis has demonstrated geographic variations in epidemiological trends in the burden of diverticulitis that will hopefully stimulate hypotheses into the aetiology of diverticular disease. Prospective studies are needed to determine if there is an association between diverticulitis and obesity, dietary intake, and other environmental factors, particularly among younger adults. From a clinical perspective, these findings drive the need for increased vigilance for diverticular disease among younger adults presenting with abdominal pain.

COMMENTS

Background

The incidence of diverticular disease increases with age. Between 10%–25% of individuals with colonic diverticula develop diverticulitis. Recent epidemiologic studies have shown a male predominant increase in diverticulitis in those under 45 years.

Research frontiers

The nationally representative analysis indicates that the burden of diverticulitis and the time trends in admissions vary by geographic region and may be associated with a decreased intake of insoluble dietary fibre and increasing obesity.

Innovations and breakthroughs

The steep rise in diverticulitis admissions among young adults is striking. From a clinical perspective, these findings drive the need for increased vigilance for diverticular disease among younger adults presenting with abdominal pain.

Applications

Prospective studies are needed to determine if there is an association between diverticulitis and obesity, dietary intake, and other environmental factors, particularly among younger adults.

Peer review

While this study is not entirely unique, it is well-written and would make a useful addition to the current literature on diverticular disease.

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Is spleen circulation impaired in systemic sclerosis and what is the role of liver fibrosis?

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doppler ultraSound, transient elastography of liver and nailfold capillaroscopy were the main outcomes.

RESULTS: Transient elastography values of SSc patients were similar to those of controls; 5.2 ± 1.1 vs 4.5 ± 1 , ($P = 0.07$). Median Alanine amino transferase (ALT) concentrations of cirrhotic patients were greater than those of controls and SSc patients, i.e. 66.5 (36-89) U/L vs 29 (22-34) U/L and 31 (22-41) U/L, respectively, ($P = 0.005$). SARI determinations in cirrhotic patients, although significantly higher than those found in controls and SSc patients, showed some degree of overlap with SSc patients, i.e. 0.59 vs 0.52 and 0.57, respectively, ($P = 0.04$). Mean systolic blood pressure was significantly higher in SSc patients than in cirrhotics and controls, i.e. 142 mmHg vs 128.2 mmHg and 127 mmHg, respectively, ($P = 0.005$). Mean diastolic blood pressure behaved in a similar fashion, i.e. 84 mmHg vs 72.2 mmHg and 76.9 mmHg ($P = 0.005$). Nailfold Capillaroscopy grades and diastolic blood pressure values correlated well with SARI results.

CONCLUSION: An enhanced resistivity of the splenic artery was found in patients suffering from SSc; they did not have evidence of splenomegaly as well as no liver fibrosis or any other form of liver damage.

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Abstract

AIM: To investigate the spleen vascular involvement and the presence of liver fibrosis in a population of subjects with established systemic sclerosis (SSc).

METHODS: In a cross-sectional fashion, 17 patients with SSc were compared with 18 patients suffering from hepatitis C virus (HCV)-related liver cirrhosis, grade A and B Child-Pugh classification. Eighteen non elderly subjects, apparently healthy, were used as the control group. Splenic artery resistivity index (SARI) at

Key words: Splenic artery resistivity index; Doppler ultra-sound; Transient elastography; Nailfold capillaroscopy; Systemic sclerosis; Liver fibrosis

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INTRODUCTION

Systemic sclerosis (SSc) is a disease characterized by a complex interplay of inflammation, fibrosis and vascular damage. In fact, the arterial microvessels of SSc display rarefaction and mural thickening, including medial smooth muscle and intimal cell hyperplasia^[1]. Vasculopathy, showing surprising similarities in various areas, i.e. pulmonary, renal, cerebral and peripheral, induces pulmonary arterial hypertension (PAH), scleroderma renal crisis, severe cerebral vasculopathy and digital tip ulcers. Endothelin-1 is a potent mediator of vascular features in SSc^[2], but immunological mechanisms also participate in SSc pathogenesis. They include a Th1/Th2 imbalance in CD4+ T cells^[3], an increase of type 2 cytokine-producing T cells in target organs^[4] and the presence of autoantibodies with fibroblast-activating capacities^[5]. Finally, caveolin-1 has been thought to regulate the signaling of transforming growth factor- β ^[6], a key determinant in SSc. In fact, the core lesion of SSc is the uncontrolled fibrosis involving internal organs and derma.

There are some *a priori* reasons, mainly indirect cautions, for predicting a reasonable incidence of hepatic fibrosis in patients with SSc. The hepatic mesenchymal tissue would be expected to share enhanced synthesis and/or deposition of collagen. The liver having a double blood supply, playing a central role in the induction of immune tolerance and also being a target for immune-mediated damage, it could actively participate in systemic diseases. Surprisingly, the extensive literature on SSc provides no definitive indications of the incidence and nature of associated hepatic fibrosis in the direct, the indirect (hepatotoxicity) or the fortuitous (occult viral infection and associated autoimmune or metabolic co-morbidities) types. As the “companion” organ of the liver, the spleen plays an important role in splanchnic hemodynamics, beyond its established immunologic functions^[7]. This occurs through changes in intrasplenic microvascular tone and through reflex activation of the splenic afferent sympathetic nerves^[8]. Actually, it is recommended to perform a biopsy examination to assess the liver participation in SSc^[9,10]. However, what about unravelling hepatic fibrosis when ethical and legal issues do not permit invasive tests? Systematic reviews have demonstrated that evaluation of liver stiffness by transient elastography (TE) is clinically useful for the diagnosis of various grades of fibrosis in the course of chronic liver diseases^[11], even though it is better at excluding than at predicting cirrhosis^[12]. Parameters of doppler ultrasound (DUS) are reckoned to be the new biomarkers of the liver-spleen circulatory axis^[13,14], by way of substituting the estimation of hepatic venous pressure gradient (HVPG) that is an expensive technique^[15] based on catheterism. Among these hemodynamic indices SARI has one of the closest

correlations to HVPG < 12 (non-severe portal hypertension)^[16]. On the other hand, enlargement of spleen may be associated with portal hypertension-related events in most patients with advanced chronic liver disease^[17]. Thus, hypothesizing a diffuse vascular injury^[18], and considering that mechanisms similar to those of SSc play a role in cirrhotic portal hypertension^[19,20], it is conceivable that a hemodynamic alteration of the spleno-portal axis could be present in SSc patients. Therefore, in a population of subjects with established SSc, the presence of liver fibrosis was investigated by TE and assessment of the spleen vascular involvement was carried out by DUS; the results of DUS were compared with those found in subjects with an advanced chronic liver disease characterized by a hyperdynamic circulation and increased resistance in the hepatic and spleen vascular bed. Furthermore, we tried to track the possible association between DUS findings and other vascular parameters such as blood pressure and nailfold capillaroscopy (NFC).

MATERIALS AND METHODS

Patients

Seventy-two subjects formed the initial study population. Twenty-six consecutive patients who fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for SSc^[21], having been classified as having limited cutaneous (lc) SSc or diffuse cutaneous (dc) SSc according to the criteria described by LeRoy *et al.*^[22], were selected. Three SSc patients were disallowed as they were on penicillamine therapy. Six other patients were excluded, two of whom due to contextual HCV infection, one to alcohol abuse and three to non-alcoholic fatty liver disease (NAFLD) presence in diabetics. The remainder (17 patients), who were on low doses of prednisone \leq 10 mg and vasodilators, acted as the SSc group. Mean duration of disease was 3.5 years.

Twenty-six patients suffering from HCV-related liver cirrhosis, genotype 1b, grade A and B Child-Pugh classification, were selected to be compared with the SSc patients. They were diagnosed on the basis of liver biopsy (8 cases), the remainder on the grounds of appropriate tests. Briefly, the presence of cirrhosis was established by appropriate clinical (spider nevi, hepato-splenomegaly), laboratory (low serum total cholesterol, prothrombin activity and pseudocholinesterase levels, reduced white blood cell and platelet count, globulin/albumin ratio > 1) as well as imaging data of the liver (coarse echo-texture, nodularity presence, increased caudate/right lobe ratio, hypertrophy of the left lobe, characterized by a rounded inferior marginal edge, and portal vein enlargement with decreased flow velocity, absence of a normal doppler waveform, hepatofugal flow). Eight patients were excluded as they were cryoglobulinemic and likely suffering from vasculitis. Eighteen patients were finally considered as forming the cirrhotic group. A cohort of non elderly subjects (18 cases), apparently healthy, was used as a control group. Laboratory data and instrumental measurements were strictly

carried out within one month of each other. The protocol was consistent with the principles of the Declaration of Helsinki, and participants gave their informed consent. The study had no external funding source.

Transient elastography

Liver stiffness was assessed by means of a Fibroscan (Echosens, France). At least 10 measurements per patient were obtained, using the standard probe. These were averaged and reported as kPa. A success rate of at least 60% was considered necessary. In our population, special care was taken in order to make sure there was no A-shaped wave on the elastogram, which indicates an incorrectly accepted (non-automatically rejected) measurement, leading to an overestimation of the stiffness produced by influence of the surrounding rib bone and soft tissue.

Doppler ultrasonography

Spleen longitudinal diameter (SLD) was performed by postero-lateral scanning at US, using a Tecnos (EsaOte, Italy). The Maximum Length (ML, the optically greatest overall longitudinal dimension obtained from one of the two poles) and the Cranio-Caudal Length (CCL, the optically maximal transversal dimension intercepting one of the two poles) were measured; the resulting values were then averaged, since the two measurements do not always coincide. SLD (ML+CCL/2) was also measured in controls to set reference intervals as mean values of TE.

SARI values at DUS were automatically calculated with the following formula: RI = peak systolic velocity-end systolic velocity/peak systolic velocity. The probe was positioned below the left costal arch or in the left costal spaces. Color Doppler allowed identification of the main branches of the splenic artery. Antihypertensive drugs were withdrawn at least three days before the test.

Nailfold capillaroscopy

All nailfold capillaroscopy (NFC) procedures were performed in a stereomicroscope (Olympus-SZ40) under 10-20x magnification according to the protocol proposed by Andrade *et al.*^[23]. All the ten digits of the hands were examined, except when prevented by extremely poor visibility. The following parameters were analyzed: (1) number of capillary loops/mm; (2) vascular deletion score; (3) number of enlarged loops (about four times the normal afferent, transition, and efferent limb widths); and (4) number of giant capillary loops (10 or more times the normal width of capillary limbs). Enlarged and giant loops were counted together. The vascular deletion score was assessed according to a well-known method^[24], in which a deletion area is defined as the absence of two or more consecutive loops. Each finger was rated from 0 to 3: grade 0, no deletion area; 1, one or two discrete deletion areas; 2, more than two discrete deletion areas; and 3, extensive and confluent deletion areas. For each patient the NFC parameters were calculated as the average obtained in all analyzed digits.

Transthoracic echocardiography

Because transthoracic echocardiography (TTE) is a reproducible method, it was used as a non-invasive diagnostic tool to determine pulmonary arterial pressure and helped us exclude any secondary causes of PAH in SSc patients with unexplained dyspnea. According to a peak velocity of tricuspid regurgitation (VTR) ≥ 3.0 m/s at TTE, which is consistent with international guidelines^[25], PAH was diagnosed. Once the diagnosis of PAH was established, together with several parameters (ventricular function, exercise parameters, peak oxygen uptake or peak systolic blood pressure), NYHA functional classes were selected to predict prognosis in these patients.

Blood pressure measurements

The systolic/diastolic blood pressure (SBP, DBP) was the average of three consecutive readings taken by the physician during the day, during usual practice hours, after subjects had rested for five minutes in the sitting position.

Laboratory data

ALT activity was determined by in-house standard procedures. Sera samples were tested for anti-nuclear antibodies (ANA) using indirect immunofluorescence and HEp-2 cells as antigen substrate (Antibodies Inc., Davis, CA). Anti-centromere antibodies (ACA) were determined by their distinctive IIF pattern on HEp-2 cells. Autoantibodies to topoisomerase I (ATA) were determined by passive immunodiffusion against calf thymus extract (Inova Diagnostics, San Diego, CA).

Statistical analysis

Liver stiffness, age, SARI, SLD, systolic blood pressure and diastolic blood pressure data derived from a normally distributed population (Shapiro-Wilk test (S-W), $P = 0.94$; S-W, $P = 0.98$; S-W, $P = 0.23$; S-W, $P = 0.064$; S-W, $P = 0.26$; S-W, $P = 0.11$, respectively) were expressed as mean plus SD. A variable not normally distributed, such as ALT (S-W, $P = 0.001$) was expressed as median (range). NFC grades were considered ordinals and managed in the same way. The difference of means was evaluated by Two-Sample t test, two-tailed probability. Frequencies were evaluated by χ^2 , or in case of more than two groups by χ^2 for trends. Tracking the degree of association between single parameters, i.e. NFC scores and SARI determinations, Spearman's rho for non uniform intervals was used. The Pearson's coefficient (r) was employed to analyze the correlation between blood pressure values and SARI determinations. One-way analysis of variance (ANOVA) with the Student-Newman-Keuls test for all pairwise comparisons was performed to examine differences among groups; when dealing with a variable not normally distributed the Kruskal-Wallis test with post-hoc analysis was adopted. To predict the presence of PAH, logistic regression (enter method), with relative odds ratio (OR) and 95% confidence intervals (CI), was employed, utilizing as independent variables ATA positivity. Following the clinical and laboratory standards

Table 1 Demographic, clinical and instrumental data of the population (mean \pm SD)

Variables	SSc	Liver cirrhosis	Controls	P-value
The number of patients	17	18	18	
Age (yr)	47.9 \pm 12.6	49.7 \pm 9.5	44.4 \pm 9.8	0.060
Females	15	16	14	0.380 ¹
Type diffuse/cutaneous	12/5	ND	ND	
GI involvement	9	0	ND	0.003
PAH presence	ND	3	ND	
C-P score A/B	ND	12/6	ND	
SLD at US (mm)	102.3 \pm 9.7	130.4 \pm 4.6	100.9 \pm 6.6	0.000
SARI at DUS	0.56 \pm 0.06	0.59 \pm 0.02	0.52 \pm 0.01	0.040
TE kPa	5.2 \pm 1.1	ND	4.5 \pm 1	0.070
NFC grade 0/1/2/3	3/7/2/5	ND	ND	
Hypertension	6	0	0	0.001 ¹
ALT (U/L), median (range)	31 (22-41)	66.5 (36-89)	29 (22-34)	0.005
ATA/ACA/ANA positivity	3/4/2010	ND	ND	

PAH: Pulmonary arterial hypertension; ATA: Anti-topoisomerase I antibodies; ACA: Anti-centromere antibodies; ANA: Anti-nuclear antibodies; GI: Gastro-intestinal motility disorders; DUS: Doppler ultrasonography; US: Ultrasonography; SLD: Spleen longitudinal diameter at US; ALT: Alanine amino-transferase; TE: Transient elastography; NFC: Nailfold capillaroscopy; SD: Standard deviation; C-P: Child-Pugh classification; ND: Not determined; ¹: χ^2 for trend.

institute 2008 guidelines (C28-A3) for smaller samples, the 90% CIs were estimated to set our normal range of TE and SLD at US. The concordance correlation coefficient (ρ_c), which measures precision and accuracy, was adopted to evaluate the degree of pair observations at US. Statistical analysis was performed operating on medcalc version 10.4.8[®] (Frank Schoonjans) software package.

RESULTS

In order to allow readers to gauge the internal validity of this study, we emphasize that the minimal required sample size, with a type I error of 0.05 and power of 68%, when breaking the population into two groups (i.e. controls and SSc patients) by SARI values (means = 0.56 and 0.52 with a pooled SD of 0.06), was calculated in 18 subjects. Additionally, to weigh how well the study findings apply to the patients (external validity) we stress that the fifty-three subjects, divided into three cohorts well balanced for gender and age, were studied in a cross-sectional fashion.

Demographic, clinical and instrumental characteristics of the full population are represented in Table 1. TE values of SSc patients were similar to those of controls, 5.2 ± 1.1 *vs* 4.5 ± 1 , $P = 0.07$, two-sample *t* test, two-tailed probability.

Median ALT concentrations of cirrhotic patients were greater than those of controls and SSc patients, i.e. 66.5 (36-89) U/L *vs* 29 (22-34) U/L and 31 (22-41) U/L, respectively, $P = 0.005$, Kruskal-Wallis with post-hoc analysis (Figure 1).

In cirrhotic individuals, SLD showed values superior to those of control subjects and SSc patients, i.e. 130.4 mm *vs* 100.9 mm and 102.3 mm, respectively, $P = 0.0001$, ANOVA with Student-Newman-Keuls test (Figure 2). In contrast, SARI determinations in cirrhotics, although significantly higher than those found in controls and SSc patients, showed some degree of overlap with SSc patients, i.e. 0.59

vs 0.52 and 0.57, respectively, $P = 0.04$, ANOVA with Student-Newman-Keuls test (Figure 3). Successively, we failed to find much higher values of SARI in the SSc subjects suffering from PAH, this being present in only three out of 17 individuals (18%), at least at the time of observation.

Mean systolic blood pressure was significantly higher in SSc patients than in cirrhotics and controls, i.e. 142 mmHg *vs* 128.2 mmHg and 127 mmHg, respectively, $P = 0.005$, ANOVA with Student-Newman-Keuls test (Figure 4). Mean diastolic blood pressure behaved similarly, i.e. 84 mmHg *vs* 72.2 mmHg and 76.9 mmHg, respectively, $P = 0.005$, ANOVA with Student-Newman-Keuls test (Figure 5).

Associations and prediction

There was a substantially good association between SARI measurements and NFC grades, i.e. Spearman's $\rho = 0.51$, $P = 0.04$ (Figure 6).

Diastolic blood pressure values correlated well with SARI results (i.e. Pearson's $r = 0.57$, $P = 0.01$); meanwhile, systolic blood pressure did not (i.e. Pearson's $r = 0.25$, $P = 0.05$).

SARI data were not correlated with SLD measurements, both in cirrhotics and SSc patients (i.e. Pearson's $r = -0.20$, $P = 0.43$ and Pearson's $r = 0.19$, $P = 0.45$, respectively). At univariate analysis, ATA positivity did not predict PAH presence, OR 1.5, 95% CI: 0.1 to 20.7, $P = 0.7$.

Agreement

The intra-observer and inter-observer errors at US and DUS, measured as concordance correlation coefficients, were found more than acceptable, i.e. $\rho_c = 0.89$ and 0.88, respectively.

Reference Intervals

The non-parametric percentile methods of TE and SLD yielded the following normal ranges, i.e. 2.4-6.1 kPa and 83-110 mm, respectively.

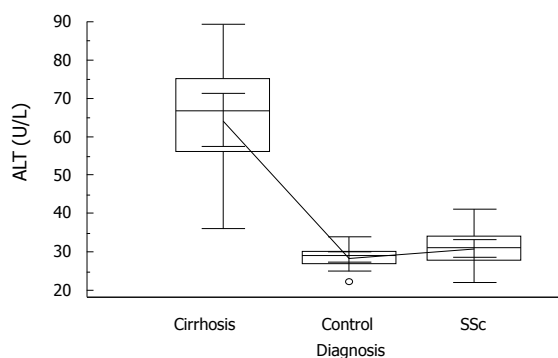


Figure 1 Behavior of alanine amino transferase activity in the various groups. Cirrhotic patients clearly showed a median value higher than that of scleroderma (SSc) patients and control subjects, both of whom were characterized by values within normal range.

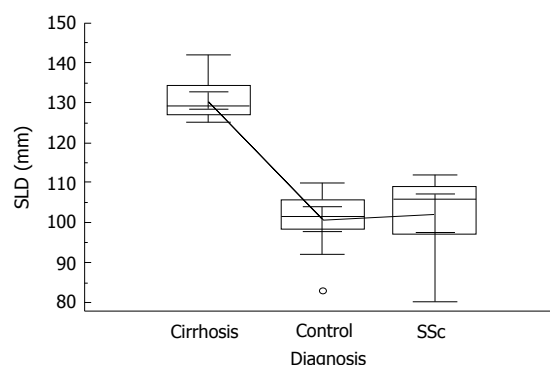


Figure 2 Behavior of spleen longitudinal diameter in the various groups. As is evident, cirrhotic patients showed a mean value significantly higher than that of scleroderma (SSc) and control subjects, both of whom were characterized by normal values.

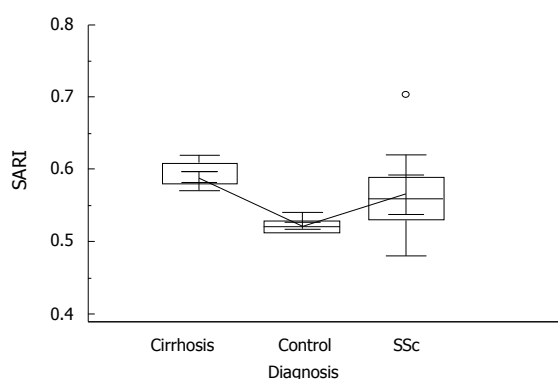


Figure 3 Behavior of splenic artery resistivity index in the various groups. Scleroderma (SSc) patients clearly showed a mean value significantly higher than that of control subjects, whose values were within the normal range, but significantly lower than that of cirrhotic patients and somewhat overlapping with them.

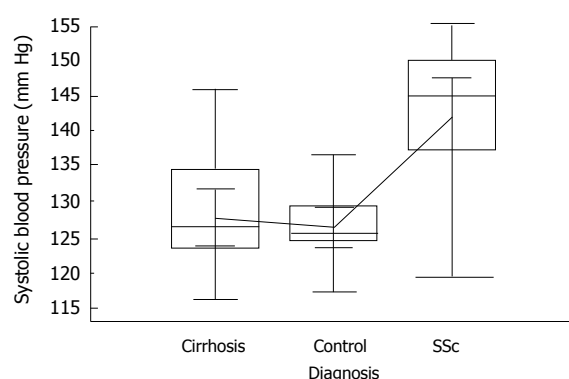


Figure 4 Behavior of systolic blood pressure, expressed in mmHg, in the various groups. Scleroderma (SSc) patients clearly showed a mean value significantly higher than that of control subjects and cirrhotic patients, both groups showing values within the normal range.

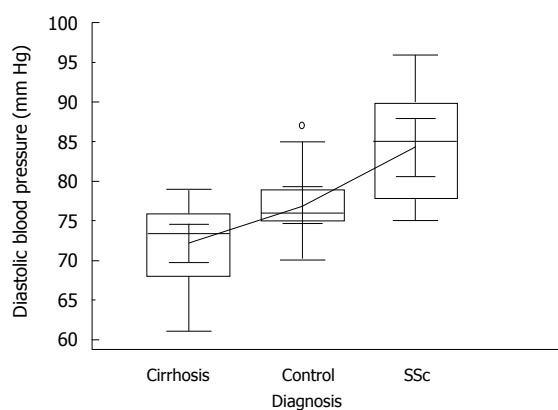


Figure 5 Behavior of diastolic blood pressure, expressed in mmHg, in the various groups. Scleroderma (SSc) patients clearly showed a mean value significantly higher than that of control subjects, but a little overlapping, as were cirrhotic patients; both groups showing values within the normal range.

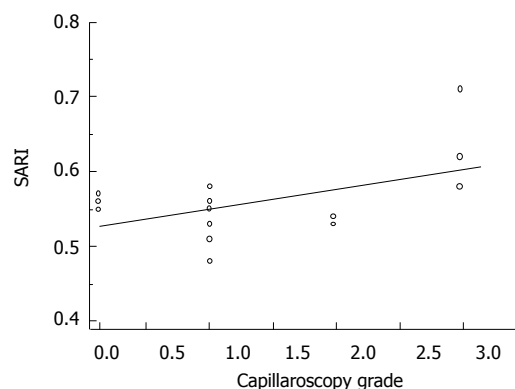


Figure 6 Good correlation between nailfold capillaroscopy grades (0-1-2-3) and splenic artery resistivity index in scleroderma patients.

DISCUSSION

To provide a brief synopsis of key findings, we stress the following: (1) an enhanced resistivity of the splenic artery was found in patients suffering from SSc; and (2) they did not demonstrate splenomegaly as well as liver

fibrosis or any other form of liver damage.

Our data agree with the body of present knowledge that provides evidence for the idea that, in addition to inflammatory infiltrates and an accumulation of extracellular matrix proteins, vascular changes are a hallmark in the pathogenesis of SSc. Consistent with the systemic vascular damage (the vasoactive endothelin-1 system?) we find important evidence, i.e. the strict association between SARI

values and the severity of features at NFC and between the same DUS parameter and diastolic blood pressure values. Surprisingly, SARI values were somewhat overlapping with those found in patients suffering from liver cirrhosis.

Considering possible discrepancies of our findings, such as why SARI does not keep up with increased spleen volume in SSc, we hypothesize that high values of this parameter are due to an intrinsic spleen vascular damage and not to portal hypertension as canonically present in cases of marked liver fibrosis following chronic liver injury. This divergence requires a distinct involvement of the two organs, i.e. spleen and liver in SSc; the former characterized by a vascular change and the latter apparently not damaged. However, what is the significance of the splenic artery in determining the increased spleen volume in the cirrhotic patients group? It is believed that passive venous congestion is the major cause of splenomegaly in advanced chronic liver disease, even though blood hyper-afflux plays an important part. Our data do support the concept of venous congestion. In fact, there was no correlation between spleen size and SARI measurements in cirrhotics, shedding further light on the mechanisms of portal hypertension in these patients with a quite compensated form.

Returning to the possible deposition of connective tissue in liver, beyond the reliance on a preliminary impression that has basis in the fragmentary published data, hepatic involvement in SSc is generally considered rare. As previously emphasized, treatment with potentially hepatotoxic drugs, coincident viral chronic hepatitis or NAFLD have usually been implicated as the main causes of liver disease in patients with connective tissue diseases. However, even after careful exclusion of these etiologies, the question remains whether to classify the patient as having a primary liver disease with associated autoimmune disorder or as having liver disease as a manifestation of generalized connective tissue disease. Now, when addressing the hepatic entanglement in our cohort of SSc patients, the absence of liver damage had been sufficiently documented, at least until the study period, even if it could not be rejected with certainty, due to the lack of histology. Obviously, if we take into consideration that nearly all the hepatic co-morbidities were excluded, we would end up in reinforcing the hypothesis that the vascular changes of the spleen are not linked to a concomitant chronic liver disease. It is therefore natural to ask whether the normal volume of spleen could play a role in explaining the absence of a liver disease. First of all, it is necessary to stress that a light-moderate chronic injury could not always be linked to an increased spleen volume. On the other hand, normal spleen size excludes an idiopathic portal hypertension complicating SSc^[26].

Commenting on drawbacks of the present study and the methods used to compensate for the main ones, we pinpoint that, although liver biopsy remains the 'best standard' to assess liver fibrosis presence, limitations are considerable, including patient discomfort and rare, but serious, complications such as bleeding or pneumothorax and a mortality rate of 1/10000 to 1/12000. Moreover, only 1/50000 of the liver volume is investigated, resulting in

sampling error^[27]. Furthermore, this tool, representing an 'instant snapshot' of a dynamic process, does not reflect a long-lasting assessment. For this reason, other authorities employ substitute 'gold standards' as relate to the natural history of the disease. Alternative attempts to diagnose and follow-up the collagen deposition in liver range from routine biochemistry (AST/platelets calculated ratio, APRI) to surrogate fibrosis markers in serum^[28], hepatic clearance tests, various imaging techniques and, more recently, the use of non-invasive TE. The reported values of this tool give a good correlation with various markers of fibrosis and increase proportionally with the progression of the hepatic fibrosis stage^[29]. There are no evidence-based data justifying biopsy as a first line estimate of liver fibrosis. Health authorities in some countries have already approved validated biomarkers as the first line procedure for the staging of liver fibrosis^[30]. However, HVPG should have been employed in our SSc cohort to exclude increased portal hypertension, but this is based on an invasive technique and X-ray exposure; thus, heavy ethical restrictions did not permit its use in our patients without evidence of serious liver chronic diseases. In this respect, DUS examination is gaining widespread consensus in order to provide patients, suspected of having portal hypertension, with important diagnostic/prognostic information. Moreover, it is important to pinpoint the lack of data from TE in cirrhotic patients; but this tool, as previously emphasized, is very good at excluding advanced chronic disease, not at confirming its presence. Final flaws could be the small sample size due to the fact that this study was carried out using "strict" inclusion criteria and the fact that SSc is a heterogeneous disease; clinical presentations are highly variable among the patients. This may result in a wide range of SARI in patients with SSc. We should have analyzed which disease subset (diffuse *vs* limited cutaneous SSc, short *vs* long disease duration, or the presence *vs* absence of individual organ involvement) was associated with high SARI in SSc, although the number of patients enrolled was too small to perform sub-analysis.

In conclusion, as we have revealed spleen vascular changes, the crucial future research direction should zero in on other vascular areas, such as retinal artery^[31] or renal artery^[32], by means of the simple and reliable tool that is DUS, to evaluate to what extent vasculopathy is represented in SSc. If these data are confirmed and expanded in a larger population, physicians will be advised to investigate the spleen circulation at the "earliest possible time" in the progression of disease to provide another window on systemic vasculopathy of these patients. How significant a role can this clinical study play? The clinical implications of this work summarized in a straightforward and circumspect manner are that our data support the possibility of a new tool (SARI) to evaluate this ongoing process in SSc, mainly before and after disease-modifying treatment.

COMMENTS

Background

Systemic sclerosis is a disease characterized by a complex interplay of inflam-

mation, fibrosis and vascular damage. In fact, the arterial microvessels of systemic sclerosis display rarefaction and mural thickening, including medial smooth muscle and intimal cell hyperplasia.

Research frontiers

There are some *a priori* reasons, mainly indirect causations, for predicting a reasonable incidence of hepatic fibrosis in patients with systemic sclerosis. The hepatic mesenchymal tissue would be expected to share enhanced synthesis and/or deposition of collagen.

Innovations and breakthroughs

The key findings in this study were the following: (1) an enhanced resistivity of the splenic artery was found in patients suffering from systemic sclerosis; and (2) they did not demonstrate splenomegaly as well as liver fibrosis or any other form of liver damage.

Applications

Having revealed spleen vascular changes, the crucial future research directions should zero in on other vascular areas, such as retinal artery or renal artery, by means of the simple and reliable tool that is doppler ultraSound, to evaluate to what extent vasculopathy is represented in systemic sclerosis. If these data are confirmed and expanded in a larger population, physicians will be advised to investigate the spleen circulation at the "earliest possible time" in the progression of disease to provide another window on systemic vasculopathy of these patients.

Peer review

The authors investigated the spleen vascular involvement and the presence of liver fibrosis in patients with established SSc. In order to proceed as described above, they have included in the present study seventeen patients with SSc compared with eighteen patients suffering from hepatitis C virus-related liver cirrhosis and eighteen non elderly subjects as a control group. As findings, the authors relate an enhanced resistivity of the splenic artery in patients suffering from SSc, who did not demonstrate splenomegaly as well as liver fibrosis or any other form of liver damage.

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Survivin isoforms and clinicopathological characteristics in colorectal adenocarcinomas using real-time qPCR

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RESULTS: Wild type survivin mRNA isoform was expressed in 48% of the 52 tumor samples, survivin-2b in 38% and survivin-ΔEx3 in 29%, while no expression was found in normal tissues. The mRNA expression of wild type survivin presented a significant correlation with the expression of the ratio of survivin-2b, survivin-ΔEx3, survivin-2b/wild type survivin and survivin-ΔEx3/wild type survivin ($P < 0.001$). The mRNA expression of wild-survivin and survivin-ΔEx3 was related with tumor size and invasion ($P = 0.006$ and $P < 0.005$, respectively). A significant difference was found between survivin-2b and morphologic cancer type. Also, the ratio of survivin-ΔEx3/wild-survivin was significantly associated with prognosis. No association was observed between the three isoforms and grade, metastasis, Dukes stage and gender. The three isoforms were not correlated with CEA and CA19-9.

CONCLUSION: Survivin isoforms may play a role in cell apoptosis and their quantification could provide information about clinical management of patients suffering from colorectal cancer.

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Abstract

AIM: To investigate three isoforms of survivin in colorectal adenocarcinomas.

METHODS: We used the LightCycler Technology (Roche), along with a common forward primer and reverse primers specific for the splice variants and two common hybridization probes labeled with fluorescein and LightCycler-Red fluorophore (LC-Red 640). Real time quantitative polymerase chain reaction (PCR) was performed on cDNAs from 52 tumor specimens from colorectal cancer patients and 10 unrelated normal colorectal tissues. In the patients group, carcinoembryonic antigen (CEA) and CA19-9 tumor markers were also measured immunochemically.

Key words: Survivin; mRNA isoforms; Apoptosis gene; Colorectal adenocarcinomas; Real time quantitative polymerase chain reaction; Lightcycler

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INTRODUCTION

Apoptosis is a tightly controlled procedure of cellular death, which is crucial for tissue homeostasis^[1]. Inhibition of apoptosis results in tumorigenesis by cell survival allowing the accumulation of genetic mutations that promote transformation of normal tissues^[2]. Among key regulators of apoptosis are proteins of the bcl-2 family and the inhibitors of apoptosis (IAP) family of proteins^[3-5].

Survivin was originally identified by structural homology to IAPs in human B-cell lymphomas. It is a multifunctional protein implicated in the control of cell proliferation, inhibition of apoptosis and the promotion of angiogenesis^[6,7]. Survivin, as an inhibitor of apoptosis directly inhibits caspase-3 and -7 activity and regulates the cell cycle in the G2/M phase^[8].

Survivin is expressed during embryonic and fetal development, is down regulated in normal adult tissues and is overexpressed in a variety of human cancers^[9,10]. A strong association between expression of survivin mRNA and aggressive tumor behavior has been found in many types of cancer such as colorectal, breast, non-small lung cancer, gliomas and B-cell lymphomas^[11-15].

In addition to the wild type survivin full length transcript, five other splice variants have been identified: survivin-ΔEx3 that arises from the removal of exon 3, survivin-2b^[16] that originates from the inclusion of intron 2, survivin-3b that stems from the inclusion of intron 3^[17] survivin-2a made from the insertions of exon 1 and 2 at the 5' end of intron 2^[18], and the recently described survivin-2b + 32 which combines intronic sequence 2b and an insertion of 32 additional nucleotides from intron 2^[19] (Table 1).

Wild type survivin and survivin-ΔEx3 have been shown to act as inhibitors of apoptosis while survivin-2b presents pro-apoptotic functions by dimerizing with wild type survivin and reducing the anti-apoptotic effects of wild type survivin^[16,20,21]. The ratio survivin-2b/wild-survivin is higher in tumor samples compared to normal tissues. Survivin-2a is also expressed at high levels in malignant cells and attenuates the antiapoptotic effect of wild-survivin^[22]. Inhibition of apoptosis by survivin predicts a poor prognosis and a shorter survival in patients suffering from carcinomas^[23-28].

Till now, there has only been one other study in the literature investigating the expression of survivin's isoforms in colorectal cancer using quantitative real time-PCR^[24]. In this study, we obtained quantitative data for the distribution of the most described transcripts, which are wild type survivin, survivin-ΔEx3 and survivin-2b in colorectal adenocarcinomas by using the accurate and sensitive real time-qPCR in the Lightcycler platform. Then, we investigated their correlation with clinicopathological characteristics of colorectal cancer, and evaluated their prognostic significance.

MATERIALS AND METHODS

Patients

Fifty-two tissues from patients with colorectal cancer and another 10 unrelated normal samples were obtained from

Table 1 cDNA structure of survivin splice variants

Transcript variants	Exons or segments
Wild type survivin	1, 2, 3, 4
Survivin-2b	1, 2, 2b, 3, 4
SurvivinΔEx3	1, 2, 4
Survivin-2a	1, 2
Survivin-3b	1, 2, 3, 3b, 4
Survivin-2b + 32	1, 2, 2b + 32nt, 3, 4

b: Sequence originated from intron.

Table 2 Clinical characteristics of 52 patients with colorectal cancer *n* (%)

Variables	Values
Gender	
Men	21 (40.4)
Women	31 (59.6)
Tumor size	
≤ 5 cm	32 (65.3)
> 5 cm	17 (34.7)
Grade	
I	3 (6.3)
II	36 (75)
III	7 (14.6)
IV	2 (4.2)
Dukes stage	
A	8 (16.3)
B	11 (22.4)
C	22 (44.9)
D	8 (16.3)
Morphologic cancer type	
Polypoid (ecblastetic)	28 (57.1)
Ulcerous	21 (42.9)

the 4th Department of Surgery, Attikon University General Hospital. Part of the resected specimens at surgery was immediately stored in RNA Later (Ambion, USA) for at least 2 d at 4°C and then stored at -80°C until total RNA extraction. Our sample population included 21 males and 31 females [mean age: 63 years, standard deviation (SD) ± S13, range: 36-93 years]. The histological type and grade were reviewed and classified according to the World Health Organization classification criteria, and the disease stage was determined according to the Dukes staging system. The clinical characteristics of 52 patients with colorectal cancer are shown in Table 2. No patients underwent chemotherapy or radiation therapy before surgery.

Total RNA isolation

Total RNA was extracted by the Illustra RNAspin Mini RNA isolation kit (GE Healthcare, USA) according to the manufacturer's instructions. DNA contamination was eliminated by the use of RNase-free DNase I (GE Healthcare). The extracted total RNA was dissolved in free diethylpyrocarbonate (DEPC) treated water and stored at -80°C until further manipulations. RNA concentration was determined by the Quant-iT RNA Assay kit in Qubit fluorometer (Invitrogen, USA).

Table 3 Sequences of primers and hybridization probes

	Name	Oligonucleotide sequence, 5'-3'
Forward primer	F1	CCACCGCATCTCTACATTCA
Reverse primers	WT	TATGTTCTCTATGGGGTCG
	Sur2B	AGTGCTGGTATTACAGGCGT
	SurΔEx3	TTTCCTTTCATGGGGTC
Hybridization probes		CAAGTCTGGCTCGTTCTCAG TGGG-FITC ¹ LCRed640-CAGTGGATGAAG CCAGCCTCG-Ph ²

¹: 3'-end-labeled with fluorescein isothiocyanate (FITC); ²: 5'-end-labeled with LC Red 640; phosphorylated on the 3' end to avoid extension of the probe.

Reverse transcription- quantitative real-time PCR analysis

For complementary DNA (cDNA) synthesis, the Transcriptor First strand cDNA synthesis kit (Roche Applied Science, USA) was used according to the manufacturer's instructions. In each reaction, 3 µg of quantitated total RNA was included. The cDNAs were stored at -20°C until real time quantitative PCR was performed in the Light-Cycler 1.5 platform (Roche Applied Science). One µL of cDNA mixture was subjected to amplification in 10 µL total volume reaction mixtures in glass capillaries. Real time PCR was performed with the FastStart DNA Master Hybprobe kit (Roche Applied Science) and cycling conditions were: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing for 10 s at 62°C, extension at 72°C for 10 s, respectively. Standard curves for each of the three transcripts were generated from purified and quantified amplicons in serial dilutions from total RNA extracted from MCF-7 cells, according to previous experience^[29]. Primer pairs and hybridization probes for the three transcripts of survivin, survivin-2B, survivin-ΔEx3 were described previously^[24] and are shown in Table 3.

Real time PCR products were additionally checked for their proper size and purity by electrophoresis on 2% agarose gels containing ethidium bromide and visualized under UV transillumination. Briefly 10 µL of real-time PCR products were run along with 10 µL of a 100 bp ladder MW marker (New England Biolabs, USA).

Normalization

Real time PCR is widely used to quantify biologically relevant changes in mRNA levels but a number of problems exist and are associated with its use due to the inherent variability of RNA, variability of extraction protocols, different reverse transcription and PCR efficiencies. It is important that an accurate method of normalization is chosen to control for these errors. Unfortunately, normalization remains one of real time PCR's most difficult problems^[30]. In our protocol, we ensured that our tissues had similar size (approximately 50 mg) and performed normalization against the same amount of total RNA (3 µg), since when dealing

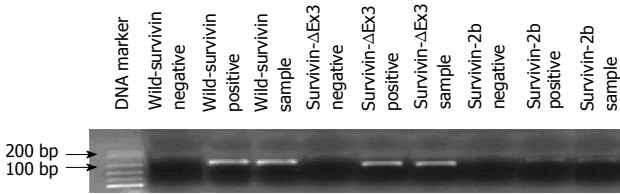


Figure 1 Electrophoresis of the survivin transcripts cDNA (wild survivin 185 bp, survivin-ΔEx3 184 bp, and survivin-2b 214 bp).

with *in vivo* samples it is not possible to predict which of the housekeeping genes is stable and appropriate^[31,32].

Determination of immunochemical parameters

The cancer antigens carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) were measured using Elecsys 2010 (Roche, USA) with electro-chemiluminescent immunoassays. The reproducibility of controls 1 and 2 (Roche) were for CEA, CV₁ = 3.6% and CV₂ = 3.0% and for CA19-9, CV₁ = 4.8% and CV₂ = 3.8%. The analytical sensitivity for CEA was 0.25 ng/mL and for CA19-9 < 0.85 U/mL. The cut-off values for CEA and CA19-9 were 5.0 ng/mL and 37 U/mL, respectively.

Statistical analysis

Statistical analysis of the data was performed using SPSS® version 17 for Windows statistical software package. Initially data were assessed through simple cross-tabulations and by using χ^2 test for categorical variables, *t*-test for normally distributed variables and Mann-Whitney *U* test for not normally distributed variables. Normality hypothesis was tested by Kolmogorov-Smirnov test, measures of asymmetry and Shapiro-Wilk test. In order to compare means of cases amongst different subgroups, one-way ANOVA test for normally distributed variables or Kruskal-Wallis test for not normally distributed variables were conducted. Post hoc pairwise comparisons were performed using the Bonferroni method. The Pearson or Spearman correlation coefficients (*r*) were used as measurements of correlation for continuous normally or not normally distributed variables respectively. In order to estimate survival functions, Kaplan-Meier curves were generated and subsequently compared using the log-rank test, the Breslow test or the Tarone-Ware test. For all tests performed, a two-sided *P* value of less than 0.05 was considered as significant.

RESULTS

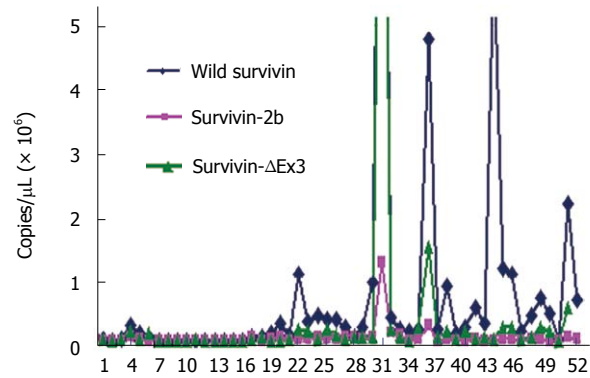
Expression of mRNA survivin transcripts variants in cancer and normal tissues

The specificity of amplification products obtained by real time qPCR was confirmed by agarose gel electrophoresis, which revealed distinct bands for all PCR products. PCR for full length survivin mRNA produced a band of 185 bp, while those of survivin-2b and survivin-ΔEx3 were 214 and 184 bp, respectively (Figure 1). Among the 52

Table 4 Quantitative measurements obtained from tissue mRNA and serum tumor markers from 52 patients with colorectal cancer (mean \pm SD)

Variables	Values
Wild-survivin (copies/ μ L)	842.5 \pm 2380.3
Survivin-2b (copies/ μ L)	58.1 \pm 192.3
Survivin- Δ Ex3 (copies/ μ L)	393.9 \pm 1988.8
Survivin-2b/wild-survivin (copies/ μ L)	0.12 \pm 0.21
Survivin- Δ Ex3/wild-survivin (copies/ μ L)	0.36 \pm 0.66
CEA (μ g/L)	191.59 \pm 934.62
CA 19-9 (μ g/L)	892.48 \pm 2991.78

CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9.

**Figure 2** Expression of mRNA wild type survivin, survivin-2b and survivin Δ Ex3 isoforms in cancerous tissues ($n = 52$).**Table 5** Spearman correlation coefficients of laboratory variables in patients with colorectal cancer ($n = 52$)

Variables	Sur2b	Sur Δ Ex3	Sur2b/wildsur	Sur Δ Ex3/wildsur	CEA	CA19-9	Age
Wildsur	0.694 ^b	0.799 ^b	0.408 ^b	0.485 ^b	0.136	0.102	0.051
Sur2b		0.623 ^b	0.527 ^b	0.471 ^b	0.238	0.212	0.165
Sur Δ Ex3			0.409 ^b	0.651 ^b	0.208	0.063	0.229
Sur2b/wildsur				0.613 ^b	0.021	0.203	0.0234
Sur Δ Ex3/wildsur					-0.054	0.084	0.320 ^a
CEA						0.522 ^b	0.395 ^a
CA 19-9							0.247

^a $P < 0.05$, ^b $P < 0.001$. CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9.

tumor samples, wild type survivin expression was detected in 25 samples (48%), survivin-2b expression was detected in 20 samples (38%) and survivin- Δ Ex3 was expressed in 15 samples (29%). Eighteen samples were positive for both the expression of wild type survivin and survivin-2b (34.6%), 14 samples were positive for both the expression of wild-survivin and survivin- Δ Ex3 (26.9%) and 14 samples were positive for both the expression of survivin-2b and survivin- Δ Ex3 (26.9%). All 3 survivin isoforms were found in 13 samples (25%). None of the 3 variants of survivin mRNA were expressed in the 10 unrelated normal samples, indicating a significant difference in survivin expression between cancerous and healthy tissues ($P < 0.001$).

Relationship between the levels of expressed survivin transcripts and clinicopathological parameters

Wild type survivin was significantly more expressed than the other two splice variants in the 52 samples, as shown in Figure 2. Quantitative data from tissue mRNA and serum tumor markers from the 52 patients are shown in Table 4. The 3 survivin isoforms were not correlated with CEA and CA19-9 (Table 5). In general, no association was found between the 3 isoforms and tumor size, survival, Dukes stage, grade and gender, although wild survivin was almost present in tumors more than 5 cm in diameter ($P = 0.088$), and the ratio survivin- Δ Ex3/wild-survivin was almost significantly higher in tumors less than 5 cm ($P = 0.097$) and in advanced Dukes stage ($P = 0.097$) (Table 6). The mRNA expression of wild type

survivin presented a significant correlation with the expression of survivin-2b and survivin- Δ Ex3 ($P < 0.001$) and the ratio of survivin-2b/wild-survivin was strongly related to the ratio of survivin- Δ Ex3/wild-survivin ($P < 0.001$). Wild type survivin and survivin- Δ Ex3 expression levels presented an association with T ($P = 0.006$ and $P = 0.041$ respectively, Table 5). Mean survivin-2b expression levels were significantly higher in ulcerous carcinomas than in polypoid (ecblastetic) ones ($P = 0.030$, Table 6).

Association of survivin isoforms with prognosis

We examined the association of survivin isoforms with prognosis in 52 patients using the Kaplan-Meier survival curve. The ratio of survivin- Δ Ex3/wild-survivin presented a significant association with patient prognosis ($P = 0.015$, Figure 3). Specifically, when the ratio of survivin Δ Ex3/wild-survivin was ≥ 0.24 , patients survived a shorter time (mean survival 41 mo) compared to patients with a ratio of survivin- Δ Ex3/wild-survivin < 0.24 (mean survival 62.2 mo). No other association was found between survivin isoforms and the ratio of survivin-2b/wild-survivin with overall patient survival.

DISCUSSION

Although survivin is cytoprotective, it is also an essential protein for cell division. In vitro survivin interacts directly with the chromosomal passenger proteins, aurora-B kinase, borealin and INCENP. These proteins are mutually dependent upon each other, and together they form

Table 6 Association of mRNA expression survivin splice variants with clinicopathological characteristics in 52 patients suffering from colorectal cancer (mean ± SD)

Clinical characteristics	n	Wild-sur (copies/μL)	P value wild-sur	Sur-2b (copies/μL)	P value sur-2b	Sur-ΔEx3 (copies/μL)	P value sur-ΔEx3	Sur-2b/ wildsur (copies/μL)	P value sur-2b/ wildsur	Sur-ΔEx3/ wildsur (copies/μL)	P value sur-ΔEx3/ wildsur
Gender			0.978		0.685		0.631		0.570		0.387
Male	20	1216.1 ± 3438.6		98.0 ± 299.7		834.8 ± 3085.6		0.08 ± 0.09		0.47 ± 0.39	
Female	32	589.5 ± 1259.1		31.1 ± 33.2		85.3 ± 117.1		0.17 ± 0.24		0.36 ± 0.41	
Tumor size			0.088		0.438		0.869		0.544		0.097
≤ 5 cm	32	308.4 ± 491.3		28.6 ± 34.6		96.2 ± 116.1		0.17 ± 0.24		0.48 ± 0.41	
> 5 cm	17	1949.4 ± 3956.2		119.3 ± 330.9		1055.8 ± 3531.9		0.09 ± 0.09		0.23 ± 0.24	
Dukes stage			0.469		0.737		0.544		0.351		0.097
A	8	212.4 ± 403.4		27.7 ± 27.7		51.7 ± 66.1		0.37 ± 0.40		0.44 ± 0.35	
B	11	476.9 ± 743.4		30.8 ± 45.8		137.7 ± 185.7		0.08 ± 0.12		0.37 ± 0.37	
C	22	1556.8 ± 3533.1		95.4 ± 292.5		782.6 ± 3019.7		0.09 ± 0.10		0.30 ± 0.39	
D	8	226.7 ± 146.7		35.6 ± 32.9		120.7 ± 66.5		0.15 ± 0.15		0.63 ± 0.35	
T			0.006		0.125		0.041		0.103		0.492
0-2	8	212.4 ± 403.4		27.7 ± 27.7		51.7 ± 66.1		0.37 ± 0.40		0.44 ± 0.35	
3	29	340.0 ± 521.1		26.5 ± 35.4		92.9 ± 127.2		0.10 ± 0.13		0.39 ± 0.37	
4	12	2620.8 ± 4579.4		162.9 ± 389.8		1413.2 ± 4056.1		0.10 ± 0.08		0.40 ± 0.44	
Histologic type			0.584		0.030		0.731		0.061		0.892
Polypoid (ecblastetic)	28	520.1 ± 1006.4		45.7 ± 56.2		141.2 ± 313.7		0.19 ± 0.25		0.31 ± 0.25	
Ulcerous	21	1354.5 ± 3550.4		79.3 ± 298.5		769.5 ± 3083.3		0.06 ± 0.06		0.54 ± 0.51	

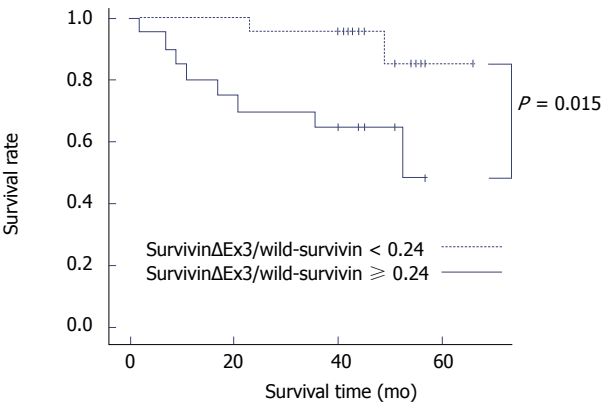


Figure 3 Kaplan-Meier survival curve for the ratio of survivinΔEx3/wild-survivin in 52 patients suffering colorectal adenocarcinomas.

a complex during mitosis, the chromosomal passenger complex, which is required for chromosome movements, proper spindle checkpoint control, and cell division^[33]. The overexpression of survivin in cancer may obliterate this apoptotic checkpoint and allow aberrant progression of transformed cells through mitosis. Survivin expression has been associated with increased aggressiveness and decreased patient survival in a number of different malignancies^[34]. Despite their ability to interact with wild type survivin, survivin-2b and survivin-ΔEx3 don't act as competitors during mitosis or have an essential function^[33]. They could be responsible for the fine tuning of wild type survivin's function. Survivin-ΔEx3 exhibits pronounced antiapoptotic properties, whereas survivin-2b has largely lost its antiapoptotic activity^[24].

Real-time technology has significantly extended the use and scope of Real time-PCR assays, with the potential

for quantification of mRNA targets being a particular advantage^[35]. Our study is the first to examine the expression of survivin and its splice variants by using quantitative Real time-PCR exclusively in colorectal adenocarcinomas. As far as the expression of the mRNA of survivin and its splice variants is concerned, previous researchers have performed mainly qualitative RT-PCR analysis or Northern blot analysis^[10,36-38]. In some studies, TaqMan quantitative real Time-PCR was used with only wild type survivin mRNA determination^[39-41]. Only recently, Mahotka *et al*^[2] made the first successful quantitative analysis of survivin splice variants in renal cell carcinoma specimens. We investigated the expression of survivin's transcript variants mRNA in patients with colorectal adenocarcinomas, while until now there have been other studies concerning colorectal cancer that used western immunoblotting, Real time PCR with SYBR Green^[42,43], microarray expression analysis and immunofluorescence^[44].

In our experiments, the prevalence of wild-survivin mRNA expression compared to the other splice variants is similar to the previous study that used specific hybridization probes^[24] raising the question if those two isoforms might act as antagonists of wild-survivin or play a role in tumor progression. The mean copy numbers of quantitative RT-PCR amplification of wild survivin, survivin-2b and survivin-ΔEx3 in tumor samples were respectively 842.5 ± 2380.3, 58.1 ± 192.3 and 393.9 ± 1988.8, while Suga *et al*^[24] found 3554.1 ± 3513.7, 1233.4 ± 1280.9 and 482.0 ± 568.1. Suga *et al*^[24] didn't find any significant association between the expression of survivin and its splice variants with gender, location, size, age, macroscopic type, histologic type, lymphatic vessel invasion, blood vessel invasion, lymph node metastasis and serosa invasion.

On the contrary, they observed a significant correlation of survivin and its splice variants with histologic disease stages, a correlation not found in our study. The differences in the results of our study with the results of Suga *et al.*^[24] might be due to race difference, genetic alterations or method performance.

The presence of transcripts of survivin isoforms has been associated with cancer. The expression of survivin in normal and cancerous samples is controversial since in previous studies data have indicated that survivin is expressed during fetal development, but not in most normal adult tissues. Using reverse transcription PCR, Mahotka *et al.*^[2] found that wild type survivin and survivin-ΔEx3 were expressed in cell lines derived from renal carcinomas but not normal renal cells. The previous isoforms are elevated in a wide variety of tumors but are not expressed to a significant extent in normal tissues^[44]. Studies using sensitive quantitative Real time-PCR revealed that survivin mRNA was expressed in normal fetal tissue panels and at a very low level in the normal adult brain template. In contrast, data from a recent study have demonstrated survivin expression in normal adult tissues, such as those of skin, endometrium, endothelial cells, normal blood lymphocytes, pancreas, spleen, colon, stomach, small intestine, large intestine, lung, kidney, prostate, pancreas, heart, and thymus^[45]. By using sensitive quantitative Real time-PCR analysis, low levels of total survivin mRNA were detectable in normal tissue adjacent to soft tissue sarcoma tumor cells (e.g. muscle) or in lymphocytes of blood donors. Further studies are needed in order to find out whether expression of survivin in normal tissue is due to mitotically active cells^[7]. The expression of survivin mRNA by semi-quantitative Real time PCR was detected in a significantly greater proportion of colorectal carcinoma samples than in adjacent normal colorectal tissues (67.3% *vs* 25%, $P < 0.01$)^[25]. With the use of tissue microarrays, survivin was detected in 147 of 230 cases of colorectal adenocarcinoma (63.9%) and no expression of survivin was observed in normal tissues^[34]. Our study showed that the expression of survivin's transcript variants mRNA was detected only in colorectal cancer tissues while it was absent in normal samples. On the contrary, Suga *et al.*^[24] found out that 60% of the normal tissues expressed survivin.

In our study, there was no significant correlation between the expression of the three isoforms and grade, Dukes stage, lymph metastasis, sex, N (TNM staging system) as well as CEA and CA19-9, two serum tumor markers not examined in any other study. Suga *et al.*^[24] found no correlation between survivin immunoreactivity and age, sex, tumor size and site, morphologic subtype, tumor grade and clinical stage ($P > 0.05$). On the other hand, our study was the only one showing that the ratio of survivin-ΔEx3/wild-survivin was strongly related with age ($P < 0.05$), while wild-survivin and survivin-ΔEx3 were associated with T (TNM staging system) and their mRNA expression was highly elevated in patients who belonged to the group T4 ($P = 0.006$ and $P = 0.041$ respectively). Another important finding of our study is that survivin-2b

was associated, for the first time in colorectal adenocarcinomas, with morphologic type, particularly overexpressed in ulcerous colorectal adenocarcinomas ($P < 0.05$). A differential expression of survivin-2b was also reported in gastric carcinomas with a negative correlation with disease progression. Other reports have linked the expression of survivin isoforms with poor patient prognosis^[33]. In our study, we examined the association of survivin transcript variants with overall patient survival showing for the first time a significant correlation of the ratio of survivin-ΔEx3/wild-survivin with prognosis. Therefore, the ratio survivin-ΔEx3/wild-survivin might be a novel prognostic marker for colorectal carcinomas.

Despite the relative rarity of colon cancer in the Greek population and the application of expensive molecular biology methodology, we implemented a sufficiently powered study, which albeit its modest size, was adequate to generate statistically significant associations with the above variables. We also examined tissues from patients with no cancerous diseases in order to investigate the expression of survivin's isoforms in healthy samples.

Further larger studies are required in order to examine the three other isoforms of survivin, for an overall view of survivin's function. Moreover, other methodology could be used, such as Northern blot analysis, microarray expression analysis, nested PCR, *etc* to further validate the results of our study.

Recent studies showed that the phenomena of chemoradioresistance were prevalent in survivin positive tumors and indicated that survivin may play an important role in chemoresistance of cancer cells. The survivin expression might guide chemotherapy and radiation therapy decisions. The clinical importance of survivin expression remains unclear in patients with colorectal cancer, since there are few studies on the subject. Survivin might provide important predictive and prognostic perspectives, and could offer new therapeutic alternatives for cancer^[25]. Our results suggest that survivin splice variants might be related with the action of survivin in colorectal carcinogenesis but further studies are required to identify the potential role and mechanism of survivin's isoforms in the development of colorectal cancer.

COMMENTS

Background

Survivin may provide prognostic information, because of its relation with increased tumor aggressiveness. The authors investigated three isoforms of survivin in colorectal adenocarcinomas (wild type survivin, survivin-2b and survivin-ΔEx3).

Research frontiers

Survivin expression has been associated with increased aggressiveness and decreased patient survival in a number of different malignancies but little has been done so far in colorectal cancer. In this study, the authors used for the first time quantitative real time-polymerase chain reaction (PCR) in order to detect three isoforms of survivin in colorectal adenocarcinomas. The authors found a significant correlation of the ratio survivin-ΔEx3/wild-survivin with prognosis. Therefore, the expression level of survivin-ΔEx3/wild-survivin might be a novel prognostic marker. Another important finding was that wild-survivin and survivin-ΔEx3 were associated with TNM staging system, underscoring their association with increased tumour aggressiveness.

Innovations and breakthroughs

Till now, there is only one other study dealing with survivin's isoforms in colorectal carcinomas using the technology of real time-qPCR. Using the same technology in colorectal adenocarcinomas the authors obtained different results.

Applications

The clinical importance of survivin expression remains unclear in patients with colorectal cancer, since there are few studies on the subject. Survivin might provide important predictive and prognostic perspectives and could offer new therapeutic alternatives for cancer. Further studies are required to identify and clarify the potential role and mechanism of survivin in the development of colorectal cancer.

Peer review

The authors have performed a nice study on the different forms of survivin and a lot of work has gone into producing the manuscript.

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Pyogenic liver abscess: An audit of 10 years' experience

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Abstract

AIM: To describe our own experience with pyogenic liver abscesses over the past 10 years and investigate the risk factors associated with failure of initial percutaneous therapy.

METHODS: A retrospective study of records of 63 PLA patients presenting between 1998 and 2008 to Australian tertiary referral centre, were reviewed. Amoebic and hydatid abscesses were excluded. Demographic, clinical, radiological, and microbiological characteristics, as well as surgical/radiological interventions, were recorded.

RESULTS: Sixty-three patients (42 males, 21 females) aged 65 (± 14) years [mean \pm (SD)] had prodromal symptoms for a median (interquartile range; IQR) of 7 (5-14) d. Only 59% of patients were febrile at presentation; however, the serum C-reactive protein was elevated in all 47 in whom it was measured. Liver function tests were non-specifically abnormal. 67% of patients had a solitary abscess, while 32% had > 3 abscesses with a median (IQR) diameter of 6.3 (4-9) cm. Causative organisms were: *Streptococcus milleri* 25%,

Klebsiella pneumoniae 21%, and *Escherichia coli* 16%. A presumptive cryptogenic cause was most common (34%). Four patients died in this series: one from sepsis, two from advanced cancer, and one from acute myocardial infarction. The initial procedure was radiological aspiration \pm drainage in 54 and surgery in two patients. 17% underwent surgical management during their hospitalization. Serum hypoalbuminaemia [mean (95% CI): 32 (29-35) g/L vs 28 (25-31) g/L, $P = 0.045$] on presentation was found to be the only factor related to failure of initial percutaneous therapy on univariate analysis.

CONCLUSION: PLA is a diagnostic challenge, because the presentation of this condition is non-specific. Intravenous antibiotics and radiological drainage in the first instance allows resolution of most PLAs; However, a small proportion of patients still require surgical drainage.

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Key words: Pyogenic liver abscess; Image guided drainage; Surgical drainage; C-reactive protein; Hypoalbuminaemia

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INTRODUCTION

The management of pyogenic liver abscesses is changing. Depending on the cause and local expertise, a varying proportion of patients are treated with antibiotics

alone, surgical therapy or radiological intervention, or a combination of the above. However, there appears to be an overall trend for antibiotics and radiological intervention (either drainage or aspiration) to be the initial treatment of choice^[1]. Surgical management is increasingly limited to cases of failed radiological management or to the management of complications^[2-5].

Furthermore, there is an increasing recognition that pyogenic liver abscesses seen in Western and Asian countries differ in demographic characteristics, aetiological factors, and clinical behaviour. Many of the recent large case series are from South East Asian institutions, where a large proportion of pyogenic liver abscesses are caused by *Klebsiella pneumoniae*. These abscesses are typically associated with diabetes or a cryptogenic aetiology^[5-7]. This contrasts with the predominance of *Escherichia coli* pyogenic liver abscesses of biliary aetiology found in Western institutions.

Continued changes to the management and aetiology of this disease entity prompted us to review it and describe our own experience with pyogenic liver abscesses over the past 10 years. In addition, we aim to investigate the risk factors associated with failure of initial percutaneous therapy.

MATERIALS AND METHODS

A retrospective study of all patients treated at Royal North Shore and affiliated hospitals for pyogenic liver abscesses was conducted. A list of patients was generated by accessing the ICD-10 codes of the hospital admissions database, which dated back to 1996. This data was augmented with data from individual (upper gastrointestinal) surgeons' databases to ensure that all possible treated patients during the study period were captured. A total of 63 patients were identified during the study period of August 1998 to November 2008. The diagnosis of pyogenic liver abscess was based upon clinical features, evidence from imaging studies (ultrasound or computed tomography), as well as microbiology (blood or aspirate culture results). Liver abscesses with demonstrated positive amoebic or hydatid serology were excluded.

Basic demographic characteristics (history, examination and investigation findings at presentation), radiological/microbiological results, antibiotic use, and surgical/radiological intervention were reviewed and recorded on a standardised data sheet.

For the purposes of data recording, the examination findings of the patients were defined by the findings from the first examination by senior surgical or medical staff (registrar or above). The degree of peritonism and the location of tenderness were recorded. The presence of fever was defined as a temperature taken within the first 24 h of presentation of greater than 37.5°C. All other vital signs were defined by the first available set of observations after presentation. Tachycardia was defined as a heart rate greater than or equal to 100 beats per minute, and hypotension as a systolic blood pressure of less than 100 mmHg. For blood test results, the first available test

within the first 48 h of presentation was used for analysis. Reference ranges of laboratory tests were defined by the reference ranges supplied by the local laboratory, Pacific Laboratory Medicine Services-these are included in results of Table 1. Acute renal failure was defined as an increase in the serum creatinine level of 50% over the baseline. With regards to imaging findings, the size of the abscess was defined by its greatest diameter, and in cases with multiple abscesses, as the greatest dimension of the largest abscess. Radiological intervention was subdivided into drainage and aspiration, depending on whether a drain was left in situ. The presumptive aetiology of the liver abscess was a subjective judgement made by either the treating team at the time or retrospectively by the investigators. A standard protocol for investigation of the underlying cause of the liver abscess was not used.

At our unit, patients with suspected or proven pyogenic liver abscesses are managed initially by empirical broad spectrum intravenous antibiotics, usually a combination of a third generation cephalosporin, such as ceftriaxone, along with metronidazole. The penicillin-based combination with ampicillin, an aminoglycoside (such as gentamicin), and metronidazole is also commonly used. The antibiotic regimen is modified according to response to initial therapy and available microbiology results, in consultation with the local microbiology team. Antibiotics are generally continued for 4-6 wk, with the initial 2 wk administered intravenously. First line intervention is radiological drainage of the liver abscess. This is performed with sedation and local anaesthesia under computed tomography or ultrasound control. Typically, a "pigtail" drain (8-12 French) is placed into the abscess, which is then left on free drainage with or without periodic normal saline flushes. Repeated procedures may be indicated. In some cases, rather than placing an indwelling drain, radiological guided aspiration was used as the first line treatment. The choice of indwelling drain or aspiration depends on clinician preference, but aspiration is generally only used when the abscess is small or multiple. Indications for surgical intervention or drainage include - signs of peritonitis, ruptured abscesses, abscesses not responding to antibiotics and drainage, and multiloculation.

All statistical analysis and plotting were performed with Intercooled Stata version 9.1 (Statacorp, Texas, USA). Note that, because of missing data for certain characteristics, some denominators of proportion statistics are not consistently equal to 63 (the full cohort of subjects).

RESULTS

Demographic characteristics and presentation

Sixty-three patients (42 males and 21 females) were identified for this study, with a mean (SD, range) age of 64 (14, 31-97) years. The peak incidence was in the 8th decade of life (70-79 years). Almost a quarter of patients (15/62, 24%) had a history of gallstones or cholecystectomy. Nearly a quarter of patients ($n = 14$) had a history (past or current) of malignancy at presentation. Of these, 10 (16%)

Table 1 Summary of laboratory results at initial presentation

	Reference range	No. of patients measured	mean \pm SD	% of patients outside reference range
White cell count ($\times 10^9/L$)	4.0-11.0	62	15.0 \pm 6.3	74%
Neutrophil count ($\times 10^9/L$)	2.0-8.0	62	12.9 \pm 5.9	82%
Haemoglobin (g/L)	135-180	62	118 \pm 21	24% Hb < 100 g/L
Platelets ($\times 10^9/L$)	150-400	62	320 \pm 202	42%
C-reactive protein (mg/L)	< 5	47	272 \pm 119	100% (47/47)
Urea (mmol/L)	3.1-8.1	62	8.2 \pm 5.96	29% Urea > 8.1 mmol/L
Creatinine (μ mol/L)	60-100	62	105 \pm 60	42% Cr > 100 μ mol/L 16% Cr > 150 μ mol/L
Albumin (g/L)	35-46	62	30 \pm 7.0	73%
AST (U/L)	12-36	61	127 \pm 301	67%
ALT (U/L)	5-40	62	118 \pm 222	73%
ALP (U/L)	41-119	61	209 \pm 140	71%
GGT (U/L)	5-65	62	191 \pm 201	75%
Bilirubin (μ mol/L)	3-18	61	30 \pm 39	54% Bil > 18 μ mol/L 13% Bil > 50 μ mol/L
Glucose (mmol/L)	3.5-5.5	47	7.8 \pm 2.6	15% (7/47) Glucose > 10 mmol/L
INR	\leq 1.5	53	1.3 \pm 0.56	13% (7/53)

AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; Bil: Serum bilirubin; GGT: γ glutamyl transpeptidase; INR: International normalized ratio; RR: Reference range. Reference ranges supplied by local pathology laboratory.

Table 2 Summary of physical examination findings on presentation

Clinical finding	n (%)
Fever (T > 37.5)	33 (59)
Hypotension (SBP < 100)	7 (13)
Tachycardia (HR \geq 100)	29 (52)
Peritonism	
None	49 (86)
Localized guarding	8 (14)
Generalized peritonism	0 (0)
Location of tenderness	
Non tender abdomen	20 (35)
Right upper quadrant	22 (39)
Other abdominal tenderness	15 (26)

HR: Heart rate in beats per minute; SBP: Systolic blood pressure in mmHg; T: Body temperature in degrees Celsius.

had a current diagnosis of malignancy: three with cholangiocarcinoma, three with pancreatic cancer, and one each with colon cancer, gastric cancer, prostate cancer, and follicular dendritic cell tumour of the liver. Only 13% (8/62) had a history of diabetes mellitus.

The median (IQR) duration of symptoms prior to presentation was 7 (5-14) d. Table 2 summarises the presentation of these patients. Note that the presence of fever and tachycardia was found in only slightly more than half of the patients. It was uncommon for patients to be hypotensive on presentation. Abdominal signs were non-specific in most cases.

The initial laboratory results were available for all but one patient. They are summarised in Table 2. Markers of inflammation [white cell count, neutrophil count, and the serum C-reactive protein (CRP)] were generally raised. In particular, CRP was elevated in all of the 47 tested patients.

Forty-two percent of patients had elevated serum creatinine levels, but only 16% had a serum creatinine of greater

than 150 μ mol/L at presentation. Liver enzymes were each elevated in about 70% of the patients. Serum bilirubin was elevated in slightly more than half of the patients, but only 13% had a clinically significant hyperbilirubinaemia of greater than 50 μ mol/L. Only 15% of the subjects had a blood glucose level of greater than 10 mmol/L.

Imaging

Computer tomography was used in all patients, but the initial diagnosis was made with abdominal ultrasonography in 14 cases. Detailed imaging reports or films were available for 60 patients. Sixty-seven percent had solitary liver abscesses: one patient had two abscesses and 32% had three or more abscesses. The median (IQR) maximum dimension of liver abscesses was 6.3 (4.0-9.0) cm. Note that in five cases multiple "microabscesses" (< 1 cm diameter) were identified. Sixty-six percent had involvement of the right liver only, 19% had left liver involvement only, and 15% (9/58) had bilateral involvement. Twelve percent demonstrated evidence of gas formation within the abscess and 15% had imaging evidence of biliary dilatation.

Microbiology and aetiology

Although all 63 patients had either culture of blood or of the abscess, the causative organism was identified in only 50 (79%). The yield from liver abscess aspirate, 67%, was higher than from blood cultures (48%). The most common causative pathogens were *Streptococcus milleri* 25%, *Klebsiella pneumoniae* 21%, and *Escherichia coli* 16% (Table 3). Note that many patients had more than one set of cultures taken during the study period. In one patient the abscess culture grew *Corynebacterium urealyticum* but blood cultures grew *Staphylococcus aureus*; however, that was likely to be a skin contaminant.

The aetiology of the abscess was only evident in 59 patients (Table 4). *Streptococcus milleri* was associated with

Table 3 Summary of microbiology results for blood culture results, liver abscess aspirate culture results, and presumed causative pathogen

Organism	n (% of patients with culture results)		
	Positive blood cultures	Positive liver aspirate cultures	Causative pathogen
<i>Streptococcus milleri</i>	8 (14)	12 (24)	16 (25)
<i>Klebsiella species</i>	9 (16)	8 (16)	13 (21)
<i>K. pneumoniae</i>	7	8	11
<i>K. oxytoca</i>	1	0	1
<i>K. terrigena</i>	1	0	1
<i>Escherichia coli</i>	6 (11)	3 (5.9)	10 (16)
Mixed ¹	2 (3.6)	6 (12)	5 (8)
<i>Fusobacterium necrophorum</i>	1 (1.8)	1 (2.0)	2 (3.2)
<i>Pseudomonas aeruginosa</i>	0 (0)	1 (2.0)	1 (1.6)
<i>Lactobacillus</i>	0 (0)	1 (2.0)	1 (1.6)
<i>Corynebacterium urealyticum</i>	0 (0)	1 (2.0)	1 (1.6)
<i>Citrobacter koseri</i>	0 (0)	1 (2.0)	1 (1.6)
<i>Staphylococcus aureus</i> ²	1 (1.8)	0 (0)	0 (0)
Causative organism not identified	-	-	13 (21)
Total negative	29 (52)	17 (33)	-
Total positive	27 (48)	34 (67)	50 (79)
Total number of patients with available culture results	/55	/51	/63

¹Includes 2 cases of *Clostridium perfringens*; ²Discordant with liver abscess microbiology. Likely contaminant.

Table 4 Summary of the presumed aetiology of the patients' pyogenic liver abscesses

Aetiology	n (%)
Cryptogenic	20 (34)
Biliary	14 (22)
Portal (e.g. appendicitis, diverticulitis)	12 (20)
Haematogenous (other bacteremic source)	7 (11)
Direct (e.g. pericholecystic abscess, RFA)	6 (9.4)

RFA: Radiofrequency ablation.

portal sepsis in 4/12, *Escherichia coli* was associated with biliary sepsis in 5/14, and *Klebsiella species* was associated with cryptogenic sepsis in 7/20.

There were two cases on *Clostridium perfringens*-both were isolated as mixed growths on cultures. Organisms isolated with *Clostridium perfringens* in these cases were *Escherichia coli* and *Enterococcus faecalis* in one case, and *Enterococcus faecalis* and *Citrobacter freundii* in the other. One of the patients had rapidly progressing sepsis and underwent a laparoscopy; however, the patient rapidly deteriorated. The second patient developed sudden onset of severe sepsis following radiofrequency ablation (RFA) of a liver metastasis. She underwent radiological guided drainage of the liver abscess and eventually thoracotomy for decortication and drainage. She returned to good health but died of progression of her pancreatic cancer six months later.

Intervention

Five patients were treated by intravenous antibiotics followed by oral antibiotics, without any need for other intervention (Table 5). Radiological drainage was the most frequent initial intervention, which was used in 43 (68%) cases. Of these, almost a third required a second drain-

Table 5 Summary of interventions

Initial intervention (%)	→	Secondary intervention (%)	→	Further interventions (%)
AB 5 (7.9)	→	RA 1/5 (20)		
		Other 1/5 (20)		
RD 43 (68)	→	RA 1/43 (2.3)		
		SG 5/43 (12)		
		RD 13/43 (30)	→	SG 1/13 (7.7)
				RD 3/13 (23)
RA 11 (18)	→	RD 2/11 (18)		
		SG 2/11 (18)		
SG 2 (3.2)	→	-		
Other 2 (3.2)	→	SG 1/2 (50)		

AB: Antibiotics only; RA: Image guided aspiration of abscess; RD: Image guided insertion of indwelling drain; SG: Surgical intervention. Note that all patients received intravenous antibiotics before any interventional treatment.

age/aspiration procedure and of these, three required further drainage procedures. In this group, surgery was eventually required in six (14%) cases. Eleven patients were initially treated with image-guided aspiration of the liver abscess. Of these, two required drain placement, and a further two required surgical drainage. Surgical drainage was performed as the initial procedure in two (3.2%) of patients. In total, 11 patients (17%) underwent surgical drainage or resection.

Two patients had other types of initial intervention. One had endoscopic retrograde cholangiopancreatography (ERCP), sphincterotomy, and removal of choledocholithiasis for biliary sepsis. Another patient had a laparoscopic appendicectomy. In both cases, the pyogenic liver abscesses were small and thought to be secondary to biliary/portal pyaemia. The patient with biliary sepsis eventually required a laparoscopic cholecystectomy and

Table 6 Complications occurring in 21 patients

Complication	n
Acute renal failure	7
Abdominal collection/sepsis	4
Thoracic empyema/large pleural effusion requiring surgical drainage	4
Multiple organ failure	2
Biliary fistula	2
Hemorrhage from percutaneous drainage	2
Portal vein thrombosis	1
Hepatic vein thrombosis	1
Subcapsular hematoma from percutaneous drainage	1
Myocardial infarction	1
Necrosis of terminal phalanges	1

bile duct exploration due to failed endoscopic retrieval of choledocholithiasis.

Other forms of surgical interventions were performed because of pleural sepsis requiring drainage of pleural empyema and decortications of lung ($n = 4$).

Indications for surgery as the initial management in two cases were as follows: peritonitis on clinical examination requiring laparoscopy (which was converted to laparotomy), and sepsis in a patient on cyclosporin, methotrexate, and prednisolone for rheumatoid arthritis.

Indications for surgical intervention as a subsequent procedure included: leakage of abscess from a percutaneous drainage, haemorrhage from percutaneous drainage ($n = 2$), failure of resolution or recurrence of the abscess ($n = 4$), and laparoscopic cholecystectomy for an intrahepatic extension of a pericholecystic abscess ($n = 1$).

Complications and outcomes

Twenty-one patients (33%) developed complications (Table 6) that were directly related to the sepsis or abscess and which required specific intervention.

In two patients, the abscess ruptured through the upper surface of the liver into the pleural cavity: one was related to the placement of a drain and the other was related to a subphrenic abscess. A further patient developed a large thoracic empyema related to her subphrenic abscess and one developed a large fibrinous pleural effusion with no pus.

Two patients had severe haemorrhage associated with the radiological intervention (ultrasound guided drain placement and computed tomography (CT) guided aspiration/biopsy) and required resection of the liver segment that contained the abscess.

The systemic effects of the sepsis were severe enough to cause acute renal failure in seven patients and multi-organ failure in two. A patient who presented with severe shock was treated in Intensive Care with antibiotics and inotropes and he gradually recovered; however, he developed necrosis of the terminal phalanges of his hands and feet. He was well enough to be discharged to the ward, but because of recurrence of sepsis, surgical opinion was sought. He required drainage of the abscess and associated cholecystectomy, which was successfully treated by laparo-

scopic approach, following which he rapidly recovered and was discharged.

Two patients developed biliary fistulae post radiological drainage. One patient underwent biliary decompression with ERCP and nasobiliary drainage, which resolved the fistulae. The second patient had a history of a cholangiocarcinoma resected with roux-en-Y and hepaticojejunostomy eight months prior to the development of her multiple liver abscesses. Three CT guided percutaneous drains were inserted following which she developed a controlled biliary fistula through one of the drains. Following choledoschoscopy, which showed no tumour recurrence, the drain was removed but recurrent abscesses occurred. A percutaneous drain guided surgical debridement was undertaken and sepsis settled; however, a chronically low-volume draining fistula remained. She was discharged home to independent living, but after five months became unwell again and decided to accept palliative care.

Four patients died during the hospital admission. One patient who had a severe sepsis due to *Clostridium perfringens* underwent a desperate surgical procedure but, not unexpectedly, the resuscitation failed. Two died from progression of pancreatic cancer managed in a palliative fashion. A further patient, a 97 year old lady, died from a cardiac arrest secondary to acute myocardial infarction six days after the insertion of a percutaneous drain.

Risk factors predicting failure of initial percutaneous therapy

Table 7 summarises the results of univariate analysis of factors that predict the failure of initial percutaneous therapy. A low serum albumin level was the only statistically significant factor found to be associated with failure of initial percutaneous therapy ($P = 0.045$). Importantly, the initial type of percutaneous therapy (drain *vs* aspiration) was not associated with initial success.

Multivariate analysis was not performed as the small number of treatment failures would mean that the event per variable rate would be unacceptably high.

DISCUSSION

Consistent with other recent studies, most patients with liver abscesses are elderly, with a mean age of 64 years^[2,8-10]. This is about 10 years older than subjects in previous reports^[4,5,11]. Again, a preponderance of males was noted.

The presentation of pyogenic liver abscesses is often non-specific and its diagnosis requires a high degree of clinical suspicion. This is reflected in the finding that the median duration of symptoms of a week, with a further quarter of patients having symptoms between 1-2 wk, prior to presentation. Clinical abdominal findings were largely unhelpful, with localised peritonism found in only 14% and localised right upper quadrant tenderness in less than 40%. The literature indicates that these signs are found in 15%-55% of cases^[2,4,9,11].

Laboratory tests were equally non-specific, apart from

Table 7 Univariate analysis of factors that may lead to failure in initial percutaneous therapy

	Treatment successful	Treatment failure	P-value
Demographic characteristics and history			
Age (yr), mean (95% CI)	67 (62-73)	63 (57-69)	0.26
Sex (M:F)	20:11	15:08	1.00
Prodrome (days), median (IQR)	7 (3-20)	7 (5-14)	0.88
History of gallstone disease, <i>n</i> (%)	8 (27)	5 (22)	0.76
History of diabetes mellitus, <i>n</i> (%)	4 (13)	3 (13)	1.00
Chronic renal failure, <i>n</i> (%)	1 (3.3)	1 (4.4)	1.00
Current cancer, <i>n</i> (%)	6 (20)	5 (22)	1.00
Examination findings, <i>n</i> (%)			
Fever	16 (53)	12 (63)	0.56
Tachycardia	13 (43)	11 (58)	0.39
Hypotension	4 (13)	1 (5.3)	0.64
Laboratory findings, mean (95% CI)			
Leucocytes ($\times 10^9/L$)	15.2 (13.1-17.4)	13.6 (11.2-16.0)	0.30
Neutrophil ($\times 10^9/L$)	12.8 (10.8-14.9)	11.7 (9.5-13.8)	0.43
Haemoglobin (g/L)	114 (106-121)	120 (110-130)	0.26
Platelet ($\times 10^9/L$)	341 (255-426)	319 (250-388)	0.70
Urea (mmol/L)	9.3 (6.8-11.9)	6.5 (5.3-7.7)	0.07
Creatinine ($\mu\text{mol/L}$)	110 (83-138)	103 (86-121)	0.70
CRP (mg/L)	287 (236-338)	258 (193-324)	0.47
Albumin (g/L)	32 (29-35)	28 (25-31)	0.045
AST (U/L)	187 (30-344)	70 (43-97)	0.19
ALT (U/L)	149 (37-260)	84 (55-114)	0.33
GGT (U/L)	193 (115-271)	183 (96-269)	0.85
ALP (U/L)	201 (154-249)	203 (143-262)	0.97
Bilirubin ($\mu\text{mol/L}$)	29 (13-45)	26 (19-32)	0.75
Glucose (mmol/L)	7.9 (6.7-9.0)	7.6 (6.4-8.9)	0.79
INR	1.2 (1.1-1.3)	1.4 (1.0-1.8)	0.24
Microbiology			
Positive blood culture, <i>n</i> (%)	12 (43)	9 (47)	0.78
Positive aspirate culture, <i>n</i> (%)	17 (61)	17 (85)	0.11
Organism, <i>n</i> (%)			0.19
<i>Streptococcus milleri</i>	8 (26)	8 (35)	
<i>Klebsiella species</i>	5 (16)	7 (30)	
<i>Escherichia coli</i>	6 (19)	0 (0)	
Other	6 (19)	4 (17)	
Negative cultures	6 (19)	4 (17)	
Imaging findings			
Multiple abscesses, <i>n</i> (%)	7 (24)	10 (45)	0.14
Side of liver, <i>n</i> (%)			0.10
Left	5 (17)	2 (10)	
Right	23 (77)	13 (62)	
Bilateral involvement	2 (7)	6 (29)	
Abscess diameter, median (IQR)	6.0 (3.8-9.5)	6.5 (5.0-8.4)	0.83
Diameter > 5 cm	11 (41)	7 (35)	1.00
Presence of gas, <i>n</i> (%)	5 (17)	1 (5)	0.38
Biliary duct dilatation, <i>n</i> (%)	6 (20)	2 (10)	0.45
Intervention			
Percutaneous drain insertion (<i>vs</i> percutaneous aspiration)	24 (77)	19 (83)	0.74
Cause			
Presumptive cause, <i>n</i> (%)			0.60
Cryptogenic	8 (29)	9 (41)	
Biliary	7 (25)	5 (23)	
Portal	6 (21)	4 (18)	
Haematogenous	5 (18)	3 (14)	
Direct	2 (7)	1 (5)	

AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; CRP: C-reactive protein; GGT: Gamma glutamyl transpeptidase; INR: International normalized ratio.

demonstrating signs of inflammation. Notably, not only did all tested patients have a raised serum C-reactive protein, but the levels were also significantly raised, with a mean of 272 mg/L. By comparison, only 82% of patients demonstrated neutrophilia. In addition, consistent with a

systemic inflammatory response, the serum albumin was depressed in 73% of patients. CRP is not a commonly measured laboratory parameter documented in older studies: Rintoul *et al*^[3] reported that all nine of their patients with PLA had a raised CRP level. However, a recent paper

by Foo *et al.*^[12] demonstrated that the CRP was elevated in all 133 tested patients. Whilst each of the “liver enzymes” was abnormal in about 70% of cases, neither a “cholestatic” or “hepatic” pattern of LFT derangement predominated. In addition, mild hyperbilirubinaemia occurred in more than half of the patients, but clinically significant hyperbilirubinaemia was uncommon. These results are largely consistent with recent large case series (from both Asian and “Western” countries)^[2,8,12,13].

It is interesting to note that four large Asian case series^[2,5,8,12], with a total of more than 600 cases over the last ten years, demonstrated a large proportion of patients with *Klebsiella* species: accounting for 43% to 66% of cases. In each of these studies, the biliary tree was the most commonly identified aetiology. However, the most common aetiology overall was “cryptogenic” in the three studies, where *Klebsiella* species accounted for more than half of all cases. Indeed, it has been suggested that *Klebsiella* is associated with “cryptogenic” liver abscesses^[9]. It is noted that *Klebsiella* liver abscess may represent a new syndrome in diabetic patients in Taiwan^[5] or perhaps in Asia. Recent “Western” series, however, suggested that *Escherichia coli* is still the most common causative organism^[10,13-15]. A new finding that has come to light in two papers published in 2010 from Western institutions with about 60 patients each, is the high prevalence (48% and 55%) of polymicrobial infections^[14,16]. The latter study was characterised by a very large proportion of patients with malignancy (88%).

The microbiological results of the current study fall somewhere in between Asian and Western series. We found that *Streptococcus milleri* was the most common organism, followed by *Klebsiella* species, and then *Escherichia coli*. This perhaps reflects the most common presumptive aetiology of liver abscesses in this series: cryptogenic, biliary, and portal. It is interesting to compare our results with another Australian study published a decade ago, which found that *Escherichia coli*, *Streptococcus milleri*, and *Enterococcus faecalis*, in this order of frequency, were the most commonly isolated organisms. Thirty-eight percent of the cases in that study had a cryptogenic origin, and 48% had a biliary aetiology^[4]. Interestingly, our series also had a large proportion of cryptogenic abscesses. Only 8% of all cases in our study had a polymicrobial culture result.

The first line treatment for all pyogenic liver abscesses is broad spectrum antibiotics. Our empirical antibiotic choice and duration of treatment are similar to those reported elsewhere in the literature^[1,2]. Shorter durations of treatment have also been described^[17].

There has been a trend for increasing use of radiological management of pyogenic liver abscesses, although the proportion of patients treated with radiological intervention varies greatly between centres^[3,11,13]. Successful use of radiological intervention as the first line intervention is reflected in three large series: Wong *et al.*^[2] described 80 patients who were all treated either with antibiotics alone or antibiotics and percutaneous aspiration/drainage with no patients requiring surgical drainage. Two other series from Taiwan described 111/128 (87%) and 79/86 (92%)

patients, respectively, who underwent radiological drainage/aspiration^[5,9]. This continued trend away from surgical therapy is confirmed by three studies published this year (2010), with only 11/377 (2.9%), 1/61 (1.6%), and 5/51 (9%) requiring surgical therapy^[12,14,16].

However, Tan *et al.*^[8] suggested that radiological aspiration may not be appropriate in all patients, and that the use of surgical drainage is advantageous in patients with abscesses larger than 5 cm, due to likely multiloculation. They found no difference in mortality or morbidity between the surgical and percutaneous drainage groups. This finding was confirmed by Alvarez Pérez *et al.*^[13] who described 133 consecutive patients in which they found no difference in mortality or morbidity between treatment groups (open *vs* percutaneous). Interestingly, the latter study also found that abscess size did not predict morbidity or mortality, which would appear to contradict the conclusion of Tan *et al.*^[8]. Consistent with this, we have found that abscess diameter did not predict failure of percutaneous therapy.

Image guided catheter drainage was favoured over radiological needle aspiration at our institution. There is no consistently favoured option in the literature, with some groups preferring needle aspiration over catheter drainage, and some *vice versa*. An early randomised study suggested that catheter drainage in patients with liver abscesses (pyogenic and amoebic) had a higher success rate compared with needle drainage alone^[18]. However, a more recent randomised study did not find any statistically significant difference between the two groups^[19]. Therefore, it is likely that both radiological interventions appear to be equally effective.

The need for a secondary intervention is similar between the drainage (19/43, 44%) and the aspiration groups (4/11, 36%) in this study. Indeed, no statistically significant difference in failure rate was found between the two groups. It may be noted that this “failure” rate is high compared to what is described in the literature. This may relate to the fact that approximately 90% of patients received radiological drainage as the initial procedure. It may also be related to the definition of a secondary procedure—we included repositioning and increasing the size of drains, as well as re-aspiration, as secondary procedures, whereas, in other case series, this may be considered an extension of the primary procedure. The overall rate of surgical drainage of 17% (11/63) in our series is consistent with the abovementioned studies.

Over the past few decades, the mortality associated with liver abscesses has decreased gradually. Prior to 1980, the mortality in published case series was consistently greater than 50%^[1]. Improvements in mortality in the 1980s were attributed to the use of effective antibiotics. Case series with study periods in the 1980s demonstrated a mortality rate of between 13%–18%^[11,20]. Further improvements in mortality then occurred due to the advent of cross sectional imaging with computed tomography and ultrasonography. The mortality rate for studies with study periods during and after 1990s was between 4%–10%^[2,4,5,8,9,19]. The mortality rate in the current study was 6.3%, continuing this trend for improving mortality.

This can be compared with the mortality of 8% in another Australian case series by Barakate *et al*^[4], which had a study period of a decade earlier than our current study. The deaths that occurred in both these studies principally occurred in already infirm patients, such as those with advanced cancer. The association of mortality with malignancy is reflected in the high mortality rate of 29% in a recent study by Mezhir *et al*^[16] where 88% of patients had a history of malignancy.

Many studies have investigated the risk factors for morbidity and mortality of the disease. However, as the mortality (and to a lesser extent, morbidity) has decreased, the focus has shifted to identifying patients who would successfully be treated with percutaneous therapy. Mezhir *et al*^[16] have found that the presence of yeast and communication with an untreated biliary obstruction were associated with failure of percutaneous therapy. Our current study has focused on the failure of initial percutaneous therapy and has found hypoalbuminaemia to be associated with failure. Hypoalbuminaemia may be associated with failure of initial therapy owing to its association with the severity of the underlying septic process.

Unlike mortality, morbidity is more difficult to compare between studies, because of differences in completeness of data collection in retrospective studies and therefore tends to be under-reported. Complications such as biliary fistula, leakage, and haemorrhage following percutaneous drainage or aspiration are known, but uncommon, complications. Tan *et al*^[8] described in the percutaneous drainage group ($n = 36$), one patient (2.8%) with fistula formation and four patients (11%) with peritonitis post drainage. This is similar to our reported rates of bile fistula and abdominal sepsis. Hemoperitoneum after needle aspiration has also been described-Yu *et al*^[19] had one such patient in a series with 64 patients. Furthermore, there are large case series, such as Wong *et al*^[2] with 80 patients who underwent percutaneous aspiration, that report no "major" complications. Operative management of liver abscesses may be associated with significant morbidity. In a study of 32 patients who underwent operative management (either for failed drainage or presenting as a surgical emergency), Christein *et al*^[21] reported a morbidity rate of 41%. This included two patients with persistent bile leaks. A similarly high morbidity rate of 27% was described by Tan *et al*^[8] in their surgical drainage group.

This is a retrospective review and was not undertaken with a standardized protocol variance in management, which could distort the conclusions; however, the surgeons involved in this study kept prospective records of their experience. The results, however, do reflect a changing trend towards a more conservative treatment protocol with an improving outcome. It provides the basis for a standardized protocol to be prospectively applied to our institution.

In conclusion, pyogenic liver abscess can be a diagnostically and therapeutically challenging problem. Often, the presentation and investigations are non-specific, although the serum CRP appears to be the simplest and most sensitive test. However, it is not specific. CT or magnetic reso-

nance scans allows imaging of the abscess for diagnosis and assessment of progress, and also allows percutaneous image guided drainage. A combination of broad spectrum intravenous antibiotics and radiological drainage usually allows resolution of pyogenic liver abscesses. A proportion of patients will require repeat procedures-this appears to be predicted by low serum albumin at presentation. A further smaller proportion of patients will ultimately require surgical drainage.

COMMENTS

Background

Pyogenic liver abscesses are most frequently caused by *Escherichia coli*, *Streptococcus milleri*, or *Klebsiella* species often through, biliary, portal sepsis, or cryptogenic sepsis, respectively. It is a diagnostic and therapeutic challenge because patients usually present with a non-specific septic illness. Although the mortality rate has greatly improved, the occurrence of a liver abscess threatens a patient's survival if not adequately treated.

Research frontiers

Improving the frequency of early diagnosis will depend on education of clinicians about the need for clinical suspicion aided by the finding of an elevated C-reactive protein and confirmed by radiological imaging. Early cases may be treated by antibiotics, but larger abscesses also frequently require image guided percutaneous aspiration or drainage. Surgery is only required in complex cases. Cryptogenic infections with *Klebsiella* are the most common cause in Asian series, while biliary sepsis and a portal source of sepsis are most common in Western society. This series was a mixture of the two patient groups.

Innovations and breakthroughs

This article describes a moderate sized case series from an Australian tertiary referral centre, where patient presentation and outcome appear to be similar to other Western case series. There was a large proportion of *Klebsiella* organisms, indicating that the aetiology appears lie between the Asian and Western case series. This study also confirms the value of percutaneous therapy.

Applications

This article confirms that elevated C-reactive protein level is an important guide to the diagnosis, which is frequently delayed. Intravenous antibiotics in combination with percutaneous image guided aspiration or drainage is usually a successful treatment.

Peer review

This is a retrospective case series of 63 Australian patients with pyogenic liver abscesses. The text is generally well written, with a structured abstract and organized sections. The manuscript has scientific value since it includes relevant information about an important condition with multiple therapeutic options. I believe it can be considered for publication, provided that the authors adequately address some key issues and suggestions.

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Trefoil factors: Tumor progression markers and mitogens via EGFR/MAPK activation in cholangiocarcinoma

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METHODS: *TFF* mRNA levels, gene copy number and protein expression were determined respectively by quantitative reverse transcription polymerase chain reaction (PCR), quantitative PCR and immunohistochemistry in bile duct epithelium biopsies collected from individuals with CCA, precancerous bile duct dysplasia and from disease-free controls. The functional impact of recombinant human (rh)TFF2 peptide treatment on proliferation and epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK) signaling was assessed in the CCA cell line, KMBC, by viable cell counting and immunoblotting, respectively.

RESULTS: *TFF1*, *TFF2* and *TFF3* mRNA expression was significantly increased in CCA tissue compared to disease-free controls, and was unrelated to gene copy number. TFF1 immunoreactivity was strongly increased in both dysplasia and CCA, whereas TFF2 immunoreactivity was increased only in CCA compared to disease-free controls. By contrast, TFF3 immunoreactivity was moderately decreased in dysplasia and further decreased in CCA. Kaplan-Meier analysis found no association of *TFF* mRNA, protein and copy number with age, gender, histological subtype, and patient survival time. Treatment of KMBC cells with rhTFF2 stimulated proliferation, triggered phosphorylation of EGFR and downstream extracellular signal related kinase (ERK), whereas co-incubation with the EGFR tyrosine kinase inhibitor, PD153035, blocked rhTFF2-dependent proliferation and EGFR/ERK responses.

CONCLUSION: *TFF* mRNA/protein expression is indicative of CCA tumor progression, but not predictive for histological sub-type or survival time. TFF2 is mitogenic in CCA via EGFR/MAPK activation.

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Abstract

AIM: To investigate trefoil factor (*TFF*) gene copy number, mRNA and protein expression as potential biomarkers in cholangiocarcinoma (CCA).

Key words: Cholangiocarcinoma; Trefoil factors; Liver fluke; Epidermal growth factor receptor; Mitogen-activated protein kinase

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from the biliary tract and classified according to the site of formation as either intrahepatic or extrahepatic type^[1,2]. CCA accounts for 3% of all gastrointestinal cancers and is the second most common primary hepatic tumor^[3]. The Southeast Asian liver fluke, *Opisthorchis viverrini* (*O. viverrini*) is a trematode parasite that infects the bile duct^[4]. Previous studies have shown a strong positive correlation between CCA incidence and the prevalence of *O. viverrini* infection, as measured by anti-*O. viverrini* antibody titers in the general population residing in the northeast Thailand^[5-7], where the Khon Kaen Province has shown the highest incidence of CCA in the world^[8]. The truncated age-standardized incidence of CCA in Khon Kaen in age ranges older than 35 years varied between 93.8 and 317.6 per 100 000 population, depending on geographical location^[5].

Several lines of evidence implicate an interaction between chemical carcinogens, especially nitrosamines, and *O. viverrini* infestation in the development of CCA in Thailand^[9,10]. Mechanical injury to bile duct epithelial cells from feeding activity and migration of the liver fluke may also contribute to biliary damage in the human host. In addition, secretion or excretion of metabolic products from the liver fluke results in chronic irritation, hyperplasia and adenomatous changes of the bile duct epithelium^[9]. Subsequent DNA damage in the biliary epithelium may drive malignant transformation^[7,11].

To date, knowledge of the molecular basis of carcinogenesis and pathogenesis of CCA is limited. Previous studies in CCA patients exploring the fine mapping of chromosome region 21q22-qter showed an amplification with a frequency of more than 30% in the markers D21S1890-D21S1893, and *TFF3*, a member of the trefoil factor (*TFF*) gene family^[12]. Kaplan-Meier survival curves demonstrated that patients who have amplifications at D21S1893, D21S1890 and the *TFF* locus had a poor prognosis, whereas patients who had deletions showed a favorable prognosis, indicating that this region may harbor candidate genes involved in the tumorigenesis and pathogenesis of CCA.

TFF genes are involved in restitution and repair of the gastric and intestinal epithelium and are rapidly up-regulated in response to mucosal injury^[13,14]. In particular, *TFF1* has been shown to function as a gastric tumor sup-

pressor gene^[15]. However, *TFF* peptides are overexpressed in other solid tumors such as esophagus, breast, and pancreas and in some circumstances may function as tumor progression factors^[16-18]. Prolonged inflammation caused by parasitic infection frequently occurs in liver fluke-related CCA. *TFFs* may exert beneficial effects during the early steps of bile duct epithelial injury and inflammation, but have undesirable effects during subsequent chronic inflammation and neoplastic progression.

The molecular basis of *TFF* activity remains enigmatic and a specific *TFF* receptor has not been identified. The epidermal growth factor receptor (EGFR) is a type- I transmembrane glycoprotein receptor with tyrosine kinase activity in its cytoplasmic domain. EGFR is activated following the binding of multiple cognate ligands and plays a significant role in initiating the signaling that directs growth, proliferation, survival, and differentiation in mammalian cells^[19]. Several ligands of EGFR have been identified^[20], and there is some evidence for the transactivation of EGFR by *TFFs*. EGFR is a key signaling pathway for *TFF1*- and *TFF2*-mediated cellular invasion in kidney and colonic cancer cells^[21], suggesting the capability of *TFFs* to directly, or indirectly, transactivate the EGFR. While EGFR signaling has been shown to be important in CCA, there has been no report of *TFF*-mediated EGFR pathway activation in CCA.

With the available evidence in mind, we hypothesized that *TFFs* play important roles in the molecular pathogenesis of CCA, and mediate their actions, at least in part, through the transactivation of EGFR. We also hypothesized that an increase in *TFF* gene copy number resulting in the inappropriate overexpression of mRNA and the corresponding protein, contributes to the progression of CCA. Accordingly, we have measured *TFF* copy number, mRNA and protein in CCA patients, and have analyzed their association with clinicopathological parameters. In addition, *TFF*-mediated proliferation, EGFR transactivation and downstream signaling *via* the mitogen-activated protein kinase (MAPK) cascade was also investigated in a human CCA cell line.

MATERIALS AND METHODS

Patients and sample processing

Frozen liver tissues were obtained from 110 CCA patients undergoing surgical resection at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. CCA cases were diagnosed by physicians using clinical findings, laboratory investigations and histological examinations. Clinicopathological data, such as age, gender, histological type, and tumor invasion, were obtained from medical records. The protocols were approved by the Ethical Committee of Khon Kaen University (HE480229). Informed consent was obtained from all patients who participated in the project.

Serum anti *O. viverrini* antibody was tested for association between liver fluke infection and CCA by enzyme-linked immunosorbent assay (ELISA) as described previously^[22]. The cut-off value was 0.200 optical density for

Table 1 Cholangiocarcinoma samples tested for serum anti *Opisthorchis viverrini* antibody

No.	Code	Anti-OV titer (OD) ¹	OV positive
1	M009	0.804	+
2	M012	0.303	+
3	M030	0.820	+
4	M043	0.182	-
5	M055	0.185	-
6	M068	0.356	+
7	M071	0.257	+
8	M097	0.188	-
9	M101	0.235	+
10	M111	0.088	-
11	M114	0.285	+
12	M117	0.345	+
13	M119	0.168	-
14	M132	0.095	-
15	M137	0.330	+
16	M139	0.239	+
17	M142	0.344	+
18	M148	0.305	+
19	M152	0.108	-
20	M155	0.642	+
21	M157	0.482	+
22	M208	0.952	+
23	M214	0.188	-
24	M229	0.165	-
25	M240	0.669	+
26	M265	0.314	+
27	M279	0.502	+
28	N004	0.218	+
29	N021	0.232	+

¹Cut-off optical density (OD) = 0.200. OV: *O. viverrini*.

O. viverrini-positive subjects^[23]. There were 20/29 (69%) *O. viverrini*-positive cases (Table 1), which agreed well with general screening for anti *O. viverrini* antibody in other sample set (68%) (unpublished data) and a previous study^[5].

DNA preparation

DNA of CCA patients was prepared from frozen liver tissues using the Puregene™ DNA purification system (Gentra System, Minneapolis, MN, USA). Because establishing standard curves for analysis of *TFF* gene copy number by quantitative polymerase chain reaction (qPCR) required a large amount of DNA, DNA prepared from placental tissue which was collected from a patient after normal labor (postpartum) was used in this study.

Gene expression and copy number analysis

Total RNA was prepared from frozen liver tissues using RNeasy mini kits (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocols. For quantitative reverse transcription PCR (QRT-PCR), oligo-dT primed first strand cDNA was synthesized from 1 µg template RNA using Omniscript Reverse Transcriptase reagents (QIAGEN) according to the manufacturer's protocols. RNA was also prepared from stomach and colon tissues collected from cancer patients who underwent surgical resection at Srinagarind Hospital, and was used to establish standard curves for analysis of *TFF* mRNA expression.

Oligonucleotide primer sequences for DNA copy number and QRT-PCR expression analysis, together with corresponding amplicon sizes are shown in Table 2. Primers sequences were designed using pDRAW32 (<http://www.acaclone.com/>).

Quantitative PCR amplification was performed on a Rotor Gene 3000 Real-time Amplification (Corbett Research, Australia) using a standard curve and SYBR Green I dye method (Amresco, Solon, OH, USA). The standard curve of each primer was generated using serial dilutions of placental DNA (for genomic *TFF1*, *TFF2*, and *TFF3*), stomach cDNA (for *TFF1* and *TFF2* mRNA), and colon cDNA (for *TFF3* mRNA). The standard curve was constructed in each PCR run and the copy number or cDNA expression of genes in each sample was interpolated using these standard curves. A coefficient of variation (CV) of each sample was determined based on triplicate test. The sample with a CV higher than 15% was re-tested. The melting curves of the PCR products were performed for each reaction to demonstrate that there were no nonspecific products or primer dimers.

TFF copy number was determined from a standard curve. The relative copy number of *TFF* was determined in comparison to the reference gene, *β-actin*, which is not amplified in CCA. The normal reference range was derived as we described previously^[12]. The relative copy number was interpreted as loss when the ratio was less than the mean - 2SD of the normal reference range, and as gain when the ratio was greater than mean + 2SD.

Immunohistochemistry

Tissue samples were processed for histology examination using standard procedures^[24]. A 4-µm thick section was cut from each paraffin block. TFF1, TFF2 and TFF3 proteins were detected by immunohistochemical staining using rabbit polyclonal anti-TFF1 and -TFF3 antibodies (Gifts from Professor Andrew Giraud, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, VIC 3052, Australia) at a dilution of 1:1000, and mouse monoclonal anti-NCL-HSP (anti-TFF2) (clone GE16C, Novocastra Lab, Newcastle, UK) at a dilution of 1:200. In brief, deparaffinized sections were boiled in 0.01 mol/L citrate buffer (pH 6.0) for 3 min for antigen retrieval. Sections were then incubated with 5 mL/L hydrogen peroxide in absolute methanol for 30 min to block endogenous peroxidase activity, then incubated with 50 mL/L horse serum (Seromed Biochrom, Berlin, Germany) for 30 min and incubated overnight at 4°C with anti-TFF antibodies. The sections were incubated with the DAKO EnVision™ solution (rabbit or mouse/horseradish peroxidase; DAKO Corporation, Carpinteria, CA, USA) for 30 min. Histochemical reaction for peroxidase visualization was carried out with 0.5 mL/L 3,3'-diaminobenzidine tetrahydrochloride and 1 mL/L hydrogen peroxide, and was then counterstained with hematoxylin. The amount of TFF was classified into 4 groups based on the percentage of positive staining tumor cells: score 0, for percent positive staining cells < 1%;

Table 2 Primer sequences of genomic and reverse transcription polymerase chain reaction, and polymerase chain reaction fragment sizes

Primer name	Forward (5→3)	Reverse (5→3)	Product size (bp)
Genomic-PCR			
<i>TFF1</i>	CAGGGATCTGCCTGCATC	ATCGATCTCTTTAATTTTAGGCC	219
<i>TFF2</i>	GAAGAATCTCCGAACCAAGG	GTCACACTCAAAAACTAGAGG	123
<i>TFF3</i>	CAGGCACTGTTCATCTCAGC	TATTCGTTAAGACATCAGGCTCC	129
<i>β-actin</i>	TCACCCACTGTGCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	375
RT-PCR			
<i>TFF1</i>	CCCGTGAAAGACAGAATT	GATCCCTGCAGAAGTGCTCT	169
<i>TFF2</i>	CTCCTGGCAGCGCTCCTCGTC	GATGCCCGGTAGCCACAGTTTCT	223
<i>TFF3</i>	AACCGGGCTGCTGCTTTG	GAGGTGCCTCAGAAGGTGC	92
<i>RPLP0</i>	CTTCCCACTGCTGAAAAG	CCAAATCCCATATCCTCGT	168

PCR: Polymerase chain reaction; RT-PCR: Reverse transcription PCR; TFF: Trefoil factor.

score 1, 1% to 25%; score 2, 26% to 50%; and score 3, > 51%. Slides were examined independently by two pathologists. Results in agreement were considered to be valid. All studies were accompanied by a negative (no primary antibody) and a positive control (gastric tissues for *TFF1* and *TFF2*, and colonic tissues for *TFF3*). In addition, we also observed TFF immunostaining in normal bile ducts and dysplasia of 110 CCA patients to evaluate protein expression and stepwise carcinogenesis in CCA.

Cell viability and proliferation

The KMBC cell line, derived from extrahepatic CCA, was maintained in RPMI-1640 medium supplemented with 5 mL/L fetal bovine serum (FBS); 50 IU/mL penicillin; 50 µg/mL streptomycin, at 37°C in 50 mL/L CO₂/air. To determine cell viability and proliferation rates, cells were serum starved overnight, then seeded (5×10^4 cells/well) in 24-well plates containing RPMI-1640 medium supplemented with 5 mL/L FBS; 50 IU/mL penicillin; 50 µg/mL streptomycin, and incubated with recombinant human (rh)*TFF2* (Gift from Professor Andrew Giraud, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, VIC 3052, Australia) (5, 50, 500 µg/mL) or EGF (Promega, Indianapolis, IN, USA) (5, 25, 50 ng/mL). Viable cell numbers were counted with 0.4% trypan-blue dye exclusion on a slide hemocytometer. For EGFR blockade the EGFR tyrosine kinase inhibitor, PD153035 (10 µmol/L), was added to abrogate EGFR 1 h before adding rh*TFF2* or EGF. All cell viability assays were performed in triplicate for each concentration and each experiment was repeated twice ($n = 6$ /group).

Immunoblotting

For immunoblotting, cell lysates were size fractionated by 10%-15% SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and blocked in 5% non-fat milk powder/Tris-buffered saline pH 7.4, 0.1% Tween-20 as described. Membranes were incubated with primary antibodies overnight at 4°C. Detection was performed with anti-rabbit/anti-mouse IgG-HRP conjugates (DAKO Corporation, Carpinteria, CA, USA) and enhanced chemiluminescence re-

agents (GE Healthcare Bio-Sciences). Sources of primary antibodies: rabbit polyclonal anti-human EGFR (#2232) diluted 1:250; anti-human phospho-tyrosine 1173 EGFR (#4407) diluted 1:500; rabbit polyclonal anti-human extracellular signal-related kinase (ERK)1/2 (#9102) diluted 1:1000; anti-human phospho-threonine 202, phospho-tyrosine 204 ERK (#9101) diluted 1:1000; mouse monoclonal anti-human cyclin E (#4129) diluted 1:1000 (all Cell Signalling Technology, Danvers, MA, USA); rabbit polyclonal anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #ab9485) diluted 1:3000 (Abcam, Cambridge, MA, USA).

Statistical analysis

Correlations between *TFF* DNA copy number and mRNA expression were analyzed using linear regression. Associations between *TFF* copy number, mRNA expression and protein expression, and clinicopathological parameters of CCA patients were evaluated using χ^2 tests. The difference in protein expression in normal bile ducts, dysplasia and CCA was assessed by Wilcoxon's rank sum test. Survival curves of patients (with *vs* without abnormal DNA copy number, normal *vs* high mRNA expression, and negative *vs* positive protein expression of *TFF* genes) were calculated using the Kaplan-Meier method. Differences in survival between two groups were assessed by the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of TFF mRNA in CCA

To evaluate the role of TFFs in CCA, we measured *TFF* mRNA levels in tumor tissues of CCA patients using quantitative reverse transcription PCR (QRT-PCR). For comparison, tissues were also collected from tumor-free margin areas of 16 individuals. *TFF* mRNA level was normalized to that of *RPLP0* mRNA. The relative mRNA expression level of *TFF1*, *TFF2* and *TFF3* in normal samples ranged from 0.000-0.052 (mean, 0.004), 0.000-0.056 (mean, 0.008), and 0.000-0.329 (mean, 0.048), respectively. Relative mRNA expression level of *TFF1*, *TFF2* and *TFF3* in 46 tumor samples ranged from 0.000-9.773 (mean, 1.178),

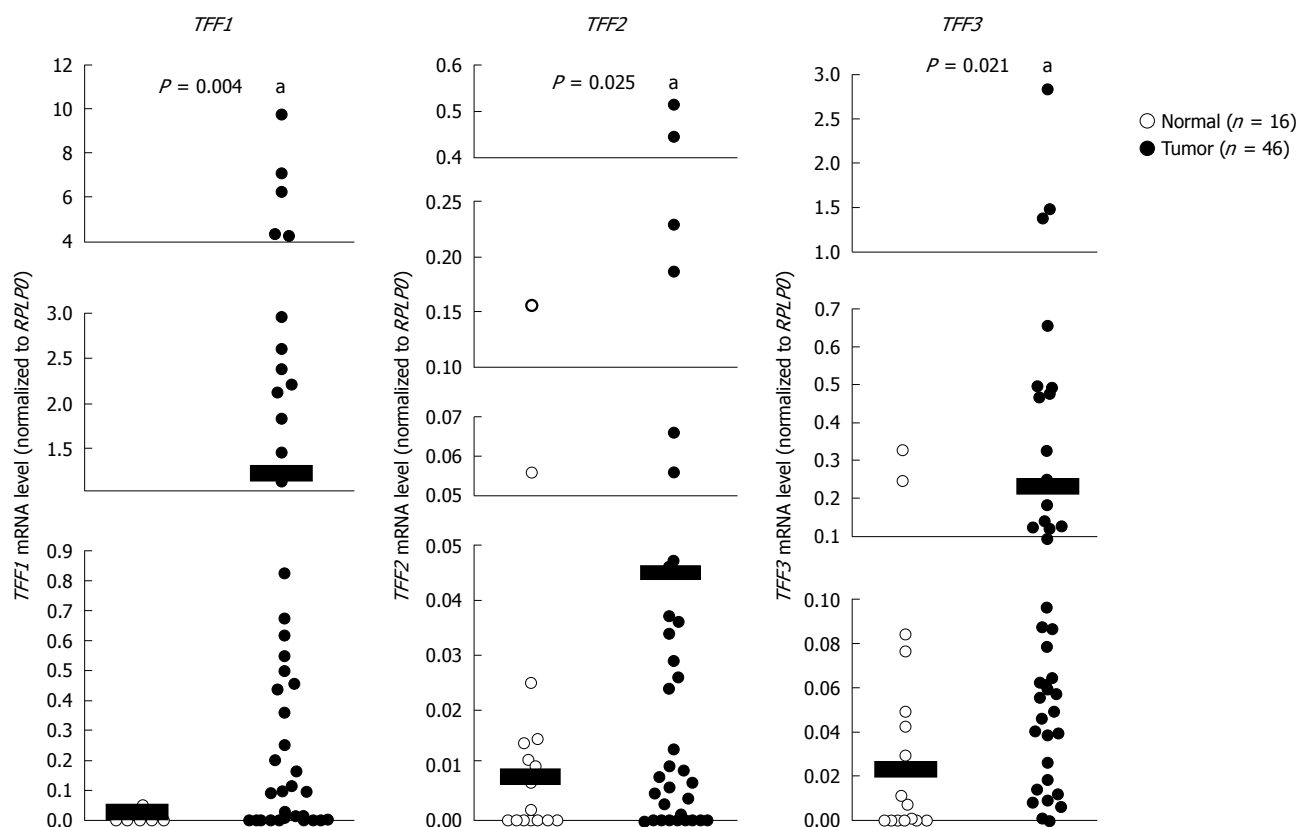


Figure 1 The mRNA expression levels of Trefoil factor genes in normal and cholangiocarcinoma tissues. Quantitative reverse transcription polymerase chain reaction analysis of trefoil factor (*TFF1*, *TFF2* and *TFF3*) mRNA levels in cholangiocarcinoma tumors ($n = 46$) compared with normal controls ($n = 16$). Scatter plots show mRNA abundance for each data point normalized to the internal reference gene *RPLP0*. Horizontal black bars show mean mRNA levels. $^aP < 0.05$. A statistical outlier in the *TFF2* normal control data set is +3.58 standard deviations from the mean and failed a Z-test, which requires that values must fall within 3 standard deviations of the mean for statistical validity. This sample (shown in bold font) was excluded from all statistical calculations for this data set.

0.000-0.516 (mean, 0.045), and 0.000-2.827 (mean, 0.229), respectively. The expression of *TFF1*, *TFF2* and *TFF3* mRNA in tumor tissues was significantly higher than in normal tissues ($P < 0.05$) (Figure 1). These results demonstrate that increased *TFF* gene expression correlates with tumor progression in CCA.

TFF protein expression in CCA

Immunohistochemistry was performed to determine TFF protein levels and cellular distribution in tissues collected from CCA patients. Examination of subcellular distribution revealed that all TFFs were present in fine cytoplasmic vesicles and at the apical/luminal surface of the epithelial cells. Faint and scattered *TFF1* immunoreactivity was observed in normal bile ducts with low frequency (24.6%). However, *TFF1* immunoreactivity was intense (Figure 2A) and markedly frequent in dysplasia. *TFF2* immunoreactivity was present mainly in peribiliary glands and rarely expressed in normal bile ducts and dysplasia (Figure 2B). *TFF3* immunoreactivity was distributed widely and expressed faintly in normal bile ducts but more intensely in dysplasia (Figure 2C) with high frequency in each case.

In tumor samples, *TFF1* was present mostly in the cytoplasm (Figure 3A), but in three cases, *TFF1* staining was also found in secreted mucus. *TFF1* immunoreactiv-

ity was detected in 56/110 (50.9%). Its frequency was markedly increased compared with normal ($P < 0.001$) (Figure 4A). *TFF2* was detected mostly in the cytoplasm with less frequency in 28/110 (25.5%). Interestingly, some positive staining was found at the invasion front (Figure 3B). *TFF3* immunoreactivity was observed in 54/110 (49.1%) of patients analyzed. Two patterns of *TFF3* staining were found in tumor cells: in cytoplasm as fine granules and in the apical/luminal surfaces, and as a goblet cell pattern in which *TFF3* was diffusely distributed as coarse granules (Figure 3C). In addition, *TFF3* immunoreactivity was highly frequent in disease-free control tissue (68.2%) but less frequent in dysplasia (55.5%) and CCA (49.1%) (Figure 4C). Although *TFF2* expression in CCA was less frequent compared to those of *TFF1* and *TFF3*, its frequency was markedly increased in comparison with those of normal bile ducts and dysplasia ($P < 0.01$) (Figure 4B) suggesting a role in tumor progression. Moreover, we found a significant correlation between *TFF1* and *TFF3* expression ($P = 0.014$), whereas *TFF2* expression was not correlated with either *TFF1* or *TFF3* expression (Table 3).

TFF copy number in CCA

The normal range of gene copy number derived from our previous study is 0.54-1.34 under a 95% CI^[12]. The

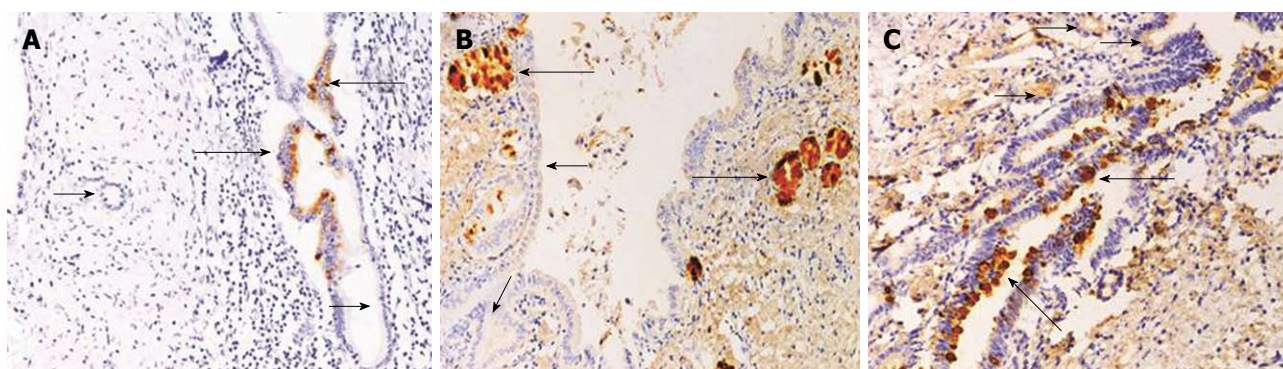


Figure 2 Immunohistochemical detection of trefoil factor proteins in normal and dysplasia bile ducts (Original magnification $\times 400$). A: Small and large normal bile ducts (short arrows) showing no trefoil factor (TFF)1 expression, whereas dysplasia showed moderately increased expression (long arrows); B: TFF2 expression was strongly positive in peribiliary glands (long arrows) but rarely present in dysplasia (short arrows); C: TFF3 was distributed widely at low level in small and large normal bile ducts (short arrows) and markedly increased in dysplasia (long arrows).

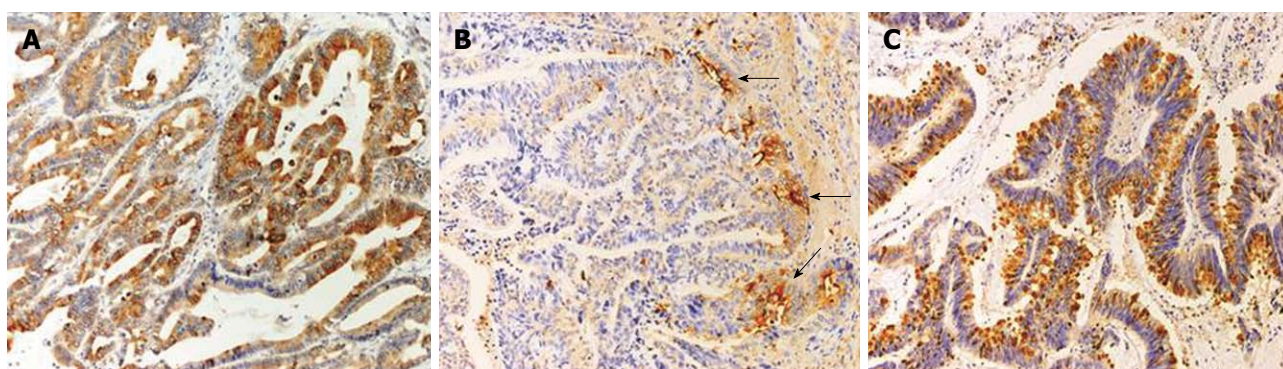


Figure 3 Immunohistochemical examinations of trefoil factor proteins in cholangiocarcinoma (Original magnification $\times 400$). A: Trefoil factor (TFF)1 staining was markedly positive and was mostly seen in the cytoplasm; B: TFF2 staining was mostly in the cytoplasm and at the invasion front (arrows); C: TFF3 staining showing a goblet cell pattern manifested as diffusely distributed coarse granules.

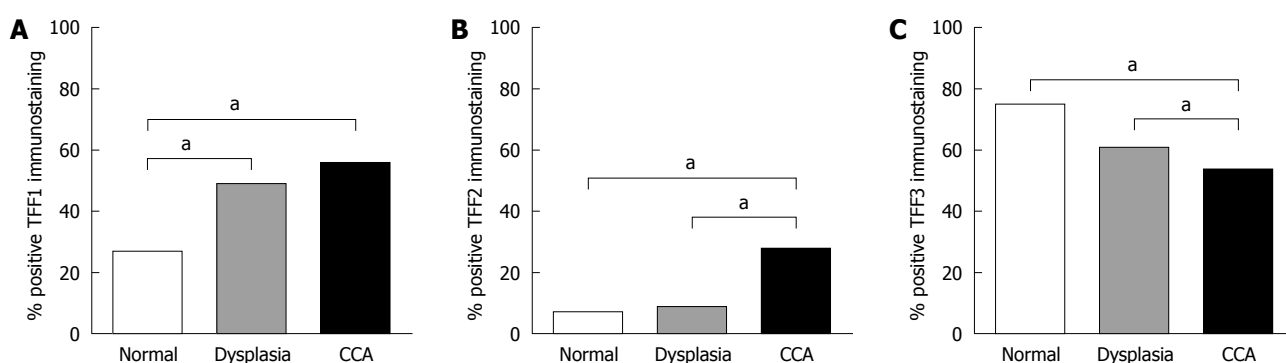


Figure 4 Frequencies of trefoil factor immunostaining in normal bile ducts, dysplasia and cholangiocarcinoma. A: Trefoil factor (TFF)1 positive immunostaining was markedly frequent in dysplasia and cholangiocarcinoma (CCA) compared to normal; B: Frequency of TFF2 positive immunostaining was significantly higher in CCA than in dysplasia and normal controls; C: TFF3 positive immunostaining was less frequent in CCA than in dysplasia and controls. $^*P < 0.05$.

percentage of CCA patients who had relative amplification at *TFF1*, *TFF2*, and *TFF3* was 25%, 12%, and 30%, respectively while that of CCA patients who had relative deletion at *TFF2* was 5%, and deletion of *TFF3* was 4% (Table 4). Relative amplification of these genes ranged from 1.35-3.67 and deletion from 0.37-0.51. Statistical analysis showed no associations of *TFF* mRNA, protein and copy number with age, gender, histological type, and survival time of the patients (Figure 5).

TFF2 stimulates CCA cell proliferation via EGFR-mediated MAPK

Our data showed increased *TFF2* expression in CCA tumor tissue compared to normal bile ducts and dysplasia, suggesting an important role in tumor progression. To address this question in greater detail, we next utilized an *in vitro* culture model to manipulate *TFF2* levels in CCA. The KMBC cell line is derived from extrahepatic CCA and is atypical in that it does not express significant lev-

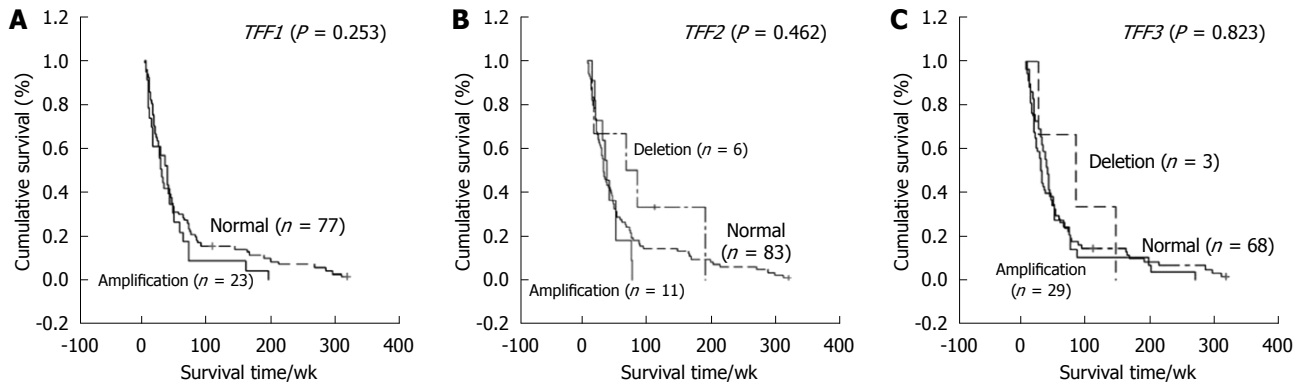


Figure 5 Kaplan-Meier survival curves with regard to trefoil factor copy number in cholangiocarcinoma. No association between trefoil factor (TFF) copy number and survival time (week) was found for A: TFF1; B: TFF2; C: TFF3.

Table 3 Associations of trefoil factor protein expression in cholangiocarcinoma

TFF protein expression	n	TFF2			TFF3		
		Negative	Positive	P-value	Negative	Positive	P-value
TFF1	110						
Negative	54	42	12	NS	34	20	0.014
Positive	56	40	16		22	34	
TFF2	110						
Negative	82			NS	44	38	NS
Positive	28				12	16	

TFF: Trefoil factor.

els of TFF2. With this in mind, we designed a series of experiments to compare the proliferation rate of KMBC cells treated with or without recombinant human (rh)TFF2 peptide. EGF is a well-known mitogen and was used to validate KMBC proliferation as a positive control. Treatment with rhTFF2 (and EGF) increased the proliferation rate of KMBC cells (Figure 6A). While signaling receptors for TFFs have not been identified, studies in other cancer cells have shown that TFF signals can be transduced by transactivation of the EGFR^[21]. To determine whether rhTFF2 mediates proliferation responses *via* the EGFR, a specific EGFR antagonist, PD153035 (10 μ mol/L), was used to abrogate EGFR activity at least 1 h before treatment with 5 μ g/mL rhTFF2 or 50 ng/mL EGF. PD153035 treatment abolished both rhTFF2 and EGF dependent proliferative responses (Figure 6B). We next determined cyclin-E protein levels as a molecular endpoint of proliferation, finding that cyclin-E abundance was directly correlated with the viable cell count data (Figure 6C). Collectively, these results indicate that TFF2 stimulates CCA cell proliferation *via* transactivation of EGFR. To confirm this finding, we next determined the levels of EGFR activity by immunoblotting for EGFR (tyrosine-1173) phosphorylation. Consistent with expectation, rhTFF2 treatment triggered a significant increase in EGFR phosphorylation (tyrosine 1173). A similar effect was obtained by treatment with EGF. On the other hand tyrosine EGFR phosphorylation was significantly attenuated after treatment with PD153035 (Figure 6D).

The MAP-kinase cascade is the predominant signal

transduction pathway utilized by the EGFR. To confirm the identity of the intracellular mediator(s) of EGFR-dependent TFF2 activity we next investigated the response of the terminal MAP-kinases, p44/p42 or ERK 1/2 to rhTFF2 treatment in KMBC cells. Consistent with the effect on EGFR activity, exogenous rhTFF2 treatment significantly increased ERK phosphorylation compared to untreated controls ($P = 0.016$). A similar effect was obtained with EGF treatment, while both rhTFF2 and EGF-dependent activation of ERK was abolished by co-incubation with the EGFR antagonist, PD153035 (Figure 6E). These results suggest that increased TFF2 expression drives CCA tumor progression by increasing cell proliferation *via* activation of EGFR and MAPK signaling.

DISCUSSION

Previous studies have revealed differences in TFF gene expression in biliary pathologies depending upon the size of bile duct; small, medium or large^[25-27]. In addition, there are variations of TFF expression observed among these studies. They showed that TFF1 and TFF3 are moderately expressed in normal bile ducts, particularly large bile ducts and markedly increased in biliary epithelial diseases^[25-27], suggesting their significant roles in cytoprotection and biliary epithelial repair. Sasaki *et al*^[28] studied TFF1 expression in normal, biliary epithelial dysplasia, and CCA associated-hepatolithiasis. It was found that TFF1 was only modestly expressed in disease-free control tissue, yet was dramatically upregulated in dyspla-

Table 4 Relative copy number of trefoil factor genes in cholangiocarcinoma

No.	<i>TFF1</i>	<i>TFF2</i>	<i>TFF3</i>
1	2.03 ¹	1.69 ¹	2.31 ¹
2	1.43 ¹	1.37 ¹	1.72 ¹
3	1.55 ¹	1.37 ¹	2.34 ¹
4	2.00 ¹	2.19 ¹	2.91 ¹
5	1.46 ¹	1.73 ¹	1.84 ¹
6	1.79 ¹	1.78 ¹	3.21 ¹
7	1.82 ¹	2.74 ¹	3.67 ¹
8	1.44 ¹	0.96	1.38 ¹
9	1.55 ¹	0.98	1.78 ¹
10	1.55 ¹	0.99	1.56 ¹
11	1.68 ¹	1.30	2.36 ¹
12	1.68 ¹	1.10	1.40 ¹
13	1.97 ¹	0.85	2.17 ¹
14	1.61 ¹	1.33	2.32 ¹
15	1.58 ¹	1.00	1.58 ¹
16	1.62 ¹	1.22	2.89 ¹
17	1.49 ¹	1.17	1.35 ¹
18	1.52 ¹	0.97	2.25 ¹
19	1.62 ¹	1.21	1.85 ¹
20	1.14	1.10	1.55 ¹
21	1.17	0.98	1.66 ¹
22	0.83	0.92	1.57 ¹
23	1.14	1.03	1.48 ¹
24	0.98	1.14	1.41 ¹
25	1.06	1.15	1.65 ¹
26	1.06	1.33	2.13 ¹
27	1.32	0.91	1.42 ¹
28	1.20	1.01	1.57 ¹
29	1.27	1.63 ¹	1.85 ¹
30	1.32	1.89 ¹	1.58 ¹
31	1.29	2.68 ¹	2.16 ¹
32	1.28	1.38 ¹	1.87 ¹
33	0.88	1.48 ¹	1.63 ¹
34	1.49 ¹	1.42 ¹	1.24
35	1.38 ¹	0.71	0.99
36	1.77 ¹	1.03	1.12
37	1.38 ¹	0.92	1.29
38	1.51 ¹	1.17	0.94
39	1.52 ¹	1.02	1.23
40	1.46 ¹	1.12	1.34
41	1.42 ¹	0.97	1.34
42	1.37 ¹	0.73	0.79
43	0.84	0.47 ²	0.58
44	0.63	0.37 ²	0.88
45	0.68	0.53 ²	0.86
46	0.63	0.51 ²	0.83
47	0.86	0.38 ²	0.55
48	0.76	0.42 ²	0.46 ²
49	0.92	0.68	0.45 ²
50	0.82	0.85	0.51 ²
51	1.07	0.79	0.97
52	1.15	0.59	1.11
53	0.95	0.84	1.02
54	0.84	0.79	0.68
55	0.74	0.77	1.26
56	0.77	1.03	0.83
57	1.13	1.31	1.26
58	1.13	1.03	1.20
59	1.00	0.88	0.89
60	1.17	0.70	1.15
61	0.94	0.91	1.19
62	0.76	0.72	0.63
63	1.15	1.04	1.15
64	1.25	0.95	0.87
65	1.01	0.77	0.90
66	1.11	1.27	1.03

67	0.85	0.62	0.54
68	1.10	0.96	1.23
69	1.06	0.92	0.84
70	1.05	0.88	0.80
71	1.12	1.09	1.10
72	1.33	0.87	1.22
73	0.87	1.01	0.71
74	0.75	0.88	0.76
75	1.04	0.74	1.14
76	1.10	0.67	0.93
77	1.06	0.98	1.14
78	1.10	0.97	1.30
79	1.22	0.87	1.21
80	1.16	0.95	1.32
81	1.13	1.07	1.32
82	1.10	0.88	1.24
83	0.92	1.05	1.31
84	1.14	0.97	1.09
85	1.01	0.98	1.14
86	1.13	1.07	1.26
87	0.91	0.59	1.23
88	0.97	0.69	1.16
89	0.91	0.78	0.91
90	1.06	0.71	1.06
91	1.15	0.69	0.88
92	0.96	0.58	0.57
93	1.03	1.00	1.30
94	1.20	0.93	1.06
95	1.25	0.76	0.77
96	1.28	1.21	1.24
97	1.28	1.15	0.94
98	1.10	0.82	0.99
99	1.28	0.73	0.71
100	0.87	0.59	0.91
101	1.05	0.78	1.28
102	1.00	0.70	0.99
103	0.66	0.61	0.61
104	0.89	0.74	0.80
105	0.88	0.57	0.60
106	0.82	0.56	0.58
107	1.04	1.05	1.04
108	0.90	0.98	0.88
109	0.65	0.65	0.64
110	1.18	1.21	1.20

Figures indicate relative copy number of trefoil factor (*TFF*) genes (*TFF1*, *TFF2*, *TFF3*) compared to the reference gene, β -actin. ¹Amplification; ²Deletion. Other figures are normal.

sia and noninvasive CCA. Paradoxically, *TFF1* expression was significantly decreased in invasive CCA with a positive rate of 60% due to promoter hypermethylation^[28]. In this context, it seems that *TFF1* expression positively regulates CCA tumor growth, but negatively regulates tumor invasion, potentially by acting as a tumor suppressor in the latter. In contrast, we found that *TFF1* mRNA expression was significantly increased in tumor tissue compared to adjacent disease-free tissues. Progressive increases in *TFF1* expression in dysplasia and CCA in our study strongly argues for a role in the stepwise carcinogenesis of liver fluke-associated CCA. Moreover, Vestergaard *et al*^[29] showed that *TFF1* and *TFF3* promoter hypomethylation correlates with increased mRNA expression in prostate cancer compared to benign prostatic hyperplasia. It is therefore conceivable that increased *TFF1* expression in our CCA cohort was due to promoter hypomethylation.

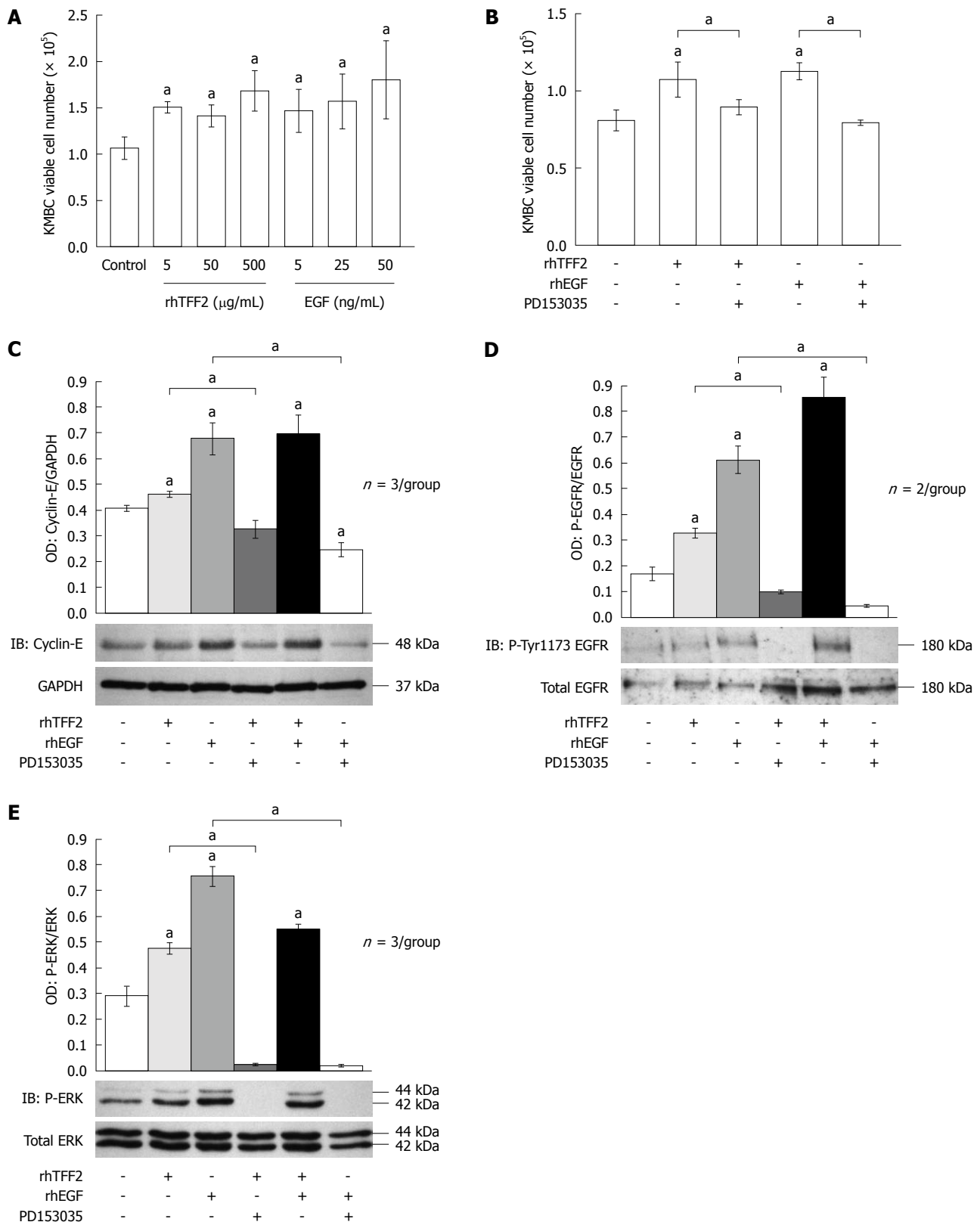


Figure 6 Trefoil factor 2 drives proliferation of KMBC cholangiocarcinoma cells via epidermal growth factor receptor tyrosine kinase and mitogen-activated protein kinase pathway activation. A: Proliferation of KMBC human cholangiocarcinoma cells determined by viable cell number counting with recombinant human trefoil factor (rhTFF)2 dose response (5, 50, 500 µg/mL) and epidermal growth factor (EGF) (5, 25, 50 ng/mL). Histograms show mean cell number per well. Error bars show standard error of the mean (SE). ^a*P* < 0.05; B: Proliferation of KMBC cells determined by viable cell number counting following treatment with combinations of 5 µg/mL rhTFF2, 50 ng/mL EGF, and 10 µmol/L PD153035 [EGF receptor (EGFR) antagonist]; C-E: Immunoblot analysis of intracellular signaling events and cell cycle regulators in KMBC cells treated with combinations of 5 µg/mL rhTFF2, 50 ng/mL EGF, and 10 µmol/L PD153035; C: Cyclin-E (48 kDa) protein abundance relative to GAPDH abundance; D: EGFR (180 kDa) phosphorylation level at tyrosine 1173 relative to total EGFR protein; E: extracellular signal related kinase (ERK; 42-44 kDa)1/2 phosphorylation at threonine 202/tyrosine 204 relative to total ERK1/2 protein. Histograms show mean normalized optical density (OD) of protein bands relative to untreated control (*n* = 3/group). Representative protein bands are shown. Error bars show standard error of the mean (SE). ^a*P* < 0.05.

The pathological features common to liver fluke infestation and other CCA are chronic inflammation of the biliary tract, bile stasis, and increased biliary epithelial cell turnover^[7]. However, the molecular mechanisms of carcinogenesis and pathogenesis of liver fluke- and non-liver fluke-associated CCA have not been well characterized and may differ according to the background of hepatobiliary lesions, and also to the stage or progression of CCA. This contradiction has been recently clarified by differential gene expression profiling of liver fluke- and non-liver fluke-associated CCA^[30]. We revealed that TFF1 protein expression in liver fluke-associated CCA was 50.7% compared with 20% in non-liver fluke-associated group suggesting that different underlying etiologies may lead to different mechanism of tumorigenesis and progression.

In contrast to previous studies^[25-27], we found down-regulation of TFF3 during stepwise carcinogenesis in liver fluke infestation. *TFF3* mRNA expression in tumors was significantly higher than in normal ($P = 0.021$) (Figure 1), whereas the positive rate of TFF3 expression in tumors was significantly lower than normal ($P = 0.003$) (Figure 4C). This suggests that TFF3 expression levels may be modified by posttranscriptional processing events. The substantially decreased TFF3 expression in CCA may reduce tumor suppressor activity, which leads to increased cell proliferation and tumor development. We found a significant association between TFF1 and TFF3 expression in our CCA cases ($P = 0.014$), suggesting that TFF1 and TFF3 were co-expressed in response to tumor progression and may adversely affect CCA patients. Although median survival time of CCA patients with the co-expression of TFF1 and TFF3 (29.43 wk) was shorter than that of CCA patients without co-expression (45.28 wk), no statistically significant difference was found between these two groups analyzed by Kaplan-Meier. Poulson *et al.*^[31] reported the co-expression of *TFF1* and *TFF3* mRNA in normal, hyperplasia, and neoplasia human breast epithelium, implicating them in the growth and progression of mammary carcinoma.

TFF2 was rarely expressed in normal and pre-malignant bile ducts but expressed mainly in peribiliary glands and markedly increased in CCA. Srivatsa *et al.*^[26] reported that TFF1 and TFF3 are induced in biliary diseases but not TFF2. However, Sasaki *et al.*^[25] showed that TFF2 expression was correlated with the degree of small bile duct damage, not with the disease etiology. Taken together, TFF2 expression in our cases was not responsible for stepwise carcinogenesis but rather was involved in the pathogenesis of CCA.

The proliferative effects of TFF2 have been investigated in other organ systems. Most significantly *TFF2*-deficient mice showed reduced gastric proliferation compared to wild-type littermate controls^[32]. Furthermore, it has been shown that TFF2 acting *via* the EGF signaling pathway promotes cell invasion and can be blocked by an EGFR inhibitor (ZD1839) in kidney and colon cell lines^[21]. Recently, Dubeykovskaya *et al.*^[33] have identified ectopically expressed CXCR4 chemokine receptor as a signaling receptor for TFF2 in lymphocytic and gastric cell lines. This study also demonstrated a distinct proliferative effect of TFF2 protein in gastric cancer cells

expressing CXCR4. Our study showed that rhTFF2 mediated CCA cell proliferation *via* the EGFR and MAPK activation, suggesting that TFF2 could act as a proliferative factor *via* the EGFR and intracellular MAPK pathway. An important conclusion of our study is the usefulness of these signaling molecules, acting downstream of TFF2, as potential therapeutic targets in invasive CCA.

This is the first study to directly analyze copy numbers of the three *TFF* genes and their relationship with mRNA and protein levels. Although a correlation among these parameters was not found, our study suggests that several mechanisms such as transcriptional and post-transcriptional processes are involved in the modulation of TFF gene expression in CCA, which plays an important role in the pathogenesis of this cancer.

Although *TFF* genes are involved in epithelium restitution and repair processes, the expression of *TFF* family members is tissue-specific. For example, TFF1 and TFF2 are expressed predominantly in the stomach, whereas TFF3 is predominantly expressed in the intestinal epithelium^[34]. The differential expression of *TFF* genes is consistent with distinct functional roles in different cell types. However, a “switch” in TFF gene expression patterns has been observed in other cancers, as exemplified by the upregulation of TFF3 in the intestinal metaplasia lineage and its association with poor prognosis in gastric cancer^[35]. Our data, showing aberrant expression and mitogenic effects of TFFs, suggests not only a mechanism for tumor progression in CCA, but also highlights the potential usefulness of TFFs as clinical biomarkers or even therapeutic targets using small molecule inhibitors.

COMMENTS

Background

Cholangiocarcinoma (CCA) in northeast Thailand is related to liver fluke infection. Previous study showed that > 30% of CCA patients have amplification of D21S1893, D21S1890 and the trefoil factor (*TFF*) gene family, which is associated with poor prognosis suggesting a role of TFFs in tumor progression. The authors hypothesized that an increase in *TFF* gene copy number resulting in the inappropriate overexpression of mRNA and corresponding protein, contributes to the progression of CCA in which TFFs mediate their actions, at least in part, through the transactivation of epidermal growth factor receptor (EGFR).

Innovations and breakthroughs

This is the first study to directly analyze copy numbers of the three *TFF* genes and their relationship with mRNA and protein levels. This is also the first report showing that TFF2 is mitogenic in CCA *via* activation of EGFR and the mitogen-activated protein kinase pathway.

Applications

Aberrant expression and mitogenic effects of TFFs suggests not only a mechanism for tumor progression in CCA, but also highlights the potential usefulness of TFFs as clinical biomarkers or even therapeutic targets using small molecule inhibitors.

Peer review

The authors clearly demonstrate that TFF2 peptide increased cell proliferation in a CCA cell line, and they first show that TFF2 activated EGFR and the ERK1/2 MAP kinase in CCA cell line, showing both the function and its mechanism of TFF2 in CCA.

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Comparison between surgical outcomes of colorectal cancer in younger and elderly patients

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The perioperative serum total protein and albumin levels were significantly lower in the elderly than in the younger group. The serum carcinoembryonic antigen level was lower in the elderly than in the younger group, and there was a significant decreasing trend after the operation in the elderly group.

CONCLUSION: The short-term outcomes of surgical treatment in elderly patients with colorectal adenocarcinoma were acceptable. Surgical treatment in elderly patients was considered a selectively effective approach.

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Key words: Colorectal tumor; Elderly patient; Morbidity; Carcinoembryonic antigen; C-reactive protein

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Jin L, Inoue N, Sato N, Matsumoto S, Kanno H, Hashimoto Y, Tasaki K, Sato K, Sato S, Kaneko K. Comparison between surgical outcomes of colorectal cancer in younger and elderly patients. *World J Gastroenterol* 2011; 17(12): 1642-1648 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i12/1642.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i12.1642>

Abstract

AIM: To compare the outcome of surgical treatment of colorectal adenocarcinoma in elderly and younger patients.

METHODS: The outcomes of 122 patients with colorectal adenocarcinoma who underwent surgical treatment between January 2004 and June 2009 were analyzed. The clinicopathological and blood biochemistry data of the younger group (< 75 years) and the elderly group (\geq 75 years) were compared.

RESULTS: There were no significant differences between the two groups in operation time, intraoperative blood loss, hospital stay, time to resumption of oral intake, or morbidity. The elderly group had a significantly higher rate of hypertension and cardiovascular disease.

INTRODUCTION

Colorectal adenocarcinoma is one of the most common malignant diseases worldwide, and colorectal adenocarcinoma is also the leading cause of cancer-related deaths in developed countries. Moreover, the incidence of colorectal adenocarcinoma has been reported to increase with age in the some countries^[1-3]. According to the published statistical data, the incidence of colorectal adenocarcinoma in elderly patients has been increasing slightly over

the past several decades, and especially in people aged > 75 years old in Japan^[4]. Surgery has always been the main curative treatment for colorectal adenocarcinoma, which can be resected curatively for primary tumor lesions and lymph node dissection. As a result of preexisting comorbidity in elderly patients with colorectal adenocarcinoma, there is controversy as to whether surgical treatment is beneficial. The average life expectancy in many developed countries is reported to be > 80 years^[5], and the number of colorectal adenocarcinoma patients is expected to increase in the future, therefore, the issue of the treatment of elderly patients with colorectal adenocarcinoma needs to be addressed. Some studies on surgery for colorectal adenocarcinoma in the elderly have been published^[6-12], but not a sufficient number.

The survival rate and quality of life after surgical treatment of colorectal adenocarcinoma are affected by postoperative complications. To clarify the impact of surgery on elderly patients with colorectal adenocarcinoma, we investigated and evaluated the short-term results of surgical treatment for colorectal adenocarcinoma in elderly patients aged ≥ 75 years, in comparison with those < 75 years.

MATERIALS AND METHODS

We studied 122 patients with pathologically diagnosed colorectal adenocarcinoma who were treated surgically at the Northern Fukushima Medical Center in Japan between January 2004 and June 2009. The patients were divided into two groups according to age: an elderly group of 54 patients aged ≥ 75 years, and a younger group of 68 patients aged < 75 years. All operations were performed by experienced surgeons in our hospital, and none of the patients received chemoradiotherapy before surgery. Operation time, intraoperative blood loss, length of postoperative hospital stay, morbidity, and mortality were analyzed to compare the risks and benefits of surgery in the two groups, and the two groups were compared with regard to blood examination data before and after surgery.

All of the patients were investigated routinely by physical examination, colonoscopy, colorectal contrast radiography, and thoracoabdominal computed tomography. Preoperative risk and preexisting comorbidity was evaluated using the American Society of Anesthesiology (ASA) scoring system. The results of surgical treatment and tumor staging were evaluated according to the International Union against Cancer tumor-node-metastasis classification, followed by pathological examination of the surgical specimen.

Information concerning the clinical characteristics of the patients upon admission was collected from the medical records. The general nutritional status of the patients was assessed on the basis of their body mass index (BMI), and their serum total protein and albumin levels. The tumor index, which was calculated by multiplying the longest diameter of the lesion by its shortest diameter, was used to evaluate the tumor. Serum carcinoembry-

onic antigen (CEA) level and carbohydrate antigen 19-9 (CA19-9) level were measured by enzyme immunoassay (EIA) before and after the operation. Systematic inflammatory response was assessed on the basis of the white blood cell (WBC) count and serum C-reactive protein (CRP) level at different times before and after surgery.

Laparoscopic-assisted colectomy for patients with colorectal adenocarcinoma was still assessed. The anastomoses were made with a circular stapler when the single-stapler technique was used, and with gastrointestinal anastomotic devices when functional end-to-end anastomosis was performed after both laparoscopic-assisted and open colectomy.

Postoperatively, oral intake of clear liquids was started after flatus was detected, provided the patient did not have severe nausea, vomiting, or abdominal distention, and a liquid diet was given, provided the patient had no trouble with the clear liquid regimen. Patients were discharged when a solid diet was well accepted and no postoperative complications were detected. Their length of postoperative hospital stay was measured from the day of surgery to the day they were discharged.

All data are reported as mean \pm SD. Statistical analyses for the categorical variables were performed by means of the χ^2 or Fisher's exact test, and the Mann-Whitney *U* test was used for measured variables. $P < 0.05$ was regarded as statistically significant.

RESULTS

One hundred and twenty-two patients with colorectal adenocarcinoma were treated by surgery, and 115 (94.3%) patients were evaluated by postoperative pathologically. The clinicopathological characteristics of the patients in the two groups are shown in Table 1. Of the 68 patients in the younger group, 40 (58.8%) were male, and 28 (51.9%) patients in the elderly group were male. The age range was 40-74 years in the younger group and 75-94 years in the elderly group. BMI was 22.23 ± 3.25 kg/m² in the younger group and 21.08 ± 3.58 kg/m² in the elderly group, and it was significantly lower in the elderly group than in the younger group ($P < 0.05$). There were no significant differences between the two groups for site of colon tumor, lesion type, tumor index, operation time, intraoperative blood loss, or pathological stage. Laparoscopic-assisted colectomy was performed in 35 (64.8%) patients in the elderly group as opposed to 19 (27.9%) in the younger group, and the percentage of patients treated by laparoscopic-assisted colectomy was significantly higher in the elderly group. Only one patient in the elderly group had 30-d surgical mortality. The numbers of lymph nodes harvested, proximal and distal to the tumor site are shown in Table 2, and there were no significant differences between the two groups.

Surgical procedure

The surgical procedures used and pathological characteristics of the resected tumors are also shown in Table 2. There were no significant differences between the two

Table 1 Clinicopathological data of the patients with colorectal adenocarcinoma *n* (%)

		Younger group (<i>n</i> = 68)	Elderly group (<i>n</i> = 54)	<i>P</i> value
Sex				NS
Male/female		40 (58.8)/28 (41.2)	28 (51.9)/26 (48.1)	
Age (yr) (mean ± SD)		62.2 ± 8.6	80.4 ± 5.0	NS
BMI (kg/m ²) (mean ± SD)		22.2 ± 3.3	21.1 ± 3.6	0.035
Localization of tumor lesion				NS
C		3 (4.4)	3 (5.6)	
A		9 (13.2)	18 (33.3)	
T		8 (11.8)	8 (14.8)	
D		3 (4.4)	1 (1.9)	
S		26 (38.2)	11 (20.4)	
R		20 (29.4)	14 (25.9)	
ASA				0.01
I		39 (57.4)	12 (22.2)	
II		22 (32.4)	31 (57.4)	
III		6 (8.8)	11 (20.4)	
IV		1 (1.5)	0 (0)	
Type of tumor				NS
0		6 (8.8)	1 (1.9)	
1		6 (8.8)	7 (13.0)	
2		52 (76.5)	44 (81.5)	
3		5 (7.4)	4 (7.4)	
Histological type				NS
Papillary adenocarcinoma		1 (1.5)	2 (3.7)	
Tubular adenocarcinoma (tub1/tub2)		29 (42.6)/33 (48.5)	17 (31.5)/28 (51.9)	
Poorly differentiated adenocarcinoma		3 (4.4)	5 (9.3)	
Mucinous adenocarcinoma		2 (2.9)	2 (3.7)	
T Stage				NS
T1 (m, sm)		0 (0)/7 (10.3)	1 (1.9)/2 (3.7)	
T2 (mp)		15 (22.1)	8 (14.8)	
T3 (ss, a)		16 (23.5)/14 (20.6)	19 (35.2)/9 (16.7)	
T4 (se, si, ai)		13 (19.1)/3 (4.4)/0 (0)	11 (20.4)/4 (7.4)/0 (0)	
N Stage				NS
N0		39 (57.4)	30 (55.6)	
N1		21 (30.9)	13 (24.1)	
N2		4 (5.9)	5 (9.3)	
NX		4 (5.9)	6 (11.1)	
Metastases				NS
M0		61 (89.7)	49 (90.7)	
M1		7 (10.3)	5 (9.3)	
Stage				NS
0		0 (0)	1 (1.9)	
I		17 (25.0)	8 (14.8)	
II		20 (29.4)	22 (40.7)	
III		23 (33.8)	17 (31.5)	
IV		8 (11.8)	6 (11.1)	
Comorbidity				
Overall	Present	27 (39.7)	36 (66.7)	0.003
	Absent	41 (60.3)	18 (33.3)	
Cardiovascular diseases	Present	6 (8.8)	14 (25.9)	0.011
	Absent	62 (91.2)	40 (74.1)	
Hypertension	Present	10 (14.7)	19 (35.2)	0.008
	Absent	58 (85.3)	35 (64.8)	
Diabetes mellitus	Present	5 (7.4)	3 (5.6)	NS
	Absent	63 (92.6)	51 (94.4)	
Cerebrovascular diseases	Present	3 (4.4)	5 (9.3)	NS
	Absent	65 (95.6)	49 (90.7)	
Other diseases	Present	6 (8.8)	7 (13.0)	NS
	Absent	62 (91.2)	47 (87.0)	

BMI: Body mass index; NS: Not significant; C: Cecum colon; A: Ascending colon; T: Transverse colon; D: Descending colon; R: Rectal colon.

groups for surgical procedure. After resecting the lesion, the double-stapler technique, single-stapler technique, and functional end-to-end anastomosis were performed for reconstruction in both groups, and colostomy was

performed after the Mile's approach. There were no statistically significant differences between the groups in reconstruction methods and no statistically significant differences between them in operation time, intraoperative

Table 2 Data related to surgical treatment of patients with colorectal adenocarcinoma between the two groups (mean \pm SD) *n* (%)

	Younger group (<i>n</i> = 68)	Elderly group (<i>n</i> = 54)	<i>P</i> value
Tumor size index (cm ³)	15.7 \pm 12.0	22.8 \pm 20.2	NS
Lesion circulation (%)	74.0 \pm 26.9	75.3 \pm 27.7	NS
Operation time (min)	137.4 \pm 45.7	120.4 \pm 35.1	NS
Operative blood loss (mL)	138.4 \pm 166.2	140.4 \pm 198.8	NS
Postoperative stay (d)	23.2 \pm 14.3	24.6 \pm 14.5	NS
Blood transfusion			0.001
Yes	0 (0)	8 (14.8)	
No	68 (100.0)	46 (85.2)	
Procedure of surgery			NS
Cecum resection	1 (1.5)	0 (0)	
Right hemicolectomy	16 (23.5)	24 (44.4)	
Transverse colon resection	2 (2.9)	4 (7.4)	
Left hemicolectomy	4 (5.9)	4 (7.4)	
Sigmoid colectomy	22 (32.4)	9 (16.7)	
Anterior resection	16 (23.5)	9 (16.7)	
Hartmann's resection	2 (2.9)	0 (0)	
Mile's resection	5 (7.4)	4 (7.4)	
Lymph node dissection			NS
D0	4 (5.9)	7 (13.0)	
D1	10 (14.7)	7 (13.0)	
D2	31 (45.6)	30 (55.6)	
D3	23 (33.8)	10 (18.5)	
Number of lymph nodes resected	12.1 \pm 9.0	11.3 \pm 8.6	NS
Distal margin distance (mm)	61.0 \pm 40.4	62.2 \pm 40.9	NS
Proximal margin distance (mm)	96.8 \pm 47.6	93.4 \pm 57.9	NS
Morbidity			NS
Surgical site infection			NS
Yes	5 (7.4)	7 (13.0)	
No	63 (92.6)	47 (87.0)	
Anastomotic leakage			NS
Yes	4 (5.9)	3 (5.6)	
No	64 (94.1)	51 (94.4)	
Others			NS
Yes	8 (11.8)	6 (11.1)	
No	60 (88.2)	48 (88.9)	

NS: Not significant.

blood loss, surgical site infection, or anastomotic leakage. Although the difference between the intraoperative blood loss in the two groups was not significant, a significantly higher percentage of patients in the elderly group required a postoperative blood transfusion ($P < 0.01$).

Resumption of oral feeding

After surgical treatment, the percentage of patients who could drink water before postoperative day 3 was 78% (53/68) in the younger group, compared to 72.2% (39/54) in the elderly group. The number of patients who received the liquid diet before postoperative day 4 was 46 (67.7%) in the younger group and 34 (63.0%) in the elderly group. There were no significant differences between the two groups for the time after surgery when a clear liquid or a liquid diet was started.

Assessment of comorbidity and surgical outcome

There was a preexisting comorbidity, such as cardiovas-

cular disease, hypertension, diabetes mellitus, or cerebrovascular disease, in 36 (66.7%) patients in the elderly group. Eleven (20.4%) patients in the elderly group were assessed as worse than ASA II at the time of operation, compared to seven (10.3%) patients in the younger group (Table 1), and the difference between the groups was statistically significant. The mean length of postoperative hospital stay was 23.24 ± 14.27 d in the younger group and 24.59 ± 14.48 d in the elderly group. Although the comorbidity rate was higher in the elderly group, the length of postoperative hospital stay was not significantly longer than in the younger group.

Right hemicolectomy and sigmoid colon resection were performed in one patient in the elderly group, because the lesions were located in the ascending and sigmoid colon. Furthermore, six patients in the younger group underwent a synchronous operation, including cholecystectomy, inguinal hernia operation, ovariectomy, and partial liver resection for liver metastasis, and two patients in the elderly group underwent synchronous cholecystectomy. Postoperative complications in the younger group consisted of surgical site infection in five patients, anastomotic leakage in four, and other morbidity, such as anastomotic bleeding, abscess in the peritoneal cavity, and wound rupture, whereas in the elderly group they consisted of anastomotic leakage in three patients, surgical site infection in seven, and anastomotic bleeding, abscess in the abdominal cavity, pancreatitis, necrosis of the stoma, and ileus in one each. There was no significant difference in the operation-related morbidity between the two groups. We compared the proximal and distal distances from the tumor lesion, lesion circulation rate, total number of lymph nodes dissected, and times after surgery when the clear liquid and liquid diets were begun, but there were no significant differences between the two groups in surgical outcomes evaluated on this basis.

Perioperative systematic inflammatory response level

The severity of the systematic inflammatory response before and after surgery was assessed on the basis of WBC count and serum C-reactive protein level in the younger and elderly groups (Figure 1). On postoperative day (POD) 1, the mean WBC count and C-reactive protein level were 10092.39 ± 3258.60 /mL and 6.88 ± 3.02 mg/dL, respectively, in the younger group and 10866.67 ± 3480.92 /mL and 5.98 ± 2.84 mg/dL, respectively, in the elderly group. Although the WBC count on POD 1 was higher in the elderly group, the difference between the two groups was not significant. After POD 1, the WBC count and CRP level gradually decreased in both groups, and there were no significant differences between the two groups in either parameter on POD 4 or POD 7.

Perioperative nutritional status

The perioperative nutritional status of the patients was assessed on the basis of their serum total protein level, serum albumin level and BMI. The total serum protein and serum albumin levels before and after surgery are shown in Figure 1, and the BMI values before the opera-

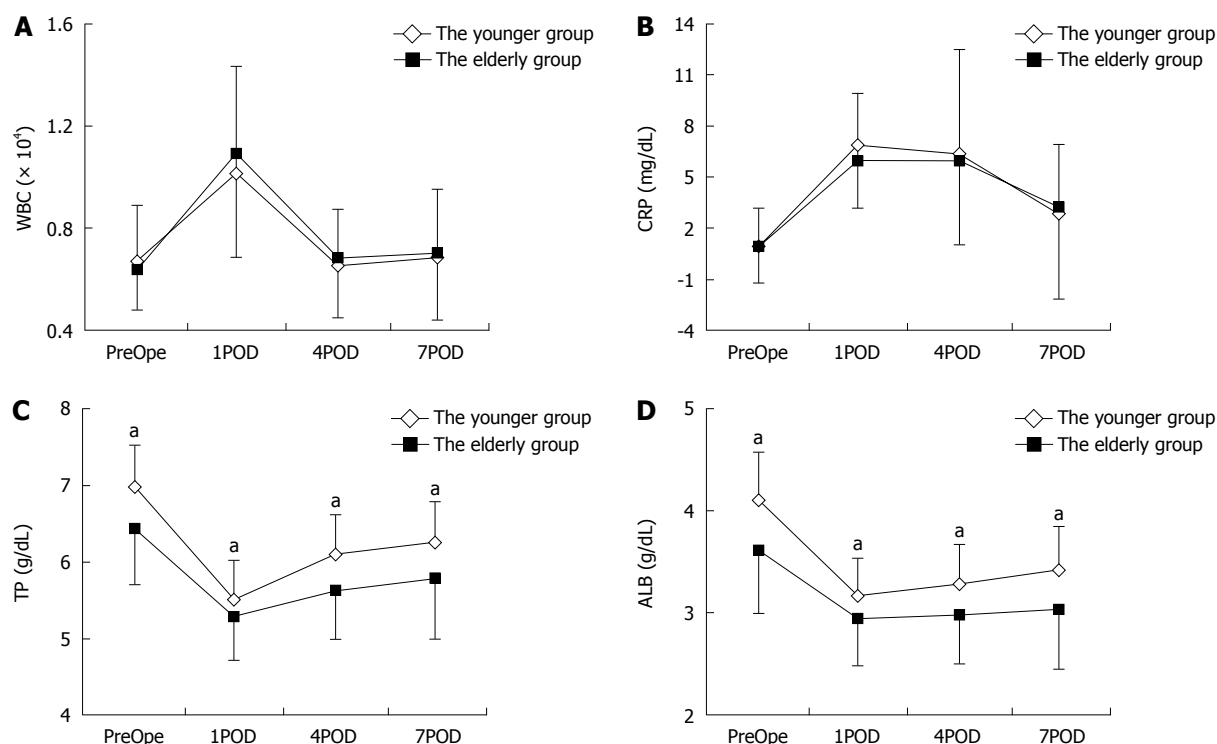


Figure 1 Changes in laboratory data of elderly and younger patients with colorectal adenocarcinoma at different times perioperatively: white blood cell count (A), serum C-reactive protein level (B), serum total protein level (C), and serum albumin level (D). Serum total protein (TP) and albumin (ALB) levels in the elderly group were significantly lower than in the younger group at different times perioperatively. The white blood cell (WBC), C-reactive protein (CRP), TP and ALB data are expressed as mean \pm SD. Preop: Before surgery ($^aP < 0.05$). POD: Postoperative day.

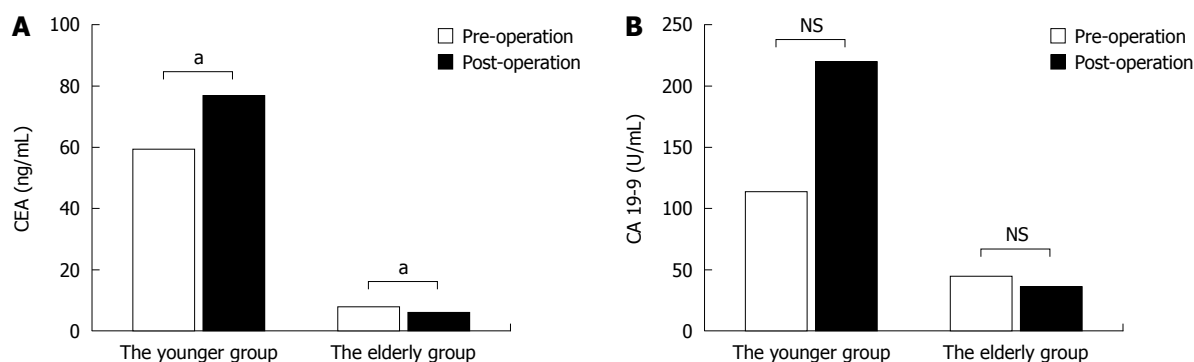


Figure 2 Serum carcinoembryonic antigen (A) and carbohydrate antigen 19-9 (B) levels measured by enzyme immunoassay in patients with colorectal adenocarcinoma were compared pre- and postoperatively between the younger and elderly groups. Postoperative carcinoembryonic antigen (CEA) level in the younger group was significantly higher than the preoperative level, and the postoperative level in the elderly group was significantly lower than the preoperative level. However, there were no significant differences for the change in carbohydrate antigen 19-9 (CA19-9) levels pre- and postoperatively in the two groups. CEA and CA19-9 data are expressed as mean. $^aP < 0.05$. NS: Not significant.

tion are shown in Table 1. The serum total protein and albumin levels preoperatively were 6.97 ± 0.55 and 4.08 ± 0.49 g/dL, respectively, in the younger group and 6.42 ± 0.73 and 3.61 ± 0.61 g/dL, respectively, in the elderly group, and the nadirs of both of these parameters in both groups were recorded on POD 1. The nutritional status of the patients based on their BMI, serum total protein level, and serum albumin level was poorer in the elderly group than in the younger group, and the differences in all three parameters between the younger group and the elderly group were statistically significant.

Perioperative changes in tumor marker values

The serum CEA and CA19-9 levels were measured before and after surgery (Figure 2). The mean serum CEA and CA19-9 levels in the younger group were 59.52 ± 374.69 ng/mL and 113.24 ± 381.16 U/mL, respectively, before surgery and 77.05 ± 494.09 ng/mL and 221.85 ± 1186.01 U/mL after surgery. In the younger group, the serum CEA and CA19-9 levels were higher after the operation than before. By contrast, the mean serum CEA and CA19-9 levels in the elderly group were 8.22 ± 15.37 ng/mL and 45.24 ± 194.35 U/mL, respectively,

before surgery and 6.65 ± 18.83 ng/mL and 36.78 ± 137.65 U/mL, respectively, after surgery, and both were lower after surgery than before. There were significant differences in the level of CEA in the younger and elderly groups perioperatively.

DISCUSSION

We compared the short-term outcomes of surgical treatment of colorectal adenocarcinoma in younger and elderly groups. Of the 122 patients included in this study, 54 (44.3%) were aged ≥ 75 years old, and the oldest patient was 94 years old. The patients in the elderly group had a high incidence of preexisting comorbidity, therefore, the effect of surgery for colorectal adenocarcinoma was unclear, except in those who had colon obstruction or bleeding caused by the tumor. The patients in the elderly group had other diseases, including hypertension, cardiovascular disease, diabetes mellitus, and cerebrovascular disease. Although preoperative status evaluated by the ASA scoring system was worse in the elderly group than in the younger group, almost all of the patients with colorectal adenocarcinoma could be treated with surgery.

There were no significant differences between the younger and the elderly groups with regard to operative factors, including bowel-margin-positive rate, tumor index, number of lymph nodes harvested, extent of lymph node dissection, intraoperative blood loss, or operation time. This could partly explain why the elderly patients with colorectal adenocarcinoma could be treated curatively by surgery if they were managed appropriately before and after the operation. Curative colectomy could not be performed in four patients in the elderly group because of distant metastasis and local deep invasion. Thus, the rate of diagnosis of colorectal adenocarcinoma in the early stage needs to be improved, especially in high-risk populations.

It has been reported that preoperative nutritional status can greatly affect the postoperative complications of the colorectal carcinoma patients after surgery^[13]. The serum albumin and total protein levels, and BMI were assessed to evaluate the nutritional status of the patients with colorectal adenocarcinoma, and there was a significantly higher prevalence of hypoproteinemia and hypoalbuminemia in the elderly group than in the younger group, before and after surgery. Although the nutritional status of the patients in the elderly group was poorer than in the younger group, the percentages of patients with anastomotic leakage and wound infection in the elderly group were not significantly higher. Our results suggest that hypoproteinemia and hypoalbuminemia do not affect the recovery of elderly patients with colorectal adenocarcinoma after surgical treatment, if they are appropriately managed during the perioperative period. Dixon *et al.*^[14] have found that patients with a low albumin level have a significantly shorter survival time after surgery for colorectal adenocarcinoma, and that the long-term outcomes of surgery need to be examined to establish

the essential benefits of surgery in elderly patients with colorectal adenocarcinoma.

A relationship between the systematic inflammatory response and survival has been reported in colorectal cancer patients who have undergone curative surgery^[15]. In the present study, the systematic inflammatory response was evaluated on the basis of the perioperative WBC count and serum CRP level between the two groups, and there was no significant difference between the severity of the systematic inflammatory response in the elderly and younger groups. These results suggested that the postoperative survival rate of the elderly patients was no worse than that of the younger patients, and that surgical treatment was acceptable for patients who could endure the operation, regardless of their age.

CEA and CA19-9 are the tumor-associated antigens that are most commonly used in the management of colorectal adenocarcinoma. It has been reported that CEA is a high-molecular-weight glycoprotein member of the immunoglobulin superfamily that plays a pivotal role in such biological phenomena as adhesion, immunity, and apoptosis of tumor cells, and in the assessment of sensitivity to antitumor agents^[16,17]. Moreover, some previous studies have shown an association between high preoperative serum CEA level and poor outcome^[18,19] of colorectal adenocarcinoma patients who underwent surgical treatment. In the present study, the serum CEA levels were significantly lower in the elderly group preoperatively and postoperatively. Moreover, the serum CEA levels exhibited a significantly decreasing trend after surgical treatment in the elderly group ($P < 0.05$), whereas the serum CEA levels in the younger patients were significantly higher after surgery than before ($P < 0.05$). These results suggest that surgical treatment might be beneficial and be one of the selectively effective strategies for elderly patients with colorectal adenocarcinoma. Some studies have found that CA19-9 level is a prognostic factor for colorectal cancer patients^[20,21]. In the present study, the serum CA19-9 levels in the elderly group were lower than in the younger group, and the levels in the elderly group showed a decreasing trend after surgical treatment, but not in the younger group. These findings also suggested that the elderly patients benefited more from surgical treatment.

The elderly population was considered to be a high-risk group for malignant diseases, and the prognosis of elderly patients with colorectal adenocarcinoma treated by surgery was estimated to be worse than for younger patients, especially for emergency surgery. The relation between emergency and elective surgery in patients with colorectal adenocarcinoma was not compared in this study. Furthermore, the number of the patients enrolled in this study was small, therefore, the results were limited. A larger sample study is needed to establish the effect of surgical treatment in elderly and younger patients.

In conclusion, the short-term outcomes of surgical treatment of elderly patients with colorectal adenocarcinoma in this study were acceptable. Surgical treatment of elderly patients with colorectal adenocarcinoma was considered a selectively effective approach.

COMMENTS

Background

Colorectal adenocarcinoma is one of the most common malignant diseases worldwide, and colorectal adenocarcinoma is also the leading cause of cancer-related deaths in developed countries. Moreover, the incidence of colorectal adenocarcinoma has been reported to increase with age in some countries. Some articles on surgery for colorectal adenocarcinoma in the elderly have been published, but their number has not been sufficient.

Research frontiers

To clarify the impact of surgery on elderly patients with colorectal adenocarcinoma, the authors investigated and evaluated the short-term results of surgical treatment for colorectal adenocarcinoma in elderly patients ≥ 75 years old in comparison with patients < 75 years old.

Innovations and breakthroughs

There were no significant differences between the two groups with regard to surgery-related factors. However, serum carcinoembryonic antigen level was lower in the elderly than in the younger group, and there was a significant decreasing trend after surgery in the elderly group.

Peer review

The present study is helpful in initiating further clinical studies to validate the results. Furthermore, the use of a comprehensive geriatric assessment instrument to determine operative risk could assist with selection of elderly patients who are most likely to benefit from surgery, and make the results of future studies more generalizable.

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Application of MPVR and TL-VR with 64-row MDCT in neonates with congenital EA and distal TEF

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Abstract

AIM: To assess the application of multiple planar volume reconstruction (MPVR) and three-dimensional (3D) transparency lung volume rendering (TL-VR) with 64-row multidetector-row computed tomography (MDCT) in neonates with congenital esophageal atresia (EA) and distal tracheoesophageal fistula (TEF).

METHODS: Twenty neonates (17 boys, 3 girls) with EA and distal TEF at a mean age of 4.6 d (range 1-16 d) were enrolled in this study. A helical scan of 64-row MDCT was performed at the 64 mm × 0.625 mm collimation. EA and TEF were reconstructed with MPVR and TL-VR, respectively. Initial diagnosis of EA was made by chest radiography showing the inserted catheter in the proximal blind-ended esophageal pouch. Manifestations of MDCT images were compared with the findings at surgery.

RESULTS: MDCT showed the proximal and distal esophageal pouches in 20 cases. No significant difference was observed in gaps between the proximal and distal esophageal pouches detected by MPVR and TL-VR. The lengths of gaps between the proximal and distal esophageal pouches detected by MPVR and TL-VR correlated well with the findings at surgery ($R = 0.87$, $P < 0.001$). The images of MPVR revealed the orifice of TEF in 13 cases, while TL-VR images showed the orifice of TEF in 4 cases.

CONCLUSION: EA and distal TEF can be reconstructed using MPVR and TL-VR of 64-row MDCT, which is a non-invasive technique to demonstrate the distal esophageal pouches and inter-pouch distance in neonates with EA and distal TEF.

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Key words: Children; Computed tomography; Congenital malformation; Esophagus; Tracheoesophageal fistula

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Wen Y, Peng Y, Zhai RY, Li YZ. Application of MPVR and TL-VR with 64-row MDCT in neonates with congenital EA and distal TEF. *World J Gastroenterol* 2011; 17(12): 1649-1654 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i12/1649.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i12.1649>

INTRODUCTION

Esophageal atresia (EA), with or without tracheoesophageal fistula (TEF), is the most important and common congenital malformation of the esophagus^[1,2]. The reported incidence of EA and TEF is approximately one in 3000-4500 live births^[1]. The majority of patients

have an associated fistula between the trachea and distal esophageal pouch. In neonates with EA and distal TEF, the anatomy of esophageal pouch, inter-pouch gap and up-growth of tracheobronchial tree, especially inter-pouch gap, not only influences the surgical approach and postoperative strategy but also predicts prognosis of such patients^[1,3,4]. If a short inter-pouch gap (e.g. shorter than 2 cm) with two longer pouches is detected, esophageal reunion with a primary anastomosis is relatively easy and postoperative complications are few. On the contrary, surgical procedure may be difficult for a longer inter-pouch gap with hypogenetic pouches, postoperative complications such as anastomotic leak may occur and ventilatory support is possibly required.

The application of multidetector-row computed tomography (MDCT) in reconstruction of 3D volume rendering (VR) has increased due to its ability to demonstrate the anatomy of hollow viscera. MDCT can generate VR images of the trachea and esophagus quickly and non-invasively, show the anatomy and 3D relation between the two structures more clearly and accurately, and provide valuable guidance for any surgical approach^[5]. Multiple planar volume reconstruction (MPVR) by MDCT is a reliable and straightforward technique to show hollow viscera in a multidirectional view and their adjacent tissues and organs clearly.

In this study, the ability of 3D TL-VR and MPVR to reconstruct EA and distal TEF in neonates was assessed by 64-row MDCT.

MATERIALS AND METHODS

The study was approved by the local ethics committee. Written informed consent was obtained from the parents of patients. Twenty neonates (17 boys and 3 girls) with EA and distal TEF were included in this study. Their birth weight was 1900-4000 g (mean 2888 g). Of the 20 neonates, 18 were full-term and 2 were pre-matured at a gestational age of 35 and 36 wk, respectively. Their age at the time of CT scan was 1-16 d with a mean age of 4.6 d. All patients had different degrees of respiratory distress and were not able to swallow food and saliva. Initial diagnosis of EA was made according their clinical symptoms and chest radiography showing the inserted catheter in the proximal blind-ended esophageal pouch and intestinal gas in the upper abdomen. Nineteen cases had different degrees of aspiration pneumonia, and only one had no inflammation of the lungs. Before CT scan, the proximal pouch was suctioned with an aspirating catheter, and then air (5 mL) was hand-injected into the proximal pouch. To avoid respiratory distress, the patients were not given sedatives. Body straps were used to immobilize the patients and decrease the motion artifacts. After CT scan, all patients underwent operation, and CT manifestations were compared with the findings at surgery.

CT data were collected with a 64-row MDCT scanner (GE Lightspeed VCT64, GE Healthcare, Wis, USA). All scans were performed from the level of larynx to the dia-

phragm. CT scan protocol was as follows: 120 kVp, z-axis automatic tube current modulation (ATCM) (AutomA; GE Healthcare) for all children with the noise index (NI) of 10 with a mean calculated CTDI dose of 1.57 mGy, a 64 mm × 0.625 collimation, 1.375:1 pitch, 0.8 s rotation time, and 512 × 512 matrix. The scanning time was 2-3.5 s. Images were reconstructed in the axial plane at a 0.625 mm interval with a standard reconstruction algorithm. CT images were transferred to an independent workstation (Advantage Workstation 4.3; GE Medical Systems) for further image reconstruction. Chest MPVR images were collected at the axial and sagittal and coronal view with a minimum intensity projection (MinIP), and 3D transparency lung VR models of the tracheobronchial system and the esophagi with a lower threshold of -704 HU and an upper threshold of -280 HU. Gaps between the proximal and distal esophageal pouches were measured at the workstation by two consultant radiologists with experiences in pediatric radiology and pediatric computed tomography. The average findings were recorded. The total image processing time was 20-30 min in each patient. Because the proximal and distal esophageal pouches were not frequently observed in the same plane of sagittal or in coronal view of multiple plane reconstruction (MPR), pulmonary air leaks were not discovered in all cases, chest MPVR with minimum intensity projection (MinIP) was used instead of MPR. However, it should be emphasized that MPR could provide more information about esophageal walls and the surrounding tissues of esophageal pouches than MPVR or TL-VR. View of the original axial and MPR images was indispensable before the measurement of MPVR and TL-VR.

The 20 patients underwent operation within 30 h (average 19 h) after CT scan. The inter-pouch distance and caliber of fistulae were measured with a silk thread between two artery forceps during operation. Then different lengths (centimeters) of silk thread were measured with a ruler.

RESULTS

The trachea and bronchial systems including the major lobe bronchi, proximal and distal esophageal pouches, and inter-pouch gaps in 20 patients could be clearly observed on the 3D TL-VR and MPVR images. The MPVR images showed the distal fistulae including their orifice in 13 cases, while the TL-VR images revealed the distal fistulae including their orifice in only 4 cases. Zigzag artifacts were trifling in most patients because of their motion and respiration. Different lengths of the gaps and different degrees of the fistulae are shown in Figures 1-4, and Figure 2 revealed that the lower fistula opened into the trachea within 1 cm of the carina.

The CT data and findings at surgery in the patients are summarized in Table 1. The inter-pouch gap was measured at surgery after the fistulae were divided in 17 cases and before the fistulae were cut in 3 cases. The diameter of fistulae was measured at surgery before the fistulae were ligated and divided in the 20 cases.

The inter-pouch distance was measured in 17 cases

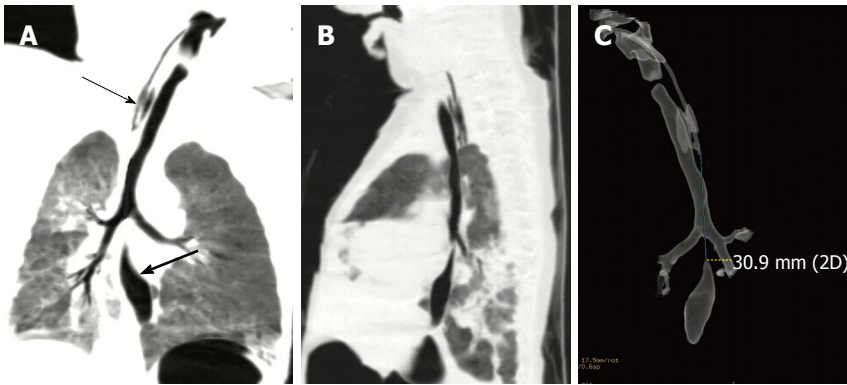


Figure 1 The reconstruction techniques of multidetector-row computed tomography show a case of esophageal atresia and tracheoesophageal fistula with a long inter-pouch gap. A: Coronal view of multiple planar volume reconstruction showing a long gap between the proximal pouch (thin arrow) and distal pouch (thick arrow) as well as the tracheobronchial tree with an aspirating catheter in the proximal esophageal pouch; B: Oblique sagittal view of multiple planar volume reconstruction showing a long inter-pouch distance with a tenuous fistula; C: Posteroanterior projection of transparency lung volume rendering demonstrating the distance between esophageal segments and three-dimensional anatomy of esophageal pouches and tracheobronchial tree after removal of lungs in case 3 (a 3-d old male neonate).

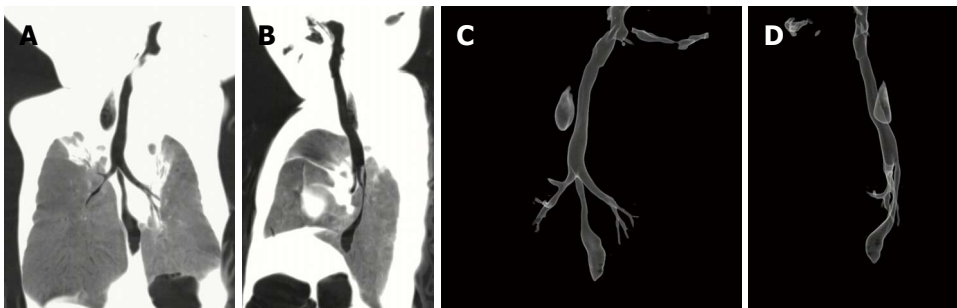


Figure 2 The reconstruction techniques of multidetector-row computed tomography clearly demonstrate not only inter-pouch distance but also the distal fistula. A: Coronal view of multiple planar volume reconstruction showing esophageal pouches and tracheobronchial tree; B: Oblique sagittal view of multiple planar volume reconstruction demonstrating inter-pouch distance and tracheal connection with the distal esophageal pouch; C, D: Transparency lung volume rendering showing anteroposterior (C) and sagittal projection (D) in three-dimensional anatomy of esophageal pouches and fistula in case 8 (a 5-d old male infant).

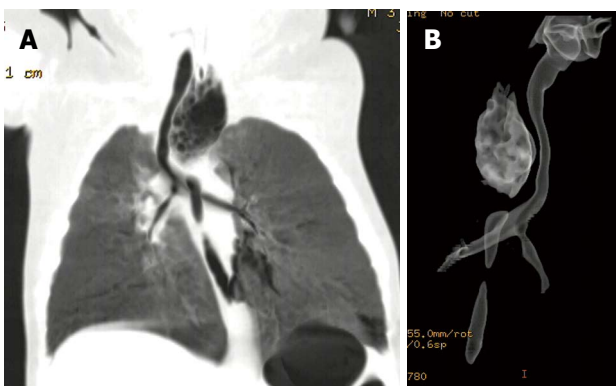


Figure 3 The proximal esophageal pouch full of air was distended and pressed the trachea rightwards. A: Coronal view of multiple planar volume reconstruction showing a short inter-pouch gap, a distended proximal esophageal pouch, and rightward-shifted trachea; B: Oblique posteroanterior view of transparency lung volume rendering demonstrating the three-dimensional anatomy of esophageal pouches and shifted trachea case 6 (a 3-d-old female neonate).

after the fistulae were cut. The inter-pouch gaps measured by MPVR and TL-VR correlated well with the findings at surgery ($R = 0.87$, $P < 0.001$). Scattered plots of the gaps in 17 patients after MPVR and surgery, and after TL-VR and surgery are shown in Figure 5A and B. Nevertheless,

the inter-pouch gaps measured by MPVR and TL-VR were shorter than those measured at surgery except for cases 3 and 11. Paired t -test demonstrated that the preoperative CT data detected by MPVR and TL-VR were significantly different from those observed at surgery ($P < 0.001$) in 17 patients. On the other hand, the inter-pouch distance in 3 cases 9, 13 and 16 was measured after initial dissection of the fistulae to define the esophageal pouches, but not measured before ligation and division of the fistulae. In cases 9 and 13, the inter-pouch distance on CT images was very coincident with that observed at surgery. The findings of CT in case 16 were slightly less than those at surgery.

The inter-pouch gaps in 20 patients measured by MPVR correlated well with those measured by TL-VR ($R = 0.98$, $P < 0.001$). Statistical analysis demonstrated no significant difference in inter-pouch gaps measured by MPVR and TL-VR. A scattered plot of the gaps was determined by MPVR and TL-VR in the patients (Figure 5C).

The 13 fistulae detected by MPVR were thinner than those observed at surgery. The 13 fistulae on CT images did not correlate with those observed at surgery.

DISCUSSION

EA with or without TEF is one of the most challenging

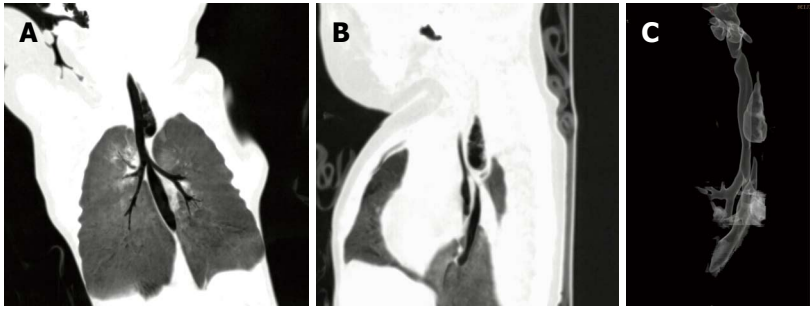


Figure 4 Multiple planar volume reconstruction revealed the inter-pouch distance and the thin fistula which was not visualized by transparency lung volume rendering. A: Coronal view of multiple planar volume reconstruction showing esophageal segments and tracheobronchial tree; B: Oblique sagittal view of multiple planar volume reconstruction showing inter-pouch distance and tracheal connection with the distal esophageal pouch; C: Oblique sagittal view of transparency lung volume rendering demonstrating the three-dimensional anatomy of esophageal pouches but no fistula in case 20 (a 4-d old male infant).

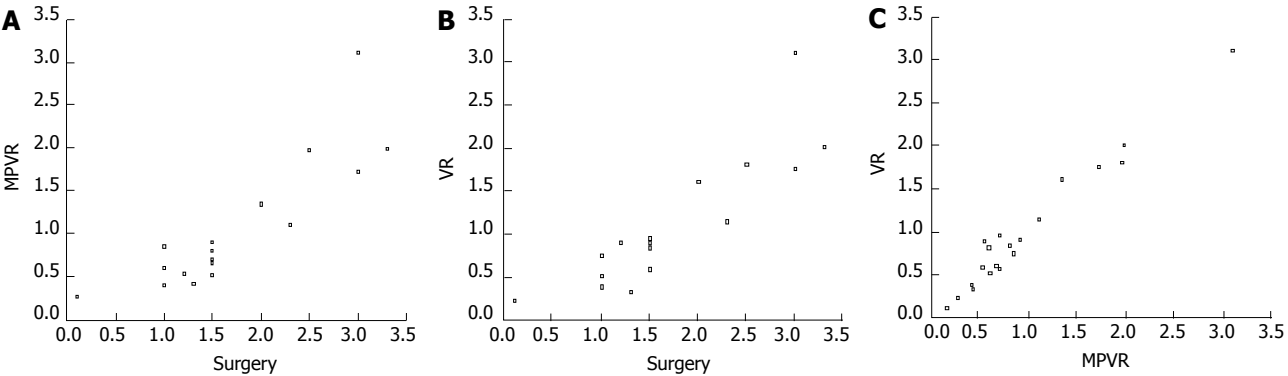


Figure 5 Scattered plots about the findings of the reconstruction techniques of multidetector-row computed tomography and surgery. Scattered plot showing gaps determined by multiple planar volume reconstruction (MPVR) (y-axis) and observed at surgery (x-axis) (A), by transparency lung volume rendering (TL-VR) (y-axis) and observed at surgery (x-axis) (B) in 17 patients, and by MPVR and TL-VR (C) in 20 patients.

Table 1 Esophageal inter-pouch gaps and distal fistulae prior to and at surgery								
Case	Sex	Age (d)	EI gaps (cm)			Caliber of TF (cm)		
			MPVR	TL-VR	Operation	MPVR	TL-VR	Operation
1	M	2	0.40	0.38	1.0	IN	IN	0.4
2	M	6	0.52	0.57	1.5	IN	IN	0.5
3	M	3	3.10	3.10	3.0	0.1	IN	0.5
4	M	3	0.84	0.74	1.0	IN	IN	0.6
5	M	3	0.54	0.89	1.2	0.2	IN	0.7
6	F	3	0.42	0.32	1.3	0.2	IN	0.7
7	M	2	1.96	1.80	2.5	IN	IN	1.0
8	M	5	1.72	1.75	3.0	0.1	0.2	0.4
9 ¹	M	6	0.58	0.81	0.6	0.2	IN	0.3
10	F	3	0.90	0.90	1.5	0.1	0.1	0.5
11	M	5	0.26	0.22	0.1	0.1	IN	0.6
12	M	11	0.80	0.83	1.5	IN	IN	0.5
13 ¹	M	5	0.70	0.56	0.7	IN	IN	0.4
14	M	1	0.60	0.51	1.0	0.1	IN	0.8
15	M	16	1.98	2.03	3.3	IN	IN	0.8
16 ¹	M	2	0.15	0.10	0.5	0.2	IN	0.5
17	M	1	0.70	0.94	1.5	0.2	0.2	0.4
18	M	6	1.34	1.60	2.0	0.1	IN	0.6
19	F	1	1.10	1.14	2.3	0.2	0.2	0.4
20	M	4	0.66	0.59	1.5	0.1	IN	0.4

The age stated is the age when computed tomography was performed. ¹The inter-pouch distance was measured before division of the fistula. EI: Esophageal inter-pouch; TF: Tracheoesophageal fistula; IN: Invisible (fistulae not shown); MPVR: Multiple planar volume reconstruction; TL-VR: Transparency lung volume rendering.

congenital anomalies in pediatric surgery because of its high morbidity and mortality^[1,4]. The distance between

esophageal segments and anatomy of the esophageal pouch as well as the tracheobronchial tree is highly valuable before operation. Frontal and lateral chest radiography can make unambiguous diagnosis of EA by inserting a catheter into the upper blind-ended esophageal pouch, and may give an estimate of the proximal pouch length. Nevertheless, they cannot show distal esophagus and fistulae in most patients. Although combined endoscopy and radiography can show distal esophagus and esophagus pouches^[6], it is highly invasive with a low resolution^[1]. Although the small size of airway in neonates naturally renders images poor in resolution, and cardiac and respiratory motion artifacts are relatively bigger in neonates than in adults, application of CT in reconstruction of EA with or without TEF has achieved encouraging results^[1,2,5,7].

Our study concentrated on the application of MPVR and TL-VR with 64-row MDCT in neonates with congenital EA and distal TEF. TL-VR is a novel technique using insufflated air as a negative contrast medium and can produce high quality, more intuitive and reproducible 3D VR images^[5]. MPVR technique is simple and efficient and has been widely used in demonstration of the anatomy of multi-organs. In this study, MPVR and TL-VR with MDCT clearly demonstrated the gaps between the proximal and distal esophageal pouches, tracheobronchial tree, as well as shape, size and position of the pouches. Furthermore, the fistulae and their orifice were observed on the images of some cases.

The inter-pouch gaps measured by MPVR and TL-VR were shorter than those observed at surgery in most cases (Table 1), which is not consistent with the reported findings^[1,2,7]. Ratan *et al*^[3] found that the shortest inter-pouch distance is increased to 5 mm after the fistula is divided. In our study, the inter-pouch distance was measured only once at surgery after and, before the fistula was cut. The CT data and findings at surgery were normally distributed in the 17 cases. The mean difference in MPVR and findings at surgery, and in TL-VR and findings at surgery was 0.67 ± 0.43 cm and 0.64 ± 0.42 cm, respectively, which is closely coincided with the reported findings^[3]. Moreover, the lengths of gaps measured by MPVR and TL-VR correlated well with the findings at surgery ($R = 0.87$, $P < 0.001$) in 17 cases (Figure 5A and B). In addition, the inter-pouch distance in 3 cases was measured at surgery before the fistula was cut. The CT data about cases 6 and 13 were almost identical with the findings at surgery, only the CT manifestations of case 16 were slightly less than the findings at surgery.

Besides division of the fistula, there were some possible causes for the less CT data than the findings at surgery. First, about 5 mL air was injected into the proximal esophageal pouch through the catheter in order to demonstrate the pouch, which may extend the proximal pouch and shorten the inter-pouch distance. For example, the proximal pouch was distended due to filling of air and pressed the trachea rightwards in case 6 (Figure 3). It was reported that CT data correspond well with the findings at surgery although no gas is injected into the proximal esophageal pouch^[1,3,7], indicating that less or

no air should be injected into the proximal pouch. As an apparent exception, distention of the proximal esophageal pouch was not seen in case 3 (Figure 1) because it was short, thin and immovable as confirmed at surgery. Second, selection of opacity threshold level for TL-VR techniques can affect the measurement of diameters and lengths of lumens, and visualization of thin tracts, so does selection of window width and location of MPVR. We selected the parameters and settings according to our experience. However, the selection was not doubtlessly optimal. Considering this drawback, we correlated the inter-pouch distance, diameters and lengths of proximal and distal esophageal pouches might measured by TL-VR and MPVR with those observed on MPR images at multidirectional view to avoid erroneous results.

On the other hand, the inter-pouch gaps measured by MPVR correlated well with those measured by TL-VR ($R = 0.98$, $P < 0.001$) in the 20 cases. Statistical analysis demonstrated no significant difference in them, indicating that the two different reconstruction techniques with MDCT do not differ in evaluation of the inter-pouch distance in neonates with EA and distal TEF.

Certainly, demonstration of the fistulae is also very significant. MPVR showed the distal fistulae and their orifice in 13 cases, whereas TL-VR revealed the fistulae and their orifice in only 4 cases. The 13 fistulae shown by MPVR were thinner than those observed at surgery. The findings in 13 fistulae on CT images did not correlate with those at surgery. MDCT demonstrated the invisible and thinner fistulae because of no interior gas, as well as peristalsis and shrinkage of esophagi, mucous plugs or secretions of respiratory tracts in the fistulae, and narrower lumen of fistulae than their diameters and walls seen at surgery. In addition, selection of threshold level for TL-VR and display of MPVR in lung window may interfere with visualization of the fistula orifice. Fitoz *et al*^[11] reported that shaded surface display (SSD) and virtual bronchoscopy reconstruction techniques can satisfactorily show the distal fistulae, which is consistent with the report of Lam *et al*^[2]. We used TL-VR instead of SSD, and virtual bronchoscopy was not performed in our study because it is inconvenient to measure inter-pouch gaps.

The results of this study demonstrate that 3D TL-VR and MPVR with MDCT can provide valuable preoperative information about neonates with EA and distal TEF. Nevertheless, due to expenses and concept of parents, gastrostomy and multiply-staged esophageal elongation are rarely performed in our institution. Therefore, the information acquired before operation in our study mainly helped the Department of Surgery to decide whether anastomosis should be performed by an experienced surgeon or an ordinary surgeon with/without supervision, anticipate the need for ventilatory assistance after operation, decrease the anesthesia and surgery time and its attendant morbidity, and predict outcome of a case. Because we focused on visualization of distal esophageal pouches and fistulae, possible consistency of measurements between MDCT reconstruction techniques and surgery, further operative procedures and follow-up were not discussed.

TL-VR and MPVR techniques are useful for neonates with EA and distal TEF. However, in those types of esophageal atresia without distal TEF in which the distal lumen is lack of air, it seemed difficult to visualize distal pouch with MPVR and TL-VR. The distal pouch might be observed to some extent on the original axial scan images or the MPR images.

The primary pitfalls of this study are lack of measurements of inter-pouch gaps at surgery before and after ligation and division of the fistulae, the number of neonatal cases was insufficient, and selection of technical parameters and preparations before scan might not be optimal.

With increased detector rows of MDCT and development in scan and reconstruction techniques, MDCT has been increasingly applied in demonstration of the anatomy of hollow viscera. However, its application in neonates is not common. Although the number of patients was limited, our findings suggest that MDCT plays a complementary role in diagnosis of congenital EA and distal TEF. MPVR and 3D TL-VR reconstruction of MDCT are useful and noninvasive for demonstrating the distal esophageal pouches, can evaluate the distance between two esophageal pouches in rough and show distal fistulae. 3D TL-VR images can show the complex anatomic features of EA, thus enabling a better orientation before operation.

COMMENTS

Background

Esophageal atresia (EA) with distal tracheoesophageal fistula (TEF) is the most important and common congenital malformation of the esophagus. The anatomy of esophageal blind pouches and inter-pouch gaps not only influence the surgical approach and postoperative strategy, but also predict prognosis of EA patients. Conventional radiography cannot show distal pouches and inter-pouch gaps, for which reconstruction techniques of multidetector-row computed tomography (MDCT) are highly valuable.

Research frontiers

The reconstruction techniques of MDCT can visualize esophageal pouch, inter-pouch gap and tracheoesophageal fistula in neonates with EA and distal TEF. Whether inter-pouch gaps determined with CT correlate well with operating measurements remains unclear.

Innovations and breakthroughs

Air was used as a negative contrast medium for visualizing esophageal pouches and fistulae by multiple planar volume reconstruction (MPVR) and 3D TL-VR reconstruction techniques of MDCT. To our knowledge, the number of cases in this study is the largest.

Applications

The reliability and accuracy of reconstruction techniques of MDCT for evaluation of EA and distal TEF still need to be confirmed by further researches. However, they are really useful and play a complementary role in diagnosis of EA and distal TEF.

Terminology

EA and distal TEF: The most common congenital malformation of esophagus, in which esophagus only has the proximal and distal blind-ended pouch and distal pouch communicates with trachea through a fistula; VR: Volume rendering, a 3D reconstruction technique of MDCT; TL-VR: Transparency lung VR model, a type of VR, which utilizes air as a negative contrast medium for visualizing hollow viscera; MPVR: Multiple planar volume reconstruction, a reconstruction technique of MDCT; MinIP: Minimum intensity projection, a reconstruction pattern of MDCT.

Peer review

The authors of this paper assessed the application of multiple planar volume reconstruction (MPVR) and three-dimensional (3D) transparency lung volume rendering (TL-VR) with 64-row multidetector-row MDCT in congenital EA and distal TEF in neonates, showing that MDCT plays a complementary role in diagnosis of congenital EA and distal TEF. MPVR and 3D TL-VR reconstruction of MDCT are useful and noninvasive for demonstrating the distal esophageal pouches, can evaluate the distance between two esophageal pouches in rough and show distal fistulae, thus enabling a better orientation before operation.

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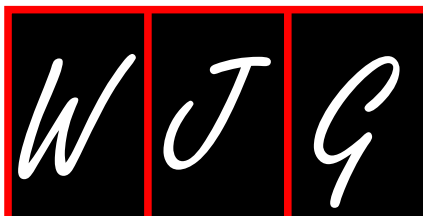
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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



Instructions to authors

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S,** Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

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

Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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Contents

Weekly Volume 17 Number 13 April 7, 2011

EDITORIAL

- 1655 Natural orifice transluminal endoscopic surgery: Progress in humans since white paper
Santos BF, Hungness ES
- 1666 Isolated lymphoid follicles in colon: Switch points between inflammation and colorectal cancer?
Sipos F, Múzes G

TOPIC HIGHLIGHT

- 1674 Patterns of local recurrence in rectal cancer after a multidisciplinary approach
Enríquez-Navascués JM, Borda N, Lizerazu A, Placer C, Elosegui JL, Ciria JP, Lacasta A, Bujanda L

GUIDELINES FOR CLINICAL PRACTICE

- 1685 Therapeutic options for intermediate-advanced hepatocellular carcinoma
Zhang ZM, Guo JX, Zhang ZC, Jiang N, Zhang ZY, Pan LJ

REVIEW

- 1690 How to assess the severity of atrophic gastritis
Dai YC, Tang ZP, Zhang YL

ORIGINAL ARTICLE

- 1694 Legalon-SIL downregulates HCV core and NS5A in human hepatocytes expressing full-length HCV
Mehrab-Mohseni M, Sendi H, Steuerwald N, Ghosh S, Schrum LW, Bonkovsky HL
- 1701 Endoscopic submucosal dissection for premalignant lesions and noninvasive early gastrointestinal cancers
Hulagu S, Senturk O, Aygun C, Kocaman O, Celebi A, Konduk T, Koc D, Sirin G, Korkmaz U, Duman AE, Bozkurt N, Dindar G, Attila T, Gurbuz Y, Tarcin O, Kalayci C
- 1710 Discovery and validation of prognostic markers in gastric cancer by genome-wide expression profiling
Zhang YZ, Zhang LH, Gao Y, Li CH, Jia SQ, Liu N, Cheng F, Niu DY, Cho WCS, Ji JF, Zeng CQ
- 1718 Differential expression of Bcl-2 and Bax during gastric ischemia-reperfusion of rats
Qiao WL, Wang GM, Shi Y, Wu JX, Qi YJ, Zhang JF, Sun H, Yan CD

BRIEF ARTICLE

- 1725 Intrahepatic natural killer T cell populations are increased in human hepatic steatosis
Adler M, Taylor S, Okebugwu K, Yee H, Fielding C, Fielding G, Poles M
- 1732 Gastrotomy closure with a new tissue anchoring device: A porcine survival study
Guarner-Argente C, Córdova H, Martínez-Pallí G, Navarro-Ripoll R, Rodríguez-d'Jesús A, Rodríguez de Miguel C, Beltrán M, Fernández-Esparrach G
- 1739 MR-arteriportography: A new technical approach for detection of liver lesions
Rennert J, Jung EM, Schreyer AG, Hoffstetter P, Heiss P, Feuerbach S, Zorger N
- 1746 Carbachol promotes gastrointestinal function during oral resuscitation of burn shock
Hu S, Che JW, Tian YJ, Sheng ZY
- 1753 Gastroesophageal reflux in cirrhotic patients without esophageal varices
Zhang J, Cui PL, Lv D, Yao SW, Xu YQ, Yang ZX
- 1759 Association between polymorphism rs6983267 and gastric cancer risk in Chinese population
Guo Y, Fang J, Liu Y, Sheng HH, Zhang XY, Chai HN, Jin W, Zhang KH, Yang CQ, Gao HJ
- 1766 EUS for choosing best endoscopic treatment of mesenchymal tumors of upper gastrointestinal tract
Zhou XX, Ji F, Xu L, Li L, Chen YP, Lu JJ, Wang CW, Huang W
- 1772 β -catenin accumulation in nuclei of hepatocellular carcinoma cells up-regulates glutathione-s-transferase M3 mRNA
Li YS, Liu M, Nakata Y, Tang HB
- 1779 Nutrition support in surgical patients with colorectal cancer
Chen Y, Liu BL, Shang B, Chen AS, Liu SQ, Sun W, Yin HZ, Yin JQ, Su Q

CASE REPORT

- 1787 Application of a wire-guided side-viewing duodenoscope in total esophagectomy with colonic interposition
Yi CY, Chou JW, Peng YC, Chow WK

Contents

World Journal of Gastroenterology
Volume 17 Number 13 April 7, 2011

ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

APPENDIX I Meetings
I-VI Instructions to authors

ABOUT COVER Santos BF, Hungness ES. Natural orifice transluminal endoscopic surgery: Progress in humans since white paper.
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Natural orifice transluminal endoscopic surgery: Progress in humans since white paper

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Abstract

Since the first description of the concept of natural orifice transluminal endoscopic surgery (NOTES), a substantial number of clinical NOTES reports have appeared in the literature. This editorial reviews the available human data addressing research questions originally proposed by the white paper, including determining the optimal method of access for NOTES, developing safe methods of luminal closure, suturing and anastomotic devices, advanced multitasking platforms, addressing the risk of infection, managing complications, addressing challenges with visualization, and training for NOTES procedures. An analysis of the literature reveals that so far transvaginal access and closure appear to be the most feasible techniques for NOTES, with a limited, but growing transgastric, transrectal, and transesophageal NOTES experience in humans. The theoretically increased risk of infection as a result of NOTES procedures has not been substantiated in transvaginal and transgastric procedures so far. Development of suturing and anastomotic devices and advanced platforms for NOTES has progressed slowly, with limited clinical data on their use so far. Data on

the optimal management and incidence of intraoperative complications remain sparse, although possible factors contributing to complications are discussed. Finally, this editorial discusses the likely direction of future NOTES development and its possible role in clinical practice.

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Key words: Natural orifice transluminal endoscopic surgery; Outcomes; Complications; Endoscopic; Surgery

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INTRODUCTION

The concept of natural orifice transluminal endoscopic surgery (NOTES[®]) has generated intense interest in the surgical and gastroenterology communities. Accessing the peritoneal or thoracic spaces through internal, transvisceral incisions instead of transabdominal incisions has the potential benefits of decreasing postoperative pain, wound complications, improving cosmesis, decreasing the physiologic and immune response to surgery, decreasing

anesthesia requirements, accelerating patient recovery and return to normal function, and improving access to organs that are currently difficult to reach with conventional open or laparoscopic approaches (e.g. esophagus, rectum). Given the intense interest in NOTES and its potential to revolutionize current surgical therapy, several working groups throughout the world have been formed to help guide NOTES research and clinical development. These groups include EURO-NOTES, EATS (European Association for Transluminal Surgery™), D-NOTES, ASIA-NOTES, NOSLA (Natural Orifice Surgery Latin America), Japan-NOTES, India NOTES, NOTES Research Group Brazil, and NOSCART, which published a white paper in 2006 outlining the perceived barriers to the clinical adoption of NOTES^[1]. These barriers included determining the optimal orifice to access the peritoneal cavity, developing a reliable means to close a viscotomy, minimizing the risk of infection as a result of access through a non-sterile orifice, developing an endoscopic suturing device, addressing difficulties with spatial orientation inherent to a NOTES technique, developing multi-tasking platforms to perform NOTES procedures, managing intraoperative complications, and developing NOTES training to allow safe, widespread adoption of the techniques. Although there have been numerous studies addressing some of these questions in animal and cadaver models, reports of clinical NOTES procedures in humans, and human data addressing these questions have only started to appear since 2007. This editorial will discuss the progress made on these questions by reviewing the currently available human outcomes data and clinical NOTES publications in the literature.

ACCESS TO THE PERITONEAL CAVITY

A comprehensive review of the human NOTES literature was conducted using PubMed to search the MEDLINE database with the search terms of “human natural orifice surgery, human transvaginal, human transrectal, human transgastric, or human NOTES surgery,” for articles published between January 1, 2004 and September 1, 2010. Manuscripts describing clinical human NOTES procedures include the use of transgastric, transvaginal, transrectal, and transesophageal approaches. Currently, the most frequently used orifice for NOTES is the vagina, with cholecystectomy accounting for the highest number of cases in the published literature^[2]. Transvaginal access has the longest history of use for intraperitoneal procedures, prior to the recent description of NOTES. In 1949, Bueno described a series of transvaginal appendectomies performed with open instruments (without an endoscope) at the time of hysterectomy^[3]. Since then, transvaginal access for intraperitoneal procedures in the form of culdoscopy has developed as an accepted, safe procedure in the gynecology community^[4-7]. Transvaginal access can be established using a posterior colpotomy created under direct vision with open instruments, or with the use of

direct trocar insertion under laparoscopic guidance. Establishment of transvaginal access does not require the use of a flexible endoscope or transanal endoscopic microsurgery (TEM) platform, unlike transgastric, transrectal, and transesophageal approaches that have been described to date. Likewise, closure of transvaginal access sites is performed with direct suturing using open instruments.

While transvaginal access is the most frequently used NOTES approach to date and can be safely performed, the potential for complications should not be overlooked. The close proximity of the rectum posteriorly, the ureters laterally, and the tendency for the small intestine to occupy the pelvis should be kept in mind while performing transvaginal NOTES. Reported complications of NOTES transvaginal access include rectal and colonic injuries, small bowel injuries, ureterovaginal fistula formation, vulvar lacerations, and bladder injuries^[8-14]. Given the possibility of these complications, assistance from a gynecologist experienced in transvaginal access should be considered, at least initially, in the performance of transvaginal NOTES. In addition, simultaneous visualization of colpotomy creation with a transumbilical laparoscope, along with the use of a uterine manipulator to anteriorly retract the uterus may minimize the likelihood of rectal, bladder, or bowel injuries during the creation of transvaginal access. Most cases reported so far have utilized a “hybrid” NOTES approach, with at least one laparoscopic port used for initial visualization, retraction, and assistance with the dissection. Until instruments for NOTES improve, a “hybrid” NOTES approach may be preferable to a “pure” NOTES approach (without any percutaneous or laparoscopic assistance) in order to increase the safety of the procedures.

Transgastric access is the second-most frequently reported access route after transvaginal access in the literature. Experience with transgastric NOTES includes at least 70 transgastric peritoneoscopy procedures reported by Nau *et al.*^[15,16] and Nikfarjam *et al.*^[17], as well as several series which have reported at least 42 cholecystectomies, 15 appendectomies, PEG rescue, and 6 cases of transgastric, stapled cystogastrostomy^[11,15,16,18-24]. Transgastric access in all of these cases was obtained in the anterior stomach (antrum or body) using needle knife cautery and balloon dilation through a flexible endoscope, except in cases of PEG-rescue and cystogastrostomy. Most cases were performed with placement of a laparoscopic port prior to gastrotomy creation to allow laparoscopic guidance and insufflation, while some were performed without any previous laparoscopic ports or insufflation. It is interesting to note that although no bowel injuries were recorded in transgastric peritoneoscopy cases performed without prior laparoscopic port placement, the authors noted there were instances of cautery burns to the anterior peritoneum or the under surface of the liver that were discovered after subsequent abdominal inspection with a laparoscope^[15]. As such, it is not surprising that the majority of transgastric cases have been performed in a hybrid fashion, with laparoscopic visualization of

the access point in order to prevent injuries to surrounding organs or the gastropiploic vessels, which may be difficult or impossible to see from inside the stomach^[2].

Transesophageal access has been used to perform esophageal myotomies in a series of 17 patients with achalasia, reported by Inoue *et al*^[25]. This procedure, termed Per-Oral Esophageal Myotomy (POEM), incises the inner circular muscle layer of the distal esophagus and lower esophageal sphincter, while completely avoiding the hiatal dissection and disruption of the phrenoesophageal ligament that occurs during laparoscopic Heller myotomy. Transesophageal access begins at the anterior, mid-esophagus with the creation of a submucosal bleb using a sclerotherapy needle. The submucosal bleb is then incised using electrocautery and the endoscope is advanced through the incision to create a submucosal tunnel distally past the gastro-esophageal (GE) junction onto the cardia of the stomach. The inner circular muscle distal to the mucosal incision is then incised. Currently transesophageal access has been used to perform only procedures on the esophageal wall. In one case, full separation of the outer longitudinal esophageal muscle layer occurred, exposing the mediastinum. Per the authors, however, this patient did not have any adverse consequences as a result; this suggests that as long as closure of the proximal mucosal incision is ensured, transesophageal mediastinal or thoracic access through a submucosal tunnel may be clinically feasible in the future. However, no clinical studies have been performed to date investigating the safety of transesophageal mediastinal or thoracic access.

In contrast to other forms of NOTES access, transrectal access has been the least reported in the literature. The only two published cases are of a proctosigmoidectomy for cancer^[26], and a transanal pull-through for Hirschsprung's disease^[27]. The proctosigmoidectomy was performed using a TEM platform, with a circumferential rectal dissection proceeding cephalad from the distal rectum, assisted by laparoscopy. The transanal pull-through was performed in an infant, without the use of the TEM; instead the authors reported using trocars inserted directly through the rectal wall to allow passage of a rigid laparoscope and rigid instruments. Although no complications were reported to have occurred in either case, further data is needed in order to accurately determine the risks of this approach.

Although so far various access points for a variety of NOTES procedures have been attempted, the specific indications that are best suited for each orifice will need to be defined. For example, the ideal indications for transoral access may end up being limited to therapeutic esophageal or gastric procedures, or diagnostic procedures in the intraperitoneal cavity. Transoral access may be poorly suited to advanced therapeutic intraperitoneal procedures given the requirement for complex, flexible instrumentation, as well as the small native diameter of the esophagus which makes extraction of large, bulky specimens potentially hazardous. Similarly, transrectal access may be best suited

to colorectal applications, and transvaginal access may end up being ideally suited for gynecologic indications. However, if these two approaches prove to be the most forgiving in terms of ease of access, ability to reach the upper abdomen, complications, and the ability to introduce both flexible and rigid instruments through the orifice, it is possible that these approaches may become "workhorse" approaches for intraperitoneal NOTES procedures or specimen removal in female and male patients, respectively.

VISCERAL CLOSURE

Transvaginal closure is currently the most feasible closure method for NOTES, as the incision is closed by direct suturing. Aside from potential injuries to surrounding structures as previously mentioned, there have been no reports of vaginal dehiscence or herniation through the vaginal incision. Also, the consequences of a vaginal wound dehiscence would likely not be as potentially dangerous as a gastric leak or a rectal leak, which would introduce highly caustic or infectious luminal contents into the abdomen.

In contrast to transvaginal closure, transgastric closure currently requires the use of flexible endoscopic clips or tissue anchors, with or without laparoscopic sutures to buttress the closure. Although several groups have reported successful performance of transgastric closures without leaks, data on the true safety of current transgastric closure techniques are sparse at best. In 2010, Zorron *et al*^[14] reported results from a prospective, multi-center NOTES registry, including data from 43 transgastric operations (29 cholecystectomies and 14 appendectomies), in which the stomach was closed using laparoscopic suturing. No gastric leaks were reported in this study. Similarly, reports of transgastric closure by other groups using endoscopic clips or anchors, with or without laparoscopic sutures, accounting for a total of approximately 30 patients, did not include any postoperative gastric leaks^[18-21,23,24]. However, there has been at least one reported complication of gastric closure: a pneumothorax which occurred due to the aberrant placement of a tissue anchor through the diaphragm^[24]. Innovative solutions for transgastric closure that have been reported in humans include the creation of a gastric valve mechanism made with tissue anchors, through which a gastrotomy is created^[21]. The gastrotomy is then closed with additional tissue anchors once the procedure is finished. Although this technique has been successfully used in 5 patients so far, the majority of transgastric cases reported in the literature continue to rely on laparoscopic suturing alone or in combination with endoscopic instruments. Completely endoscopic means for closing gastrotomies will need to be developed and evaluated in human studies for transgastric NOTES to become feasible without laparoscopic assistance. Numerous prototype closure devices and techniques have been developed and tested in pre-clinical models. However, a detailed discussion of these devices and their results in animals are beyond the scope of this editorial.

Transesophageal NOTES closure has so far been reported using endoscopic clips to close the longitudinal mucosal incision at the entrance to the submucosal tunnel during POEM. No esophageal leaks or mediastinitis were reported in a series of 17 patients^[25]. These clips slough off into the GI tract, with healing of the mucosal incision demonstrated on follow-up endoscopy.

Closure of transrectal NOTES access has so far been accomplished by incorporating the rectotomy into a hand-sewn coloanal anastomosis. This technique increases the safety of transrectal NOTES since it uses currently accepted anastomotic techniques, but it is limited to resections of the left colon and rectum. The safety of transrectal closures left in situ (not incorporated into the anastomosis) remains to be determined, although there is evidence from the TEM literature suggesting that intraperitoneal rectal closures can be performed as safely as those without peritoneal entry during full-thickness rectal tumor excision^[28]. Research to test closure techniques for transrectal surgery will ultimately need to be performed on human tissue rather than porcine models for it to be useful. However, initial closure tests should be attempted on tissues that are already targeted for removal, such as in portions of the colon that will be removed following colectomy, to ensure patient safety.

RISK OF INFECTION

Concerns about potentially higher rates of infection have repeatedly been raised in regards to NOTES. The notion of introducing surgical instruments through non-sterile orifices into the normally sterile peritoneal cavity runs counter to years of established surgical dogma. Many groups performing clinical NOTES have adopted the routine use of preoperative intravenous (IV) antibiotics combined with local application of antibiotic or antiseptic solutions such as povidone-iodine at the site of visceral entry as a precaution. Although the data are currently limited, concerns about increased infectious risk with a transvaginal approach compared to conventional laparoscopy have not been substantiated. The best data so far are from a large, prospective NOTES registry including 488 patients that underwent transvaginal cholecystectomy^[13]. Complications reported in this registry included urinary tract infection, abscess in the pouch of Douglas, wound infection, vaginal mycosis, and bacterial vaginitis, with a combined incidence of 1%, which is comparable to the rate of infectious complications seen with conventional laparoscopic cholecystectomy^[29].

Bacterial contamination has been quantified during performance of laparoscopic roux-en-y gastric bypass (LRYGB) as a surrogate for NOTES, and during actual transgastric NOTES peritoneoscopy by investigators at Ohio State University^[30,31]. These authors measured contamination in 50 patients undergoing LRYGB given preoperative IV Cefazolin alone without additional luminal decontamination, and showed that native levels of bacte-

ria in the stomach were higher (mean 22 303 CFU/mL) compared to that of the peritoneum after the operation (1102 CFU/mL), with significant correlation between these levels. These results indicate that some cross-contamination occurs during transgastric peritoneoscopy, but that the degree of contamination is not dependent on the pre-existing level of bacteria in the stomach. In addition, despite the documented levels of contamination, no clinically obvious infections were found with a minimum of 30 d of follow-up for all patients. A follow-up study in patients undergoing transgastric NOTES peritoneoscopy prior to a planned pancreaticoduodenectomy also showed minimal cross-contamination with insignificant levels of intra-operative peritoneal contamination (160 CFU/mL), and no infectious complications in a group of 10 patients with 30 d follow-up. An observation requiring further investigation, though, was that patients on proton-pump inhibitors (PPIs) had significantly higher levels of bacteria in the stomach (median 33 000 CFU/mL) compared to those not on PPIs (median 0 CFU/mL). Differences in post-operative peritoneal contamination between patients with or without PPI use preoperatively approached, but did not reach, statistical significance due to a limited number of patients in the study. Thus, while the risk of clinically significant infection as a result of transgastric NOTES appears to be low, the optimal perioperative management of patients on PPIs undergoing NOTES requires further study.

In contrast to transvaginal and transgastric NOTES access, transesophageal and transrectal access have a theoretically higher risk of infectious complications due to their proximity to the oropharyngeal and colonic flora, respectively. Unfortunately, to date no human studies have directly quantified the levels of bacterial contamination from either of these NOTES approaches, or the true incidence of clinically significant infections. Nevertheless, in the reported series of 17 POEM patients who received preoperative IV antibiotics and irrigation of the submucosal tunnel with dilute antibiotic solution prior to closure, no infectious complications were noted with a mean follow-up period of 5 mo (minimum 1 mo)^[25]. In addition, the two transrectal NOTES procedures reported in the literature to date did not report any infectious complications. The rectosigmoid resection patient underwent preoperative mechanical bowel preparation with oral sodium phospho soda, received preoperative IV Cefoxitin, and had a dilute Betadine irrigation of the rectum^[26]. The 5-d-old patient who underwent a NOTES transanal pull-through received perioperative systemic antibiotics for 24 h following the case with no reported complications. In short, more data are needed to accurately estimate the risk of infectious complications with transrectal and transesophageal NOTES approaches.

The fear of increased infectious risk from NOTES procedures has so far not been substantiated by examining available clinical outcomes and bacteriologic studies. It is likely that IV antibiotics alone for transgastric proce-

dures, along with some form of luminal disinfection for transvaginal, transrectal or transesophageal procedures will be the ultimate strategy adopted clinically.

DEVELOPMENT OF ENDOSCOPIC SUTURING OR ANASTOMOTIC DEVICES

The development of endoscopic suturing and anastomotic devices was deemed by the white paper to be necessary in order for NOTES to ultimately be applied to the wide spectrum of current surgical therapy^[1]. However, the development of these devices and their use in clinical trials has proceeded slowly since 2005. Currently, two types of endoscopic suturing devices have been approved: OverStitch™ (Apollo Endosurgery, Inc., Austin, TX, USA) and the Tissue Apposition System (TAS, Ethicon Endosurgery, Cincinnati, OH, USA). However, only use of the TAS has so far been reported clinically to approximate partial colonic wall defects at the time of laparoscopic-assisted polypectomy^[32]. The TAS system works by sequentially deploying a threaded T-tag through the bowel wall on each side of a defect using an endoscopic hollow bore needle; once two threaded T-tags have been placed on either side of a defect, the two threads are cinched together and trimmed by a one-way locking mechanism in order to approximate both sides of the luminal defect. A similar endoscopic T-tag closure technique using instruments from Cook Medical (Bloomington, IN, USA) was employed by Park *et al.* to close gastrotomy defects during transgastric NOTES^[24]. One of the difficulties with existing endoscopic T-tag systems, however, is the inability to directly visualize deployment of the T-tag from the extraluminal side of the defect without a laparoscope. This impaired visualization may contribute to the risk of inadvertent injury to surrounding organs, or deployment through a vessel. As mentioned previously, inadvertent deployment of a T-tag through the diaphragm during a gastric closure was reported to have resulted in a pneumothorax discovered post-operatively^[24]. The OverStitch™, in contrast to T-tag based systems, employs a lateral needle-passing mechanism more similar to conventional suturing techniques. However, the OverStitch™ still requires assistance from an endoscopic grasper, and may be limited by the visual and mechanical constraints of conventional flexible endoscopes. Human use data will be needed to adequately evaluate the potential of the OverStitch™ for use in luminal closures.

The development of endoscopic anastomotic devices for NOTES has proceeded even more slowly than the development of suturing devices. The only reports of NOTES procedures with anastomoses have utilized hand-sewn coloanal anastomoses during colorectal resections, or a flexible, powered surgical stapler (SurgAssist™ SLC 55, Power Medical Interventions, Langhorne, PA, USA) during cystogastrostomy^[33]. In the cystogastrostomy cases, the stapler was passed down the esophagus through an

overtube, alongside a flexible gastroscope. Although this stapler was used successfully to create a cystogastrostomy, the authors reported significant difficulty in passing the rigid part of the stapler through the esophagus, even through a previously placed overtube. In addition, the authors reported significant difficulty directing the stapler into the appropriate angle once inside the stomach. Unfortunately, since the publication of the study, the stapler has been removed from the market due to the acquisition of Power Medical, Inc. by Covidien (Mansfield, MA) and is not currently available for use. Development of flexible, articulating, low-profile staplers is needed to make creation of anastomoses or luminal closures during NOTES more feasible. Additional features which may make application of stapling technology to NOTES more feasible include the addition of visualization and steering capabilities. These features might allow staplers to be more precisely directed into difficult to reach areas and fired with more confidence.

SPATIAL ORIENTATION

The difficulty with correct spatial orientation during NOTES and its consequences in hindering the performance of advanced procedures was foreseen in the white paper. These difficulties are inherent to the use of current flexible endoscopes to perform NOTES, and have the potential to create not only a difficult operation, but may also increase the risk of complications during NOTES. Perretta *et al.*^[34] reported a case of misinterpretation of biliary anatomy during transgastric NOTES which was fortunately recognized, preventing the occurrence of a common bile duct injury. The authors in this case converted to a laparoscopic view temporarily to clarify the unclear biliary anatomy. As emphasized by the authors, current NOTES techniques may alter the usual surgical anatomy that is seen due to the difficulty in achieving adequate retraction without laparoscopic instruments, and the spatial confusion created by retroflexion when using a flexible endoscope. A solution to the problem of difficult spatial orientation during NOTES may be the use of rigid endoscopes whenever possible. However, the use of rigid endoscopes is potentially feasible only through transvaginal or transrectal approaches, or through the umbilicus in the case of transgastric surgery. Short of using a rigid endoscope routinely, surgeons performing NOTES with flexible endoscopes should have a low threshold to convert to a laparoscopic view, even temporarily, to resolve any confusion in regards to the surgical anatomy. Although image-guided systems have been described as having potential applications for NOTES, none of these systems have been applied in a clinical setting so far^[35]. Future solutions to the problem of spatial orientation may also involve the use of small, wireless cameras that are able to provide a wider, overhead view of the surgical field, and can be moved to the appropriate location as needed. Use of this type of camera has been described for human single-incision laparoscopy (SIL) cases^[36]. However,



Figure 1 The Transanal Endoscopic Operations device from Karl-Storz allows the insertion of rigid or flexible instruments through the anus and is currently used for performing transanal endoscopic microsurgery excisions of rectal tumors. It also has the potential to serve as a stable transrectal natural orifice transluminal endoscopic surgery (NOTES®) platform. Image used with permission (©Karl Storz).



Figure 2 The TransPort™ multi-channel access device from USGI has been used as a transgastric natural orifice transluminal endoscopic surgery platform. It has a steering mechanism similar to a flexible endoscope, along with multiple, large-diameter channels to accommodate a small-diameter flexible endoscope and other large caliber flexible endoscopic instruments (g-Prox® tissue anchor device is shown). Image used with permission (©USGI Medical).

it should be noted that these cameras are not currently FDA-approved.

DEVELOPMENT OF A MULTITASKING PLATFORM

The creation of a multitasking platform to allow the performance of multiple NOTES procedures with the same platform continues to be an issue of the highest priority in the development of NOTES as a viable technique. Although several types of advanced operations (nephrectomy^[12,37], partial gastrectomy^[38,39], sigmoidectomy^[40,41], and splenectomy^[42]) have been reported using a NOTES technique, many of these procedures have relied heavily on laparoscopic instruments for visualization and dissection. In order for “pure” NOTES (without laparoscopic or percutaneous assistance) to become feasible, a multi-tasking platform that balances flexibility and maneuverability with



Figure 3 The Anubis® platform from Karl-Storz is an advanced flexible natural orifice transluminal endoscopic surgery platform (in development), with a tip that opens to expose working instruments capable of multiple degrees of freedom controlled by the surgeon. Image used with permission (©Karl Storz).

the ability to provide powerful retraction and instrument mobility, as well as an intuitive interface for the surgeon will need to be developed. Examples of the novel application of multi-lumen operating platforms for NOTES include use of a TEM platform (Figure 1) to perform proctosigmoidectomy, and the use of the TransPort™ (USGI Medical, San Clemente, CA, USA) multi-channel access port for transgastric NOTES (Figure 2). Use of a TEM platform for transrectal surgery allows the simultaneous use of rigid instruments with a flexible or rigid endoscope to perform intra-abdominal surgery. This combination of operating instruments permits strong retraction, while allowing flexible visualization and dissection capabilities through the flexible endoscope. Current limitations of such a system, however, include the difficulty of reaching beyond the sacral promontory with rigid instruments, and the limitations of dissection performed with current flexible endoscopes. The TransPort™ device is a flexible, multi-channel device which allows passage of a flexible endoscope through one channel as well as additional flexible instruments through the other channels. This device is flexible enough to be passed transgastrically and has the ability to retroflex and assume a rigid configuration independent of the endoscope. While it has been used for transgastric cholecystectomy and appendectomy^[19,21,23], use of the flexible instruments through its channels is similar to the use of accessories through a conventional flexible endoscope in that the instruments have limited degrees of freedom and lack the ability to make lateral or vertical movements independent of the endoscope in an intuitive fashion. The limitations of both of these platforms make them less than ideal multi-tasking platforms for NOTES. However, the development of a system combining aspects of both platforms, along with robotic control may greatly facilitate the performance of NOTES procedures and may be the crucial enabling technology that would allow NOTES development to proceed exponentially, similar to the way the development of the charge-coupled device (CCD) camera revolutionized laparoscopy. Examples of experimental platforms with some of these



Figure 4 EndoSamurai is a prototype, advanced platform in development by Olympus. To operate the system, a surgeon uses an intuitive, bi-manual interface to control instruments with multiple degrees of freedom (inset shows close-up of endoscope tip with working instruments). Image used with permission (©Olympus Medical Systems Corp.).

capabilities that may be seen in future clinical reports include Anubis (Karl-Storz, Tuttlingen, Germany, Figure 3), EndoSamurai (Olympus, Tokyo, Japan, Figure 4), and the Direct-Drive Endoscopic System (Boston Scientific, Natick, MA, USA, Figure 5). Economic concerns continue to be an issue with regard to the development of these platforms, however. The emergence of single-incision laparoscopy (SIL) has caused a tremendous amount of resources to be redirected away from NOTES, towards the development of SIL technology. Although some of this technology may end up being adapted for NOTES, the development of SIL will likely delay the development of NOTES-specific technology such as advanced multitasking platforms. Both industry and innovators in minimally-invasive surgery need to not lose sight of the potential promise of NOTES, while SIL occupies the spotlight.

TRAINING

There are currently not enough data from human studies to make quantitative recommendations in regards to the ideal amount of previous endoscopic or laparoscopic clinical or laboratory NOTES training prior to the performance of clinical NOTES procedures. Nevertheless, a conservative approach described in the white paper recommends that NOTES procedures be performed by multi-disciplinary teams after a period of laboratory training in a properly equipped facility in order to maximize patient safety and ensure continuing regulatory acceptance of early NOTES development.

Future NOTES practitioners will likely need some form of fundamental surgical training, along with platform-specific and procedure-specific training once the field has undergone significant development. The current paradigm of performing NOTES primarily with flexible endoscopes is reaching the limits of practicality and safety, and will arguably become quickly obsolete with the availability of advanced multitasking platforms. Thus, rec-



Figure 5 Direct-Drive Endoscopic System from Boston Scientific is a prototype, advanced multi-channel platform currently in development, featuring instruments with multiple degrees of freedom controlled through a bi-manual user interface. Inset figure shows close-up of device tip with a small diameter flexible endoscope in place. Image used with permission (©Boston Scientific).

ommendations made in regards to training surgeons for NOTES using currently available instruments and techniques may quickly become obsolete.

COMPLICATIONS OF NOTES

Complications are an inevitable part of surgical practice, especially during the application of new techniques such as NOTES. Intraoperative complications inherent to all procedures, such as bleeding, will need to be managed appropriately to ensure patient safety. Along with the development of better endoscopic instruments to manage hemorrhage, surgical decision-making will need to evolve based on laboratory and clinical data. Although the method of hemorrhage control will always depend on the situation and surgeon judgment, it will be useful to determine what is realistically manageable using a pure versus hybrid NOTES technique, and when it would be most beneficial to convert to a full laparoscopic procedure. Unfortunately, data from human studies to answer this question are currently limited. The reported incidence of bleeding in a prospective NOTES registry of 488 transvaginal cholecystectomy patients was 0% for intraoperative bleeding, and 0.6% for postoperative bleeding, comparable with the incidence of bleeding during laparoscopic cholecystectomy^[13]. However, it should be kept in mind that the dominant technique in these cases did not include the use of any flexible endoscopes or accessories, and relied heavily on dissection through a transumbilical laparoscopic port. These results may thus only apply to NOTES performed exclusively with rigid instruments, as opposed to NOTES performed using a combination of flexible and rigid instruments. A more accurate picture of NOTES outcomes from operations performed primarily with flexible endoscopes (with or without laparoscopic assistance) may be derived from the registry by Zorron *et al.*^[14], which reported the incidence of intraoperative bleeding to be approximately 2% for transvaginal cholecystectomy (all from

the cystic artery), 8% for transvaginal appendectomy (all from the appendiceal artery), and a combined incidence of 4.7% for all transgastric procedures (7% for the appendiceal artery and 3.4% from the gastroepiploic artery)^[14]. Although these rates of bleeding may seem high, it should be kept in mind that all of these bleeding complications occurred intraoperatively and were managed laparoscopically or endoscopically with the exception of 1 instance of gastroepiploic bleeding during transgastric access which required conversion to an open procedure. In addition, no cases of delayed postoperative bleeding were reported. Future research on the optimal method to control hemorrhage during NOTES will likely need to be performed in animal models (for ethical reasons), and should involve both the development of new instruments and algorithms to help guide intraoperative decision-making.

In addition to bleeding, the authors of the white paper foresaw the possibility that physiologic complications and compression syndromes might be more frequently seen during NOTES procedures, compared to existing laparoscopic procedures. So far the incidence of these complications in the literature has been low (0.8% of 362 NOTES procedures), however, as reported by Zorron *et al*^[14] in a large, multi-institutional registry. These complications consisted of two episodes of intraoperative abdominal hypertension which resolved with desufflation of gas and fluid therapy, as well as one episode of facial and cervical subcutaneous emphysema following transvaginal, retroperitoneal cyst excision from the kidney^[14,43]. This complication was reported to have been managed with oxygen therapy and observation in the intensive care unit, without requiring re-intubation. Although it was reported that most groups in the registry used laparoscopic insufflators through a transabdominal port or Veress needle, the case with subcutaneous emphysema used a laparoscopic carbon dioxide (CO₂) insufflator connected to one of the flexible endoscopic channels with pressure maintained between 12 to 16 mmHg. Although the overall incidence of physiologic and compression syndrome complications was low in this registry report, surgeons performing NOTES should be aware that these complications may still occur and the risk of their occurrence may depend on the insufflation gas or insufflators used, and the anatomic compartment where dissection is performed.

Although the use of pressure-controlled CO₂ insufflation is likely to continue being a key component of NOTES procedures, lower insufflation pressures compared to conventional laparoscopy may be feasible, further reducing the risk of compression syndromes and subcutaneous emphysema.

More serious complications during NOTES cases have been reported that would otherwise be rare in the corresponding laparoscopic operations. These are worth noting to caution those who might be tempted to prematurely or over-enthusiastically adopt this still nascent approach to intra-abdominal surgery, and also to prioritize areas for potential improvement through better patient selection or

technical modifications to NOTES procedures. Reported complications of this kind during transvaginal cholecystectomy include 4 bladder injuries (0.8%), 2 rectal injuries (0.4%), and 1 small bowel injury (0.2%). All bladder injuries were reported to have occurred in older, obese women. However, it was not clear from the report whether these injuries occurred during the establishment of transvaginal access using a transvaginal trocar inserted under laparoscopic guidance or whether they occurred during the latter parts of the procedures. The occurrence of these complications emphasizes the extreme care that should be taken when establishing transvaginal access, closing the defect, and with the use of rigid transvaginal laparoscopic instruments. Similarly, the registry report by Zorron *et al* noted 2 esophageal hematomas, 1 esophageal laceration, and 1 esophageal perforation during 29 transgastric cholecystectomies, accounting for a combined rate of 13.7% esophageal complications. This is an unacceptably high rate of complications compared to conventional laparoscopic cholecystectomy, which is normally performed with minimal morbidity and mortality. Investigators from the International Multicenter Trial on Clinical Natural Orifice Surgery (IMTN) investigators addressed this high rate of esophageal complications and recommended the use of esophageal overtubes to protect the esophagus during the procedures, especially during specimen extraction. In agreement with this recommendation, a study conducted by our group found that preoperative ultrasound measurements of gallbladder stones can be used to help predict which gallbladders are able to be extracted through an esophageal overtube^[44]. Gallbladders found to be full of multiple small stones, in which the size of the largest stone cannot be determined, as well as those in which the largest gallstone is greater than or equal to 10 mm, are unlikely to pass through an overtube. Patients with these ultrasound findings may be better managed with conventional laparoscopic cholecystectomy. Criteria such as these may help improve patient selection for transgastric cholecystectomy, for example.

Ultimately, once more human data on the risks and benefits of NOTES procedures become available surgeons will have to decide whether the benefits of NOTES are worth the risks. It should be kept in mind that just because a procedure has an inherently higher rate of a specific complication doesn't mean it is not worthwhile. Laparoscopic cholecystectomy, for example, has been shown have increased rates of common bile duct injury compared to open cholecystectomy^[45,46]. However, this risk is acceptable given that the other benefits of laparoscopic cholecystectomy (decreased postoperative pain, decreased wound complications, improved cosmesis, and a faster rate of recovery for patients) outweigh its potential for harm. The same type of analysis weighing the risks and benefits of NOTES will need to be applied to determine its ultimate role in surgical practice.

NOTES MOVING FORWARD

In the five years since the publication of the NOTES

white paper, there has been a substantial proliferation of clinical NOTES publications. Progress has been made addressing the questions originally foreseen as the likely barriers to the introduction of NOTES into clinical practice. However, NOTES development in the next 10-15 years is likely to change surgical practice through a series of small incremental gains, rather than through an overnight revolution as in the case of laparoscopic surgery. This evolution may involve first a change in the practice of specimen extraction, with the use of natural orifices instead of the abdominal wall. Finally, once advanced platforms reach the clinical arena surgeons may shift to using the natural orifice not only for specimen extraction, but also for dissection. The evolution may also occur more quickly for some indications than others. For example, esophageal and colorectal NOTES applications may evolve more quickly compared to hernia, solid-organ, biliary, and general intra-abdominal applications given the already excellent outcomes and ease of laparoscopic techniques in performing these later procedures. NOTES may be able to more easily establish a niche in the thoracic esophagus, distal colon and rectum, or other anatomic locations where laparoscopic approaches are currently challenging or where there is still significant morbidity with traditional approaches. For example, limited resections performed through a natural orifice may replace the current practice of removing a large segment of colon or rectum for endoscopically unresectable polyps, assuming that oncologic outcomes can be maintained or equaled using alternative methods to assess or treat the possibility of disease in regional lymph nodes.

Given this likely evolutionary pattern for NOTES, the original goals of NOTES should be re-thought and re-prioritized. Transgastric NOTES in the near-term is unlikely to be useful to perform advanced therapeutic procedures or operations requiring removal of bulky specimens, even with the appearance of suturing devices for conventional flexible endoscopes. Rather transgastric NOTES may be better suited for diagnostic peritoneoscopy, using lower profile endoscopes that are able to traverse the gastric wall without the need for complicated closures of large, potentially dangerous gastrostomies. In addition to a decreased primary emphasis on transgastric NOTES as the access route of choice, the NOTES community should re-think its primary emphasis on flexible endoscopy as the preferred platform for NOTES, and instead be open to the use of rigid, pre-bent, or articulating instrumentation either in concert or instead of flexible instruments, until more advanced platforms become available.

CONCLUSION

In summary, since the first description of the concept of NOTES, many clinical NOTES cases have been reported in the literature, adding to the body of human data with which to begin to answering questions raised by the white paper. So far, transvaginal access has been the most feasible access route for NOTES procedures, although there

is growing experience with transgastric, transesophageal, and transrectal approaches. Luminal closure appears to be most feasible with a transvaginal approach, with smaller but nevertheless good outcomes also reported for transgastric and transesophageal closures. Data on the feasibility of true, intraperitoneal transrectal closures remain limited by the fact that the only closures performed to date have been hand-sewn coloanal anastomoses. Infection appears to be a non-issue with regard to transvaginal and transgastric surgery with the use of preoperative IV antibiotics (and local disinfection in the case of transvaginal procedures), with additional data required to more accurately estimate the risk with transesophageal and transrectal procedures. Development of suturing and anastomotic devices for NOTES has progressed slowly, with limited clinical data on their use so far. Likewise, the development of true multitasking platforms for NOTES has been slow and has not yet reached the clinical arena. The optimal management of intraoperative complications has still not been determined, but the data suggest that intraoperative hemorrhage may not automatically require conversion to laparoscopy. The incidence of compression syndromes appears low, as long as procedures are performed primarily with controlled, laparoscopic insufflation using CO₂. Additional major complications specific to NOTES procedures that would normally not occur during the corresponding laparoscopic operations have been noted in the literature. These types of complications absolutely need to be reported in order to constructively analyze the current status of NOTES and optimize patient selection and techniques to minimize their occurrence. As far as recommendations for NOTES training, there are no data to provide more specific recommendations outside of previous recommendations in the white paper and those from large NOTES registries. Finally, it may be useful to re-prioritize the development of NOTES to focus on high-yield colorectal and esophageal applications that are more likely to succeed in the near-term, instead of seeking the holy-grail of being able to perform entire, complicated procedures through transgastric access alone.

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Isolated lymphoid follicles in colon: Switch points between inflammation and colorectal cancer?

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Abstract

Gut-associated lymphoid tissue is supposed to play a central role in both the organization of colonic repair mechanisms and colorectal carcinogenesis. In inflammatory conditions, the number, diameter and density of isolated lymphoid follicles (ILFs) increases. They are not only involved in immune surveillance, but their presence is also indispensable in normal mucosal regeneration of the colon. In carcinogenesis, ILFs may play a dual role. On the one hand they may support tumor growth and the metastatic process by vascular endothelial growth factor receptor signaling and producing a specific cytokine and cellular milieu, but on the other hand their presence is sometimes associated with a better prognosis. The relation of ILFs to bone marrow derived stem cells, follicular dendritic cells, subepithelial myofibroblasts or crypt formation, which are all involved in mucosal repair and carcinogenesis, has not been directly studied. Data about the putative organizer role of ILFs is scattered in scientific literature.

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Key words: Isolated lymphoid follicle; Colon; Mucosal repair; Colorectal cancer; Epithelial stem cell; Myofibroblast; Follicular dendritic cell; Mesenchymal-epithelial transition; Epithelial-mesenchymal transition

INTRODUCTION

The imbalance of colonic epithelial proliferation and apoptosis may lead to both ulcer- and carcinoma development of the mucosa. The final direction of this imbalance depends on complex pathogenetic pathways in which isolated lymphoid follicles (ILFs) seem to have a specific role.

Some steps of colonic epithelial regeneration are known, but the connection among them is not fully understood. The continuous reformation of the epithelial layer is important in avoiding the aggregation of pernicious mutations induced by intraluminal factors. In inflammation, the lack of regenerative factors and the disturbance of the regulation of regenerative mechanisms favour ulcer development. It has also been observed that in colonic inflammation there is a tight connection between the degree of epithelial damage and the number, diameter and cellular compounds of subepithelial lymphoid follicles^[1,2]. The more severe the epithelial destruction that develops, the higher the number of ILFs that can be found in adjacent mucosa.

It has recently been reported that lymphoid follicles are also present in carcinomas of the lung^[3], endometrium^[4], liver^[5], and colon^[1]. They are supposed to have immune-mediated anti-tumoral effects, as their elevated number is in positive correlation with a better prognosis and a longer survival^[3]. However, the density of lymphoid

follicle-associated flat dysplastic aberrant crypt foci was significantly higher compared to the rest of the mucosa in azoxymethane-treated rats^[6]. Several reports have investigated the association between lymphoid aggregates and colonic tumors in rodents^[7,8]. The results indicate that colonic crypts overlying ILFs show a significantly higher proliferative activity, which may also influence genetically defected epithelial cells. Hence, the risk of carcinoma is increased in the colonic mucosa of ILFs compared to mucosa without ILFs. It has also been shown that the incidence of ILFs in early human colorectal cancers significantly differs by gender, location, macroscopic type and histology, but moreover, their localization significantly differs by their macroscopic type^[9].

However, the exact role of ILFs in colonic epithelial repair and colorectal carcinogenesis is not yet known. Some data show^[10] that the lack of lymphoid follicles results in abnormal crypt formation in the case of epithelial destruction. On the other hand, Apc gene mutation causes impairment of developmental and apparent differentiation blockade in proliferative tissues, including those of the lymphoid follicles^[11]. Whether ILFs act as a regenerative pool containing putative stem cells in case of mucosal damage, or they are responsible only for the optimal cytokine milieu for the differentiation of immigrating stem cells or invasive carcinoma cells^[12] need to be further examined.

THE ORGANIZATION OF THE GUT-ASSOCIATED LYMPHOID TISSUE

The gut-associated lymphoid tissue (GALT) is a component of the mucosa-associated lymphoid tissue, in which approximately 70 percent of the body's immune cells are found^[13,14]. GALT differentiates between pathogens and commensal bacteria.

The majority of GALT is composed of isolated and aggregated lymphoid follicles dispersed throughout the small and large intestines^[15]. These lymphoid follicles, including Peyer's patches (PPs) of the small intestine and ILFs of the large intestine, are composed of a specialised follicle associated epithelium (FAE), which overlies a sub-epithelial dome containing numerous macrophages, dendritic cells, T, B lymphocytes, and special antigen sampling microfold/M/cells^[15-17]. The FAE has a crucial role in the initiation of the mucosal and systemic immune response^[18]. ILFs have, in general, an average diameter of 0.1-0.7 mm and number of around 30 000 in humans^[19].

ILFs are innervated sites of GALT. Functionally, antigen-triggered mast cell and eosinophil activation affects both the secretory and motor functions of the intestines^[20], and these defensive reactions can be modulated by the enteric nervous system^[21]. It has been recently recognised that there is a dense neuronal network at the level of the supra-follicular dome region, but not within the germinal centers in lymphoid follicles^[22]. Neuronal alterations of PPs and ILFs, such as nerve-eosinophil associations or increasing neuronal cell adhesion molecule expression, may have consequences on the uptake of particular pathogens^[16,23].

VASCULARIZATION OF ILFS

ILFs have rich blood and lymphatic vascularization^[14,19]. Vasculogenesis may play a dual role in mucosal organization, in that it is not only necessary for nutritional and metabolic processes, but the homing of the repopulating bone marrow derived stem cells to the site of tissue damage may happen *via* blood vessels. In the case of cancer development, the vascular system is essentially involved in tumor growth, invasion and metastasis formation.

Revascularization is a key point of colonic mucosal repair. During inflammatory stages, due to cytokine action and intercellular adhesion, molecules signalling some of the vessels differentiate into high endothelial venules (HEVs)^[24,25]. In the case of lymphocytes and neutrophils, it is supposed that they firstly reach the inflammatory sites *via* a transcellular pathway through the HEVs^[26], but an intercellular pathway is also known^[27]. Upon epithelial injury the circulating bone marrow derived cells (BMDCs) migrate to the stromal layer of the damaged colonic wall, presumably *via* HEVs at an increased number regulated by overexpressed inflammatory chemokines^[28].

Based on the result of Witmer *et al.*^[29], it has been suggested that in lymphoid tissues, including GALT, the signaling system of the vascular endothelial growth factor (VEGF) and its receptor play a permanent role in the vasculogenesis of ILFs. Whereas the inhibition of VEGF has shown promising results in sporadic colon cancer, it has been recently published that VEGF receptor signaling acts as a direct growth factor for tumor cells in colitis-associated cancer, providing a molecular link between inflammation and the development of colon cancer^[30].

BONE MARROW DERIVED STEM CELLS OF ILFS

Based on the former results^[31-33], emerging evidence suggests that bone marrow derived stem cells contribute to tissue regeneration partly by promoting neovascularization or arteriogenesis. After human hematopoietic cell transplantation epithelial tissue chimerism appears^[34-36].

The bone marrow origin of epithelial cells may be supposed by observations in which epithelial cell markers and leukocyte markers showed that double positive cells were found in inflamed mucosa adjacent to lymphoid aggregates^[2,37-39]. The presence of cytokeratin, epithelial growth factor receptor, hepatocyte-derived growth factor receptor or CDX2 co-expression in CD45+ cells of ILFs may support the mesenchymal origin of epithelial stem cells. Based on these results, it seems that ILFs are involved in the homing and differentiation of BMDCs in the case of colonic mucosal damage (Figure 1).

The cause of metastasis remains elusive despite a vast amount of information on cancer cells. According to recent research, cancer cell fusion with macrophages or immigrating BMDCs provides an explanation^[40,41]. BMDCs fused with tumor cells were present not just in animal tumor xenografts where they were associated with metastases, but in

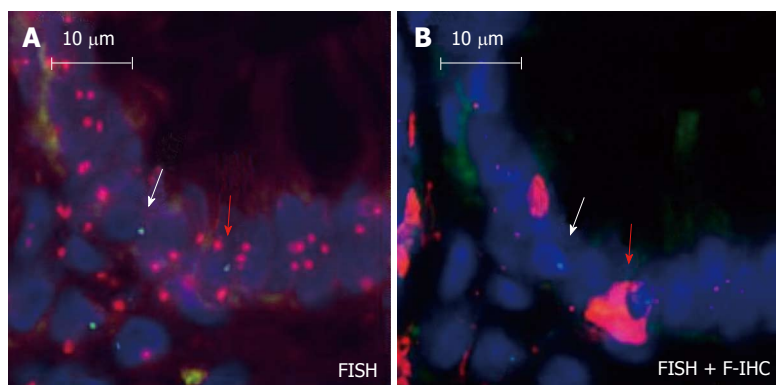


Figure 1 Intraepithelial male donor bone marrow origin CD45/Y-FISH+ cell (white arrow) and CD45/Y-FISH+ intraepithelial lymphocyte (red arrow) in the colonic biopsy specimen of a female acceptor. A: Chromosomal detection (green: Y-chromosome, red: X-chromosome; fluorescence *in situ* hybridization); B: CD45 and cytokeratin (green: cytokeratin, red: CD45; fluorescence immunohistochemistry; 130 × magnification).

human carcinomas, including colon cancer. BMDC-tumor cell fusion explains the epidermal-mesenchymal transition in cancer since BMDCs express mesodermal traits and epithelial-mesenchymal transition regulators (i.e.: Twist, SPARC). If BMDC-tumor cell fusion underlies invasion and metastasis in human cancer, new therapeutic strategies would be mandated.

DENDRITIC CELLS IN ILFS

Follicular dendritic cells (FDCs) in lymphoid follicles retain native antigens in the form of immune complexes on their membrane for months, and present these antigens to B cells during the secondary response^[42,43]. The origin and cell lineage of FDCs are controversial. Whereas their immune functions and expression of hemopoietic cell-associated antigens suggest that they belong to the hemopoietic lineage^[44], their spindle-shaped morphology “*in vitro*”, lack of CD45, and presence of antigens expressed by fibroblasts^[45] indicate that FDCs may be mesenchymal cells. Based on studies with mouse radiation chimeras, Humphrey *et al.*^[46] concluded that FDCs were not derived from the bone marrow, but came from a local mesenchymal precursor. However, Kapasi *et al.*^[44], using mice homozygous for the SCID mutation, which lack T, B lymphocytes, and FDCs, demonstrated that after reconstitution with bone marrow from donor mice, the FDCs of the reconstituted mice expressed the donor phenotype. These authors concluded that FDC precursors came from bone marrow.

According to the results of Muñoz-Fernández *et al.*^[47], FDCs seem to be a specialized form of myofibroblasts and derive from bone marrow stromal cell progenitors. The authors were able to isolate and culture 18 follicular dendritic cell lines from human tonsils. These cells were CD45-negative and expressed antigens associated with FDCs (CD21, CD23, CD35, CD40, CD73, BAFF, ICAM-1, and VCAM-1) and antigens specific for FDC (DRC-1, CNA.42, and HJ2). These cell lines were also able to bind B cells and secrete CXCL13, and they had functional activities characteristic of FDCs. Nevertheless, the additional expression of STRO-1, together with CD10, CD13, CD29, CD34, CD63, CD73, CD90, ICAM-1, VCAM-1, HLA-DR, al-

kaline phosphatase, and α -smooth muscle actin (α -SMA) indicated that FDCs are closely related to bone marrow stromal cell progenitors. The expression of α -SMA also relates FDCs with myofibroblasts. Like myofibroblasts, FDC lines expressed stress fibers containing α -SMA and were able to contract collagen gels under the effect of TGF β 1 and platelet-derived growth factor.

In various inflammation models, tissue-derived dendritic cells have been shown to migrate from the inflammatory site *via* lymphatics to secondary lymphoid organs where they interact with lymphocytes^[48]. Based on their dual phenotype, follicular dendritic cells may represent a transformation switch point among immigrating bone marrow derived stem cells in ILFs and the surrounding subepithelial myofibroblasts.

The origin of dendritic cells (DCs) in tumors remains obscure. Recent studies indicate that conventional DCs in lymphoid tissues arise from a distinct population of committed conventional DC precursors (pre-cDCs) that originate in bone marrow and migrate *via* blood. Diao *et al.*^[49] showed that pre-cDCs are precursors for conventional DCs in tumors, and they migrate from blood into the tumor where they generate conventional DCs. The chemokine CCL3, which is markedly upregulated in tumors (including colon cancer) and in tumor-infiltrating stromal and immune cells, promotes pre-cDC recruitment. Both pre-cDCs and their conventional DC progeny actively proliferate within the tumor, and have the ability to mature and stimulate Ag-specific lymphocytes. This finding suggests that in several cases the migration of pre-cDCs to tumors may represent a normal response to inflammation. Further studies are needed to delineate the role of pre-cDCs in other inflammatory processes and to compare them with monocytes, which are currently considered the main source of inflammatory DCs in peripheral tissues^[50,51].

MYOFIBROBLASTS SURROUNDING ILF ADJACENT EPITHELIUM

Subepithelial myofibroblasts (SEMFs) exist as a syncytium that extends throughout the colonic lamina propria, merg-

ing with the pericytes surrounding the blood vessels^[52,53]. SEMFs are involved in two epithelial repair processes^[54,55]. One process is called restitution^[56]. This is an important response to minor to moderate injury. The other process is observed when the wound is deep, and the subepithelial tissues and the basement membrane need to be reconstituted^[55].

According to recent studies^[54,57,58], myofibroblasts are thought to derive from two major sources, bone marrow or locally activated fibroblasts, in response to transforming growth factor- β 1. In the case of serious tissue injury (i.e. active ulcerative colitis) the regeneration capacity of local stem cells is not enough to complete tissue repair. In this case, bone marrow derived mesenchymal stem cells migrate into the gastrointestinal wall where they may contribute to the repair progress^[59,60] as differentiated mesenchymal cells (e.g. myofibroblasts)^[61].

Despite the increasing number of publications illustrating the role of tumor-associated stromal cells in cancer progression, there still exists a significant ambiguity with respect to the identification of cancer-associated fibroblasts, myofibroblasts and peritumoral fibroblasts in the cancer tissue. SEMFs appear early in the cancer's development. The mutual interaction (through direct cell-cell contacts and paracrine signals) between cancer cells and SEMFs is essential for invasive growth and is translated into a poor clinical prognosis^[62].

TOLL-LIKE RECEPTOR EXPRESSION IN ILFS

Beside immune functions, PPs and ILFs are supposed to be involved in mucosal repair *via* Toll-like receptors (TLRs). In ILFs, TLRs are expressed on the cells of the monocyte/macrophage system, on some kinds of T cells, as well as on intestinal epithelial, endothelial and stromal cells^[63]. Using the dextran sodium sulfate (DSS) model of colitis, mice lacking TLR2, TLR4 or MyD88 all developed more severe colitis than wild type mice when exposed to orally administered DSS^[64]. These findings suggest that signaling from commensal bacteria throughout TLRs resulted in protection from DSS colitis through enhanced epithelial cell proliferation, and worked as a compensatory factor against epithelial damage^[64].

TLRs can also bind endogenous ligands including necrotic cells, heat shock proteins, and extracellular matrix components^[65-67]. Necrotic cells may activate NF- κ B through TLR2, leading to the expression of tissue repair-associated genes^[65]. It is supposed that necrosis induced inflammation in tissue damage may provide danger signals functioning as inducers of tissue repair responses through TLRs. The TLR ligands released from necrotic cells have not been identified, although heat shock proteins produced by damaged cells are known to be TLR ligands^[66]. Components of the extracellular matrix, such as hyaluronan, can be an endogenous ligand for TLR4^[67]. Increased hyaluronan production has been demonstrated in both DSS colitis in mice and in human Crohn's disease^[68]. It

is possible that TLR activation may occur in the absence of microbial products^[68]. In the case of inflammatory mucosal damage, ILFs may induce repair mechanisms *via* endogenous TLR activation.

TLR4 was also shown to be expressed on human colon carcinoma cells and functionally active. It may play important roles in promoting immune escape of human colon carcinoma cells by inducing immunosuppressive factors and apoptosis resistance, and it may also promote the proliferation and migration of cancer cells^[69,70].

The analysis of isolated tumor cells from primary colon cancers showed co-expression of TLR7 and TLR8 with CD133 and gave evidence for a subpopulation of colon cancer-initiating cells^[71]. Persistent TLR-specific activation of NF- κ B in colorectal cancer, particularly in tumor-initiating cells, may sustain further tumor growth and progression through perpetuated signaling known from inflammatory and tissue repair mechanisms with consecutive self-renewal in pluripotent tumor cells. Activation through self-ligands or viral RNA fragments from tumor-associated lymphoid aggregates may putatively maintain this inflammatory process, suggesting a key role in cancer progression.

THE EFFECT OF THE PRESENCE OF ILFS ON MUCOSAL REPAIR

The epithelia of intestinal crypts associated with ILFs and PPs have an increased proliferation rate^[10,14]. Saxena *et al*^[10] showed that PPs in rats have a facilitative effect on the healing of intestinal wounds by promoting both epithelial cell migration on the defect and epithelial cell proliferation in the crypts adjacent to the wound and by decreasing the rate of wound contraction.

In rats, a difference in epithelial apoptosis between the FAE of PPs and intestinal villi was described^[72]. Onishi *et al*^[72] showed that the progression of the apoptotic process in the epithelial cells of FAE occurs later than in the intestinal villi, so the possibility of epithelial differentiation might remain in FAE, unlike in intestinal villi. PPs are supposed to have a regulatory effect on the epithelial proliferation as well^[73].

The Wnt signaling pathway is critical for regulating a number of basic cell functions, such as cell proliferation, cell fate, polarity, differentiation, and migration, leading to morphogenesis and organogenesis^[74,75]. There is strong genetic evidence that Wnt signaling play critical roles in the regulation of epithelial stem cells in the intestinal tract^[76]. The Wnt target gene *Lgr5* has been recently identified as a novel stem cell marker of the intestinal epithelium and the hair follicle^[77]. In the intestine, *Lgr5* is exclusively expressed in cycling crypt base columnar cells^[77]. Many Wnt family proteins are expressed in hematopoietic tissues, and can also be secreted by lymphoid cells^[78,79]. The Wnt-*Lgr5* pathway may be a potential switch between the ILFs and colonic epithelial renewal. Lymphoid cells of ILFs may produce Wnts which are essential components of a milieu in which bone marrow derived stem cells immigrated to ILFs to engage in epithelial differentiation (Figure 2).

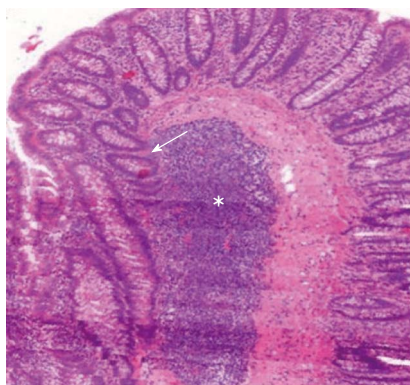


Figure 2 3D reconstruction of a human colonic surgical sample (MIRAX Viewer, 3D, 3DHISTECH Ltd., Budapest). A large subepithelial isolated lymphoid follicle (white star) can be seen. Colonic crypts (white arrow) with no connection to the luminal surface “outgrow” from the isolated lymphoid follicle.

THE EFFECT OF THE PRESENCE OF ILFS ON COLORECTAL CARCINOGENESIS

Results from experimental colon cancer studies indicated that ILFs might promote the development of adenocarcinomas^[7,8]. However, studies in experimental animals have also shown that the intestinal lymphoid system plays an important role in immunologic defense mechanisms; that is, antigenic stimuli result in germinal center formation, antibody production, and finally enlargement of the follicles^[80]. In humans, the presence of tumor-infiltrating lymphocytes is associated with an improved prognosis in colorectal cancers, as does the presence of high level DNA microsatellite instability^[81]. These results suggest that ILFs in early colorectal neoplasms play an important role in defense rather than in promotion.

In a recent study, Fu *et al.*^[9] found that the incidence of ILFs in early human colorectal neoplasms significantly differs by gender, location, macroscopic type, and histology, but moreover, their localization significantly differs by macroscopic type.

In squamous cell carcinomas of the esophagus, cyclin A expression in the germinal center cells of ILFs beneath the superficial tumorous lesions was shown to be an immunological signal toward the proliferation and progression of the tumors^[82]. Gutfeld *et al.*^[83] found that the cells of colonic ILFs, inflammatory cells, ganglion cells, and endothelial cells express serum amyloid A, an acute phase reactant, whose level in the blood is elevated in response to trauma, infection, inflammation, and neoplasia, on both mRNA and protein levels. The serum amyloid A mRNA expression in epithelial cells was found to gradually increase as it progressed through different stages of dysplasia to overt carcinoma. While expression of the serum amyloid A1 and -4 genes in colon carcinomas was confirmed by RT-PCR analysis, this expression was barely detectable in normal colon tissues. Their findings indicate local and differential expression of serum amyloid A in human colon cancer and tumor-associated ILFs, and suggest its role in colorectal carcinogenesis.

MESENCHYMAL-EPITHELIAL AND EPI-THELIAL-MESENCHYMAL TRANSITION IN ILFS

Epithelial-mesenchymal transition (EMT) is a physiological mechanism present during development, and is also encountered in several pathological situations such as renal interstitial fibrosis, endometrial adhesion, and cancer metastasis^[84]. A reverse phenomenon, mesenchymal-epithelial transition (MET) also takes place during normal development in processes such as somitogenesis, kidney development and coelomic cavity formation^[85]. In adult organisms, it has been proposed that restrictive mechanisms repress EMT and MET^[86]. During tumor development, these mechanisms appear to fail, allowing EMT described in metastasis generation^[87].

In inflammation, MET can also be altered because mesenchymal stem cells are mobilized to these sites of injury and consequently subjected to the inflammatory response^[88]. BMDCs could differentiate into mature-appearing epithelial cells in response to tissue damage^[89]. It was recently published that versican, a large chondroitin sulfate proteoglycan, mediates MET^[90]. The results of Hirose *et al.*^[91] indicate that versican can bind specific chemokines through its chondroitin sulfate chains and that the binding tends to down-regulate the chemokine function. This raises the possibility that versican may act as a regenerative factor in colonic mucosa, and may be an important switch point between ILFs and MET. The presence of CDX2 and cytokeratin positive subepithelial cells in the marginal zone of ILFs also suggests that MET may take place in these immune formations^[2].

Stroma-tissue, including lymphoid aggregates and ILFs surrounding the cancer cells, plays an important role in the tumor behavior. Mesker *et al.*^[92] analyzed the expression of markers involved in pathways related to stroma production and EMT (β -catenin, TGF- β -R2, SMAD4) in high-risk colorectal cancer patients, and found that patients with stroma-high and SMAD4 loss are of high risk. The anti-EMT effect of SMAD4 was also proven in colon carcinoma cells^[93].

CONCLUSION

Based on the summarized results of literature, it seems that ILFs act like a switch between colonic mucosal regeneration and colorectal carcinogenesis.

Subepithelial revascularization after mucosal damage takes place partly under the direction of ILFs with the prominent help of vascular endothelial growth factor and its receptors. Immigrating stem cells from bone marrow may leave circulation *via* high endothelial venules in ILFs and their surroundings. Their differentiation throughout mesenchymal-to-epithelial transition may also happen in ILFs, and follicular dendritic cells, as well as the subepithelial myofibroblasts, seem to be crucial parts of colonic crypt formation and epithelial renewal.

Vasculogenesis in ILFs supports not just tumor growth

and the metastatic process, but the VEGF receptor signaling acts like a direct growth factor for tumor cells. The fusion of BMDCs immigrating to ILFs with tumor cells may explain EMT in colorectal cancers. The presence of ILFs, dendritic cells and subepithelial myofibroblasts may also result in a specific milieu for tumor formation, growth and invasion.

Better understanding of the role of ILFs in mucosal repair may lead to the development of new therapeutic agents for inflammatory colon diseases that not only decrease the activity of inflammation, but also accelerate epithelial barrier recovery, hence dramatically decreasing clinical symptoms. Moreover, by revealing the exact connections between ILFs and colorectal carcinogenesis, the basis of individualized anti-cancer immunotherapies may be established.

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Patterns of local recurrence in rectal cancer after a multidisciplinary approach

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chemoradiotherapy versus extended lymphadenectomy, though there is a trend towards posterior or presacral LR in patients in the Western world and lateral LR in Asia. Nevertheless, both may arise from the same mechanism. Moreover, as well as the mode of treatment, the type of LR is related to the height of the initial tumor. Nowadays most LRs are related to the advanced nature of the disease. Involvement of the circumferential radial margin and spillage of residual tumor cells from lymphatic leakage in the pelvic side wall are two plausible mechanisms for the genesis of LR. The patterns of pelvic recurrence itself (pelvic subsites) also have important implications for prognosis and are related to the potential success of salvage curative approach. The re-operability for cure and prognosis are generally better for anastomotic and anterior types than for presacral and lateral recurrences. Overall survival after LR diagnosis is lower with radio or chemoradiotherapy plus optimal surgery approaches, compared to optimal surgery alone.

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Abstract

Improvements in surgery and the application of combined approaches to fight rectal cancer have succeeded in reducing the local recurrence (LR) rate and when there is LR it tends to appear later and less often in isolation. Moreover, a subtle change in the distribution of LRs with respect to the pelvis has been observed. In general terms, prior to total mesorectal excision the most common LRs were central types (perianastomotic and anterior) while lateral and posterior forms (presacral) have become more common since the growth in the use of combined treatments. No differences have been reported in the current pattern of LRs as a function of the type of approach used, that is, neo-adjuvant therapies (short-term or long-course radiotherapy, or

Key words: Rectal cancer; Local neoplasm recurrence pelvis; Pattern of recurrence multidisciplinary approach

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INTRODUCTION

Over the past two decades, there have been improvements in the management of rectal cancer in terms of postoperative death (falling from 10% to 2%), locoregional failure (dropping from 30%-40% to less than 15%), conservative surgery rates (increasing from 20% to 60%) and survival, with advances made in the understanding of the biology of this type of tumor as well as staging and the use of combined therapies^[1]. The anatomical and technical basis of tumor recurrence within the pelvis has been extensively investigated by surgeons and pathologists, and this has led to major improvements in surgical therapy. Nonetheless, although surgery remains the mainstay of treatment aimed at achieving locoregional control, nowadays the therapeutic approach to rectal cancer is eminently multidisciplinary.

The key to successful surgery is complete excision of the tumor proximally, distally and around its circumference with sufficient margin of normal tissue (R0 resection). In total mesorectal excision (TME) surgery the rectum and its perirectal lymphatic and fatty tissue (mesorectum) is completely mobilized by sharp dissection, as an intact package surrounded by an undamaged peri-mesorectal layer of proper rectal (visceral) fascia, avoiding spillage and growth of residual tumor cells into the pelvis and subsequent development of a local recurrence (LR). Educational programs aimed at training surgeons in this pelvic dissection technique have demonstrated reproducible results in achieving a reduction of the LR of rectal cancer rate by 40%^[2] and even greater when associated with radiotherapy (RT)^[3] or neoadjuvant chemoradiotherapy (CRT)^[4].

Nevertheless, LR of rectal cancer remains a significant clinical problem, associated with severe morbidity, low rates of success of salvage procedures, and eventual death in the majority of patients^[5].

It is important to review the patterns of treatment failure resulting after rectal cancer management. Improvements in surgical and adjuvant therapies may affect not only the likelihood of tumor recurrence in the pelvis but also the pattern of pelvic recurrence itself (i.e. pelvic subsites). Knowledge of the pattern and natural history of LR, the associated risk factors for their development and the mechanism by which they occur may serve as the foundation for efforts to improve the results of multidisciplinary care (i.e. RT field design, suitability of lymphadenectomies, strategy in the follow-up monitoring, *etc.*).

The aim of this review is to characterize and analyze the pattern of LR today following different curative approaches for rectal cancer, with special emphasis on the correlation between subsites of pelvic recurrences and treatment modalities.

LIMITATIONS OF THIS REVIEW

Many of the studies that report patterns of pelvic recurrence have multiple limitations. Some are outdated or do not give exact anatomical information of the location of recurrent tumors in the pelvis. In particular, the diagnostic procedures, methods of documentation, acknowledge-

ment or confirmation of diagnosis, presence or absence of histology, the use of interval pain, anatomic definitions of the rectum, first site of recurrence and cumulative recurrence data, as well as the definition of the LR itself and other details all affect the analysis of incidence rates, timing, and patterns^[6].

Old literature concerning the pattern of local failure in rectal cancer was based on planned or symptomatic reoperations data or autopsy series. Planned "second look" procedures and symptomatic surgery were performed in the pre-TME surgery era and LR data obtained may be outdated. Furthermore, autopsies reveal only the end pattern of failure.

On the other hand, most recent reports are based on clinical or imaging data which can be also misleading as the methods of diagnosing and confirming LR and length of follow-up are not described consistently. The actual rate of pelvic recurrence may be somewhat higher than estimated by these reports, as some studies report only first sites of failure, and pelvic relapse later in the course of disease is not always assessed in patients under palliative chemotherapy for distant metastasis.

Trials of preoperative RT or CRT in resectable rectal cancer are characterized by multiple methodological problems because treatments are combined (RT and surgery) to address a heterogeneous condition (various populations and stages of rectal carcinoma) and to achieve a variety of goals (downstaging and improving resectability, as well as decreasing local and possibly distant recurrences and improving survival).

DEFINITION AND CLASSIFICATION OF LOCAL RECURRENCE

Although by definition the term LR is only applicable when the initial or primary surgery is expected to be curative (no remaining macroscopic evidence of disease locally, that is R0 and R1 according to the UICC (International Union Against Cancer), it must be seen as the further development of tumor cell remnants: there is a close biological similarity between a primary tumor and an LR, in contrast to the situation with corresponding organ metastases^[7].

Recurrent rectal cancer may be isolated (local or metastatic) or combined (local and metastasis). Indeed LR can be defined as any tumor located within the pelvis, either alone or in conjunction with metastases^[6]. Several authors have classified locoregional pelvic recurrence in order to facilitate treatment and compare outcomes. Specifically, the distinction between localized and diffuse pelvic recurrence is pivotal in defining subsequent management and prognosis.

The Mayo Clinic^[8] described recurrence in terms of degree of fixation both in term of site (anterior, sacral, right or left) and number of points of fixation (F0-F3). Wanebo *et al*^[9] proposed a classification based on the UICC TNM system: TR1 and TR2 corresponding to intraluminal LR, either following local excision or at the anastomosis; TR3 corresponding to LR at or around the level of the anastomosis with limited extramural spread

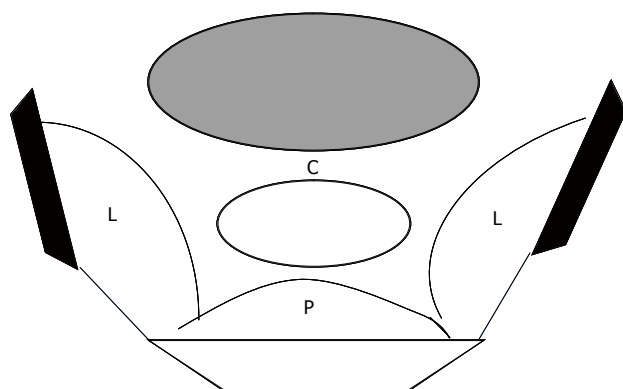


Figure 1 Types of pelvic recurrence. C: Central; L: Lateral; P: Posterior.

and without pelvic fixation; TR4 corresponding to invasion into either adjacent urogenital organs or presacral tissues with tethering but no fixation; and TR5 corresponding to invasion into the sacrum or pelvic side walls. On the other hand, others divided pelvic local failures into just three basic types of recurrence: localized (central), sacral and pelvic sidewall.

The Memorial Sloan Kettering^[10] group describe a nomenclature based on the anatomical region of the pelvis that is involved (Figure 1). Accordingly, LR is defined as either: axial, subdivided into anastomotic, mesorectal (residual mesorectum) or perirectal soft tissue within the center of the pelvis or perineum following an abdominoperineal resection (APR); anterior, involving the genitourinary tract; posterior, involving the sacrum and presacral fascia and sacral root sheaths; or lateral, involving the muscles (piriformis, elevator), soft tissue of the pelvic sidewall, lymph nodes, major iliac vessels, sacral nerve plexus and lateral bony pelvis. Lastly, the Dutch TME trial^[11] classifies LR based on the same pelvic subsites, although the perineum and anastomotic recurrences are grouped separately. We have found the latter to be the most useful classification, although it does not distinguish between two different origins of the pelvic sidewall involvement itself: true lateral involvement by growth of tumor deposit in lymph nodes along the iliac vessels and continuous extension of tumors of central origin.

DIAGNOSIS OF RECURRENT RECTAL CANCER

Patients with recurrent cancer are a heterogeneous group. To establish LR or pelvic disease after definitive resection of rectal cancer^[12], most authors accept at least one of the following major criteria: (1) Histological confirmation; (2) Palpable or evident disease with subsequent clinical progress; (3) Clear evidence of bone destruction; and (4) Positive positron emission tomography examination, and at least one of the minor criteria: (1) Progressive enlargement of soft tissue mass on repeated computed tomography (CT) or magnetic resonance (MRI) examination; (2) invasion of adjacent organs; (3) subsequent rise in tumor

markers; and (4) typical appearance in endoscopic ultrasound, CT or MRI imaging.

Note that according to these major criteria no patient should be accepted as having pelvic recurrence by diffuse pelvic pain.

RISK FACTORS FOR LR

Many factors affect the risk of local recurrence.

Pathological factors

The pelvic recurrence rate is tumor stage dependent: the more advanced the stage the more likely it is that rectal cancer will recur^[13]. Many authors have confirmed the association between advanced UICC or Dukes' stage and the likelihood of recurrence. Not surprisingly, the extent of invasion beyond the rectum affects recurrence, with an incidence of less than 1% in patients in whom no local extension is noted, compared to between 5% and 10% in patients with moderate spread and 15%-25% in those with more extensive spread^[14,15]. The number of positive lymph nodes^[16] as well as a positive circumferential resection margin (CRM) also influence both LR and survival^[11]. Even with combined treatments, the incidence of LR in patients with one of these risk factors (that is, TMN stage IV, T4 tumor, N2 disease or positive CRM in T3 disease) reaches 20%, compared to less than 5% in patients who do not have these characteristics^[11]. It is also clear that the combination of risk factors is also important: in patients at T1-T2 the incidence of LR is 1% with a negative CRM but this rises to 12% for a positive CRM, while for those at T3-T4 it is 15% for a negative CRM but 25% for a positive CRM^[11]. In patients undergoing surgery with or without preoperative RT, the combination of CRM and lymph node status has been shown to be a more effective discriminator of prognosis than TNM staging^[1,11].

Height of the tumor is also a critical factor as LR is also more likely with tumors in the lower third of the rectum (10%-15%) than in patients with tumors in either the middle third (5%-10%) or upper third (2%-5%)^[3,5,11,15]. The risk of LR is also related to the position of the tumor within the circumference of the rectum. In the series of Chan *et al.*^[17] the rate of LR was 15% (95% CI, 11-22) for tumors affecting the anterior side of the rectum but was 5.8% (95% CI, 3-11) for other locations. Anterior tumors tend to be more advanced, at least in male patients, and the anterior aspect of the TME dissection more difficult to perform in the narrow male pelvis, presenting a higher risk of LR and death than tumors in other sites^[18].

The shape (exophytic versus non-exophytic) of the tumor, the presence or absence of budding, lymphatic, venous or perineural invasion, the presence of obstruction or perforation of the tumor together with the degree of tumor differentiation, and fixity of the tumor, all influence the risk of local recurrence adversely^[13,19].

Therapeutic factors

Inadequate removal of the primary tumor is the most

Table 1 Patterns and rates of recurrences after different approaches

Authors - year	Treatment	5-year local recurrence (%)			Metastases as a first site of recurrence (%)	Median time to LR
		Total	Isolated	Combined		
Pilipshen <i>et al</i> ^[32] (1968-1976)	Pre-TME surgery	25.5	13.3	12.3	NA	16 mo
Heald <i>et al</i> ^[34] (1978-1986)	TME surgery	3.7	2.6	1.1	NA	14 mo
Swedish Trial ^[39]	Pre-TME surgery/	23.0	13.0	10.0	11	NA
(1987-1990)	sRT+ pre-TME	9.0	5.0	4.0	19	
Mohiuddin <i>et al</i> ^[67] (1976-1989)	LRT+ Pre-TME	13.0	6.0	7.0	17	25 mo
German Trial ^[25] (1995-2002)	CRT+ TME/	6.0	3.0	3.0	20	NA
	TME + CRT	13.0	7.0	6.0	20	
British Trial ^[44] (1998-2005)	sRT +TME/	4.7	2.0	2.7	19	NA
	TME+ selective CRT	11.5	6.1	5.4	21	
Dutch Trial ^[41] (1996-1999)	TME surgery/	11.3	6.6	4.7	17	18 mo
	sRT+ TME	5.8	2.3	3.5	19.3	30 mo
Guillem <i>et al</i> ^[4] (1988-2002)	CRT+TME	4.3	2.3	2.0	22	23 mo
Yu <i>et al</i> ^[54] (1989-2001)	CRT+TME	8.3	6.3	2.0	NA	NA
Kim <i>et al</i> ^[56] (2001-2005)	CRT+TME	8.0	3.7	4.3	19	24 mo central 18 mo lateral
Kusters <i>et al</i> ^[58] (1993-2002)	Unilateral EL/	15.4 (only T3,T4 tumors)			NA	NA
	Bilateral EL	8.3 (only T3-T4 tumors)				
Moriya <i>et al</i> ^[57] (1982-1991)	Nerve-sparing EL	6.2 (14 for N+ tumors)			12	17 mo

TME: Total mesorectal excision; sRT: Short-term preoperative radiotherapy; LRT: Preoperative long-term radiotherapy; CRT+TME: Pre-operative chemoradiotherapy; TME+CRT: Post-operative chemoradiotherapy; EL: Extended lymphadenectomy; NA: Not available; LR: Local recurrence.

important factor determining whether the tumor recurs^[20]. In addition to the involvement of the CRM, the plane of surgery achieved, as a measure of the quality of mesorectal excision, has been shown to be an important prognostic factor for LR^[21]. Surgeon variability is also a widely studied phenomenon. In multivariate analysis “the surgeon factor” has emerged as a critical treatment-independent variable, and is not only related to the volume of operations performed^[22].

The incidence of lateral lymph node involvement has been extensively investigated by Japanese authors. The term “extended lymphadenectomy” (EL) refers to the removal of the lymph nodes in the extra-mesenteric area. Generally rates of node involvement have been reported to be 5%-10%, but are markedly higher in stage III tumors in the lower third of the rectum, with rates of up to 15%-25%^[23]. However, it is doubtful that remaining lateral lymph nodes after an R0 resection is the major or only source of tumor regrowth^[24].

There is abundant clinical data supporting the importance of the pathologic response and downstaging to preoperative CRT. All patients who developed cancer recurrence in the German trial had positive lymph node involvement post-treatment^[25]. A pathological complete response (pCR) or greater than 95% CR in the post-CRT is a predictive factor of low LR rate and good prognosis, and several studies have shown that this is the most important independent prognostic factor in multivariate analysis for disease-free survival^[4,26].

While attention was traditionally focused solely on the optimal distal mucosal margins required to achieve an oncologically safe resection, not only have these margins been reduced significantly, but also greater importance has come to be placed on the lateral margins for achieving an R0 resection. Short (but negative) distal margins have

repeatedly been found not to be associated with pelvic recurrence^[27,28]. The risk is not so much of intramural spread as of intramesorectal spread, and probably the risk for mesorectal tumor deposit is higher in node-positive than in node-negative patients^[27].

Other factors that may increase a patient's risk of LR are related to increasing body mass index^[29]. It has been shown that obese men are more likely than normal weight males to develop an LR, and that adiposity is a strong predictor of requiring an APR^[30].

SITES OF LOCAL RECURRENCE AFTER SURGERY ALONE

In the late 1970s, areas of failure found on planned reoperation (“second look” procedures) of patients at risk of recurrence, in spite of an initial curative resection, were investigated. The seminal article by Gunderson and Sosin^[31] showed that as distant metastases alone were uncommon (7%), LR as the only type of failure occurred in nearly 50% of cases of recurrence and as some component in 92%. They also depicted the pattern of pelvic subsites LR (Table 1), showed that disease relapse rates were indeed related to the degree of bowel wall penetration and the extent of nodal disease, and paved the way for the exploration of radiation therapy.

In the era before the adoption of TME, surgery alone was associated with local failures of up to 30%-50%. The classic article of Pilipshen *et al*^[32] describes the pattern of LR at a prestigious institution (Sloan-Kettering) in the pre-TME era, with LR rates of 31% in cases at T3-T4 and 49% in cases at N2. Hruby *et al*^[33] provide a detailed analysis of the sites of LR after undergoing surgery for rectal cancer in a series of 269 patients mainly during the 1980s. As can be seen in Table 1, at that time most LR was axial

or central, in the block of fatty tissue surrounding the rectal wall, within or contiguous to the operative site, and appeared 6-16 mo before the appearance of metastasis.

In the 1980s details emerged of the first series of patients treated with TME surgery. Heald *et al.*^[34] published an accumulated local recurrence rate of 3.7% in a personal series of 115 cases of “curative” low anterior resections^[5]. Ten years later, the same author reported actuarial 5- and 10-year recurrence rates of 6% (95% CI, 2%-10%) and 8% (95% CI, 2%-14%) respectively with TME surgery alone. Another TME pioneer, Enker, published a rate of 4.1% for Dukes’ B and 8.2% for Dukes’ C patients^[35].

In the 1990s large reductions in local and distant recurrences were reported with TME and so, even without a proper randomized control trial, the TME resection became the new gold standard of surgery for rectal cancer.

Although regarded as equivalent in the pre-TME surgical era, the superiority in terms of LR of the TME low anterior resection (LAR) over the “traditionally” executed APR soon became clear. “Standard” or “traditional” APR for low rectal cancer is associated with a higher rate of positive CRM (30%-60%) and operative perforation (20%-33%), leading to higher LR and poorer survival rates than with LAR^[36]. Recently a more radical excision of the levators and puborectalis muscles, carried out in the prone jack-knife position, has been proposed (“extralevator APR”)^[37].

As the surgical techniques worldwide evolved to TME resections, European researchers focused on the delivery of a short course of high-dose preoperative RT, without chemotherapy, after ascertaining that adjuvant RT, both before and after surgery, substantially reduced the risk of LR when biologically effective doses of 30 Gy or more were used^[38].

LOCAL FAILURES AFTER SHORT-TERM PREOPERATIVE RT

The Swedish Rectal Cancer Trial^[39] randomized 1168 patients to surgery alone or to surgery following a 1-wk of pelvic RT (25 Gy in 5 daily fractions), and showed that not only was the 5-year LR rate significantly improved with preoperative RT (23% *vs* 9%, among the curatively treated patients) but also the 5-year survival rate significantly improved (58% *vs* 48%). However, this trial was conducted in the surgical era prior to the adoption of TME.

Syk *et al.*^[40] reviewed the incidence and location of LR in a group of 880 patients from Stockholm after the introduction of TME surgery, and half of the group also received short-term preoperative RT. In this study, 42% of LR originated from tumors in the upper rectum, and a majority of these patients had not received RT. In all these cases, the recurrence was at the anastomosis and virtually all had visible signs of residual mesorectal fat. Eighteen percent of the patients had LR involving the lateral wall of the pelvis, but only 6% of the tumors involved sites consistent with recurrence in iliac lymph nodes. The authors concluded that lateral pelvic lymph node metastases are not a major cause of local recurrence after TME, and

that partial mesorectal excision may be associated with an increased risk of local recurrence due to presacral and/or pelvic sidewall involvement in the upper rectum.

As surgery improved, Dutch researchers then asked whether preoperative short-term RT would still be beneficial in the setting of TME resection properly executed. In the Dutch TME trial a significant benefit was seen with preoperative RT in patients with TNM stage II and III disease, with the two-year local relapse rates decreasing from 5.6% to 1% and from 15% to 4.3%, respectively^[41]. The update of the trial reported in 2007 noted a drop in local relapse from 22% to 11% for stage III patients but no significant reduction for stage II patients, and no difference in distant metastasis rate or 5-year overall survival^[42].

Subgroup analysis showed a significant fall in patients with cancer in the lower rectum, with nodal involvement but uninvolved CRM. Although those CRM positive patients who received preoperative RT had a lower LR rate than the group with TME alone, this difference was not statistically significant (9.3% *vs* 16.4%, *P* = 0.08). The authors arguably concluded that short-term preoperative RT “hardly compensate” for involved CRM^[43].

The Dutch group has also recently published a complete and updated analysis of the pattern of LR and the most likely mechanism of recurrence in the trial^[11]. They showed that preoperative RT reduces LR in all subsites. However the appearance of LR was slower in the group who underwent RT (2.6 years *vs* 1.5 years), and if distant metastases diagnosed within 1 mo of LR diagnosis were also considered to have occurred simultaneously, the rate of combined recurrences was higher in the RT + TME group (74% *vs* 40% in the TME group). In the TME group the recurrences were predominantly anastomotic and posterior, while in the RT + TME group most were posterior and lateral. The anastomotic recurrences were significantly more common in the TME only group, suggesting that RT is especially effective in preventing anastomotic recurrence. Lateral recurrences represented more than 25% of the total in the RT + TME group and most appeared together with metastasis. After LAR, the recurrences were mostly perianastomotic while after APR they were mostly presacral. Perineal LR were found after APR in the TME only group, but not in the RT + TME group. TME alone in node positive disease resulted in considerable local recurrence when the distal margin was 2 cm or less, while RT resulted in a small number of LRs, except when distal margins were less than 5 mm. In total 17% of the patients had a positive CRM, and of those 17% developed LRs (12% in those with T1-T2 tumors, and 24% in T3-T4 tumors with positive CRM).

Given the higher LR rates with narrow CRM, the question of whether selective postoperative RT could improve outcomes in this setting was addressed by researchers in the United Kingdom. The MRC CR07 trial^[44] randomized 1350 patients to TME preceded by short-term RT (25 Gy in 1 wk) *vs* TME followed by CRT (45 Gy plus 5-fluorouracil) if the CRM was < 1 mm. The results showed that the 5-year local recurrence rate was significantly better in

the preoperative RT group (4.7%) than the postoperative CRT group (11.5%). However, in those patients with a positive CRM, the LR rates were not statistically different (16% preoperative *vs* 23% postoperative).

PATTERN OF PELVIC FAILURE AFTER LONG COURSE RT PLUS CHEMOTHERAPY

Postoperative adjuvant strategies to improve outcomes following rectal resection have mainly been explored in the United States. Indeed, in 1990, after positive trials conducted by the Gastrointestinal Tumor Study Group^[45] and the Mayo Clinic/North Central Cancer Treatment Group^[46], the NCI issued a statement declaring combined postoperative therapy the new standard of care in this setting^[47].

Researchers, however, were questioning whether preoperative combined therapy would be even more beneficial. In the 1990s, data from the Memorial Sloan-Kettering Cancer Center and the MD Anderson Cancer Center accumulated^[48] in support of that benefit. Moreover, results from three randomized trials (the Uppsala trial^[49], NSABP R03^[50] and above all the German CAO/ARO/AIO trial^[25]), demonstrated the clear superiority of preoperative RT regimens over postoperative therapy in terms of local control with better compliance to treatment and lower toxicity.

The next step was to test the hypothesis that chemotherapy plus preoperative RT significantly improved local control, tumor downsizing and downstaging compared with RT alone. Two randomized trials compared preoperative RT *vs* preoperative CRT, the study by the Fédération Francophone de Cancérologie Digestive (FFCD 9203)^[51] and the EORTC 22921 trial^[52], and similar results were reported. In the latter, the five-year results showed that chemotherapy increased the rate of pCR (14% *vs* 5.3%), translated into a 3% benefit in terms of sphincter preservation and significantly reduced LR rate from 17% without chemotherapy down to 8% with CRT. Thus chemotherapy, regardless of whether it is administered before or after surgery, confers a significant benefit with respect to local control. The main criticism that can be made of those trials is that TME resections were not uniformly implemented.

On the other hand, the favorable effect of delaying surgery after CRT on downstaging (and possibly also sphincter preservation) was shown in the Lyon R90-01 trial^[53].

There is, however, limited data on patterns of relapse in rectal cancer patients treated with TME surgery and CRT. Such information might help determine whether modifications in RT dose or field design are warranted (i.e. a local recurrence after RT may be inside or outside the RT field; recurrence outside the field requires an increase in the size of the field, and recurrence inside the field implies the need for an increase in the total dose). We have identified reports on only four series of patients that contain detailed analysis of the pattern of pelvic recurrence after CRT and TME surgery.

From the MD Anderson Cancer center, Yu *et al*^[54] presented a thorough study attempting to identify subsites of pelvic LR in an effort to correlate sites of relapse on

CT images with RT simulation films in 46 rectal cancer patients. Of all the LR, approximately two-thirds were in-field (within the radiation field) recurrences and only one-third were marginal (inside but within 1 cm of the border of the field) or out-of-field (more than 1 cm from the border) recurrences. Of the in-field recurrences, nearly 80% occurred in the low pelvic and presacral regions. Multivariate analysis showed that the risk of in-field LR was significantly associated with pathological N stage, while it was notably not with positive CRM or downstaging. The authors suggested various strategies to improve locoregional control in low pelvic and presacral regions.

Höthch *et al*^[12] published a large-scale multicenter study based in Germany to evaluate pelvic sites of recurrence with special attention to radiation ports. Nearly 80% of LR occurred within the treated volume, in the central pelvis, and the pelvic sidewall structures were involved in fewer than 5% of tumor relapses. They found no significant differences in the incidence of pelvic sidewall involvement between APR and LAR cases, however there was a significant difference in the spread of recurrent tumors in the inferior part of the pelvis.

However, a quite different picture has been reported from Korea, where Kim *et al*^[56] examined the patterns of locoregional recurrences in 366 patients with locally advanced rectal cancer who underwent preoperative CRT and curative TME surgery, and assessed the effect of clinical parameters on lateral pelvic recurrence. Eight percent of the patients had LR, of which around 20% and 80% occurred in central and lateral pelvic areas respectively. Multivariable analysis showed that lateral pelvic recurrence was significantly associated with ypN classification (lymph node status after preoperative CRT) and lateral lymph node size. The authors suggested that lateral lymph node metastasis is a risk factor for LR and could be a potentially curable regional disease rather than a sign of systemic disease. Accordingly, they suggested that patients with lateral lymph node size of > 10 mm and ypN0 or lateral lymph node size of 5 mm and ypN+ are a potential subgroup of patients who might benefit from lateral lymph node dissection.

In tumors of the middle and lower rectum, lateral lymph nodes remain a potential cause of locoregional recurrence after conventional TME because they are not removed. EL has been championed mainly by Asian surgeons, who are internationally renowned for their skills in radical surgery.

PATTERN OF RECURRENCES AFTER EXTENDED LYMPHADENECTOMY AND TME, WITH OR WITHOUT ADJUVANT TREATMENT

Around 40% of patients treated for rectal cancer present with lymph node metastases, which occur along the mesorectal nodal chain, along the inferior mesenteric artery lymph nodes or in the lateral pelvic lymph nodes (along the obturator, internal iliac or medial aspect of the external

iliac artery). Whether pelvic sidewall lymph nodes should be considered metastatic disease as suggested by the TNM classification (M1) or part of the regional lymphatics (N3) as outlined in Japanese guidelines that are amenable to curative resection, is a contentious issue. Japanese surgeons have adopted the technique of EL to supplement TME, with the aim of minimizing LR and improving survival. Western surgeons do not use EL regularly, and this might pose a risk of local recurrence in the pelvic sidewall in patients operated on without preoperative RT.

In a recent detailed topological analysis of the pattern of lymphatic spread in 605 cases of rectal cancer, 285 cases (47%) were identified as having lymph node metastases. Of this total, 71.5% were mesenteric, 21.5% were lateral and mesenteric, and only 4.7% were exclusively lateral (so-called skip metastases), while among the cases of lateral metastases slightly more than a third were bilateral. The authors^[23] concluded that lateral lymph node status is reflective of overall mesenteric lymph node status and that evidence of lateral lymph node involvement may be an ominous sign of advanced disease with an inherent dismal prognosis.

EL is associated with high degrees of urinary and sexual dysfunction and while it is possible to undertake lateral node dissection with autonomic nerve-sparing surgery (NSEL), there are problems with this surgical technique in terms of worldwide uniformity. Moriya *et al.*^[57] have studied the pattern of recurrence after NSEL surgery in 306 patients, of which 14% were in Dukes' stage C, and found an overall LR rate of 6.2%. Dukes' A and B patients with relapse had suture-line recurrence. In contrast, in the Dukes' C group, 70% of the recurrences were in patients who had had involvement of more than 5 lymph nodes and 40% in those in whom there had been lateral spread, the number of mesenteric lymph node metastases being the factor which had the strongest impact on the LR. The authors judged that the LR rate with NSEL is similar to that obtained with conventional EL.

Kusters and Van de Velde^[58] reviewed 351 patients operated for rectal carcinoma at or below the peritoneal reflection at the National Cancer Center in Tokyo. Standard TME surgery was performed for T1 and T2 ($n = 145$), and NSEL was added to TME for T3 and T4 (unilateral = 73; bilateral, when the tumor was located centrally, $n = 133$). They noted that overall there was lymph node involvement in 42% of cases, and lateral involvement in 10%, with "skip" metastases in 3% (mesorectal nodes negative and lateral nodes positive). Overall the 5-year LR rate was 6.6%, while for node-positive (N+) patients the difference between the uni- and bilateral NSEL (32% *vs* 14%, respectively) was significant.

On the other hand, studies of EL are observational and the reported outcomes are far from uniform even within series reported from Japan. Moreira *et al.*^[24] indicated that EL has no advantages for patients in Dukes' stages A and B and that for cases in stage C it does not significantly reduce LR rates compared to TME. Other series, from both Western and Asian countries, suggested that despite undergoing EL few patients survive for 5 years or more if carcinoma has spread to the pelvic lymph nodes.

Yano *et al.*^[59] proposed selective use of extra-mesenteric nerve-sparing lymphadenectomy for those cases in which lateral node metastasis is detected in the CT scan. These authors reported a high level of sensitivity and accuracy (88%) of CT scans for detecting lateral node metastasis, in marked contrast to their diagnostic accuracy for mesorectal lymph node involvement. This same group has published a recent review of lateral lymph node spread in a Japanese journal, emphasizing in particular the rates (20%) of lateral node involvement in T3-T4 cases of low rectal cancer with positive mesorectal nodes. Similarly, Min *et al.*^[60] reserved EL for cases in which high-resolution MRI detected extra-mesenteric lymph node metastasis. These authors found a positive predictive value of MRI of 86.4% and 40% for the lateral and paraaortic nodes respectively, and that the location of the lymph node metastasis was the only prognostic factor for cancer-specific survival, with disease in the paraaortic area indicating a worse prognosis than lateral or mesenteric involvement. This suggestion accords with the recommendation in the Guidelines 2000 for Colon and Rectal Cancer Surgery that dissection should be attempted to remove clinically suspected lateral lymph node disease, as far as is technically feasible^[61].

A different approach would be to consider EL in various combinations with RT. In the only randomized control trial of EL ($n = 23$) versus non-EL ($n = 22$) after preoperative RT (50 Gy) in both groups, Nagawa and colleagues^[62] reported no difference in disease-free survival or local recurrence. However, given the small sample size and the fact that the study did not include patients with lateral pelvic lymph node involvement, no safe conclusion can be drawn on the role of either RT or EL in this particular group of patients. Wanatabe *et al.*^[63] in a retrospective non-randomized study of four patient groups comparing EL with non-EL using either 50 Gy pre-operative RT or no RT found a 5-year survival advantage in the RT group. In addition, the authors reported no significant survival difference between the patients who had preoperative RT with conventional TME surgery compared with those who had EL without preoperative RT, and concluded that preoperative RT could be an alternative to EL.

A comparative non-randomized study by Kim *et al.*^[64] recently reported a higher local recurrence rate in patients with TME plus EL than in those with TME plus postoperative CRT. Among those with stage III lower rectal cancer, they successfully demonstrated that the EL group showed a 2.2-fold increase in local recurrence rate compared to the CRT group. However, the 5-year LR rate in stage III in the EL group was much higher than the rate previously reported by the same Japanese group (16.7% *vs* 7.4%), therefore either a patient selection bias or a different definition of LR cannot be ruled out in this study.

A recent meta-analysis^[65] comparing EL and non-EL TME surgery showed no overall difference in cancer-specific outcomes (5-year survival, 5-year disease-free survival and local or distant recurrence). However, as the authors state, the question of whether EL provides benefits in terms of survival or just local control in a subset of

Table 2 Relative incidence of sub-site locations of pelvic recurrences

Authors (yr)	Treatment	Axial or central (%)				Lateral (%)	Other (%)
		Anastomotic (perianastomotic)	Anterior (genitourinary)	Posterior (presacral)	Perineal		
Gunderson <i>et al</i> ^[31]	Pre-TME surgery	- ¹	40	19	31	10	
Pilipshen <i>et al</i> ^[32] (1968-1976)	Pre-TME surgery	40	10		40 ²	10	
Hruby <i>et al</i> ^[33] (1979-1996)	Pre-TME surgery	21	10.7	47	11	11	
Dutch Trial ^[41] (1996-1999)	TME surgery	24	18	32	5	18	2.5
Dutch Trial ^[41] (1996-1999)	sRT+ TME	13	16	41	-	25	2.7
Syk <i>et al</i> ^[40] (1995-1999)	sRT +TME	37 ³	30	10		18	
Yu <i>et al</i> ^[54] (1989-2001)	CRT+ CRT	-	44	28	-	10	18.0
Höthch <i>et al</i> ^[12] (1998-2001)	CRT+TME		60 ⁴	29		10	
Kim <i>et al</i> ^[56] (2001-2005)	CRT+TME		20 ⁵			80	
Kusters <i>et al</i> ^[58] (1993-2002)	Unilateral EL	25	-	16	16	40 ⁶	
Kusters <i>et al</i> ^[58] (1993-2002)	Bilateral EL	20	10	16	16	40	

APR: Abdominoperineal resection; Anast: Anastomotic; perin: Perineal; Ant: Anterior. ¹0% were APR; ²Post + perineum; ³Anast + ant; ⁴Perin + ant + anast; ⁵Axial o central; ⁶20% ipsilateral and 20% contralateral. TME: Total mesorectal excision; sRT: Short-term preoperative radiotherapy; EL: Extended lymphadenectomy; CRT: Chemoradiotherapy.

patients with advanced rectal cancer could not be safely answered by this meta-analysis.

PATTERNS OF RECURRENCE AFTER INTRA-OPERATIVE RADIOTHERAPY PLUS EXTENDED SURGERY

Several authors reported the impact of intra-operative radiotherapy (IORT) with or without preoperative external beam irradiation and surgical resection in patients with locally advanced or recurrent cancer. The overall available data on IORT showed a favorable impact on local control and in overall survival for patients resected for cure (R0, R1); However, there is a need for randomized studies of the effect of IORT. From a Dutch national referral center, the pattern of LR in 247 patients with locally advanced rectal carcinoma after IORT including multimodal treatment (preoperative CRT and extended surgery) has been analyzed in detail^[55]. The 5-year LR rate was 13.2% (7.5% after R0 resections). The most prominent sites of LR were the presacral (44%) followed by the anterior (21%) subsites and lateral spread accounted for less than 10% of recurrences. Around 50% of the LRs appeared in the IORT field, particularly high rates of infield recurrences being observed after dorsal IORT (75%). The authors hypothesize that migration of remaining tumor cells to the presacral space would explain the occurrence of this LR.

PATTERNS OF RECURRENCE FOLLOWING COMPLETE CLINICAL RESPONSE AFTER CRT

The definitive role of an initially non-surgical approach to treatment following complete clinical response (cCR) after CRT has not yet been determined and no definitive conclusions can be drawn before long-term results concerning LR and distant failure are available.

However, Habr-Gama *et al*^[66] have reported the pattern of recurrence and survival of 99 patients with distal rectal cancer (0-7 cm) and cCR following adjuvant CRT, sustained for at least 12 mo, managed by initial non-operative treatment. They observed 13 recurrences: five endorectal (limited to the rectal wall), seven systemic, one combined (endorectal and distant), and no pelvic recurrence outside the rectal wall was detected. There were no significant clinical differences either between patients with and without recurrence, or these same patients according to the location of the recurrence. Surprisingly, systemic recurrence occurred sooner than LR. The authors suggest that a change in the approach to follow-up monitoring may be necessary.

CLOSING REMARKS

The reported patterns and rates of local recurrence, after the aforementioned range of treatment approaches to rectal cancer, used in isolation and as combined therapies are summarized in Table 1. Overall, combining therapies reduces LR rates, delays the appearance of LR and means that when there is recurrence it is less often isolated than after surgery alone.

Table 2 lists the relative frequency of LRs in various pelvic subsites. In recent years, a subtle change has been observed in the distribution of LRs in terms of location within the pelvis, implying the involvement of a different mechanism in their development. In general terms, in the pre-TME years most recurrences were central, perianastomotic and anterior and since the adoption of combined therapies lateral and posterior (presacral) forms dominate. However, the LR distribution is not only related to therapy modality but also to the height of the tumor. LRs in the upper rectum are relatively rare, but when they do occur are usually perianastomotic, originating in the residual mesorectal fatty tissue (as they are treated with partial mesorectal excision, transecting the rectum at about 5 cm below the tumor), and are comparatively more common when only surgical treatment is used. This suggests that while

preoperative RT helps to prevent LR at all sites, it is especially effective in preventing anastomotic recurrences^[67]. Isolated anastomotic recurrences are also seen in select cases of very low rectal cancer treated using intersphincteric resection (ISR)^[68]. Surgical technique and attention to distal margin can also play a role in preventing this type of LR. The LR rate after ISR is higher in poorly selected cases of pT3 with no previous RT, due to accidental tumor spillage into the intersphincteric space or positive CRM^[69]. A lower LR rate has been reported with stapled coloanal anastomosis than for ISR even in T1-T2 patients^[70]. As expected, after transanal endoscopic microsurgery, intramural recurrence is the most common type of LR^[71].

Most local recurrences of mid-rectal cancers treated with RT or CRT + TME are related to the advanced nature of the disease. Tumor height of 5 cm or more is associated with a higher incidence of presacral and lateral LR^[72]. If the CRM is found to be positive after CRT, the hazard ratio for LR after surgery is significantly higher than if the CMR is involved when no preoperative CRT has been administered (6.3 *vs* 2.0), possibly because of selection of a population of tumor cells that are resistant to therapy^[73]. LR may also sometimes occur even in the absence of an involved CRM possibly owing to lymphatic spread from the distal rectum to lymph nodes in the pelvic side wall^[57]. Unilateral EL (lateral lymph nodes on one side of the pelvis are left intact) result in more LR than bilateral ELs, and it has been suggested that the mechanism for the formation of posterior-lateral recurrences may be the migration of tumor cells through the lateral lymphatic vessels to the presacral space under gravity^[55]. This would explain why presacral local recurrence is more common in advanced disease than in limited disease.

The highest rates of positive CRM are found with lower rectum tumors. Indeed, TME surgery is not a universal solution for all rectal carcinomas: in low rectal cancer TME may be insufficient to obtain the desired circumferential clearance because of this lack of mesorectum at the level of the pelvic floor. On the other hand, APR surgery mainly results in perineal and presacral LR, which may be prevented by a wider resection^[57].

The pelvic pattern of recurrence itself (i.e. pelvic sub-sites) also has important prognostic value and is related to the potential success of repeat curative intent surgery^[74,75]. The operability for cure and prognosis for anastomotic and anterior recurrence are generally better than for presacral and lateral recurrences^[75]. Moreover, the upper sacral/lateral invasive type of LR is often associated with synchronous metastatic disease^[74]. The type of pelvic invasion is also closely associated with survival after re-resection^[74-76]. Finally, it is worth noting that overall survival after LR diagnosis is lower with RT and CRT+TME approaches, than after TME surgery alone^[74].

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Therapeutic options for intermediate-advanced hepatocellular carcinoma

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on the optional therapeutic modalities for intermediate-advanced HCC.

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies, ranking the sixth in the world, with 55% of cases occurring in China. Usually, patients with HCC did not present until the late stage of the disease, thus limiting their therapeutic options. Although surgical resection is a potentially curative modality for HCC, most patients with intermediate-advanced HCC are not suitable candidates. The current therapeutic modalities for intermediate-advanced HCC include: (1) surgical procedures, such as radical resection, palliative resection, intraoperative radiofrequency ablation or cryosurgical ablation, intraoperative hepatic artery and portal vein chemotherapeutic pump placement, two-stage hepatectomy and liver transplantation; (2) interventional treatment, such as transcatheter arterial chemoembolization, portal vein embolization and image-guided locoregional therapies; and (3) molecularly targeted therapies. So far, how to choose the therapeutic modalities remains controversial. Surgeons are faced with the challenge of providing the most appropriate treatment for patients with intermediate-advanced HCC. This review focuses

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the third most common cause of cancer-related death^[1]. Patients at the early stage are those who present with an asymptomatic single HCC with the nodule < 5 cm in diameter or ≤ 3 in number. Patients exceeding these limits, but free of cancer-related symptoms and vascular invasion or extrahepatic spread, are considered at the intermediate stage. The patients with the cancer-related symptoms and vascular invasion or extrahepatic spread are deemed at the advanced stage. HCC is frequently diagnosed at the late stage and has a high mortality rate. Surgical resection is a potentially curative therapy for HCC, however, only 10%-30% of patients with HCC are eligible for curative hepatectomy. Comprehensive therapy for HCC has become the focus of interest in recent years^[2-6]. The current therapeutic modalities

for intermediate-advanced HCC are collected and evaluated as follows.

SURGICAL PROCEDURES

Radical resection is still the first choice for treatment of HCC^[7,8], even at the intermediate or advanced stage^[9,10]. If radical resection is impractical, palliative resection combined with comprehensive therapy can significantly prolong patients' survival time^[11,12]. Intraoperative comprehensive therapy includes radiofrequency ablation, cryosurgical ablation, and hepatic artery and portal vein chemotherapeutic pump placement. Two-stage hepatectomy can improve the survival rate in selected patients with advanced HCC^[13,14]. Liver transplantation has been shown to achieve excellent survival rate in appropriate HCC patients^[15,16].

Radical resection

Radical resection for intermediate-advanced HCC is indicated as follows: (1) single HCC with large or huge tumor nodule, swelling outward, clear border or pseudocapsule, and less than 30% hepatic tissue destroyed measured by computed tomography (CT) or magnetic resonance imaging (MRI) scan, or more than 50% compensatory hepatic hypertrophy; (2) multiple HCC with 3 or fewer nodules localized in one lobe or segment of the liver^[17,18]. It should be pointed out that tumor nodules limited to the liver are not the absolute operative indication. The outcome of radical resection could be affected by multicentric occurrence of HCC, tumor nodule adjacent to major blood vessel or bile duct, and the hepatic insufficiency induced by coexisting cirrhosis^[19].

With the deeper recognition of the pathology of HCC, the rational criteria of negative surgical margin are initially determined as follows: (1) > 2 cm margin free from tumors < 5 cm in diameter; (2) > 1 cm margin free from tumors 5-10 cm; and (3) > 0.5 cm margin free from tumors > 10 cm. More than 90% hepatectomies fulfilling the above-mentioned criteria can achieve negative surgical margin^[20]. Thereby, healthy hepatic tissue should be reserved as much as possible during radical resection so as to enhance the operative security, to facilitate the postoperative recovery and to help with further treatment.

Palliative resection

The indications of palliative resection for intermediate-advanced HCC are: (1) multiple HCC with 3-5 tumor nodules, exceeding half of the liver; (2) multiple HCC with nodules localized in 2-3 adjacent segments or half of the liver, more than 50% compensatory hypertrophy in the tumor-free liver demonstrated by image examinations; (3) central HCC with more than 50% compensatory hypertrophy in the tumor-free liver; (4) hilar lymph node metastasis should be cleared up during hepatectomy; and (5) invaded organs around the liver, such as colon, stomach, diaphragm, right adrenal gland, *etc.*, and single metastatic neoplasm far from the liver (e.g. lung metastasis) should be resected^[17].

Intraoperative radiofrequency ablation

Radiofrequency ablation (RFA) is a technique in which an electromagnetic energy deposition is used to thermally ablate the hepatic tumor tissue^[21]. During RFA treatment, heat energy generated by high-frequency alternating currents targeted at the living tissues causes protein denaturation at a temperature of 60-110°C through ionic vibration, resulting in coagulative necrosis of the target lesion. In addition, RFA treatment stimulates the immune system and provides an easy way to achieve *in vivo* vaccination against tumoral antigens^[22].

RFA is generally indicated for HCC patients who are not candidates for either liver resection or transplantation^[23]. HCC patients are required to have ≤ 5 nodules, each < 3 cm in diameter, no evidence of vascular invasion or extrahepatic spread, 0 score performance status of the Eastern Cooperative Oncology Group (ECOG), and liver cirrhosis in Child-Pugh class A or B. The more versatile radiofrequency probes allow ablation of nodule > 5 cm. When complete resection by major hepatectomy is dangerous because of difficult nodule location, selective use of intraoperative RFA will be helpful^[24]. The integration of intraoperative RFA into resection surgery contributes to complete removal of nodules with adequate margin, diminishes the extent of parenchymal resection, and improves the resectability rate for patients with advanced HCC^[24].

Pretreatment imaging must carefully define the location of tumor nodule with respect to the surrounding structures for RFA in HCC: nodules located on the surface of the liver can be considered; nodules adjacent to the hepatic vessels may be considered because flowing blood usually protects the vascular wall from thermal injury; nodules adjacent to the hepatic hilum represents a relative contraindication due to the risk of thermal injury of the biliary tract; and nodules adjacent to any part of the gastrointestinal tract must be avoided^[25].

Intraoperative cryosurgical ablation

Although RFA has been the most widely utilized ablation modality for HCC, cryosurgical ablation has several advantages (most significantly, the ability to produce larger and more precise zones of ablation) over RFA^[26].

Cryosurgical ablation for HCC patient relies on nonspecific tissue necrosis due to freezing as well as microvascular thrombosis. Argon-helium cryosurgical ablation is able to induce the necrosis of tumor cells through the formation of extracellular and intracellular ice crystals and then cell dehydration due to rapidly freezing (< -140°C) as well as rapidly thawing (20-40°C) the tumor tissues with argon/helium gas. Therefore, argon-helium cryosurgical ablation has become one of the major therapeutic approaches for unresectable intermediate-advanced HCC.

The indications of cryosurgical ablation for HCC patient are: (1) nodules < 5 cm in diameter, ≤ 3 in number; (2) nodule > 5 cm with irregular margin, may be given intraoperative cryosurgical ablation with or without excision of nodule. Intraoperative cryosurgical ablation offers

an effective and safe option for management of advanced HCC^[27]. HCC patients with diffuse infiltrative disease or large bilobar nodules (> 50% of liver volume) are not candidates for cryosurgical ablation because complete ablation of the nodules might induce hepatic failure.

Intraoperative hepatic artery and portal vein chemotherapeutic pump placement

The liver has a dual blood supply from the hepatic artery and the portal venous system. For HCC patients who are not suitable for hepatectomy confirmed by intraoperative exploration, two chemotherapeutic pumps could be implanted subcutaneously into the upper abdominal wall near the incision, with the tip of pump catheter separately inserted into the hepatic artery and portal vein during the operation, followed by postoperative chemotherapy. The advantage of intraoperatively implanted chemotherapeutic pump is the ability to accurately and selectively place into the main trunk or branch of hepatic artery and portal vein. For resectable intermediate-advanced HCC, the postoperative hepatic artery and portal vein dual perfusion chemotherapy *via* chemotherapeutic pumps could prevent tumor recurrence^[28].

Two-stage hepatectomy

Two-stage hepatectomy has been developed as a surgical strategy for extremely difficult patients with intermediate-advanced HCC^[7]. This strategy is applied when it is impossible to resect the tumor in a single procedure. The main principles of this strategy are: huge HCC with the remnant liver volume cannot maintain hepatic function after hepatectomy; central or hilar HCC adjacent to or invaded major blood vessel; and serious cirrhosis with possible hepatic decompensation after hepatectomy.

For unresectable HCC, preoperative intervention with transcatheter arterial chemoembolization (TACE)^[29], portal vein embolization (PVE)^[30,31], or percutaneous RFA could control tumor progression and invasion, downstage tumor status, increase remnant liver volume, and decrease tumor recurrence rate, thus making the two-stage hepatectomy possible. The indication of two-stage hepatectomy is that tumor diameter reduced to 50% of the initial size, and nontumorous liver tissue had significant compensatory hyperplasia. Sequential TACE and PVE could broaden the surgical indication and the safety of major hepatic resection for advanced HCC patient with damaged liver^[32,33].

Non-anatomic local excision of liver cancer or hepatic segmentectomy should be used in the two-stage hepatectomy so as to maximally preserve the normal liver tissue. For the patients with HCC invading the hepatic hilum and inferior vena cava, total hepatic vascular exclusion (HVE) should be prepared to avoid massive hemorrhage during hepatectomy.

Liver transplantation

Liver transplantation is an ideal treatment option, as it simultaneously cures HCC. However, up to date, there are no uniform criteria of liver transplantation for HCC

patients in China. The United Network for Organ Sharing (UNOS) criteria for liver transplantation are usually adopted in the world: single tumor ≤ 5 cm; 2-3 tumors, each ≤ 3 cm; no macrovascular invasion; and no extrahepatic spread to surrounding lymph nodes, lungs, abdominal organs, or bones^[34]. However, if the UNOS criteria are strictly adopted in China, it means that most HCC patients will lose the opportunity of liver transplantation, because more than 100 000 patients die of advanced HCC each year. For this reason, the indication of liver transplantation for advanced HCC should be relatively loose in China. For the patients with unresectable huge or multiple HCC, if no vascular invasion and no extrahepatic spread, liver transplantation is the treatment of choice. Considering the limited organ supply, high cost, and considerable risk, we suggest that only those HCC patients with a high probability of survival benefit should be selected to receive liver transplantation. The shortage of donor livers is the major constraint of liver transplantation.

INTERVENTIONAL TREATMENT

Although surgical resection has been the first choice for treatment of HCC, a simple surgical exploration could accelerate the process of disease and even cause death due to the postoperative complication of patients with unresectable HCC. With advances of medical imaging and improvement of interventional technology, interventional treatment has become an effective approach to inoperable HCC^[35-37]. The common approaches of interventional treatments for inoperable HCC include transcatheter arterial chemoembolization, portal vein embolization, and image-guided locoregional therapies.

Transcatheter arterial chemoembolization

For the treatment of inoperable HCC demonstrated by preoperative image examination, the priority is transcatheter arterial chemoembolization (TACE). The theoretical basis of TACE is the special vascular supply of liver and HCC. Liver derives dual blood supply from portal vein and hepatic artery, the former accounts for 2/3 to 3/4 while the latter for only 1/4 to 1/3. HCC derives 90% blood supply from hepatic artery and only 10% from portal vein. Thus, TACE provides a higher local concentration of chemotherapeutic drugs into tumor compared with intravenous perfusion chemotherapy, and meanwhile, it blocks blood supply of HCC, but only exerts little influence on blood supply of the liver. The consequence is that the major portion of cancer nodule becomes necrotic, while hepatic function remains unchanged or little impaired.

Better patient selection and selective segmental chemoembolization may improve the benefit-risk ratio of TACE^[38]. TACE is indicated in intermediate-advanced HCC even in the setting of portal vein involvement (excluding main portal vein)^[39]. The presence of main portal vein thrombosis, extrahepatic metastasis, Child-Pugh class C liver function, and severe hepatic arterio-portal shunts is considered as contraindications for TACE.

Portal vein embolization

Percutaneous transhepatic portal vein embolization (PVE) is a useful procedure for the preoperative intervention of advanced HCC patients selected for hepatectomy. PVE could increase the volume and function of the future remnant liver through the acceleration of hepatocyte proliferation, and embolize possible hepatic arterio-portal shunts, so as to prevent postoperative liver insufficiency.

For the treatment of intermediate-advanced HCC, the combination of TACE and PVE not only blocks most blood supply of main tumor and satellite lesions, but also increases the local concentration of chemotherapeutic drugs into tumor, so as to more effectively control the tumor growth and decrease tumor recurrence. Contraindications to PVE include distant metastases, uncontrolled coagulopathy, active cholangitis, portal hypertension, and renal failure^[40].

Image-guided locoregional therapies

Ultrasound or CT guided locoregional therapies have a therapeutic effect in advanced HCC patients by means of thermoablative therapy (radiofrequency ablation, microwave coagulation, laser ablation), cryotherapy (argon-helium knife, liquid nitrogen), or chemical therapy (ethanol injection, acetic acid injection) to destroy tumor tissues. To date, the commonly used therapies include percutaneous RFA, microwave coagulation, cryoablation therapy, and ethanol injection, especially with percutaneous RFA as the first choice to inoperable HCC. The roles of different locoregional therapies may change with further development of technology and availability of data from future prospective randomized trials^[38,41-43].

MOLECULARLY TARGETED THERAPIES

Recently, molecularly targeted therapies, including sorafenib, sunitinib, brivanib, cetuximab, erlotinib plus bevacizumab, and lapatinib, have emerged as promising therapeutic approaches for advanced HCC^[44,45]. Sorafenib, as an orally-active multikinase inhibitor targeting both tumor cells and the tumor vasculature, and the first agent to improve the overall survival status for patients with advanced HCC, has been approved for systemic therapy in patients with advanced HCC in Eastern and Western countries^[3,46-48]. Many other molecularly targeted agents of blocking epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and mammalian target of rapamycin (mTOR) are at different stages of clinical development for the treatment of advanced HCC^[49-51].

CONCLUSION

For the treatment of intermediate-advanced HCC, various surgical procedures may produce the definite therapeutic effects. The interventional treatment can also improve the prognosis to a great extent, but so far there is still lack of a special effective approach. In recent years, the model of comprehensive therapies mainly based on surgical

resection has been adopted to further enhance the curative effect, prolong the survival time, and improve the life quality of the patients. According to the indications and advantages of each therapeutic method, combined with the patient's clinical stage, the selection of therapeutic approaches to maximize the efficacy and minimize the adverse effect is very important for designing a more rational therapeutic plan for intermediate-advanced HCC.

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How to assess the severity of atrophic gastritis

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Abstract

Atrophic gastritis, is the main consequence of long-standing *Helicobacter pylori* infection, and is linked to the development of gastric cancer. The severity of atrophic gastritis is related to the lifetime risk of gastric cancer development, especially in terms of its degree and extent of mucosal damage. Therefore, it is important for clinicians to assess the severity of atrophic gastritis, interfere with the disease progress, and reverse gastric mucosal atrophy. In the article, we demonstrated some methods (conventional endoscopy, modern endoscopic technology and noninvasive methods) that may help assess the severity of atrophic gastritis and select the reasonable treatment protocols.

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INTRODUCTION

Atrophic gastritis (AG) is a histopathological entity that is characterized by chronic inflammation of the gastric mucosa with loss of gastric glandular cells and replacement by intestinal-type epithelium, pyloric-type glands, and fibrous tissue. Atrophy of the gastric mucosa is the endpoint of chronic processes, such as chronic gastritis associated with *Helicobacter pylori* (*H. pylori*) infection, other unidentified environmental factors, and autoimmunity directed against gastric glandular cells^[1]. It has been established that people with AG have a high risk for gastric cancer^[2,3], and it has been reported that about 10% of the patients with moderate-severe AG will develop gastric malignancies during a mean follow-up of 7.8 years^[4]. Thus, the assessment of the severity of AG may be an important challenge for the management of these patients because its features (i.e. extension of atrophy and intestinal metaplasia, and hypochlorhydria) may be considered as potential surrogate markers for the increased risk for gastric cancer. Here, we demonstrate some methods used to assess the severity of AG.

DEFINITION AND CLASSIFICATION OF AG

Gastric mucosal atrophy is defined as the loss of appropriate glands, which occurs when glands damaged by inflammation are replaced either by connective tissue (scarring) or by glandular structures inappropriate for location (metaplasia). Most often, as in the antral mucosa, the metaplastic transformation assumes the phenotype of the glands lined by intestinal-type epithelium (IM), but in the oxyntic mucosa, it may also take the form of mucin-secreting antral glands (pseudopyloric metaplasia)^[5]. Traditionally, AG can be divided into gastric body atrophy

and sinuses ventriculi atrophy: the former is mostly associated with autoimmune diseases, and the latter is often associated with *H. pylori* infection^[6,7]. However, in general practice, the diagnosis of atrophy and IM is troublesome due to an unsatisfactory interobserver agreement among pathologists, therefore in 2000, an international group of pathologists from Atrophy Club reviewed once again the spectrum of gastric atrophy and IM, and proposed a simplified definition of atrophy, which includes a metaplastic and a non-metaplastic category, thus making metaplasia an absolute concept to demonstrate the severity of the disease^[5].

CONVENTIONAL ENDOSCOPY AND AG

In 2003, the Chinese Society of Digestive Endoscopy established endoscopic criteria for chronic gastritis in Dalian meeting. The scar lesions were characterized by the following attributes: mucosal atrophy, granular mucosa, flattened folds, gray intestinal-type epithelium and blood vessel permeability. AG was classified into three patterns of ridges: (1) fine granular mucosa, permeability of some blood vessels and a single nodule of gray intestinal-type epithelium; (2) medium granular mucosa, permeability of blood vessels, multiple nodules of gray intestinal-type epithelium; and (3) coarse granular mucosa, blood vessels can be seen up to the surface, diffuse nodules of gray intestinal-type epithelium^[8].

MAGNIFYING ENDOSCOPY AND AG

Magnifying endoscopy has been developed to visualize the microstructure of gastrointestinal surface mucosa and mucosal vascularity, which provides a magnified image of up to 200 times^[9]. The pit patterns observed on the mucosal surface are considered to reflect the arrangement and structure of surface epithelia, morphology, number, distribution and function of glands, mucosal edema and inflammation, and vascular morphology, arrangement, number and distribution. The basic units of the microstructures on the surface of gastric mucosa are countless gastric pits that form gastric areas separated by minor gastric grooves (also called interval grooves). As the openings of glands, gastric pits are the first to undergo structural change due to gastric mucosal lesions. Yagi *et al*^[10] thought that the presentation of gastric mucosal atrophy was that gastric pit became white, expanded in size, and was surrounded by areas of erythema. In the study of Sakaki *et al*, magnifying endoscopy patterns of gastric erosion pits were classified into six types: A (round spot pits), B (short rod pits), C (sparsely and thickly linear), D (patchy), E (villous) and F (unclear or disappearance of pits or abnormal hyperplasia blood capillary)^[11]. Yuan *et al*^[12] used magnifying endoscopy in combination with methylene blue staining to examine the microstructures of gastric mucosa in 180 patients with gastric erosion. Their results showed that types A and B were found in normal gastric mucosa, while types C-F were found in gastric mucosa with active inflammation, atrophic

inflammation, intestinal metaplasia and dysplasia of varying degrees. Type E mucosa (81.8%) suggested intestinal metaplasia, type F indicated existence of dysplasia (86.3%), and type F with abnormal hyperplasia blood capillary suggested dysplasia (89.9%).

MAGNIFYING NARROW-BAND IMAGING AND AG

Narrow-band imaging (NBI) is an endoscopic imaging technique for the enhanced visualization of mucosal microscopic structure and capillaries of the superficial mucosal layer. Images are obtained using narrower bands of red, blue and green filters, which are different from conventional red-green-blue filters^[13]. Combining the NBI system and magnifying endoscopy allows for simple and clear visualization of microscopic structures of the superficial mucosa and its capillary patterns^[14]. In the study of Tahara *et al*^[15], gastric mucosal patterns seen with magnifying NBI in uninvolved gastric corpus were divided into the following categories: normal small, round pits with regular subepithelial capillary networks; type 1, slightly enlarged, round pits with unclear or irregular subepithelial capillary networks; type 2, obviously enlarged, oval or prolonged pits with increased density of irregular vessels; and type 3, well-demarcated oval or tubulovillous pits with clearly visible coiled or wavy vessels. They found that the mucosal patterns were associated with the degree of endoscopic gastric atrophy. As mucosal patterns advanced from normal to types 1, 2 and 3, the degree of endoscopic gastric mucosal atrophy increased simultaneously. The sensitivity and specificity for types 1, 2 and 3 for detection of *H. pylori* infection and type 3 for detection of intestinal metaplasia were 95.2%, 82.2%, 73.3%, and 95.6%, respectively. Uedo *et al*^[16] found in their study that the appearance of a light blue crest on the epithelial surface was correlated with histological evidence of intestinal metaplasia with a sensitivity of 89% (95% CI: 83-96), specificity of 93% (95% CI: 88-97), positive predictive value of 91% (95% CI: 85-96), negative predictive value of 92% (95% CI: 87-97), and accuracy of 91% (95% CI: 88-95).

AUTO-FLUORESCENCE IMAGING VIDEOENDOSCOPY AND AG

Auto-fluorescence imaging (AFI) produces real-time pseudocolor images based on natural tissue auto-fluorescence emitted by light excitation from endogenous fluorophores such as collagen, nicotinamide, adenine dinucleotide, flavin and porphyrins. AFI enables the detection of mucosal features not visible with conventional endoscopy, therefore, it might improve the identification and characterization of the premalignant status in gastric mucosa^[17,18].

The fluorescence is almost purple, weaker in the normal gastric gland mucosa than that in the pyloric gland mucosa.

When gastric mucosa is atrophic, the color is green, which is the same as that in the pyloric gland mucosa. Gastric biopsy is taken separately from purple and green region for pathological studies, and the green region is significantly increased in AG and intestinal metaplasia^[19]. The extent of chronic atrophic fundal gastritis (CAFG) was considered to be the green areas in the gastric body and was classified into six categories by Inoue *et al.*^[20]: AF-C-I, the entire gastric body appears purple to dark green; AF-C-II, a color border on the lesser curvature was observed at a lower part of the gastric body; AF-C-III, a color border on the lesser curvature at an upper part of the gastric body; AF-O-I, a color border between the lesser curvature and the anterior wall; AF-O-II, a color border between the anterior wall and the greater curvature; and AF-O-III, a color border on the greater curvature proximal to the lower gastric body. They found that the diagnostic accuracy of green areas in the gastric body of the patients in the activity, inflammation, atrophy and intestinal metaplasia was 64%, 93%, 88% and 81%, respectively. However, the diagnostic accuracy of AFI was not compared with that of white-light images in relation to the histology. Therefore, whether the accuracy of AFI is superior to that of white-light images is not known.

SERUM BIOMARKERS AND AG

Pepsinogen I and II

Pepsinogens (PGs) are aspartic proteinases that are mainly secreted by gastric cells. They can be immunologically classified into two major types: pepsinogen I (PG I) and pepsinogen II (PG II). PGI is secreted only from the gastric fundic mucosa, whereas PG II is secreted from the cardiac, fundic and antral mucosa of the stomach, and also from the duodenal mucosa^[21]. Patients with gastric fundic atrophy have a lower mean serum PG I concentration than those without atrophy. Both mucosal types secrete PG II, however, serum PG II levels remain stable or are increased during progression from a normal stomach to one with severe atrophy^[22]. The net effects of severe atrophy on serum PG concentrations are lower PGI and a stable or increased PG II, and this leads to a lower PG I / II ratio^[22]. Ren *et al.*^[23] have confirmed a strong association between gastric fundic atrophy and PGs, as estimated by a low serum PGI and PG I / II ratio in a prospective study. They have found that compared to the subjects with a PG I / II ratio of > 4, those with a ratio ≤ 4 had hazard ratios (HRs) of 2.72 (95% CI: 1.77-4.20) and 2.12 (95% CI: 1.42-3.16) for non-cardiac and cardiac gastric adenocarcinoma, respectively. Storskrubb *et al.*^[24] found that the phenotype of gastritis is characterized by normal levels of serum PGs (PG I ≥ 25 ng/mL and PG I / PG II ratio ≥ 3 indicate that the corpus mucosa is normal). For the diagnosis of atrophic corpus gastritis, three different criteria have been used as follows^[25-27]: Mild: PG I ≤ 70 ng/mL and PG I / II ratio ≤ 3.0; Moderate: PG I ≤ 50 ng/mL and PG I / II ≤ 3.0; Strict: PG I ≤ 30 ng/mL and PG I / II ≤ 2.0. Both cut-offs for PG I and PG I / II should be fulfilled at the same time for each criterion.

Gastrin-17

Gastrin-17 (G-17) is secreted exclusively by the G-cells of

the gastric antrum. The levels of G-17 are depressed in cases of atrophy in this area^[28]. Leja *et al.*^[29] found that G-17 < 5 pmol/L is related to atrophy in the antral region ($P = 0.007$) with a 36.8% sensitivity and a 86.5% specificity. They indicated that G-17 used for the detection of atrophy in the antral part of the stomach requires further evaluations due to its low sensitivity.

H. pylori testing

H. pylori is now recognized as a major cause of gastric cancer and is classified as a group I carcinogen by the WHO^[30,31]. *H. pylori* infection causes persistent chronic gastritis, which in susceptible individuals can progress to atrophy, intestinal metaplasia and dysplasia, and finally, intestinal-type gastric cancer^[31,32]. Nearly all infected individuals (> 90%) exhibit *H. pylori*-specific IgG antibodies. Most (70%) of these individuals also exhibit IgA antibodies and *H. Pylori* proteins, including cytotoxin-associated gene A (CagA) protein and vacuolating cytotoxin A (VacA) protein. These proteins are used for *H. pylori* testing. A combined use of the serological biomarkers (PGI, PGII, G-17 and *H. pylori* antibodies) shows a high accuracy as a noninvasive method to diagnose gastric atrophy, which is common in the general population^[23,24,33].

CONCLUSION

In the cases of AG, its severity is mainly related to the lifetime risk to develop gastric cancer, especially in terms of the degree and extension of mucosal damage. The application of conventional endoscopy, modern endoscopic technology and noninvasive methods is useful for the identification of those patients with atrophic gastritis at higher risk for gastric malignancies. Using these technologies to assess the severity of atrophic gastritis, interfering with the disease progress, and reversing gastric mucosal atrophy are the important issues for clinicians.

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Legalon-SIL downregulates HCV core and NS5A in human hepatocytes expressing full-length HCV

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Abstract

AIM: To determine the effect of Legalon-SIL (LS) on hepatitis C virus (HCV) core and NS5A expression and on heme oxygenase-1 (HMOX-1) and its transcriptional regulators in human hepatoma cells expressing full length HCV genotype 1b.

METHODS: CON1 cells were treated with 50 $\mu\text{mol/L}$ or 200 $\mu\text{mol/L}$ LS. Cells were harvested after 2, 6 and 24 h. HCV RNA and protein levels were determined by quantitative real-time polymerase chain reaction and Western blotting, respectively.

RESULTS: HCV RNA (core and NS5A regions) was

decreased after 6 h with LS 200 $\mu\text{mol/L}$ ($P < 0.05$). Both 50 and 200 $\mu\text{mol/L}$ LS decreased HCV RNA levels [core region (by 55% and 88%, respectively) and NS5A region (by 62% and 87%, respectively) after 24 h compared with vehicle (dimethyl sulphoxide) control ($P < 0.01$). Similarly HCV core and NS5A protein were decreased (by 85%, $P < 0.01$ and by 65%, $P < 0.05$, respectively) by LS 200 $\mu\text{mol/L}$. Bach1 and HMOX-1 RNA were also downregulated by LS treatment ($P < 0.01$), while Nrf2 protein was increased ($P < 0.05$).

CONCLUSION: Our results demonstrate that treatment with LS downregulates HCV core and NS5A expression in CON1 cells which express full length HCV genotype 1b, and suggests that LS may prove to be a valuable alternative or adjunctive therapy for the treatment of HCV infection.

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Key words: Hepatitis; Hepatitis C virus; Silymarin; Silybin; Genotype; Huh7.5; CON1

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INTRODUCTION

Over 170 million people are infected by hepatitis C virus (HCV) worldwide, and of these, approximately 85% will develop chronic hepatitis C (CHC). This could potentially

lead to fibrosis, cirrhosis, end-stage liver disease and hepatocellular carcinoma^[1]. The ultimate goal of antiviral treatment for hepatitis C is the sustained elimination of HCV. Currently, the standard of care for individuals with CHC is pegylated interferon (IFN) α -2a or IFN α -2b plus ribavirin. However, this protocol is far from ideal. Even under the best conditions of sponsored clinical trials, sustained virologic responses have been achieved in only 40%-50% of those with HCV genotype 1 infection^[2,3]. Furthermore, serious side effects are associated with this therapy. The paucity of effective and affordable treatments for HCV-infected patients has led scientists to seek alternative therapies. At present, novel therapeutic agents with various mechanisms of action are under development or in clinical trials.

Although the precise mechanisms underlying hepatocellular injury associated with HCV has yet to be determined, there is compelling evidence that HCV produces increased oxidative stress in human liver cells that is linked to the production of reactive oxygen species (ROS), and consequent increases in cellular lipid peroxidation and other oxidative damage. Oxidative stress appears to be an important aspect of HCV-induced hepatocellular injury^[4]. Microsomal heme oxygenase-1 (HMOX-1) is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism to release free iron and equimolar amounts of carbon monoxide and biliverdin^[5,6]. A variety of DNA-binding proteins interact with regions that contain multiple antioxidant response elements (ARE). Among these are nuclear factor erythroid 2-related factor 2 (Nrf2) and Bach1, a leucine b-zipper transcription protein, which form heterodimers with the small Maf proteins^[7,8]. Nrf2 is known to be associated with activation of HMOX-1 and numerous other antioxidant genes in response to multiple agents^[9], while Bach1 is a negative regulator of HMOX-1^[10].

Milk thistle (*Silybum marianum*) has been used since ancient times as a liver tonic. Silymarin (SI), a purified extract of polyphenolic flavonoids isolated from milk thistle, is composed mainly of silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B. After oral administration, the SI flavonolignans are rapidly metabolized^[11]. Silybin (SBN) constitutes approximately 50% of SI and is the most biologically active component^[12]. A number of studies have shown that SI has potent antioxidant and immunomodulatory effects in addition to numerous metabolic actions that may contribute to its purported hepatoprotective actions^[13-15].

We recently showed that SI downregulates HCV RNA (core region) and protein in CNS3 cells that stably express HCV RNA core to the amino terminal of NS3 proteins^[16]. Another recent study *in vitro* showed that SI exerts antiviral and antiinflammatory effects in hepatoma cell lines expressing the HCV full length genome of genotype 2a^[17]. On the other hand, a randomized, double-blind, placebo-controlled study administering oral SI to CHC patients failed to show a significant effect on either serum aminotransferase levels or quality-of-life measures^[18].

Legalon-SIL (LS) is a form of SBN which is a water-soluble formulation of the dihydro-succinate sodium salt of SBN A and SBN B in equal proportion. Recent results

from a pilot study in patients with chronic HCV using LS indicate that some SI flavonolignans may have antiviral activity^[19]. In this study we assessed the effects of LS on HCV RNA and protein levels in cell lines expressing the full length genome of HCV genotype 1b. We also determined the effects of LS on HMOX-1, Bach1, and Nrf2 expression in these cells.

MATERIALS AND METHODS

Chemicals and antibodies

LS was obtained from Rottapharm-Madaus (Italy). Dimethyl sulphoxide (DMSO) was purchased from Thermo Fisher Scientific Inc (Rockford, IL, USA). A 100 mmol/L LS stock solution (molecular weight = 726) was prepared in DMSO and filtered through a 0.2 μ mol/L nylon filter. LS was prepared fresh just prior to use in each experiment. Mouse monoclonal antibody against HCV core protein was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal antibody against HCV NS5A was purchased from Virogen Corporation (Watertown, MA, USA). Rabbit polyclonal antibody against HMOX-1 was purchased from Stress Gene (Ann Arbor, MI, USA). Goat monoclonal antibody against Bach1, rabbit polyclonal antibody against Nrf2, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL)-Plus Western blotting detection reagent was obtained from Amersham Biosciences (Piscataway, NJ, USA).

Cell cultures and treatments

Huh-7.5 and CON1 subgenomic genotype 1b HCV cell lines were from Apath LLC (St. Louis, MO, USA). Huh-7.5 is a highly permissive, IFN-cured Huh-7 human hepatocellular carcinoma cell line derivative. The CON1 cell line is a Huh-7.5 cell population containing the full-length HCV genotype 1b replicon.

Huh-7.5 and CON1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and selection antibiotic for CON1 cells (750 μ g/mL G418).

Colorimetric MTT assay

Cellular proliferation of treated CON1 cells was assessed by measuring the conversion of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] to MTT formazan (Sigma-Aldrich). The absorbance was measured on a Synergy HT microtiter plate reader (Biotek Instruments, Winooski, VT, USA), at a wavelength of 570 nm with background subtraction at 690 nm. Decreases in absorption were taken as an index of decreased cellular proliferation.

Propidium iodide assay

The viability of CON1 cells treated with LS was also confirmed by the standard propidium iodide [(PI); Invitrogen,

USA)] assay. The experiment was performed according to the manufacturer's recommended protocol. CON1 cells were plated in 12-well plates 24 h before treatment and incubated at 37°C. LS was dissolved in DMSO and added to the cell culture medium. The effects of LS on cell viability were studied at various concentrations (0, 50, 100, 200, 300, 400 and 500) $\mu\text{mol/L}$ and at different time points (4, 8, and 24 h). Percent cell viability was determined by counting cell density in drug-treated cells and in DMSO-treated cells as control in the same incubation period [percentage of cellular viability = (total cell count-PI positive cell count)/total cell count*100]. All experiments were repeated 3 times.

RNA isolation and quantitative reverse transcriptase polymerase chain reaction

RNA from treated cells was isolated by TRIZOL reagent (Invitrogen). The RNA concentration and purity were determined by measuring absorbance at 260/280 nm. Reverse transcription was performed on 1 μg of total RNA to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative real time reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed using a CFX-96 Real-Time PCR Detection System (Bio-Rad Laboratories) and iQTM SYBR Green Supermix (Bio-Rad Laboratories). Sequence specific primers for HMOX-1, Bach1, and GAPDH were designed as described^[20]. Nucleotide sequences of other primers are as follows: NS5A: Forward primer, 5'-CG-GACGTAGCAGTGCTCACTTC-3' and reverse primer, 5'-TGATGAGCTGGCCAAGGAGG-3'; Nrf2: Forward primer, 5'-CCTTCTCGCCTAGGCATCA-3', reverse primer, 5'-CCCTTCAGCTCTCCCTACCG-3'. Fold change values were calculated by comparative cycle threshold (Ct) value analysis after normalizing for the quantity of GAPDH mRNA in samples.

Protein preparation and Western blotting

Cells were grown to near confluence and washed with phosphate-buffered saline (PBS), lysed in a buffer containing 1% Triton X-100 with PBS and Halt Protease Inhibitor Cocktail (Pierce Chemicals, Rockford, IL, USA). Protein concentrations were measured using the bicinchoninic acid method. Total proteins (10 μg) were separated on 4%-12% gradient sodium dodecyl sulphate-polyacrylamide gel (Invitrogen Laboratories) and electrophoretically transferred onto an Immun-Blot PVDF (Invitrogen Laboratories). The membranes were blocked for 1 h in PBS containing 5% nonfat dry milk, and then incubated for 1 h with the primary antibody at room temperature. The dilutions of the primary antibodies were as follows: 1:1000 for anti-HCV core antibody; 1:1000 for anti-HCV NS5A antibody; 1:500 for anti-HMOX-1, and 1:1000 for anti-Bach1, anti-Nrf2, and anti-GAPDH antibody. After 4 washes with 0.1% Tween 20 in PBS (PBS-T), the membranes were incubated for 1 h with a secondary antibody (anti-rabbit, anti-goat or anti-mouse immunoglobulin G; dilution 1:10 000). Finally, the membranes were washed 4 times with PBS-T, and the

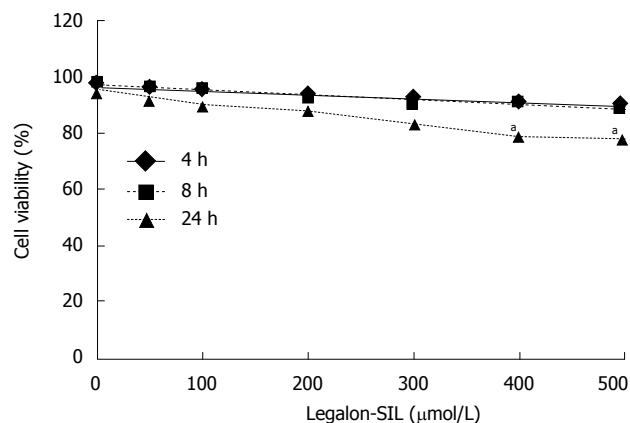


Figure 1 Effects of legalon-SIL on CON1 cell viability. Cellular viability of CON1 cells was measured using the propidium iodide assay after 4, 8, and 24 h of exposure to legalon-SIL (LS) at varying concentrations ranging from 0-500 $\mu\text{mol/L}$. The mean \pm SE from 3 independent experiments. ^a $P < 0.05$ vs DMSO control.

bound antibodies were visualized with the ECL-Plus chemiluminescence system. A computer based imaging system, LAS 3000 (Fuji Film, USA) was used to measure the relative optical density of each specific band obtained after Western blotting.

Statistical analysis

Data are expressed as mean \pm SE of the mean. Statistical differences between groups were analyzed by analysis of variance followed by Dunnett's test. $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity of Legalon-SIL in CON1

Appropriate doses of LS in the cell lines were determined. Effects of different doses of LS on cell viability in CON1 cells were assessed by PI staining (Figure 1). LS concentrations of 50-300 $\mu\text{mol/L}$ had no significant effect on cell viability, whereas concentrations equal to or more than 400 $\mu\text{mol/L}$ caused significant cytotoxicity in the cells ($P < 0.05$). Similar results were demonstrated with the MTT proliferation assay (data not shown).

Legalon-SIL downregulates HCV RNA as well as HCV core and NS5A proteins in CON1 cells

LS downregulated HCV RNA (core region) in a dose-dependent and also a time-dependent manner in CON1 cells. The HCV RNA (core region) level was decreased 21% following 6 h treatment with LS 200 $\mu\text{mol/L}$ compared with the DMSO control ($P < 0.05$, Figure 2A). HCV RNA (core region) levels were further decreased after 24 h treatment by both LS 50 $\mu\text{mol/L}$ (55% decrease, $P < 0.05$) and 200 $\mu\text{mol/L}$ (88% decrease, $P < 0.01$) when compared with vehicle (DMSO) control (Figure 2A). The HCV RNA (NS5A region) level was also decreased 43% following 6 h treatment with LS 200 $\mu\text{mol/L}$ compared with DMSO control ($P < 0.01$ Figure 2B), and was also further decreased after

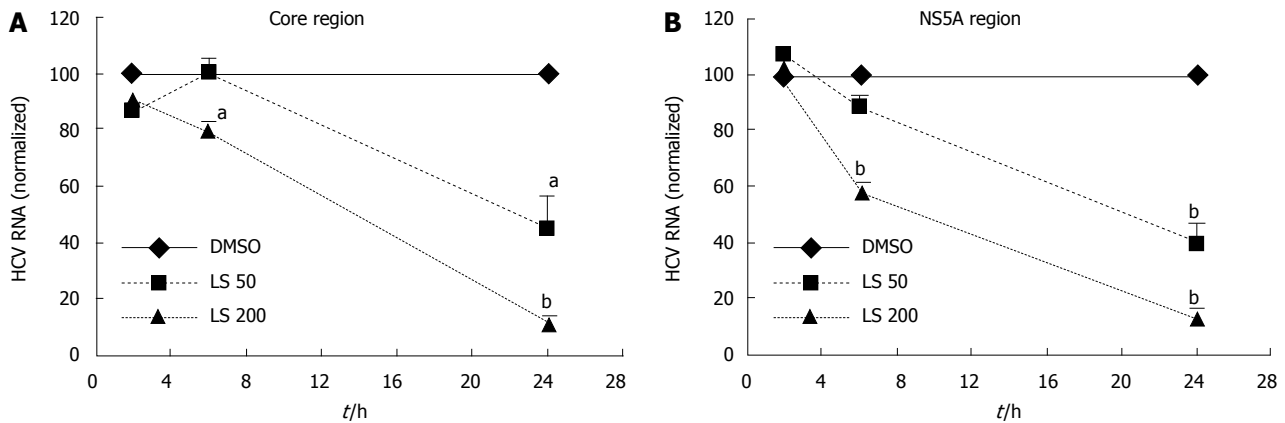


Figure 2 Time course of effects of legalon-SIL on hepatitis C virus RNA in CON1 cells. CON1 cells were grown to near confluence and the medium was changed to 5% fetal bovine serum (FBS) plus Dulbecco's modified Eagle's medium, then treated with vehicle only (DMSO) or 50 or 200 $\mu\text{mol/L}$ legalon-SIL (LS). The cells were harvested after 2, 6, and 24 h after treatment. The levels of hepatitis C virus (HCV) RNA [core region (A) and NS5A region (B)] were quantified using qRT-PCR as described in "Materials and Methods". The amounts of HCV RNA were normalized to glyceraldehyde-3-phosphate dehydrogenase. A: LS 50 $\mu\text{mol/L}$ downregulated HCV RNA (core region) after 24 h. LS 200 $\mu\text{mol/L}$ downregulated HCV RNA (core region) after 6 and 24 h; B: LS 50 $\mu\text{mol/L}$ downregulated HCV RNA (NS5A region) after 24 h. LS 200 μM downregulated HCV RNA (core region) after 6 and 24 h. Data for RNA levels are mean \pm SE ($n = 3$ independent experiments). ^a $P < 0.05$, ^b $P < 0.01$ vs DMSO control.

24 h treatment by both LS 50 $\mu\text{mol/L}$ (62% decrease, $P < 0.01$) and 200 $\mu\text{mol/L}$ (87% decrease, $P < 0.01$) (Figure 2B). LS 200 $\mu\text{mol/L}$ also downregulated HCV core (by 57%) and NS5A protein (by 49%) after 24 h of treatment although this was statistically significant only for HCV core protein, $P < 0.05$). This effect was more pronounced following 48 h of treatment: LS 200 $\mu\text{mol/L}$ decreased HCV NS5A protein expression by 65% ($P < 0.05$), while LS significantly decreased HCV core protein expression in a dose-dependent manner (52% reduction at 50 $\mu\text{mol/L}$ and 85% reduction at 200 $\mu\text{mol/L}$ $P < 0.01$) (Figure 3).

Legalon-SIL downregulates HMOX-1 and Bach1 mRNA levels in CON1 cells while it upregulates Nrf2 protein expression

HMOX-1 and Bach1 mRNA levels were significantly decreased following 24 h treatment by both LS 50 $\mu\text{mol/L}$ and 200 $\mu\text{mol/L}$ when compared with the DMSO control (HMOX-1 decreased by 40%, $P < 0.01$; Bach1 decreased by 35%, $P < 0.01$; Figure 4). LS treatment decreased Bach1 protein level, although not significantly, while it significantly increased Nrf2 protein expression ($P < 0.05$) (Figure 5).

HMOX-1 expression is increased in CON1 cells in comparison with its expression in the parental Huh7.5 cell line

For investigation of possible effects of stable transfection of the HCV genome in CON1 replicon system, we compared HMOX-1, Bach1, and Nrf2 mRNA levels between CON1 cells and Huh7.5 cells, the 'parental' cell line. In untreated CON1 cells, the HMOX-1 mRNA level was 3-fold higher than in untreated Huh7.5 cells ($P < 0.01$), while there was no significant difference in the level of Bach1 or Nrf2 mRNA between CON1 and Huh7.5 cells (Figure 6).

DISCUSSION

The current treatment for CHC is a combination of pe-

gylated IFN- α and ribavirin which is effective only in 40%-50% of treated patients infected with genotype-1, by far the most frequent HCV genotype worldwide. This therapeutic regimen is expensive, prolonged (usually at least 48 wk) and causes serious side effects. Therefore investigations continue to search for alternative treatments for hepatitis C. SI is an herbal remedy that has been used to treat acute and chronic liver diseases for millennia^[12-22]. Despite this broad use, the exact molecular mechanism by which SI confers hepatoprotection is yet to be elucidated. Ferenci *et al.*^[19] recently showed that LS, as used in our studies, significantly reduced serum HCV RNA level in patients who had not responded to combination therapy with full dose pegylated IFN and ribavirin. They showed that SBN was effective only when administered intravenously, as LS, and that the antiviral effect was dose-dependent. They reported that HCV RNA was undetectable in 7 (out of 14) patients receiving 15 and 20 mg/kg SBN as LS. In a recent study *in vitro*^[23], SBN A, SBN B, a mixture of SBN A and SBN B, and LS were shown to inhibit HCV RNA-dependent RNA polymerase (RdRP) function and inhibited HCV genotype 1b sub-genomic replicon replication and HCV genotype 2a strain JFH1 replication in cell culture. Our results extend these findings showing that LS inhibits HCV replication in human hepatoma cells expressing full-length HCV genotype 1b. In this study, we also showed that LS doses equal to 400 $\mu\text{mol/L}$ and higher are cytotoxic for human hepatoma cells similar to other recent results^[23]. The exact pharmacokinetics of SI and LS remain to be determined. However, it is suggested that oral doses of SI up to 2.1 g/d are safe and well-tolerated^[24]. In the present study, we showed that LS started to downregulate HCV RNA in a dose-dependent manner after 2 h, but the significant effect was observed after 6 h of treatment. Although LS 200 $\mu\text{mol/L}$ was found to downregulate HCV RNA and proteins significantly in CON1 cells, LS 50 $\mu\text{mol/L}$ was also found to be effective in downregulation of HCV RNA.

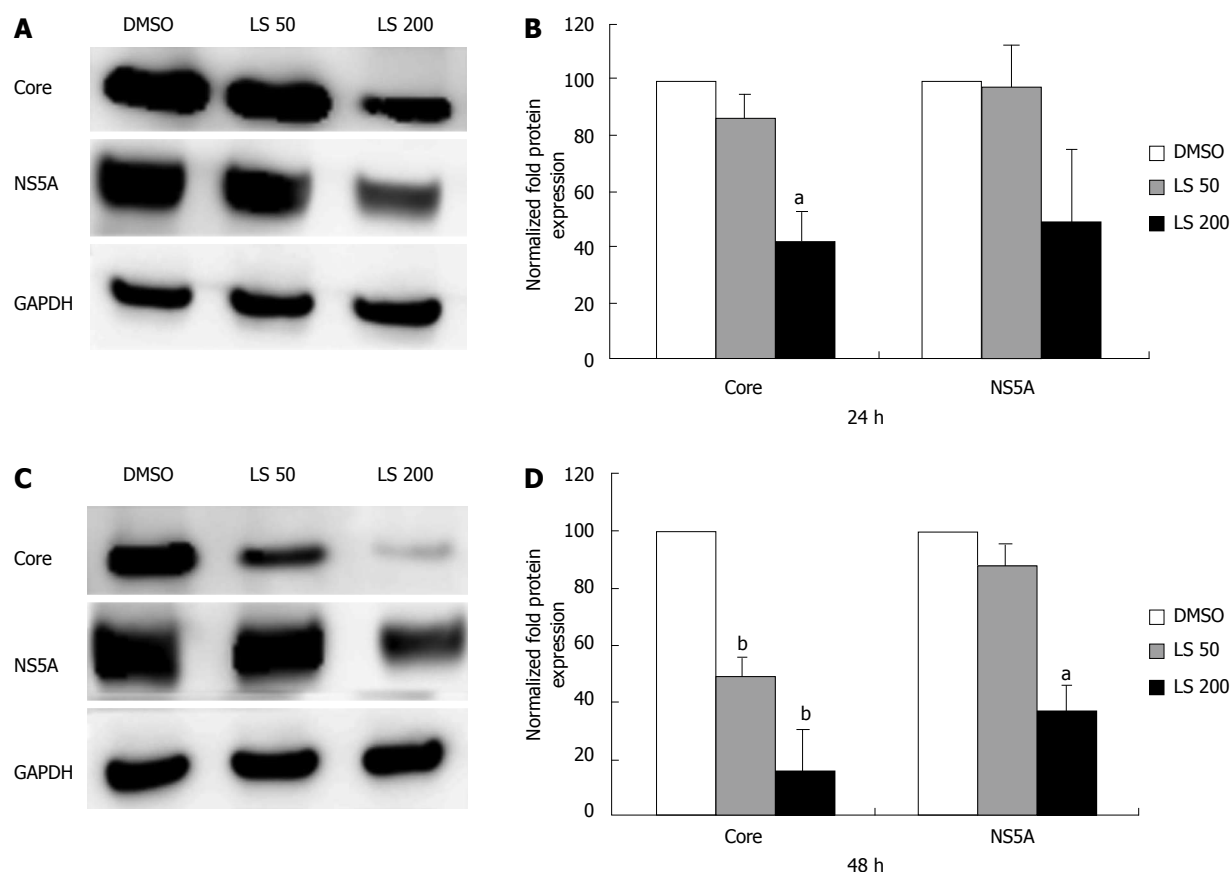


Figure 3 Effects of legalon-SIL on hepatitis C virus core and NS5A protein levels in CON1 cells after 24 and 48 h. CON1 cells were grown to near confluence and the medium was changed to 5% FBS plus Dulbecco's modified Eagle's medium, then treated with vehicle only (DMSO) or 50 or 200 $\mu\text{mol/L}$ legalon-SIL (LS). The cells were harvested 24–48 h after treatment. Total protein (10 μg) was separated on 4%–12% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred onto an Immun-Blot PVDF. Hepatitis C virus core and NS5A protein levels were assessed through Western blotting. Representative Western blots, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalized quantifications are shown after 24 h (A, B), and 48 h (C, D). Data for protein levels are mean \pm SE ($n = 3$ independent experiments). $^aP < 0.05$, $^bP < 0.01$ vs DMSO control.

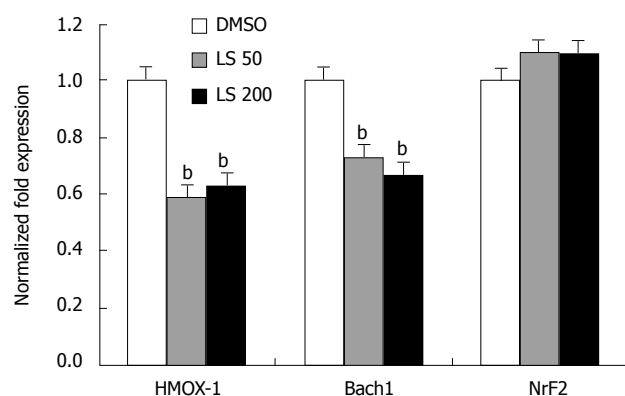


Figure 4 Effects of legalon-SIL on heme oxygenase-1, Bach1, and nuclear factor erythroid 2-related factor 2 mRNA levels after 24 h. CON1 cells were grown to near confluence and medium was changed to 5% FBS plus Dulbecco's modified Eagle's medium, then treated with vehicle only (DMSO) or 50 or 200 $\mu\text{mol/L}$ legalon-SIL (LS). The cells were harvested 24 h after treatment. The levels of HMOX-1, Bach1, and nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA were quantified using quantitative reverse transcriptase polymerase chain reaction as described in "Materials and Methods". The amounts of HMOX-1, Bach1, and Nrf2 mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase. Data are presented as the mean \pm SE experiments were performed 3 times. $^bP < 0.01$ vs DMSO control.

In addition to a direct effect of LS on HCV replication,

for instance inhibition of RdRP, LS might downregulate HCV through interaction with signaling molecules or other as yet unknown host factors. For example, Polyak *et al*^[25] reported that SI suppresses TNF- α activation of nuclear factor- κ B-dependent transcription, without affecting binding of p50 and p65 to DNA. They also reported that all SI-related compounds which they studied blocked JFH-1 virus-induced oxidative stress, including compounds that lacked antiviral activity.

In the present study, we found that LS decreased HMOX-1 mRNA in CON1 cells as well as the mRNA level of Bach1, which is its transcriptional repressor. However, no effect was observed on HMOX-1 and Bach1 protein levels, despite modest upregulation of Nrf2 protein. Bach1 and Nrf2 compete for binding to the ARE and exert their Bach1-induced repressive, or Nrf2-induced activating effects on HMOX-1 transcription. It is noteworthy to mention that for Nrf2 to bind to ARE, Bach1 needs to be dislocated from the binding sites^[26]. Therefore, a lower level of Bach1 is necessary for the maximal effect of Nrf2 on HMOX-1 transcription. As our study did not show downregulation of Bach1 protein, it is possible to hypothesize that Bach1 does not dissociate from ARE to allow Nrf2 to bind. Therefore HMOX-1 is not upregulated despite mod-

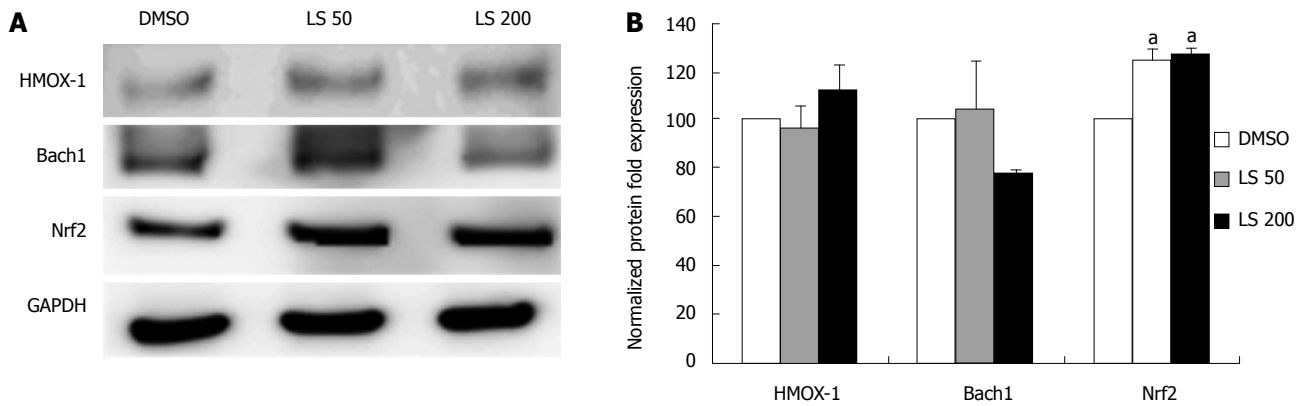


Figure 5 Effects of legalon-SIL on HMOX-1, Bach1, and Nrf2 protein levels in CON1 cells after 48 h. CON1 cells were grown to near confluence and the medium was changed to 5% FBS plus Dulbecco's modified Eagle's medium, and then treated with vehicle only (DMSO) or 50 or 200 $\mu\text{mol/L}$ legalon-SIL (LS). The cells were harvested 48 h after treatment. Total protein (10 μg) was separated on 4%-12% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred onto an Immun-Blot PVDF. HMOX-1, Bach1, and Nrf2 protein levels were assessed through Western blotting, and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A: Representative Western blotting. B: Results of these independent experiments quantified and normalized to GAPDH. Data for protein levels are mean \pm SE ($n = 3$ independent experiments). ^a $P < 0.05$ vs DMSO control.

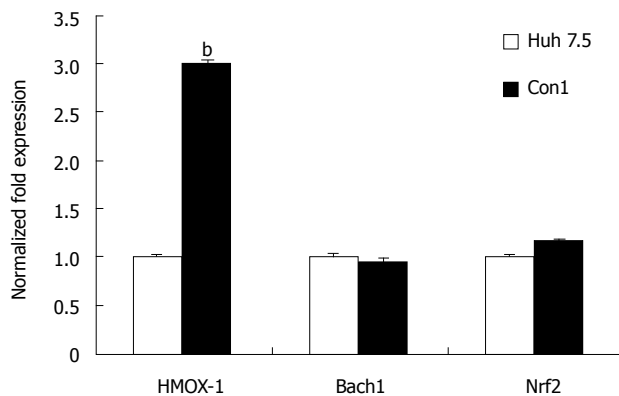


Figure 6 HMOX-1, Bach1, and Nrf2 mRNA levels in CON1 vs Huh 7.5 cells. The levels of HMOX-1, Bach1, and Nrf2 mRNA in untreated Huh 7.5 and CON1 cells were quantified using quantitative reverse transcriptase polymerase chain reaction as described in "Materials and Methods". The amounts of HMOX-1, Bach1 and Nrf2 mRNA were normalized to GAPDH. Data for mRNA levels are mean \pm SE. ^b $P < 0.01$ vs Huh7.5.

est elevation of Nrf2 protein. Additionally, other HMOX-1 transcriptional factors may be affected by LS (e.g. changes in Keap1 or Kelch, glutathione, other thiol-containing compounds, levels of heme, other metalloporphyrins, *etc.*) which were not all examined in this study^[27].

Oxidative stress plays an important role in various diseases, including viral infection and chronic inflammation. HCV gene expression, in particular HCV RNA (core region) expression, can increase the levels of ROS^[28], and therefore upregulate HMOX-1^[20,29]. We also compared HMOX-1 mRNA levels between CON1 cells and its parental cell line (Huh7.5), and we identified a 3-fold upregulation of HMOX-1 in the HCV replicon system compared with the parental cell line. Although SBN is a well-known antioxidant and cytoprotective enzyme, and upregulation of Nrf2 protein could be attributed to this antioxidant effect of LS, lack of HMOX-1 upregulation could be explained by anti-HCV activity of LS. Since HMOX-1 is already

increased by HCV infection in CON1 cells, its level is decreased toward normal levels as LS attenuates HCV infection. This downregulatory effect of LS on HMOX-1 seems to be more pronounced at the RNA level, and is compensated by an Nrf2 increase at the protein level.

In conclusion, LS downregulates HCV in hepatoma cell lines expressing full length HCV genotype 1b. LS treatment is also associated with downregulation of HMOX-1 mRNA and upregulation of Nrf2 protein. Further research is needed to further delineate mechanisms of the LS effect on HCV replication and on CHC.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection is a global health problem, and one causal factor for development of liver cirrhosis and hepatocellular carcinoma. The current therapy for HCV is interferon (IFN) α -2a or IFN α -2b plus ribavirin which is effective in only 40%-50% of those with HCV genotype 1 infection. Furthermore, serious side effects are associated with this therapy. The paucity of effective and affordable treatments for HCV-infected patients has led scientists to seek alternative therapies.

Research frontiers

Silymarin, also known as milk thistle extract, inhibits HCV infection and also displays antioxidant, antiinflammatory, and immunomodulatory actions that contribute to its hepatoprotective effects. In this study the authors demonstrated that Legalon-SIL (LS), a water-soluble formulation of silybin A and silybin B downregulates expression of HCV mRNA and protein in replicon CON1 cells which are human hepatoma cell lines stably transfected with full-length HCV genotype 1b.

Innovations and breakthroughs

This report highlighted that LS decreases HCV core and NS5A expression. Additionally, the authors demonstrated that LS modulates expression of heme oxygenase-1 and its transcriptional regulators, Bach1 and Nrf2. This is the first study examining the effects of LS using a human hepatoma cell line expressing full-length HCV genotype 1b.

Applications

Our results suggest that LS may prove to be a valuable alternative or adjunctive therapy for the treatment of HCV infection.

Terminology

LS is a form of SBN which is a water-soluble formulation of the dihydro-succinate sodium salt of silybin A and silybin B in equal proportions.

Peer review

The paper will appeal to clinicians as well as researchers involved in the field of elucidating the mechanisms of silymarin in HCV positive patients.

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Endoscopic submucosal dissection for premalignant lesions and noninvasive early gastrointestinal cancers

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had premalignant lesions or non-invasive early cancers of the gastrointestinal tract and had endoscopic and histological diagnoses. Early cancers were considered to be confined to the submucosa, with no lymph node involvement by means of computed tomography and endosonography.

RESULTS: Sixty ESD procedures were performed. The indications were epithelial lesions ($n = 39$) (33/39 adenoma with high grade dysplasia, 6/39 adenoma with low grade dysplasia), neuroendocrine tumor ($n = 7$), cancer ($n = 7$) (5/7 early colorectal cancer, 2/7 early gastric cancer), granular cell tumor ($n = 3$), gastrointestinal stromal tumor ($n = 2$), and leiomyoma ($n = 2$). En bloc and piecemeal resection rates were 91.6% (55/60) and 8.3% (5/60), respectively. Complete and incomplete resection rates were 96.6% (58/60) and 3.3% (2/60), respectively. Complications were major bleeding [$n = 3$ (5%)] and perforations [$n = 5$ (8.3%)] (4 colon, 1 stomach). Two patients with colonic perforations and two patients with submucosal lymphatic and microvasculature invasion (1 gastric carcinoid tumor, 1 colonic adenocarcinoma) were referred to surgery. During a mean follow-up of 12 mo, 1 patient with adenoma with high grade dysplasia underwent a second ESD procedure to resect a local recurrence.

CONCLUSION: ESD is a feasible and safe method for treatment of premalignant lesions and early malignant gastrointestinal epithelial and subepithelial lesions. Successful en bloc and complete resection of lesions yield high cure rates with low recurrence.

Key words: Endoscopic submucosal dissection; Premalignant gastrointestinal lesion; Noninvasive early gastrointestinal cancer; Neuroendocrine tumor; Gastrointestinal stromal tumor

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Abstract

AIM: To investigate the indication, feasibility, safety, and clinical utility of endoscopic submucosal dissection (ESD) in the management of various gastrointestinal pathologies.

METHODS: The medical records of 60 consecutive patients (34 female, 26 male) who underwent ESD at the gastroenterology department of Kocaeli University from 2006-2010 were examined. Patients selected for ESD

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INTRODUCTION

New developments in the optical technology of endoscopes allow early detection of mucosal abnormalities that are amenable to endoscopic therapies. Endoscopic therapies are used for premalignant lesions and noninvasive early cancers with low risk of lymph node metastasis. Endoscopic therapies include ablation and resection-based modalities. Resection-based modalities consist of endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD). The major advantage of resection-based modalities is the recovery of the specimen for histopathological analysis. A recent study has shown the long-term prognosis of complete en bloc EMR to be comparable to surgery for differentiated early gastric cancer with 10-year survival rates of 99%^[1]. Although lesions with a diameter of less than 20 mm can be resected in an en bloc fashion with EMR, larger lesions can only be removed in a piecemeal fashion. It is difficult to have an accurate histopathological evaluation of a lesion that is removed in a piecemeal fashion. Furthermore, the risk of local recurrence after piecemeal resection is higher than that of en bloc resection^[1-3].

ESD is a newly developed technique that allows en bloc resection of larger (usually more than 20 mm) mucosal as well as subepithelial gastrointestinal lesions above the muscularis propria with the use of cutting devices. En bloc resection rate of ESD ranges between 83%-98%, which is significantly higher than EMR. Local recurrence rates range between 0%-3%^[4]. However, compared to EMR, ESD is technically more challenging, requires higher endoscopic skills, is time consuming and has a prolonged learning curve^[5-7]. Although ESD has been accepted in the armamentarium of endoscopic management of premalignant and noninvasive early gastrointestinal cancers in Japan and Asia, the Western experience with this new modality has been quite limited. This may be related to differences in the epidemiology of certain gastrointestinal diseases (e.g. gastric cancer), differences in technical expertise, due to its prolonged procedure time, due to its long learning curve, or possibly due to legal concerns, as well as procedural reimbursement. Our study is the first series of ESD cases from Turkey and among the few studies performed in Europe. The aim of this study is to describe indications, feasibility, safety, complications, and recurrence rate of the

mucosal and subepithelial ESD cases in the upper and lower gastrointestinal tract.

MATERIALS AND METHODS

Patients

From September 2006 to June 2010, a consecutive series of patients who underwent an ESD at a tertiary referral center (Kocaeli University Hospital) were reviewed. Premalignant lesions larger than 15 mm in size and noninvasive early cancers with low risk for lymph node metastasis larger than 10 mm were included in the study. The inclusion criteria for carcinoma were histological well differentiation, diameter of ulceration \leq 30 mm, lack of submucosal invasion and lymph node involvement detected with computed tomography (CT) or endoscopic ultrasound (EUS). Prior to an ESD attempt, each case was reviewed by a team consisting of an oncologist, a general surgeon, and an anesthesiologist. ESD indications, procedural information (instruments, sedation, procedure duration, findings, interventions, outcome, and complications) were retrospectively collected and analyzed. The research protocol was approved by the local ethics committee. Both oral and written informed consents were obtained from the patients. This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and in compliance with good clinical practice.

Equipment and procedure

All ESD procedures were performed by a single operator (S.H.) who had studied ESD in 2006 for three months (under the supervision of Hironori Yamamoto, MD; Jichi Medical School, Tochigi, Japan). During his study as a visiting professor he studied the indication, techniques and other basic knowledge regarding ESD for the esophagus, stomach and colon in the endoscopy unit of Jichi Medical University. The author also joined ESD courses in porcine stomach models organized by the gastrointestinal endoscopy society during national gastroenterology weeks. In these courses he achieved more experience, but also contributed to the education of physicians interested in this area.

Prior to an ESD attempt, all lesions were examined with an optical magnifying endoscope (EG-450 ZW5; Fujinon) and colonoscope (-EC-590 Z/WL; Fujinon), with 1% Indigo Carmine used as an adjunct to magnification. The lesion size was determined upon the comparison of standard open biopsy forceps with the lesion. The invasion depth of the lesions was examined either with high frequency ultrasound mini probes (P1912-MB, P1915-MB, P2012-M, 12-20 MHz; Fujinon) or echoendoscopes (GF-UE 160-AL5; Olympus) depending on the size and location of the lesion. Superficial lesions were classified according to the Paris classification system as type I (protruding), type II (flat) a, b, c and type III (excavated)^[8]. Kudo classification was used for characterization of colonic pit patterns^[9].

ESD procedures were performed in a standardized way. The margins of the lesion were marked with an

electrocautery (30 W soft coagulation) to determine the resection border (except in the colon). Then submucosal injection was performed to lift the lesion. For the injection, a special mixture (1 unit of 2% sodium hyaluronic acid, 3 units of saline, 0.5 mL of epinephrine (1/10000) and 0.5% of indigo carmine) was used. After sufficient lifting, a flush knife (DK2618JN 20; Fujinon), insulated-tip knife (KD-610L; Olympus) or needle knife (KD-11Q-1) was used to create a circumferential incision around the lesion extending into the submucosa. After circumferential incision, a submucosal dissection was performed to remove the lesion in an en bloc fashion.

A high frequency generator with an automatically controlled system (Endo-cut mode; ERBE ICC 200, Elektromedizin GmbH, Germany) was used for dissection and coagulation. A specialized cap (EMR ST Hood DH 15CR, Fujinon) was placed on the tip of the endoscope to make the dissection easier by increasing stability. Initially marked lesions were dissected with a diathermic knife (Olympus or Fujinon) circumferentially using endo-cut mode (2-3/80 W). An insulated-tip (IT) knife (KD-610L; Olympus) was used to dissect the borders of the lesions with a high perforation risk (wide based lesions and colonic lesions between haustral folds) using endo-cut mode (3/120 W). Submucosal dissection was performed with spray coagulation (45 W). Small vessels were coagulated with spray coagulation. Larger vessels or arteries with high bleeding risk were coagulated with hemostatic forceps (Fujinon).

Circumferential incision was completed in all cases. In colonic lesions, semi-circumferential incision was performed initially. After submucosal dissection, circumferential incision was completed. A few cases were finished with snare resection, but only after 80% of ESDs were completed. Standard or therapeutic gastroscopes (Fujinon EG-530 D) were used for lesions located in the rectum and sigmoid colon. Lesions close to the anus were treated in the retroflexion position. Colonoscopes were used for lesions proximal to the splenic flexure.

The first gastric lesions treated by ESD were located at the antrum in our series, as most gastric lesions were. Later on, cardiac lesions were treated by ESD in the retroflexion position.

All of the ESD procedures were performed under deep sedation. A combination of propofol and fentanyl was provided by an anesthesiologist. Patients were continuously monitored with an electrocardiogram, and blood pressure and oxygen saturation were monitored. The position of the patient could be easily changed whenever required with the help of medical attendants under the control of the anesthesiologist.

Definitions and follow-up strategy

All the specimens were examined by a single pathologist who is specialized in gastrointestinal pathology. En bloc resection was defined as the removal of a lesion in a single piece. Piecemeal resection was defined as the removal of a lesion in more than one piece.

A recurrent disease was defined as the reappearance of neoplastic tissue at the site of initial ESD at the 6th mo

follow-up endoscopy. In the case of a perforation, hemoclips were used. Bleeding that could be managed with endoscopic intervention was considered as minor bleeding. Bleeding with hemodynamic instability and blood transfusion requirement with or without the need for surgical intervention was considered as major bleeding.

A lesion was considered to be completely removed (R0 resection), when the vertical and lateral surgical margins were 2 mm away from the lesion. When neoplastic cells were present at surgical margins, this was considered as an incomplete resection (R1). Patients found to have undifferentiated or signet cell adenocarcinoma and submucosal/lymphovascular invasion on histopathological evaluation were referred to surgery. Patients were hospitalized for observation after the procedure and underwent a control endoscopy within 2 d of the ESD procedure. Patients underwent follow-up endoscopies at 3 and 6 mo. After a normal endoscopy at the 6th mo, annual follow-up was offered.

Statistical analysis

A median of continuous variables was used to present data. The Kruskal-Wallis test was used to compare median procedure time of ESD groups. When Kruskal-Wallis test results were statistically significant ($P < 0.05$), a Mann-Whitney test using Bonferroni correction was used to compare median procedure time between ESD groups. $P < 0.01$ was accepted as statistically significant. Statistical Packages for Social Sciences version 16.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis.

RESULTS

Over the 46-mo period, 60 ESD procedures were performed by a single operator (S.H.). There were 34 female (56.6%) and 26 male (43.3%) patients. The mean age (*via* standard deviation) of patients was 54.6 (± 14.1) years.

The majority of ESD procedures (65%) were performed for intraepithelial lesions ($n = 39$) (33/39 adenoma with high grade dysplasia, 6/39 adenoma with low grade dysplasia). The indication of the remaining ESD procedures were as following: neuroendocrine tumor (NET) ($n = 7$), cancer ($n = 7$) [5/7 early colorectal cancer (ECC), 2/7 early gastric cancer (EGC)], granular cell tumor ($n = 3$), gastrointestinal stromal tumor (GIST) ($n = 2$), and leiomyoma ($n = 2$). Microscopic types of the lesions in the different locations according to Paris classification is given in Table 1. En bloc and piecemeal resection rates were 91.6% (55/60) and 8.3% (5/60), complete and incomplete resection rates were 96.6% (58/60) and 3.3% (2/60), respectively.

An adenoma (piecemeal resection is done on purpose, Figure 1), early gastric cancer located in the antrum, and three flat adenomas with lateral invasion in the colon were resected in piecemeal fashion. A patient with a NET that was incompletely resected was referred to surgery due to vascular invasion noted on histopathologic evaluation. A patient with an incompletely resected early colon cancer (ECC) due to lateral spreading was referred to surgery. Invasion into the muscularis propria was seen

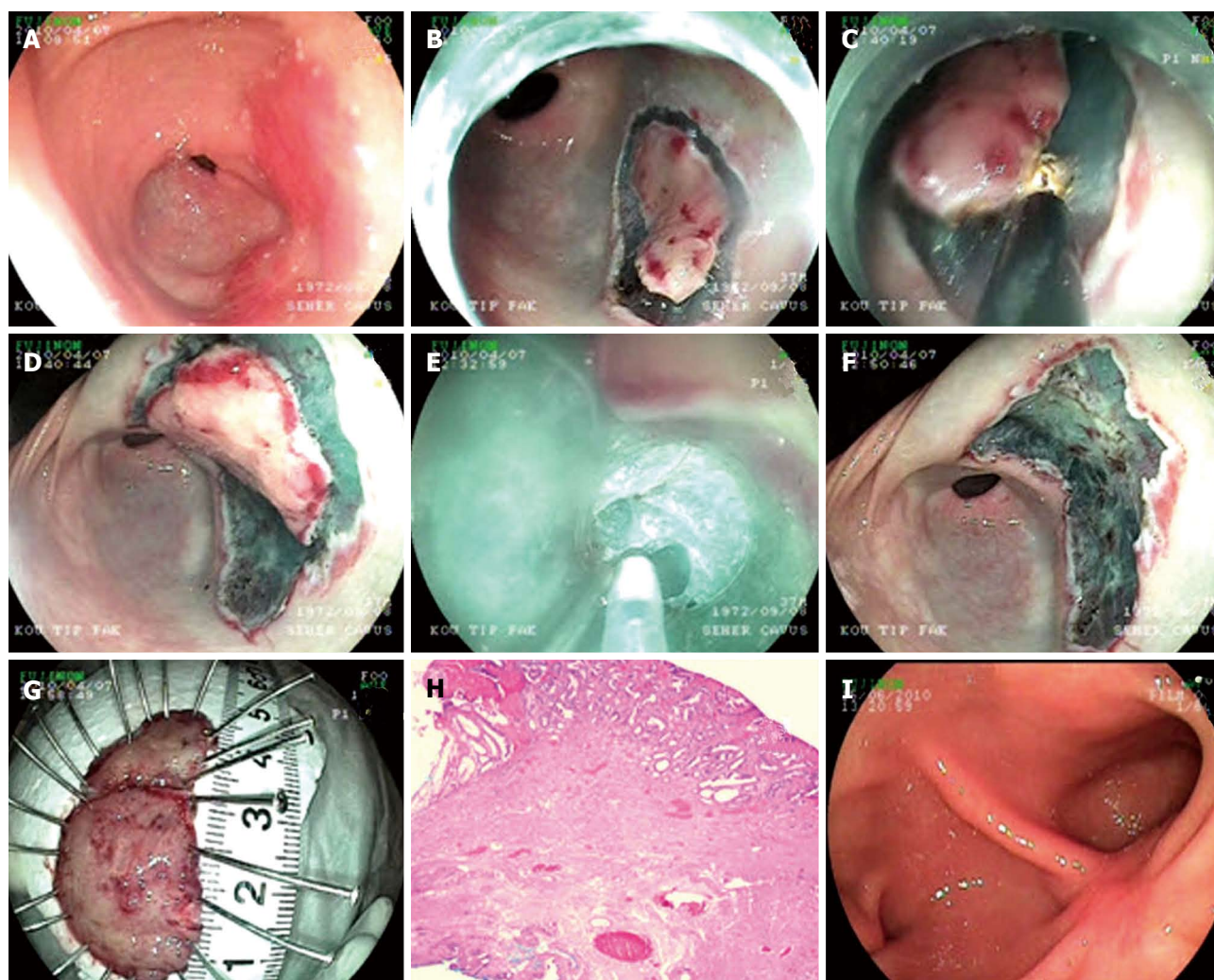


Figure 1 Endoscopic submucosal dissection procedure for adenoma with high grade dysplasia at antrum. A: Endoscopic view flat adenoma at antrum; B: Cutting of the first piece of the lesion which was decided to be extracted in two pieces; C: Submucosal dissection of the first piece; D: Cutting of the second piece of the lesion; E: Submucosal dissection of the second piece; F: Endoscopic view after the lesion is being extracted; G: Microscopic view of the lesion; H: Histology; Adenoma including fields of marked glandular atypia and distortion (HE $\times 20$); I: Endoscopic view ten weeks after the procedure.

Table 1 Paris classification according to the endoscopic imaging of lesions

	I a	II a	II b	II a + II c
Stomach	-	14 ¹	-	10
Small bowel	-	3	-	1
Colon	-	12 ²	-	13 ³
Esophagus	1	5 ¹	-	1

¹Two cases are submucosal; ²Nine granular type lateral spreading tumor(LST); ³Seven pseudo-depressed type LST.

in the surgical specimen of the patient with gastric NET referred for surgery. But there was no invasion in the surgical specimen of the patient with ECC (this patient was referred for surgery because histology revealed neoplastic cells at the surgical margins of the specimen resected in en bloc fashion). En bloc, piecemeal, complete, and incomplete resection rates are shown in Table 2.

The duration of ESD procedures per cm² were as follows; 27.8, 21.8, and 18.3 min for stomach, esophagus, and

colon, respectively. However, the mean procedure durations were as follows; 158, 90.4 and 50.5 min for colon, stomach, and esophagus, respectively. The mean procedure time of colonic and gastric lesions was significantly longer than esophageal lesions [Kruskal-Wallis test ($P < 0.01$)]. This is due to the differences in size of the lesions removed in different anatomic locations; colon (8.61 cm²) > stomach (3.25 cm²) > esophagus (2.34 cm²). Technical challenges related to the anatomic location of lesions as well as nature of lesions contribute to the procedure time (endoscopic resection should be done in the retroflexed position for lesions located in the cardia and proximal corpus, neuroendocrine tumors (NET) with rich vascularization have more bleeding).

Esophageal lesions

Seven esophageal lesions consisting of 3 granular cell tumors, 2 adenomas with high grade dysplasia, 1 gastrointestinal stromal tumor (submucosal), and 1 leiomyoma (submucosal) were treated with ESD (Table 3). No complications occurred in patients with esophageal lesions that were removed with ESD. No recurrences were not-

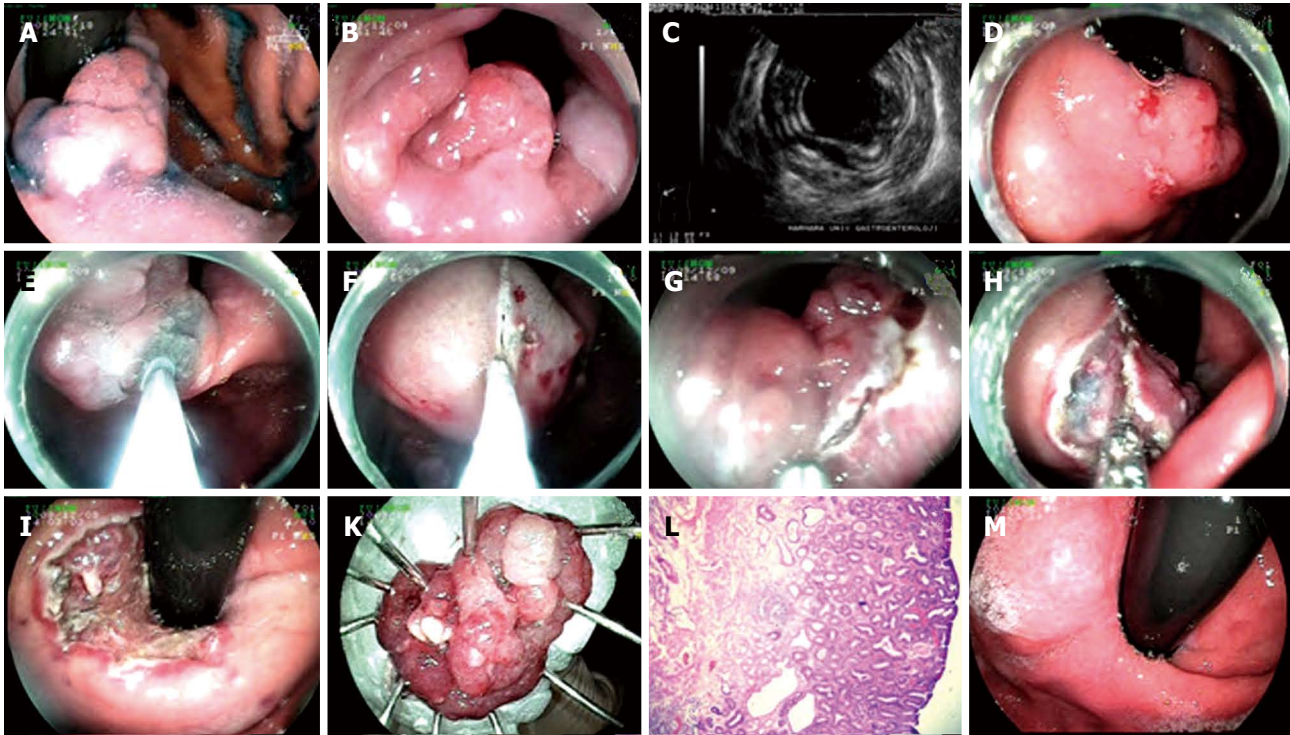


Figure 2 Endoscopic submucosal dissection procedure for adenoma with high grade dysplasia at cardia. A: Adenoma at cardia; B: View of the lesion from esophageal aspect; C: Endosonographic image of the lesion; D, E: Marking the borders of the lesion with needle knife and lifting it; F, G: Cutting the lesion circumferentially with endo-cut above Z line, in retroflexion; H: Dissection of the submucosal area; I: Appearance of the mucosa after the lesion being extracted; K: Microscopic view of the lesion; L: Histology: mucosa, muscularis mucosa and superficial submucosa of stomach (HE $\times 20$). Adenoma structure including adenomatous epithelium formed by irregular glands at mucosa; M: Endoscopic view six months after the procedure.

Table 2 En bloc, piecemeal, complete, incomplete resection rates, median follow up and recurrence rates

	En bloc res. rate	Piecemeal res. rate	Complete res. rate	Incomplete res. rate	Median follow-up (mo)	Local recurrence
Esophagus	7/7 (100%)	0	7/7 (100%)	0	15	0
Stomach	22/24 (91.7%)	2/24 (8.3%)	23/24 (95.8%)	1/24 (4.1%)	11.8	1
Small intestine and colon	26/29 (89.7%)	3/29 (10.3%)	28/29 (96.5%)	1/29 (3.4%)	12.5	0
Total	55/60 (91.6%)	5/60 (8.3%)	58/60 (96.6%)	2/60 (3.3%)	12.51	1/58 (1.7%) ¹

¹Two patients who underwent surgery were excluded. Res: Resection.

Table 3 Esophageal endoscopic submucosal dissection cases

Location	Number	Histology (n)
Proximal esophagus	1	Granular cell tumor (1)
Middle esophagus	2	Granular cell tumor (2)
Distal esophagus	4	GIST (1) HGD-A (2) Leiomyoma (1)
Specimen size (median)		2.34 cm ² (1.5-3 cm ²)
Procedure time (median)		50.5 min (21.8 min/cm ²)

GIST: Gastrointestinal stromal tumor; HGD-A: Adenoma with high grade dysplasia.

ed in the 7 patients that had follow-up data. The mean follow-up period for these 7 patients was 15 mo.

Gastric lesions

Twenty four gastric lesions, consisting of 16 adenomas (12 adenomas with HGD, 4 adenomas with LGD), 4 carci-

noid tumors, 2 early gastric cancers, 1 GIST (submucosal), and 1 leiomyoma (submucosal) were treated with ESD (Table 4) (Figures 1 and 2). One lesion was located in the cardia (adenoma with HGD), 3 lesions were located in the gastric corpus (3 carcinoid tumors), and 20 lesions were located in the antrum (11 adenomas with HGD, 4 adenomas with LGD, 2 EGC, 1 GIST, 1 carcinoid tumor, 1 leiomyoma). A patient with a gastric adenoma with HGD had recurrence at the site of prior resection. This patient had a second ESD, 12 mo after the first ESD.

Colonic and small intestinal lesions

Twenty-nine lesions including 21 adenomas (2 tubulovillous adenoma with HGD in duodenal bulb, 1 tubulovillous adenoma with HGD in cecum, 2 tubulovillous adenoma with HGD in transverse colon, 3 tubulovillous adenoma with HGD in sigmoid colon, 1 tubular adenoma in sigmoid colon, 9 tubulovillous adenoma with HGD in rectum, and 3 tubular adenoma in rectum), 3 carcinoid

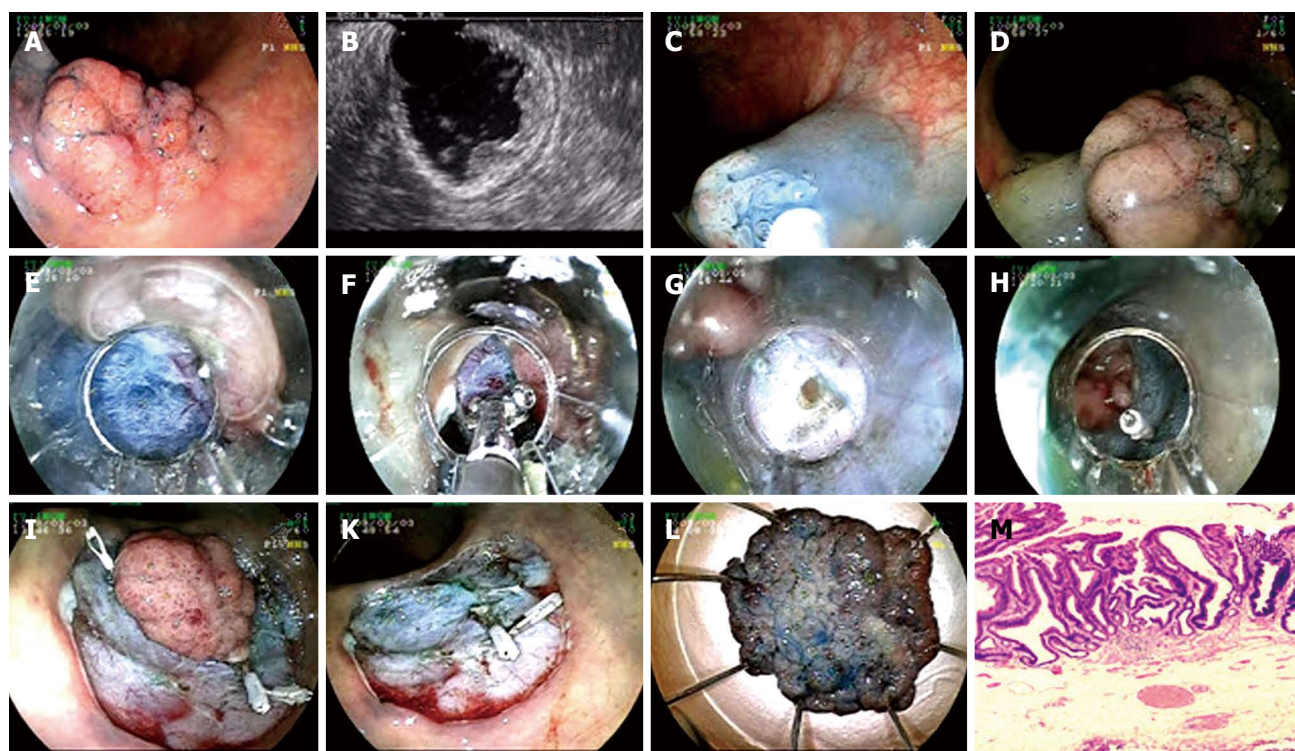


Figure 3 Endoscopic submucosal dissection procedure for tubulovillous adenoma with high grade dysplasia at sigmoid colon. A: Adenoma at sigmoid colon; B: Endosonographic image of tubulovillous adenoma; C-D: Lifting the lesion; E: Cutting with endo-cut; F: Coagulation of submucosal vein with hemostatic forceps; G: Mini perforation during the procedure; H: Fixing perforation with hemoclip; I: Hemoclip application to control bleeding that occurred after cutting the lesion circumferentially with endo-cut; K: Appearance of the mucosa after the lesion being extracted; L: Microscopic view of the lesion; M: Histology; tubulovillous adenoma with high grade dysplasia (HE × 40).

Table 4 Gastric endoscopic submucosal dissection cases		
Location	Number	Histology (n)
Cardia	1	HGD (TVA) (1)
Corpus	3	NET (3)
Antrum	20	HGD-A (11)
		LGD-A (4)
		NET (1)
		EGC (2)
		GIST (1)
		Leiomyoma (1)
Specimen size (median)		3.25 cm ² (1.5-12 cm ²)
Procedure time (median)		90.4 min (27.8 min/cm ²)

HGD: High grade dysplasia; NET: Neuroendocrine tumor; HGD-A: Adenoma with high grade dysplasia; LGD-A: Adenoma with low grade dysplasia; EGC: Early gastric cancer; LGD: Low grade dysplasia; GIST: Gastrointestinal stromal tumor; TVA: Tubulovillous adenoma.

tumors (1 in duodenal bulb, 1 in terminal ileum and 1 in rectum), and 5 early colon cancers (4 in rectum and 1 transverse colon) were treated with ESD (Table 5) (Figures 3 and 4). All early colon cancers were classified as II a + II c according to the Paris classification.

Safety

Among 5 perforations (8.3%), four were located in the colon (1 early colon cancer, 2 laterally spreading lesions and 1 large tubulovillous adenoma located on a colonic haustra) and one (carcinoid tumor) was located in the stomach.

Table 5 Colonic and intestinal endoscopic submucosal dissection cases		
Location	Number	Histology (n)
Duodenal bulb	3	HGD (TVA) (2)
		NET (1)
Terminal ileum	1	NET (1)
Cecum	1	HGD (TVA) (1)
Transverse colon	3	HGD (TVA) (2)
		ECC (1)
Sigmoid colon	4	HGD (TVA) (3)
		LGD-TA (1)
Rectum	17	ECC (4)
		HGD (9 TVA, 2 TA)
		LGD-TA (1)
		NET (1)
Specimen size (median)		8.61 cm ² (1.5-25 cm ²)
Procedure time (median)		158 min (18.3 min/cm ²)

HGD: High grade dysplasia; NET: Neuroendocrine tumor; TA: Tubular adenoma; ECC: Early colonic cancer; LGD: Low grade dysplasia; TVA: Tubulovillous adenoma.

Two colonic perforations were managed with surgical intervention and the other three were managed with hemoclip application. Those managed with hemoclip application were smaller than 5 mm in size; hence it was easy to treat them. The patients with perforation were hospitalized for an average of ten days. There was no association of perforation with scarring and fibrosis from previous procedures. No post-ESD stenosis was noted. Major bleeding occurred

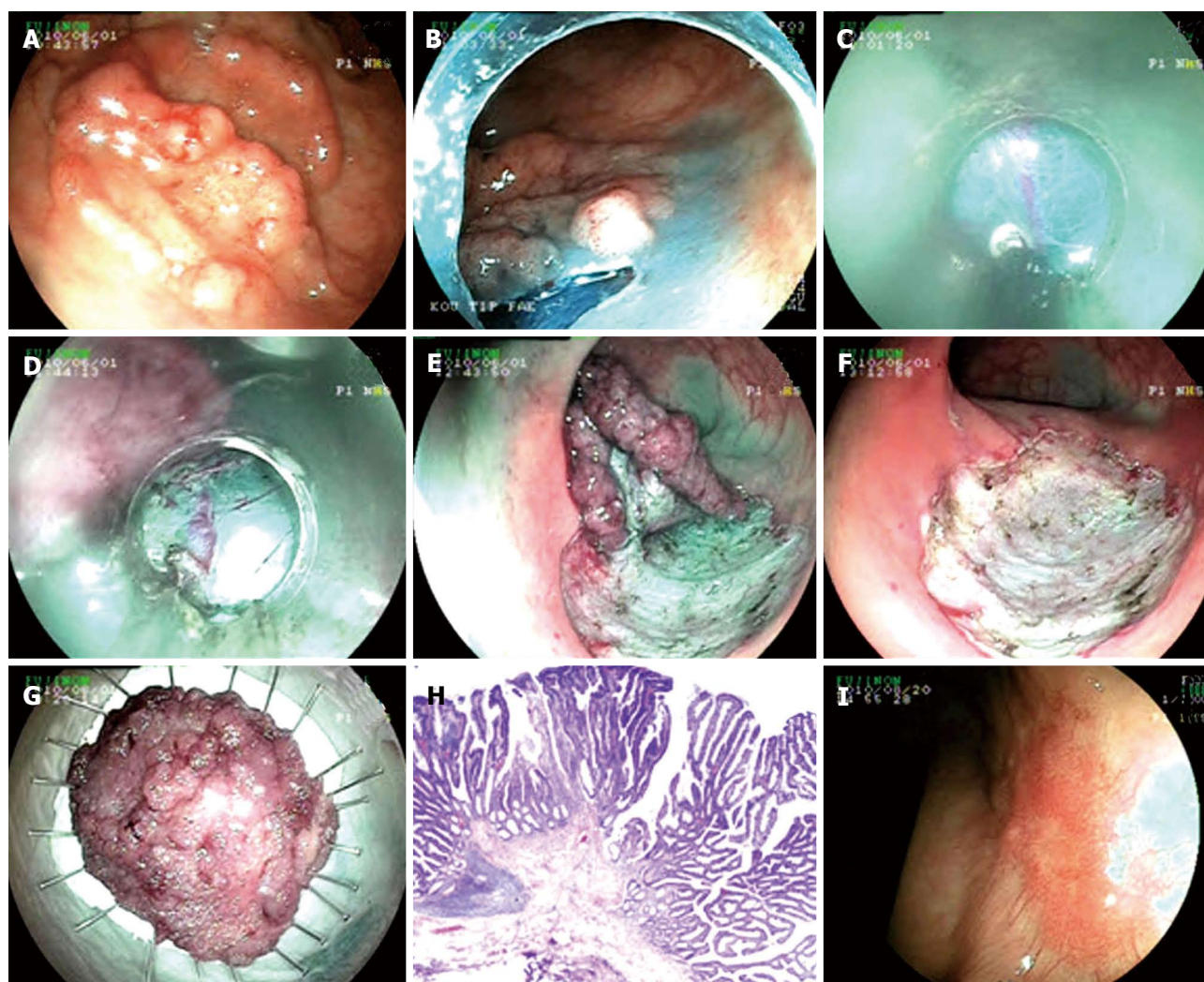


Figure 4 Endoscopic submucosal dissection procedure for pseudo-depressed type lateral spreading tumor with high grade dysplasia at rectum. A: Pseudo-depressed type lateral spreading tumor at rectum; B: Cutting the lesion circumferentially with endo-cut; C, D: Submucosal dissection with semipermeable cap; E: Endoscopic view just before completing submucosal dissection; F: Appearance of the mucosa after the lesion being extracted; G: Microscopic view of the lesion; H: Histology; tubulovillous adenoma including fields of focal pattern loss and dysplasia (HE $\times 20$); I: Endoscopic view ten weeks after the procedure.

in three patients (5%). One of these patients had a lesion located in the colon and two of them had lesions located in the stomach. Contributing factors to major bleeding may possibly be the nature as well as the location of the lesion, possibly due to the rich vascularization of the lesion. Both of the bleeding lesions in the stomach were NETs and located in the lesser curvature. Bleeding from gastric lesions was delayed and required blood transfusion. The colonic case that had a major bleed was an early colonic cancer. Minor bleeding occurred in 13 cases (7 lesions located in the colon and 6 lesions located in the stomach) (21.7%). Table 6 shows complications in detail.

DISCUSSION

Although endoscopic resection-based therapeutic modalities (EMR and ESD) are considered to be the treatment of choice for premalignant and early gastrointestinal neoplasias in Japan, they are not widely practiced by Western endoscopists^[10]. This is the first study from Turkey and among the few studies from outside of Japan and Asia on

the application of ESD in the management of premalignant and noninvasive early gastrointestinal cancers from various anatomic locations in the gastrointestinal tract.

It is difficult or impossible to remove large lesions with EMR technique in one fragment. Removal of a lesion in one piece is very important to accurately diagnose the tumor depth as well as decreasing the risk of local recurrence. A recent study has illustrated that this problem can be solved with the use of ESD for larger lesions^[11]. In early esophageal cancers with a diameter of less than 20 mm, en bloc and curative resection rate of ESD (97%) was found to be significantly higher than that of EMR using a transparent cap (71%) and 2-channel EMR (46%)^[11]. However, no significant difference was found between ESD and EMR using a transparent cap in en bloc and curative resection rate of lesions less than 15 mm in diameter. Therefore, ESD would be a better therapeutic modality than EMR for esophageal lesions with a diameter of greater than 15 mm. Given the size (median size of esophageal/gastric/colonic lesions 2.34 cm²/3.25 cm²/8.61 cm² respectively), various anatomic location of

Table 6 Complications with regard to location and diagnosis

	<i>n</i>	Diagnosis	Minor bleeding	Major bleeding	Perforation
Esophagus	7	3 granular cell tumor 1 GIST 2 Premalignant lesions 1 Leiomyoma			
Stomach	24	4 NETs 2 EGC 18 Premalignant lesions	4 2	2	1
Small intestine	4	2 Premalignant lesions 2 NETs			
Colon	25	5 ECCs 19 Premalignant lesions 1 NETs	3 4	1	1 3
Total	60		13 (21.7%)	3 (5%)	5 (8.3%)

GIST: Gastrointestinal stromal tumor; NET: Neuroendocrine tumor; EGC: Early gastric cancer; ECC: Early colorectal cancer.

lesions and subepithelial nature of some lesions, ESD would be the treatment of choice for our cases.

There are few studies from Europe that were published in full manuscript format. Dinis-Ribeiro *et al*^[12] evaluated feasibility and effectiveness of ESD in 19 gastric superficial lesions with HGD, LGD and noninvasive epithelial neoplasias. Probst *et al*^[13] evaluated ESD in 71 flat adenomas, early cancers and submucosal tumors located in various locations of the gastrointestinal tract (51 gastric, 17 rectal, 2 esophageal and 1 duodenal). In the study of Dinis-Ribeiro *et al*, complete and en bloc resection rates were 89% and 79%, respectively. Major bleeding occurred in 1 case (5%). There were no perforations. Recurrence of a lesion (5%) was noted within a mean follow-up of 10 mo. In order to evaluate ESD learning curve, Probst *et al*^[13] compared various aspects of ESD procedures performed in the first and second halves of the study.

A statistically significant increase in specimen size and decrease in procedural duration were noted between the two groups. En bloc resection rates and R0 en bloc resection rates in the first half of the study (77.1% and 65.7%, respectively) increased when compared with the second half of the study (86.1% and 72.2%, respectively), however this difference did not reach statistical significance. No recurrence occurred after R0 en bloc resection; however 38% recurrence occurred after piecemeal resection. Complications in the study of Probst included 2 perforations (gastric submucosal tumors) that required surgery (2.7%), 2 other perforations (large flat rectal lesions) (2.7%), 8 minor bleedings (10.9%) and 3 pyloric stenosis (4.1%) that were endoscopically managed.

ESD complication rates among the published studies have been variable depending on the size of the study as well as the experience of the operator. In our study, a patient (1.7%) was found to have a recurrence of an adenoma with HGD at the site of prior ESD on 12-mo follow-up endoscopy. The recurrent lesion was treated with a repeat ESD. The patient was free of disease at the 6-mo follow-up. No recurrences were noted with esophageal and colonic lesions. Compared to other studies, lower

recurrence rate in our study may be related to the relatively shorter follow up (median = 12.5 mo) of the patients after ESD. Our bleeding rate is consistent with other studies. Our perforation rate is higher than the study of Probst *et al*^[13]. However, all of our perforations occurred at the first half of the study, which is a reflection of the impact of the operator's experience with the success of ESD procedures. Eighty percent of perforations occurred with colonic cases, which may be related to the relatively thinner colonic wall thickness and larger size of colonic lesions. Most perforations took place in initial cases. In those cases needle knives were used. We believe that the use of these knives also contributed to this relatively high number of perforations. After providing IT-knives we did not encounter any perforations. As stated above we could not refuse the patients and it is true that we had to perform colorectal cases with insufficient experience in gastric ESD, resulting in relatively high perforation rates.

In a review article from Japan, the en bloc resection rate of early gastric cancers was reported to be 79%-100%, with local recurrence, bleeding and perforation rates of 0%-1%, 1.7%-38%, 0%-5%, respectively^[14]. Another study from Japan evaluating ESD in colorectal epithelial neoplasms revealed the rate of en bloc resection and en bloc resection with tumor free margins to be 91.5% and 70.5%^[15]. In this study, perforation and local recurrence rates were found to be 5% and 1.7%, respectively. The sample size of studies coming from outside of Japan and Asia is quite modest; therefore it is premature to compare Western experience with the Asian one.

Ideally one should begin with gastric cases located in the antrum. After getting sufficient experience in gastric cases they can proceed with esophageal and colorectal cases, which are more risky. Our practice seems incompatible with this idea. But ESD is only performed in our institute in Turkey and patients are referred to our hospital from the entire country. So we did not have the chance to refuse the patients and performed esophageal and colorectal cases before having sufficient experience in gastric cases.

We believe that, besides EMR and endoscopic piecemeal mucosal resection, ESD will be a good alternative in the treatment of non-epithelial esophageal lesions. We observed that neuroendocrine tumors which have rich vascularization are more likely to bleed, so more attention should be paid when operating on them. During the ESD procedures in the colon and esophagus, a needle knife should be avoided in endo-cutting because of the perforation risk. Perforation risk is even higher in laterally spreading colonic lesions so IT-knife is a better choice for those lesions.

In summary, ESD, which originates in Japan, has been gaining popularity in other parts of the world as well. Comparable outcomes of ESD to surgery play an important role in the rapid propagation of this therapeutic endoscopic modality. Although no procedure related mortality has been reported, there is considerable morbidity with this technique. There is a significant learning curve to achieve proficiency in order to acquire skills to perform ESD safely and effectively. Therefore, importance of training can not be overemphasized. Further studies from outside of Japan and Asia are needed to better determine the global role of

ESD in the management of premalignant and early malignant epithelial, as well as subepithelial lesions.

COMMENTS

Background

Advances in endoscopic diagnosis techniques allowed premalignant lesions and non-invasive cancers of the gastrointestinal system to be detected early and be treated effectively and safely by endoscopic methods. Among these methods, endoscopic submucosal dissection (ESD) is being used more and more commonly and has pleasing results.

Research frontiers

ESD is a safe and effective modality for the treatment of premalignant lesions and early non-invasive cancers of the gastrointestinal system and when compared to surgery it has the advantage of preserving the gastrointestinal system and its functions. This is the first study on ESD reported from Turkey.

Innovations and breakthroughs

Complications related to ESD are similar to other studies in the literature except for the complication rate. Recurrence rates are lower compared to other studies. In this study we observed that neuroendocrine tumors had higher bleeding rates due to hypervascularization and the use of a needle knife in colonic and esophageal lesions increased the risk of perforation.

Applications

When performed by experienced endoscopists ESD has pleasing results and can be safely performed for the treatment of premalignant lesions and early cancers of gastrointestinal system.

Terminology

ESD is being used for the treatment of premalignant and lesions (with no lymph node involvement) of the gastrointestinal tractus confined to mucosa and submucosa. After lifting the lesion by injecting the specially prepared solution (Na - Hyaluronate + Adrenalin + Saline + Indigo carmine), the basement of the lesion, along with the surrounding area, are cut with special knives and the lesion is extracted.

Peer review

This retrospective study sets out to evaluate the feasibility, safety and clinical outcomes of ESD for premalignant lesions and early gastrointestinal cancers. It establishes that the rates of en bloc and complete resection of these lesions were good, and comparable those of previous studies.

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Discovery and validation of prognostic markers in gastric cancer by genome-wide expression profiling

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Abstract

AIM: To develop a prognostic gene set that can predict patient overall survival status based on the whole genome expression analysis.

METHODS: Using Illumina HumanWG-6 BeadChip followed by semi-supervised analysis, we analyzed the expression of 47 296 transcripts in two batches of gastric cancer patients who underwent surgical resection. Thirty-nine samples in the first batch were used as the training set to discover candidate markers correlated to overall survival, and thirty-three samples in the second batch were used for validation.

RESULTS: A panel of ten genes were identified as prognostic marker in the first batch samples and classified patients into a low- and a high-risk group with significantly different survival times ($P = 0.000047$). This prognostic marker was then verified in an independent validation sample batch ($P = 0.0009$). By comparing with the traditional Tumor-node-metastasis (TNM) staging system, this ten-gene prognostic marker showed consistent prognosis results. It was the only independent prognostic value by multivariate Cox regression analysis ($P = 0.007$). Interestingly, six of these ten genes are ribosomal proteins, suggesting a possible association between the deregulation of ribosome related gene expression and the poor prognosis.

CONCLUSION: A ten-gene marker correlated with overall prognosis, including 6 ribosomal proteins, was identified and verified, which may complement the predictive value of TNM staging system.

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Key words: Gastric cancer; Gene expression profiling; Survival markers; Prognosis; Ribosomal proteins

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INTRODUCTION

Gastric cancer is the second leading cause of cancer related death worldwide^[1]. As a complex and heterogeneous disease, it comprises multiple tumor entities associated with distinctive histological patterns and biological features, as well as clinical behaviours^[2]. The 5-year survival rate of patients with advanced disease is only 20%-30%^[3]. The current treatment plan and prognosis prediction for gastric cancer mainly depend on the clinicopathologic staging of the disease, and TNM staging system is still the golden standard for survival prediction among gastric cancer patients. However, prognosis varies among patients with a similar tumor stage, therefore disease staging alone can not accurately predict the outcome for individual patients.

Although great efforts have been made in the identification of prognostic markers from gene expression profiling to improve prognosis prediction for many cancers especially breast cancer^[4], limited research has been conducted in the field of gastric cancer. To date, most studies on the selection of prognosis markers were conducted by cDNA array or quantitative RT-PCR, in which only a few thousand genes were analyzed^[5-9]. In an attempt to predict peritoneal relapse after gastrectomy for gastric cancer, the whole genome microarray consisting of 30K transcripts was employed in a very recent gene expression analysis^[10]. Such a robust approach may provide not only more signals in marker selection, but also more comprehensive information in understanding molecular mechanisms of tumor-related processes. In this study, we explored the gene expression by microarray containing over 47K probes in two batches of surgical samples from 79 Chinese gastric cancer patients. A ten-gene marker for overall survival was identified and verified in an independent batch of samples.

MATERIALS AND METHODS

Patients and samples

Seventy-nine tissue samples from patients who were surgically treated for primary gastric carcinoma were procured at the Beijing Cancer Hospital (Peking University, School of Oncology) from 1999 to 2003. No patient received chemotherapy or radiotherapy before surgery. All patients were treated with curative surgical resection, which was, in some cases, followed by second-line treatment at the time

of recurrence. Macroscopic and microscopic evaluations were conducted by pathologist according to the general rules for gastric cancer. Follow-up was performed every three month for the first two years, and every three to six months thereafter. Stage of gastric cancer was classified according to 2002 tumor-node-metastasis (TNM) classification system recommended by the American Joint Committee on Cancer.

Overall survival was calculated from the date of primary surgery to the date of last follow-up or to the date of death due to cancer relapse or metastasis. All tumor samples were obtained at the surgery, followed by fresh freezing in liquid nitrogen and stored at -80°C. Informed consent was obtained from each patient for the collection and storage of tissue samples in a tissue bank for future research. This investigation was performed after approval by Ethics Committee of Peking University.

RNA preparation and microarray analysis

Total RNA was purified from clinical samples using TRIzol reagent (GibcoBRL, Grand Island, New York, USA). And mRNA was linearly amplified by in vitro transcription using T7 RNA polymerase (MEGAscript T7 kit, Ambion, Inc, USA). The quality and integrity of total and amplified mRNA (cRNA) was monitored by both spectrophotometry (OD UV 260/280 ratio > 1.8) and agarose gel electrophoresis.

Gene-expression profiling was performed using Illumina HumanWG-6 BeadChip, which contains 47 296 transcripts. BeadChips were scanned with a BeadStation 500 GX and data are available at Gene Expression Omnibus (GSE21983). <http://www.ncbi.nlm.nih.gov/geo>.

Statistical analysis

Average normalization in BeadStudio software was conducted for probe level average normalization and background correction. A detection *P* value was used in BeadChip to calculate probability to see a certain signal level without specific probe-target hybridization. All genes and probes with *P* value > 0.01 were filtered and removed from the analysis. Among 47 296 transcripts, 18 819 were expressed with *P* values < 0.01.

The supervised principal components method was used for survival profiling^[11]. In the training set, we calculated the modified univariate Cox proportional-hazard scores for all genes (*n* = 18 819), which were measured to identify genes with their expression correlated to the duration of survival. We selected a set of genes whose absolute Cox score exceeded a threshold using cross-validation. For each iteration of the complete cross-validation, 10% of the cases were omitted, and principal components derived from the remaining 90% of the cases were included in a Cox model to predict the survival in 10% of the cases. By repeating the iteration process for 10 times, we found that a threshold of 2.6 yielded the highest average partial log-likelihood ratio. Principal component analysis (PCA) was then performed using 10 transcripts whose absolute Cox score equalled or exceeded the threshold for all cases in the training data set.

Table 1 Clinicopathological characteristics of all patients

Variables	Cases	Training dataset (<i>n</i> = 39)	Validation dataset (<i>n</i> = 33)	<i>P</i>
Sex				
Male	53	28	25	0.79
Female	19	11	8	
Age (yr) mean ± SE	72	60.9 ± 1.5	61.6 ± 1.3	0.74
Depth of wall invasion				
T2	4	3	1	0.12 ¹
T3	56	32	24	
T4	12	4	8	
Differentiation				
Well	7	5	2	0.18 ¹
Moderate	31	14	17	
Poor	27	18	9	
Undifferentiated ²	7			
Lymph node metastasis				
Negative	16	10	6	0.57
Positive	56	29	27	
Distance metastasis				
M0	66	38	28	0.09
M1	6	1	5	
TNM stages				
I + II	17	12	5	0.30 ¹
III	33	18	15	
IV	22	9	13	

¹The multiple comparisons of different subclasses; ²Data was incomplete.

Kaplan-Meier survival curves were then plotted to predict overall survival. All analysis and plotting were conducted using R package superpc (<http://www-stat.stanford.edu/~tibs/superpc>).

Based on the transcript level in the 10 transcripts and the weight assigned to each transcript from the training set, a discrete risk score (the supervised principal components risk score) was then calculated for each patient in the validation dataset.

Multivariate analysis was conducted to evaluate the prediction accuracy of our survival profile in comparison with the standard clinicopathological covariates by Cox proportional hazards regression using SPSS software.

Functional gene set enrichment analysis was performed to find the pathways associated with prolonged and poor survivals. A total of 249 sets of canonical pathways (Gene Set Enrichment Analysis-Molecular Signatures Database) were analysed to indicate their correlations with overall survival to a greater degree than expected by chance^[12].

RESULTS

Patient characteristics

Totally, 79 gastric cancer patients treated with surgical resection were recruited in this study. Samples were randomly separated into two batches with no significant differences between the two sets with respect to age, sex and other clinicopathological features. Microarray was conducted in all samples, and the data of batch one served as the training dataset for marker discovery and data of batch two as the validation dataset. In batch one, microarray Quality Control

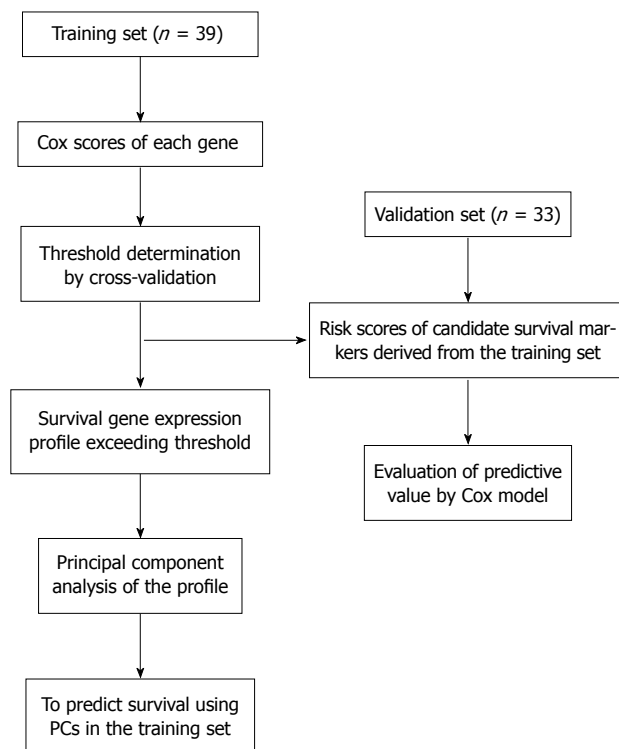


Figure 1 Overview of the strategy used for the development and validation of prognostic markers.

(QC) removed 7 samples due to failure in hybridization or failure to meet the analysis criteria, resulting in a total of 39 samples included in the training set. All of 33 samples in the second batch passed QC and were used in validation phase. The characteristics of the 72 patients are summarized in Table 1. The median overall survival time of all samples was 31 mo, ranging from 4.2 to 73.6 mo, and the 5-year overall survival was 33%.

Gene expression profile associated with the overall survival

The “semi-supervised” learning approach was used to identify the gene expression profile related to the overall survival in the training dataset^[12] (Figure 1). A total of 18 819 expression signals passed QC. First, we calculated the Cox scores of all 18 819 genes based on the survival times versus the expression levels obtained in 39 training observations. To choose the genes with the best prediction power, the threshold of Cox scores was calculated by 10-fold cross-validation. The expression profile of 10 transcripts whose Cox score equalled or exceeded the threshold was obtained (Table 2). Next, we performed PCA on the entire training set. For each case, a risk score that represents the sum of the weighted expression levels of the 10 prognostic transcripts was computed by supervised component analysis in a regression model. As shown in the Kaplan-Meier survival curves in Figure 2A and B, the patients were categorized into two groups based on their scores above or below the median risk of death. The low-risk group (*n* = 20) had a median survival of 42.1 mo, whereas the high-risk group (*n* = 19) had a median survival of only 26.5 mo.

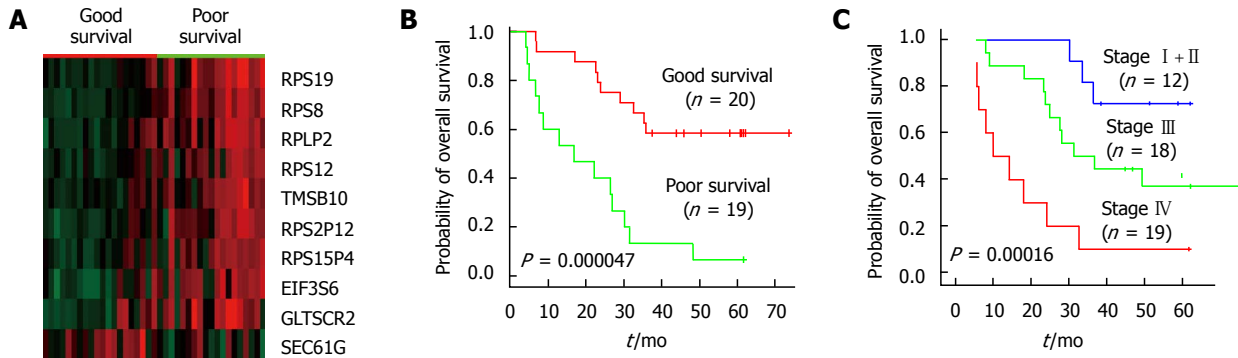


Figure 2 Overall survival curves and the expression profile of the ten-gene prognostic marker in the training dataset. A: The gene expression pattern of the ten-gene prognostic marker. Nine genes were associated with the prolonged survival and one gene with poor survival. Red, high expression; green, low expression; B: Kaplan-Meier survival curves based on the expression profile of the ten-gene prognostic marker; C: Overall survival curves according to the tumor-node-metastasis stages.

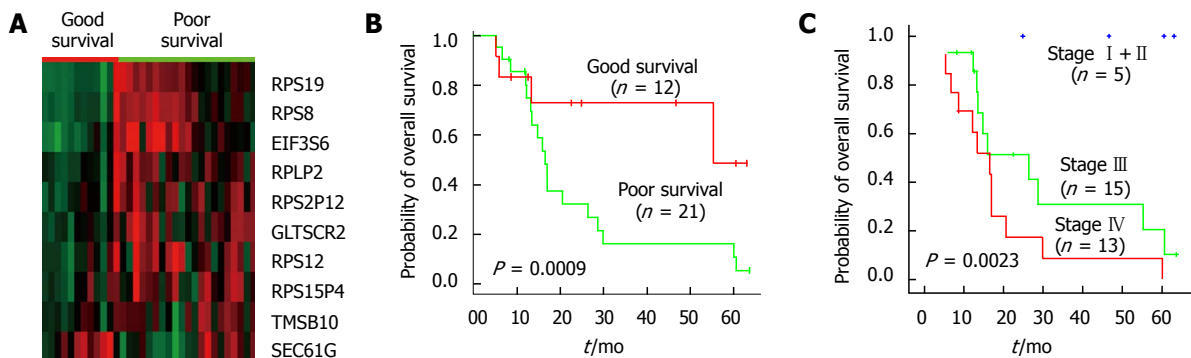


Figure 3 Overall survival curves and the expression profile of the ten-gene prognostic marker in the validation dataset. A: The gene expression pattern of the ten-gene prognostic marker. Red, high expression; green, low expression; B: Kaplan-Meier survival curves based on the expression profile of the ten-gene prognostic marker; C: Overall survival curves according to the tumor-node-metastasis stages.

Table 2 The ten-gene prognostic marker correlated to patients' survival

Symbol	Cox score	Description
RPS19	2.93	Ribosomal protein S19
RPS8	2.90	Ribosomal protein S8
RPS2P12	2.62	Predicted ribosomal protein S2
RPS12	2.59	Ribosomal protein S12
RPS15P4	2.57	Predicted 40S ribosomal protein S15
RPLP2	2.51	Ribosomal protein, large, P2
EIF3S6	2.61	Eukaryotic translation initiation factor 3
GLTSCR2	2.67	Tumor suppressor candidate region gene 2
TMSB10	2.47	Thymosin, beta 10
SEC61G	-2.98	Sec61 gamma subunit

The correlation of the risk score and the survival status ($P = 0.000047$, log-rank) indicates that this transcriptional pattern was associated with the patient outcomes. A similar classification was also seen by TNM staging ($P = 0.00016$, log-rank; Figure 3A).

Among the ten-gene prognostic markers, high expression levels of 9 genes were associated with poor survival (Figure 2B). SEC61G, a subunit of the heteromeric SEC61 complex, was the only gene with its high expression associated with prolonged survival. Interestingly, six out of the

10 genes in this profile are either identified or predicted ribosomal proteins, including RPLP2, RPS12, RPS8, RPS19, RPS2P12, and RPS15P4. The involvement of numerous ribosomal genes with survival times suggested either their regulation by tumor suppressor or oncogenes, or their direct participation in certain pathways other than protein synthesis. Furthermore, RPS8 and RPS12 were previously reported as cancer related markers in colorectal tumor and cervical squamous cell carcinoma^[13,14]. EIF3S6, a member of eukaryotic translation initiation factor 3, was identified as a prognostic factor in Stage I non-small cell lung cancers^[15]. To test if the survival categories relate to known pathways, we applied gene set enrichment analysis (GSEA) to microarray data of 18 819 transcripts using 249 canonical gene sets collected by MsigDB. Glucocorticoid receptor (GCR) pathway gene set, referring to glucocorticoid receptor-related inhibition of inflammatory response, was significantly associated with the overall survival (FDR = 0.15, $P = 0.004$).

Independent validation of prognostic markers

Next we evaluated the ten-gene prognostic marker in an independent dataset containing 33 cancer samples. As shown in Figure 3A and B, based on the expression of these 10 genes, the patients were classified into either a

Table 3 Association between different prognosis groups identified by the ten-gene marker and the clinicopathological characteristics

Variables	Cases	Prolonged survival (<i>n</i> = 12)	Poor survival (<i>n</i> = 21)	<i>P</i>
Sex				
Male	14	9	5	0.070
Female	18	3	15	
Age (yr)				
mean ± SE	33	58.1 ± 2.2	63.5 ± 1.5	0.040
Depth of wall invasion				
T2	1	1	0	0.120
T3	24	11	13	
T4	8	0	8	
Differentiation				
Well	2	1	1	0.490
Moderate	17	8	9	
Poor	9	2	7	
Undifferentiated ¹	5			
Lymph Node Metastasis				
Negative	6	4	2	0.160
Positive	27	8	19	
Distance metastasis				
M0	28	12	16	0.080
M1	5	0	5	
TNM stages				
II	5	5	0	0.002
III	15	5	10	
IV	13	2	11	

¹Data was incomplete. *P* values for stage, grade and location of tumors were derived from the Pearson χ^2 test. *P* value for age was derived from the *t* test.

“low-risk group” or “high-risk group” with significantly different survival times ($P = 0.0009$, log-rank). The low-risk group patients had a median survival of 31.7 mo whereas the high-risk group one had a median survival of 21.4 mo. The expression patterns of these 10 genes in validation set were also similar to the observation in the data training set (Figure 3B). The results in validation dataset showed the consistency of our ten-gene prognostic marker in survival prediction. For pathway analysis, no significant association was observed by GSEA.

Analysis of candidate survival markers with clinicopathological parameters

As certain clinicopathological parameters, especially TNM staging, have been used as prognosis indicators, we also compared our ten-gene prognostic marker with the clinicopathological characteristics in the validation dataset in order to assess the impact of clinicopathological factors on overall survival. First we examined the distribution of prognostic factors as a function of risk assignment based on our ten-gene prognostic marker (Table 3). Certain variation such as age was seen between high- and low-risk groups, while gender, tumor location and differentiation grade, the depth of wall invasion, and metastasis showed no significant difference between the two groups except for the TNM staging ($P = 0.0023$).

Since the TNM staging is used most widely in clinical

Table 4 Multivariate Cox regression for overall survival in validation dataset

Variables	<i>P</i>	HR	CI (95%)
Depth of wall invasion	0.370	1.66	0.55-4.90
Differentiation	0.240	4.93	0.15-1.58
Lymph node metastasis	0.780	0.77	0.13-4.53
Distance metastasis	0.120	0.32	0.08-1.35
Ten-gene prognostic marker	0.007	0.13	0.29-0.56

CI: Confidence interval; HR: Hazard ratio.

prognosis prediction, we compared the predictive power of our survival markers with the TNM staging. All the TNM stage II patients were categorized into the low-risk group and the entire high-risk group patients were in stage III or IV. A consistency between TNM staging and the staging was found by the ten-gene marker. However, two IIb patients of the low-risk group had a survival of 63.7 and 22.5 mo at last follow-up, respectively. By Kaplan-Meier survival plots and log-rank tests, we assessed the patient survival status predicted by our prognosis candidates and TNM staging. Relatively more accurate predictions were shown by the ten-gene prognostic marker in both sample groups (TNM, $P = 0.00016$ and $P = 0.0023$; survival markers $P = 0.000047$ and $P = 0.0009$, Figure 2B and C, Figure 3B and C). As a result, in multivariate analysis, our ten-gene marker was the only independent indicator in prognosis prediction with statistical significance ($P = 0.007$; Hazard ratio 0.13; 95% CI: 0.29-0.56, Table 4).

DISCUSSION

It has been known that the environment and genetic background among ethnic groups correlate to the genesis and development of gastric cancer^[16]. Up until now, very limited studies have been conducted in finding prognosis markers for gastric cancer, especially in the Chinese population. Moreover, only one set of prognosis markers was reported recently using the whole genome microarray ($> 30K$), which, however, could predict peritoneal relapse but not overall survival^[10]. In addition, in previous reports, by the classical supervised method to select survival markers, “low-risk” and “high-risk” subgroups are contrived based on survival times before analysis. Such a subjective step may result in bias for next process or lead to the classification which is not biologically meaningful. Therefore, in this study, we adopted a supervised PCA strategy to build prognosis profiles with the consideration of survival time as continuous parameters^[11]. And based on the whole genome expression profiling, we found and verified a set of ten genes as candidate survival markers from the discovery panel of 39 samples and the validation panel of 33 samples.

In these 10 survival genes markers, 3 genes (RPS12, EIF3S6, RPS19) were previously reported as candidate

markers of diagnosis or prognosis in various types of cancers^[13,17,18]. TMSB10, a migration-inducing gene, was shown to relate to cancer metastasis^[19]. Additionally, a few genes (RPS19, RPLP2, GLTSCR2) are known factors involved in cell cycle control and apoptosis^[20-22]. None of our 10 markers was reported in other sets of candidate genes for gastric cancer prognosis^[5-8]. This is not a surprise since these 10 genes were selected based on the whole genome expression profiling followed by supervised PCA, whereas much less genes were included in earlier studies with the analysis strategy of supervised classification. Patients' genetic background may also contribute to such diversity.

Unexpectedly but also interestingly, 6 out of 10 candidate markers identified are ribosomal proteins (RPs). There may be a few explanations for this phenomenon. First, RPs have been shown to be the targets of several tumor suppressors and proto-oncogenes which affect the formation of the mature ribosomes or regulate the activity of proteins^[23]. Moreover, the deregulated expression of RPs was reported to associate with the carcinogenesis and metastasis of various cancers^[14]. Therefore, besides their unknown mechanisms possibly related to p53 and MYC^[24,25], RPs appear to have various cellular roles independent of protein biosynthesis, including their functions in DNA replication and DNA repair, transcription, RNA splicing and modification, cell proliferation, apoptosis, and cellular transformation.^[26] Among 6 RPs of our candidate prognosis markers, RPLP2, RPL19, RPS8 and RPS12 were all found to be involved in the carcinogenesis and progression of various cancers^[13,17,27]. RPS12 was also seen to have significant higher expression in gastric tumors in comparison with normal tissues in Chinese^[28].

In a number of diagnosis and prognosis sets identified in expression profiling from various cancer researches, the gene profile in most panels came from various pathways with different cellular functions. The result of our ten-gene prognostic marker containing 6 RPs raised another interesting issue on the molecular composition of biomarkers, i.e. which type is more powerful and more accurate in prediction, a set consisting of single gene tags from multiple individual pathways, or a group of genes from a few and related pathways. This issue needs more tests and evaluations for convincing answers. At this point, however, a few facts shall be brought into attention. First, our prognostic marker resulted from systematic analysis of whole genome expression profiling, and our strategy of supervised PCA largely reduced subjective attribution in analysis. Thus, a group of pinpointed signals will be more representative in biological meaning, thus providing more accurate prediction. Second, obviously in comparison with individual single signatures from multi-pathways, a group of signals would significantly overcome the individual bias, in which the pathway components in tumors vary widely^[29]. Finally, it has been shown that even for genetic alterations of a large number of genes in cancer, these variations may function through a relatively small number of pathways and processes^[29]. In our prognosis marker, although the details of the interrelationship

among those 6 RPs are still unknown, they have the same elevation in high-risk group, indicating the concordance of their functions in gastric cancer.

To reduce the heterogeneity among patients and samples which may bring bias to the analysis in this study, samples were randomly separated into training and validation batches. And no significant difference with respect to age, sex and other clinicopathological factors was found between the two batches (Table 1). And, by comparing clinicopathological factors between the high-risk group and low-risk group predicted by ten-gene markers, there was no significant difference between the two groups except for TNM staging (Table 3). Then we compared this ten-gene prognostic marker with TNM staging system. Both ten-gene prognostic marker and TNM classification can predict survival with statistical significances in discovery and validation sample batches (Figure 2C and 3C), indicating that our prognosis set can effectively complement traditional clinicopathological staging (Figure 2B and C, Figure 3B and C). The applicability of a marker with only 10 genes also suggests its potential to be developed as the prognosis marker panel for pre-operative molecular staging from endoscopic biopsy. Further validation with large scale samples are warranted for clinical application.

In conclusion, based on the whole genome expression profiling, we found and validated a ten-gene prognostic marker for overall survival prognosis of gastric cancer patients, which may be used with the TNM staging system as a parallel and complementary approach. However, the predominance of ribosome protein genes in our molecular prognostic marker warrants further research on their roles in cancer progression.

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COMMENTS

Background

Gastric cancer is the second leading cause of cancer related death in China and worldwide. The 5-year survival rate of patients with advanced disease is very poor. Currently, treatment plan and prognosis prediction for gastric cancer mainly depend on the clinicopathological staging. However, prognosis varies among patients with the same clinical pathological stage. An individualized expression test for selected markers in biopsy and surgical samples will complement the current staging system, especially for prognosis prediction.

Research frontiers

The gene expression profiling has enabled researchers to quantify the biological states and consequently to uncover the subtle phenotypes in cancer. Such analyses have provided unique opportunities to develop various profiles that can distinguish, identify, and classify discrete subsets of disease, predict the disease outcome, and even predict the response to therapy.

Innovations and breakthroughs

In this study, based on the whole genome expression profiling, the authors identified and validated a ten-gene set that can be further developed as clinical prognosis markers to predict overall survival of gastric cancer patients. This marker set showed consistent prognosis results with the traditional Tumor-

node-metastasis (TNM) staging system. The findings in this study also provided new clues about the possible association between the deregulation of ribosome related gene expression and survival status of the patients after surgery.

Applications

Based on the whole genome expression profiling, a ten-gene prognostic marker set for overall survival prognosis of gastric cancer patients may be applied in combination with the TNM staging system as a parallel and complementary approach. However, the predominance of ribosome protein genes in these molecular prognostic markers awaits for further research on their roles in cancer progression.

Terminology

TNM: The TNM system is one of the most widely used staging systems in tumor classification. The system is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of distant metastasis (M). A number is added to each letter to indicate the size or extent of the primary tumor and the extent of cancer spread. **Principal component analysis (PCA):** A mathematical tool used to reduce the number of variables while retaining the original variability of the data. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. **Gene set enrichment analysis (GSEA):** A computational method that determines whether a prior identified set of genes shows statistically significant, concordant differences between two biological states. It is a method which focuses on the analysis at the level of functional related gene sets instead of a single gene. It helps biologists to interpret the DNA microarray data by their previous biological knowledge of the genes in a gene set. GSEA has been shown to efficiently identify gene sets containing known disease-related genes in the real experiments.

Peer review

A ten-gene prognostic marker, including 6 ribosomal proteins, for overall survival prognosis of gastric cancer were identified and validated based on whole genome expression profiling. By comparing with the traditional TNM staging system, this ten-gene prognostic marker showed consistent prognosis results, which may complement the predictive value of current TNM staging system.

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Differential expression of Bcl-2 and Bax during gastric ischemia-reperfusion of rats

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the early phases of reperfusion, reached its minimum at 1 h ($P < 0.05$); it then increased, reaching its peak at 24 h of reperfusion ($P < 0.05$). The pattern of Bax expression was opposite to that of Bcl-2. Bax expression increased after reperfusion, with its peak at 1 h of reperfusion ($P < 0.05$), and then it decreased gradually to a minimum at 24 h after reperfusion ($P < 0.05$). On the other hand, inhibition of activation of ERK1/2 induced by PD98059, a specific upstream MEK inhibitor, had significant effects on Bcl-2 and Bax in GI-R. Compared with GI-R treatment only at 3 h of reperfusion, PD98059 reduced the number of Bcl-2 positive cells (0.58% of R3h group, $P < 0.05$) and Bcl-2 protein level (74% of R3h group, $P < 0.05$) but increased the number of Bax-positive cells (1.33-fold vs R3h group, $P < 0.05$) and Bax protein level (1.35-fold of R3h group, $P < 0.05$).

CONCLUSION: These results indicated that the Bcl-2 and Bax played a pivotal role in the gastric mucosal I-R injury and repair by activation of ERK1/2.

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Key words: Stomach; Ischemia-reperfusion; Bcl-2; Bax; Extracellular signal-regulated kinase 1/2

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Abstract

AIM: To investigate expression of Bcl-2 and Bax in gastric ischemia-reperfusion (GI-R) and involvement of extracellular signal-regulated kinase (ERK) 1/2 activation.

METHODS: The GI-R model was established by ligation of the celiac artery for 30 min and reperfusion in Sprague-Dawley rats. Rats were assigned to groups in accordance with their evaluation period: control, 0, 0.5, 1, 3, 6, 24, 48, and 72 h. Expression and distribution of Bcl-2 and Bax proteins were analyzed by immunohistochemistry and western blotting in gastric tissue samples after sacrifice.

RESULTS: Compared with controls, the percentage of positive cells and protein levels of Bcl-2 decreased in

INTRODUCTION

Current research into gastric ischemia-reperfusion (GI-R)

has focused on its pathogenic and underlying molecular mechanism^[1-8]. GI-R injury and repair is related to the changes in gastric mucosal cellular apoptosis and proliferation induced by GI-R in rats. In our previous experiments, we have explored the time course of gastric mucosal apoptosis and proliferation induced by GI-R, and the role of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathway in GI-R-induced gastric mucosal injury and repair^[7]. We have found that serious gastric mucosal damage occurs rapidly at the early stage of reperfusion and is closely related to the suppression of ERK1/2 activation. The activity of ERK1/2 increases as the time of reperfusion is extended, and the activated ERK1/2 might inhibit apoptosis and promote proliferation in gastric mucosal cells. However, the precise mechanisms by which activated ERK1/2 accomplishes gastric mucosal apoptosis and proliferation are unknown.

The balance between apoptosis and cellular proliferation is a key in gastric injury and repair, and is regulated by several genes, including p53 and members of the Bcl-2 family such as Bax and Bcl-2^[9-11]. The Bax gene is a proliferative suppressor gene that encodes Bax protein that promotes apoptosis. On the other hand, bcl gene encodes Bcl-2 protein that blocks wild type p53-mediated apoptosis, and heterodimers with Bax, antagonizing the function of Bax^[12].

Therefore, it is conceivable that increased cellular apoptosis and proliferation, because of altered expression of the regulating proteins such as Bax and Bcl-2, may be associated with gastric injury and repair induced by GI-R. Although Bcl-2 and Bax are expressed in gastric mucosa, their presence in normal gastric mucosa is controversial. Liu *et al.*^[13] have reported that Bcl-1 mRNA and protein are expressed in the gastric gland zone at a middle level and Bax protein is expressed in the epithelial cells of normal gastric mucosa. Xia *et al.*^[14] have found that, in intact gastric tissue, Bcl-2 and Bax are localized predominantly in the glandular base region in chief cells in normal rat gastric mucosa. However, a conflicting study has found that no expression of Bcl-2 protein is detected in the glandular epithelium of normal gastric mucosa^[15]. On the other hand, Bcl-2 and Bax show significant changes in many conditions including gastric cancer, gastritis, and GI-R^[15-18]. El Eter *et al.*^[15] have reported that cytoplasmic expression of Bcl-2 protein is observed in the superficial portion of gastric mucosa sections obtained from rats subjected to GI-R injury.

The available data on expression of Bcl-2 and Bax proteins in the stomach, and their relation to apoptosis of gastric mucosal cells, seem equivocal, thus, a further study of Bcl-2 and Bax expression in the stomach is clearly important. In the present study, we used an immunohistochemical assay and western blotting to determine the changed courses of Bcl-2 and Bax at different reperfusion durations after GI-R, and whether ERK1/2 activation was involved in this process.

MATERIALS AND METHODS

Animals

Groups of six adult Sprague-Dawley rats, regardless of

sex, weighing 220-270 g, were provided by the Experimental Animal Centre of Xuzhou Medical College. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Rats were housed under controlled temperature (22-24°C) and photoperiod (12 h light/12 h dark), and allowed food and water *ad libitum*. Rats were fasted for 24 h before the experiment, but were allowed free access to tap water. Animals were randomly assigned to groups: GI-R (different reperfusion time points after 30 min of ischemia); PD98059 + R3h (PD98059 + reperfusion for 3 h after 30 min of ischemia); and vehicle control (PD98059 replaced with vehicle but otherwise the same as PD98059 + R3h). PD98059 was given 20 min before operation [150 µg/kg, administered intraperitoneally (i.p.), dissolved in dimethyl sulfoxide]. A sham group in which only the same surgical procedure without clamping the celiac artery was performed served as a control.

Reagents

PowerVision™ two-step immunohistochemistry detection kit were purchased from Zhongshan Biotech Co. (Beijing, China), anti-Bcl-2 and anti-Bax polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), alkaline-phosphorylase-tagged goat anti-rat IgG antibody, PD98059 was from Promega (Madison, WI, USA), and sodium pentobarbital was purchased from Sigma (St. Louis, MO, USA).

Preparation of GI-R model

GI-R models were induced according to the method of Qiao *et al.*^[7]. The randomly grouped rats were all anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Their abdomens were incised along the midline, and the celiac artery and its adjacent tissues were carefully isolated. The celiac artery was clamped with a small non-traumatic vascular clamp for 30 min to induce gastric ischemia and then released for 0, 0.5, 1, 3, 6, 24, 48 and 72 h to allow reperfusion. Following reperfusion, the rats were sacrificed and the stomachs were removed immediately. The stomachs were incised along the greater curvature and flushed with ice-cold PBS (0.1 mol/L). One half of the gastric mucosa was frozen at -80°C for western blotting, and the other was fixed in Bouin's fixative for immunohistochemical staining.

Immunohistochemical staining

The fixed stomach was embedded in paraffin, sliced into 4-µm-thick sections, and mounted on glass slides. The immunohistochemistry was performed with a PowerVision two-step immunohistochemistry detection kit. The sections were stained with 3,3'-diaminobenzidine (DAB), then counterstained using hematoxylin. The sections were examined with a microscope (Model IX71; Olympus, Tokyo, Japan). Gastric mucosal cells with brown granules visible in the cytoplasm or nucleus were considered positive. The number of positive cells per section was counted in 10 random lower-power (× 10) fields, and the percentage

of positive cells (positive cells/total cells \times 100%) was calculated. Three non-consecutive sections were selected from each specimen and those indexes were averaged.

Western blotting

The frozen gastric mucosa was homogenized with a Teflon glass homogenizer in 1:10 (w/v) ice-cold homogenization buffer consisting of 50 mmol/L 3-(N-morpholino) propanesulfonic acid (MOPS, pH 7.4), 50 mmol/L NaF, 20 mmol/L sodium pyrophosphate (NaPPi), 20 mmol/L b-glycerophosphate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulphonyl fluoride, 10 mg/mL leupeptin, 10 mg/mL aprotinin and 10 mg/mL pepstatin A. The homogenate was centrifuged at 800 *g* for 15 min at 4°C, and the supernatant was retained as cytoplasmic parts. Protein concentrations were determined by Coomassie brilliant blue protein assay. The proteins were heated at 100°C for 5 min with loading buffer containing 0.125 mol/L Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% mercaptoethanol and 0.002% bromophenol blue, then separated by 10% SDS-PAGE. The proteins were isolated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were incubated with 4% bovine serum albumin in TBST (10 mmol/L Tris, pH 7.5; 150 mmol/L NaCl, 0.05% Tween-20) at 4°C for 6 h and probed with primary antibodies (anti-Bcl-2 polyclonal antibody 1:500, anti-Bax polyclonal antibody 1:400) at 4°C overnight. Membranes were rinsed and incubated with secondary antibody for 2 h and were detected with an NBT/BCIP assay kit. After immunoblotting, the bands were scanned and analyzed by Image J software. The optical density (OD) of the band in each lane was expressed as the fold change versus the OD of the sham control.

Statistical analysis

All results are presented as mean \pm SD. Comparisons between two groups were made with Student's *t* test; multiple-group analyses were made by one-way ANOVA. Statistical analyses were performed with SPSS for Windows version 11.5. *P* < 0.05 was considered statistically significant.

RESULTS

Quantitative changes in Bcl-2 and Bax positive cells of the gastric mucosa for different reperfusion durations after ischemia

Immunohistochemical staining clearly showed that Bcl-2 and Bax were expressed and limited to the cytosol of the cells in the gastric mucosa (Figure 1). Bcl-2 expressing cells were found predominantly in the lower part of the gastric gland, and were present only in gland cells of the stomach fundus (Figure 1A-D). Bax positive cellular distribution was similar to that of Bcl-2, with prominent expression in the base, but staining for Bax was also noticeable in the cells of the pit. Bax appeared to be absent from the middle part of the gastric gland, and weak expression of Bax was detected in most cells of the gastric mucosa (Figure 1E-H). The control sample (sham-operated) obtained prior to the ischemic period showed a

normal appearance of Bcl-2 (Figure 1A and B) and Bax (Figure 1E and F), and there were significant differences between the GI-R (Figure 1C, D, G and H) and control groups (Figure 1A and E) in the quantities of Bcl-2 and Bax immunoreactive cells.

The percentage of Bcl-2 and Bax positive cells in various groups is shown in Figure 2. Bcl-2 and Bax positive cells decreased in the early phase of reperfusion, with a nadir (10.02% \pm 1.21%) at 1 h of reperfusion, then increased significantly after reperfusion for 3 h, with a peak (29.76% \pm 3.32%) at 24 h of reperfusion, and returned to near the base level (20.47% \pm 2.97%) at 72 h of reperfusion. The opposite pattern was observed for Bax positive cells. The percentage of Bax positive cells increased in the initial stages of reperfusion, reached the highest Bax positive cell count (49.34% \pm 3.83%) at 1 h of reperfusion, then decreased gradually, with its nadir (13.36% \pm 3.05%) at 24 h of reperfusion, and recovered to base level (24.94% \pm 2.83%) at 72 h of reperfusion.

Protein expression of Bcl-2 and Bax in gastric mucosa at different reperfusion durations after ischemia

Figure 3 shows the Bcl-2 and Bax protein levels in the gastric mucosa in different groups of the study. After reperfusion, expression of Bcl-2 protein was significantly lower than that of the controls, and the lowest level was observed at 1 h of reperfusion (0.59% of sham group, *P* < 0.05). A peak of Bcl-2 protein expression was displayed in the 24 h reperfusion group (1.36 fold *vs* sham group, *P* < 0.05). The Bax protein level increased in the early stage of reperfusion, reached its peak (1.62-fold *vs* sham group, *P* < 0.05) at 1 h of reperfusion, and then decreased gradually to its lowest levels (0.57% of sham group, *P* < 0.05) at 24 h of reperfusion. At 72 h of reperfusion, Bcl-2 and Bax protein levels were the same as that of the control group.

Effects of PD98059 on expression of Bcl-2 and Bax

PD98059 is a specific upstream inhibitor of ERK1/2. By immunohistochemical assay, we found PD98059 had significant effects on Bcl-2 and Bax expression in GI-R. Compared with the control group (R3h group), the PD98059 + R3h group showed a fall in the number of Bcl-2 positive cells (0.58% of R3h group, *P* < 0.05) but an increase in Bax positive cells (1.31-fold *vs* R3h group, *P* < 0.05) (Figure 4). To ascertain the effect of PD98059 on expression levels of Bcl-2 and Bax protein, western blotting was performed. In the PD98059 + R3h group, gastric mucosal Bcl-2 protein level was 74% of that in the R3h group (*P* < 0.05), whereas Bax protein level was 1.35-fold more than that in the R3h group (*P* < 0.05) (Figure 5).

DISCUSSION

Previous studies have shown that many stress conditions, such as hemorrhagic shock, burns, sepsis, major surgery, ischemia and trauma can lead to GI-R injury. In recent years, studies on GI-R injury have revealed that reactive

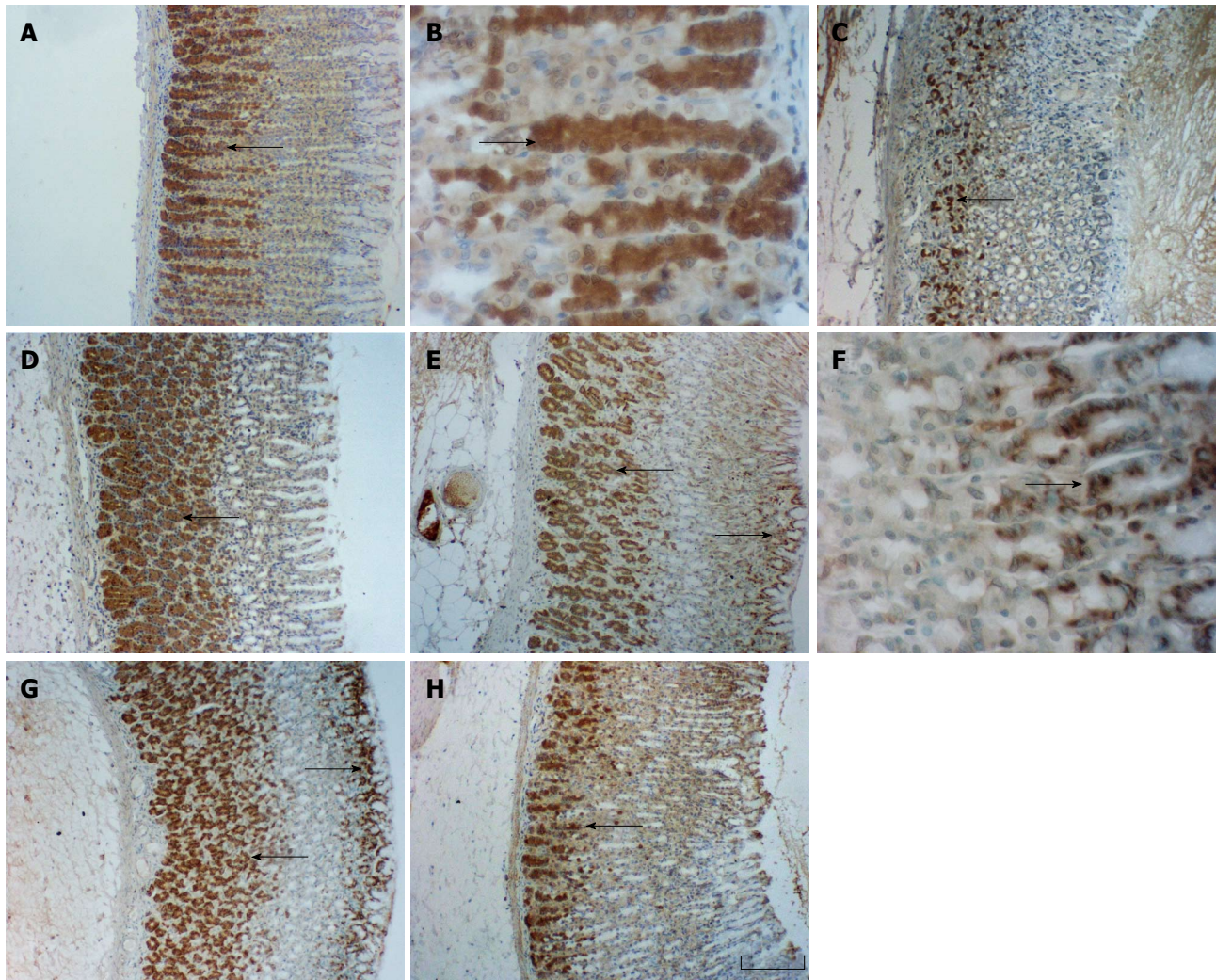


Figure 1 Histological exhibition of Bcl-2 and Bax positive cells in the gastric mucosa at different reperfusion times after ischemia, by immunohistochemical staining in rats. The Bcl-2 and Bax positive cells were respectively probed with anti-Bcl-2 and anti-Bax polyclonal antibodies in rat gastric mucosa. Nuclear counterstaining was performed with hematoxylin. The examples of immunoreactive cells are those with dark brown staining in their cytosol (arrows). A and B: Bcl-2, control; C: Bcl-2, GI-R at 1 h after reperfusion; D: Bcl-2, GI-R at 24 h after reperfusion; E and F: Bax, control; G: Bax, GI-R at 1 h after reperfusion; H: Bax, GI-R at 24 h after reperfusion. Images were obtained at $\times 100$ (A, C, D, E, G and H, Bar 100 μm) and $\times 400$ (B and F, Bar 400 μm).

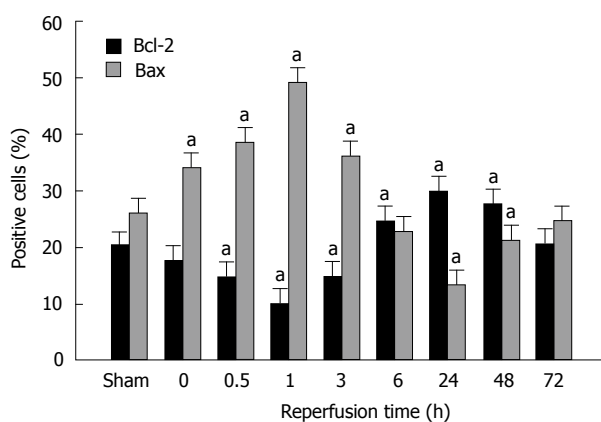


Figure 2 Quantitative changes in Bcl-2 and Bax positive cells in rat gastric mucosa for different reperfusion times after GI-R. Reperfusion was maintained for 0, 0.5, 1, 3, 6, 24, 48 and 72 h after 30 min of ischemia. Sham: sham-operated. Values are percentage of positive cells (positive cells/total cells) counted in 10 microscopic fields. Each column represents mean \pm SD, $n = 6$. ^a $P < 0.05$ vs sham.

oxygen species, endothelin, microvascular dysfunction, polymorphonuclear leukocyte infiltration, nitric oxide release, gastric acid secretion and decreased prostaglandin concentrations may play a role in the pathogenesis of gastric mucosal injury induced by GI-R^[1,5,19-25]. Although the gastric mucosa is vulnerable to damage by various factors, it can quickly repair the damage^[26]. Mucosal integrity is maintained by a balance between proliferation and apoptosis of the gastric mucosal cells. To understand better the causes of gastric lesions, it is important to study the imbalance between proliferation and apoptosis^[27,28].

In previous experiments^[7], we have shown the changed courses of gastric mucosal injury and repair induced by GI-R, and the role of ERK1/2 in this process. Our results indicated clearly that the gastric mucosal injury induced by GI-R was mainly the result of reperfusion. The serious gastric mucosal lesions occurred in the initial stages of reperfusion and the aggravating processes of mucosal lesions were at 1 h after reperfusion, which were main-

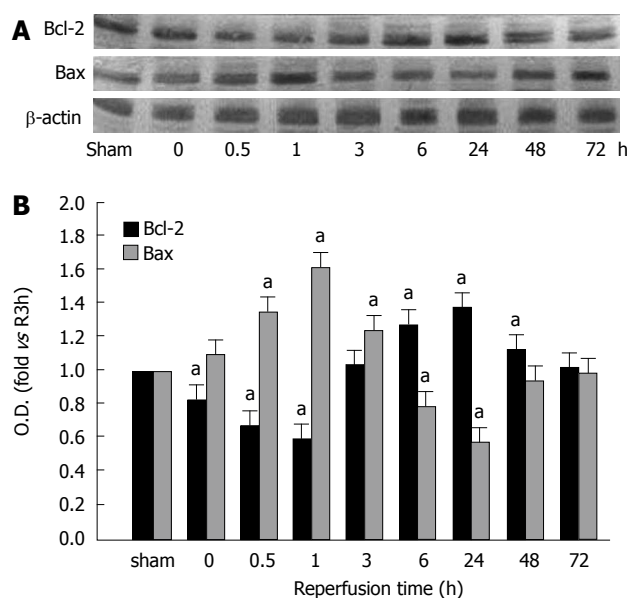


Figure 3 Expression of Bcl-2 and Bax proteins in cytoplasm extracts from rat gastric mucosa for different reperfusion durations after gastric ischemia-reperfusion. Extracts were obtained from sham-operated rats or from gastric ischemia-reperfusion rats with different reperfusion durations (0, 0.5, 1, 3, 6, 24, 48, or 72 h) after 30 min of ischemia, and were analyzed respectively by western blotting with anti-Bcl-2 and anti-Bax antibodies. A: Representative blots corresponding to expression levels of Bcl-2 and Bax proteins; B: Semi-quantitative analysis of the levels of Bcl-2 and Bax. Sham: Sham-operated. Values are means \pm SD, $n = 6$. $^aP < 0.05$ vs sham.

tained about 3 h after reperfusion. The gastric mucosal repairs started after 3 h of reperfusion, and the complete recovery took almost 3 d. Based on these facts, it indicated that gastric mucosa has an amazing self-repairing ability. ERK1/2 are important members of the mitogen-activated protein kinase family. The activation of ERK1/2 participates in the regulation of cellular injury and repair in many tissues. Our researches have also shown that the p-ERK1/2 protein level decreased at 0.5 h after reperfusion began, and then gradually increased, reaching its peak after 3 h of reperfusion. Inhibition of the activation of ERK1/2 aggravated the gastric mucosal injury, with apoptosis increased and proliferation reduced in the gastric mucosal cells at the same duration of reperfusion. Therefore, activated ERK1/2 inhibited apoptosis and promoted proliferation in gastric mucosal cells.

Apoptosis and proliferation are fundamental mechanisms for cell death and survival and differentiation in the gastric mucosa. The status of the Bcl-2 family proteins determines whether a cell will live or die through the regulation of cytochrome c release from the mitochondria^[29,30]. Bcl-2 protein mainly inhibits apoptosis and facilitates cellular survival and differentiation, whereas overexpression of Bax protein induces apoptosis and inhibits the effect of Bcl-2^[31-33]. Our data showed that Bcl-2 expression decreased significantly after the start of the reperfusion, reaching its nadir at 1 h, before increasing gradually to a peak after 24 h of reperfusion. The pattern of change in Bax expression was opposite to that of Bcl-2 expression. Bax expression increased at first, reaching its maximum

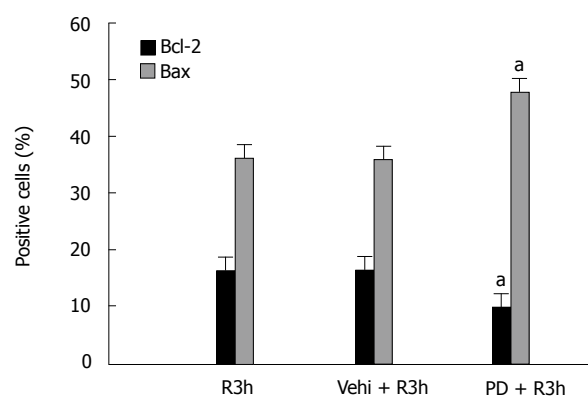


Figure 4 Effects of PD98059 (ERK1/2 inhibitor) on quantitative changes of Bcl-2 and Bax positive cells in rat gastric mucosa after gastric ischemia-reperfusion. R3h: Reperfusion for 3 h after 30 min of ischemia; Vehi + R3h: Vehicle + R3h; PD + R3h: PD98059 + R3h; Sham: Sham-operated. Values are percentage of positive cells (positive cells/total cells) counted in 10 microscopic fields. Each column represents mean \pm SD, $n = 6$. $^aP < 0.05$ vs R3h.

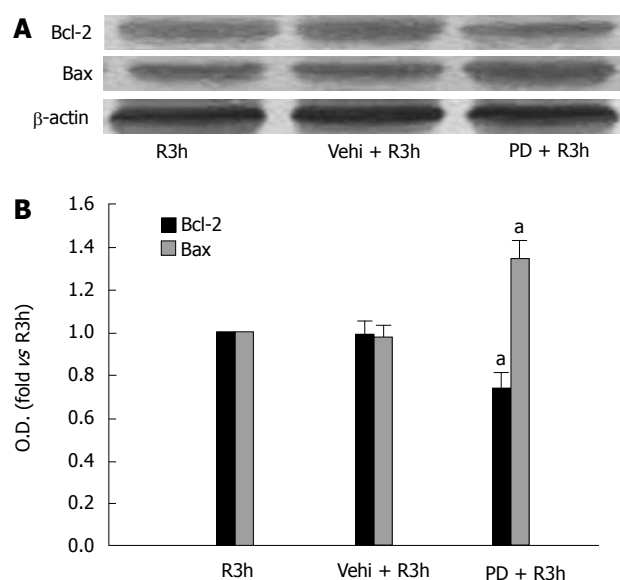


Figure 5 Effects of PD98059 (ERK1/2 inhibitor) on expression of Bcl-2 and Bax proteins in cytoplasm extracts from rat gastric mucosa after gastric ischemia-reperfusion. R3h: Reperfusion for 3 h after 30 min of ischemia; Vehi + R3h: Vehicle + R3h; PD + R3h: PD98059 + R3h. Extracts were obtained for analysis by western blotting with anti-Bcl-2 and anti-Bax antibodies. A: Representative blots corresponding to expression levels of Bcl-2 and Bax proteins; B: Semi-quantitative analysis of the levels of Bcl-2 and Bax. Values are means \pm SD, $n = 6$. $^aP < 0.05$ vs R3h.

after 1 h of reperfusion, and then decreased. Bcl-2 and Bax recovered gradually to base level at 72 h of reperfusion. PD98059, a specific upstream inhibitor of ERK1/2, downregulated expression of Bcl-2 and upregulated expression of Bax in GI-R. These results suggest that the course of expression of Bcl-2 and Bax were closely correlated with p-ERK1/2. Activation of ERK1/2 causes upregulation of Bcl-2 and downregulation of Bax.

In conclusion, Bcl-2 and Bax played a pivotal role in GI-R injury and repair by activation of ERK1/2. Bcl-2 was involved in recovery of GI-R-mediated gastric mucosa injury by promoting cellular proliferation, and Bax

was involved in gastric mucosal injury induced by GI-R by promoting apoptosis.

COMMENTS

Background

It is well known that many hemorrhagic and stress conditions lead to gastric ischemia-reperfusion (GI-R) injury. Gastric ulceration is very prevalent in humans and is usually preceded by burns, sepsis, major surgery, ischemia, trauma and other heterogeneous forms of stress. Erosions in the gastric mucosa can be demonstrated in as many as 75%-100% of patients within 24 h of admission to the intensive care unit (ICU). Clinically apparent gastrointestinal bleeding can occur in as many as 25% of ICU patients.

Research frontiers

In recent years, studies on GI-R injury have focused on its pathogenic and underlying molecular mechanism. In recent years, studies on GI-R injury have revealed that reactive oxygen species, endothelin, microvascular dysfunction, polymorphonuclear leukocyte infiltration, nitric oxide release, gastric acid secretion and decreased prostaglandin concentrations during reperfusion may play a role in the pathogenesis of gastric mucosal injury induced by GI-R. Mucosal integrity is maintained by the equilibrium between proliferation and apoptosis of the gastric mucosal cells. To understand better the pathogenesis of gastric lesions, it is of great importance to study the imbalance between proliferation and apoptosis.

Innovations and breakthroughs

This is believed to be the first study to investigate changes in expression of Bcl-2 and Bax at different times of reperfusion after gastric ischemia, and whether extracellular signal-regulated kinase 1/2 activation was involved in this process.

Applications

Not only does our study provide insights into the mechanism of gastric mucosal tissue injury and repair; it also provides information that could potentially guide development of a new therapeutic strategy.

Peer review

The quality of the paper is excellent and deserves a fast publication, considering the contribution importance.

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Intrahepatic natural killer T cell populations are increased in human hepatic steatosis

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Author contributions: Poles M was responsible for study concept design direction, supervision, formulating the hypothesis and obtaining funding; Fielding G and Fielding C were responsible for obtaining the liver samples during bariatric surgery; Adler M enrolled patients, performed flow cytometry and drafted the manuscript; Taylor S enrolled patients and performed flow cytometry; Okebugwu K performed flow cytometry; Yee H reviewed the liver biopsies for steatosis.

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study. Subjects with moderate or severe steatosis had a higher percentage of intrahepatic CD3+/CD56+ NKT cells (38.6%) than did patients with mild steatosis (24.1%, $P = 0.05$) or those without steatosis (21.5%, $P = 0.03$). Patients with moderate to severe steatosis also had a higher percentage of NKT cells in the blood (12.3%) as compared to patients with mild steatosis (2.5% $P = 0.02$) and those without steatosis (5.1%, $P = 0.05$).

CONCLUSION: NKT cells are significantly increased in the liver and blood of patients with moderate to severe steatosis and support the role of NKT cells in NAFLD.

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Key words: Nonalcoholic fatty liver disease; Natural killer T cells; Natural killer T-like cells; Lymphocytes; Hepatic steatosis

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Abstract

AIM: To determine if natural killer T cell (NKT) populations are affected in nonalcoholic fatty liver disease (NAFLD).

METHODS: Patients undergoing bariatric surgery underwent liver biopsy and blood sampling during surgery. The biopsy was assessed for steatosis and immunocyte infiltration. Intrahepatic lymphocytes (IHLs) were isolated from the remainder of the liver biopsy, and peripheral blood mononuclear cells (PBMCs) were isolated from the blood. Expression of surface proteins on both IHLs and PBMCs were quantified using flow cytometry.

RESULTS: Twenty-seven subjects participated in this

INTRODUCTION

With the epidemic of obesity burgeoning across much of the world, nonalcoholic fatty liver disease (NAFLD) has become an increasingly pressing problem. The prevalence of NAFLD in western countries is as high as 17%-33%^[1], and the more severe form of NAFLD, non-alcoholic steatohepatitis (NASH), will progress to cirrhosis in 20% of patients^[1]. Due to its increasing prevalence, NAFLD has become the third leading indication for liver transplantation^[2].

The etiopathogenesis of NAFLD/NASH involves a number of environmental, genetic, and inflammatory influences. However, many of the factors that play a role in the development of NAFLD and NASH remain unknown. What is clear is that NASH is associated with hepatic infiltration of inflammatory cells, resulting in hepatocyte injury and hepatocyte death. The prevailing two-hit theory, posited by Day *et al*^[3], proposes an initial hit whereby obesity is associated with hepatic accumulation of free fatty acids and triglycerides, followed by a second hit, whereby oxidative stress, mitochondrial dysfunction, elaboration of pro-inflammatory cytokines and inflammatory cell infiltration leads to development of NASH.

Natural killer T (NKT) cells are a highly conserved subset of lymphocytes with properties of both T cells (CD3+ expression) and NK cells (CD56+ and CD161+ expression)^[4] and have been implicated in NAFLD. NKT cells are concentrated in the liver where they serve an important role in innate immunity. In the murine liver, NKT cells comprise 30%-50% of all hepatic lymphocytes^[5]. These cells can be directly cytotoxic *via* FasL-dependent and perforin-mediated mechanisms, but also produce an array of cytokines that direct cytokine secretion by other cells within their microenvironment^[5]. These functions may be responsible for cell death seen in NAFLD. NKT cells are believed to be primarily stimulated by various glycolipids, which are presented by CD1d, an MHC-like molecule on antigen presenting cells, such as Kupffer cells, to the NKT cells' invariant T cell receptor^[6]. The role of NKT cells in immunity has yet to be fully elucidated and there have been many proposed functions for this unique cell, ranging from antitumor activity to autoimmune diseases^[7]. In addition, murine models of obesity and fatty liver disease, using leptin-deficient, *ob/ob* mice, have suggested that NAFLD is associated with depletion of NKT cells^[8]. The loss of CD4-expressing NKT cells is particularly intriguing as this cell subset is believed to primarily secrete Th2-type cytokines, including IL-4 and IL-13^[9]. This loss of Th2 cytokines might tip the inflammatory milieu of the liver into a pro-inflammatory Th1 state, leading to excessive production of TNF- α and IFN- γ . The increase in pro-inflammatory cytokines likely plays a role in hepatic oxidative stress and recruitment of additional inflammatory cells into the liver, resulting in NASH^[10]. The transfer of NKT lymphocytes back into leptin deficient mice has been shown to reduce hepatic steatosis and improves glucose intolerance^[11]. In addition, inducing expansion of the NKT cell population, by norepinephrine injection or by stimulation with glucocerebroside, has also been shown to reduce hepatotoxicity and improve hepatic fat content in murine models^[12,13].

While murine models of NAFLD clearly support a pivotal role of NKT cells in pathogenesis, data on the role of NKT cells in human NAFLD is limited. Xu and colleagues found that peripheral blood NKT cells are depleted in patients with clinically diagnosed NAFLD^[14]. Three other studies evaluated intrahepatic NKT cells and had differing results. The study by Kremer *et al*^[15] found that NKT cells

are depleted with increased steatosis, whereas the one by Tajiri and colleagues found an increase in NKT cells with steatosis^[16]. Finally a study by Syn *et al*^[17] also found an increase in NKT cells with steatosis. In this study, we sought to further investigate the changes in lymphocyte populations that occur in NAFLD.

MATERIALS AND METHODS

Patients and lymphocyte isolation

From January to November 2007, peripheral blood and hepatic tissue were collected from obese subjects undergoing laparoscopic gastric banding surgery. Patients were excluded if they were under the age of 18, infected with hepatitis B virus, hepatitis C virus, HIV, were known to have pre-existing hepatic disease, or found to have any non-NAFLD pathological processes found on histological examination of the liver biopsy material. Patients were also excluded if they had a known history of excessive alcohol ingestion. All enrolled subjects signed an informed consent form that was approved by the institutional review board of NYU Langone Medical Center.

Immediately prior to surgery, 10 mL of blood was obtained from each subject by venipuncture. During the surgery a 2 cm³ liver scissor biopsy was obtained. The liver biopsy sample was placed in 15cc of sterile RPMI 1640 (Mediatech Inc, Herndon, VA) and was transported to the laboratory with the blood sample for lymphocyte isolation. An additional portion of the biopsy was evaluated by a single hepatopathologist who made the diagnosis of NAFLD and NASH using the staging system proposed by Brunt *et al*^[18]. Mild steatosis was defined as steatosis involving up to 33% of hepatocytes, moderate steatosis involved 33%-66% of hepatocytes, and severe steatosis involved greater than 66% of hepatocytes. Steatohepatitis was defined by a number of features including steatosis, ballooning, and acinar and portal inflammation.

Once transported to the laboratory, the liver biopsy sample was washed in sterile phosphate-buffered saline (PBS) and was minced to 1 mm³ pieces in a petri dish with 30 mL of RPMI 1640 containing 0.5 mg/mL collagenase type II (Clostridiopeptidase A), 0.02 mg/mL DNase I, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L L-glutamine (all from Sigma-Aldrich, St. Louis, MO) and 10% fetal calf serum (FCS) (Invitrogen, Carlsbad CA). The minced liver was incubated in this digestion solution at 37°C for 30 min after which it was strained through a 70 mm disposable plastic strainer. Immediately after isolation, cells were washed and re-suspended in PBS. The cell solution was then pipetted onto a 30%-70% percoll (Sigma-Aldrich, St. Louis MO) gradient and was centrifuged at 2000 r/min for 30 min. The isolated lymphocytes were removed from the gradient, washed with PBS, resuspended in 1 mL PBS/10% FCS containing 2% formaldehyde (Sigma-Aldrich, St. Louis MO) and placed at 4°C. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation on a Ficoll-Hypaque density gradient (Mediatech, Herndon, VA).

Table 1 Patient characteristics

	No. steatosis (<i>n</i> = 10)	Mild steatosis (<i>n</i> = 11)	Moderate- severe steatosis (<i>n</i> = 6)	Reference values
Age	36.3 ± 13.1	44.5 ± 14.4	48.3 ± 11.3	N/A
Gender (% female)	90%	73%	66%	N/A
Body mass index (kg/m ²)	42.4 ± 3.2	44.2 ± 6.2	41.9 ± 4.3	18.5-24.9
Aspartate transaminase (U/L)	28.8 ± 13.2	42.8 ± 34.2	47.0 ± 26.9	0-40
Alanine transaminase (U/L)	36.1 ± 18.7	50.1 ± 48.2	75.8 ± 40.1	0-45
Alkaline phosphatase (IU/L)	79.2 ± 18.0	89.5 ± 16.6	82.5 ± 12.7	20-140

All results except gender presented as mean ± standard deviation.

Flow cytometric analysis of lymphocyte populations

Cell surface expression of lymphocyte antigens was identified by monoclonal antibody staining of freshly isolated IHLs and PBMcs, followed by flow cytometry using a BD LSR II (Becton Dickinson Immunocytometry Systems (BDIS), Mountain View CA) flow cytometer with analysis using CellQuest[®] software (BDIS, Mountain View CA). Monoclonal antibodies used in this study included anti-human CD3 (clone UCHT1) (BDIS, Mountain View, CA), anti-human CD4 (clone RPA T4) (Pharmingen, San Diego, CA), anti-human CD8 (clone RPA T8) (Pharmingen, San Diego, CA), anti-human CD56 (clone NCAM16.2) (Pharmingen, San Diego, CA), anti-human CD161 (clone DX12) (Pharmingen, San Diego, CA), anti-human α 24 (clone C15) (Immunotech, Fullerton, CA), and the appropriate isotype controls. During flow cytometry, lymphocytes, initially identified by their forward and side scatter characteristics, were subject to phenotypic analysis. Dead cells were excluded from analysis using 7-aminoactinomycin D (Calbiochem, La Jolla, CA).

ELISA for quantification of IFN- γ and IL-4 secretion

IFN- γ and IL-4 secretion by intrahepatic and peripheral blood lymphocytes was determined by ELISA (BD Pharmingen) after culture for 12 h. For these assays, 1×10^5 lymphocytes derived from the liver or blood were co-cultured with monocyte-derived macrophages in the presence of alpha-galactosyl ceramide at 10 μ g/mL in a 96-well flat-bottom plate.

Statistical analysis

Values are expressed as mean ± SD. Statistical comparisons were made between PBMcs and IHLs from individuals using a paired *t*-test. Statistical comparisons were made between subjects without hepatic steatosis, those with mild steatosis and those with moderate-to-severe steatosis using a two-sample, unequal variance *t*-test. All reported *P* values were two-sided at the 0.05 significance level using SPSS[™] 11.0 for Windows software (SPSS, Chicago, IL).

RESULTS

Subject cohort characteristics

Table 1 describes the clinical characteristics of the 27 patients enrolled in this study. Ten of the twenty-seven subjects (37%) had normal liver biopsies, without steatosis, while 11 of 27 (41%) had mild hepatic steatosis and 6 of 27 (22%) had moderate-severe hepatic steatosis. Of the patients with mild steatosis, 10 had increased hepatic lymphocyte infiltration, but were not felt to be severe enough to merit a diagnosis of NASH. One of the six subjects with moderate-severe steatosis had grade 3 steatohepatitis with grade 1 fibrosis. Seventy-eight percent of the subjects were female, their mean age was 42 years, and their mean body mass index (BMI) was 42.9. There were no significant differences between patient cohorts with regard to age, gender, BMI, or serum levels of liver-associated enzymes.

Moderate-to-severe hepatic steatosis is associated with increased percentages of intrahepatic and blood NKT cells

Using the most common phenotypic definition of NKT cells, we sought to compare the percentage of CD3+/CD56+ in the liver and the periphery of subjects without hepatic steatosis with those with moderate-to-severe steatosis. As shown in Table 2, the liver and blood of subjects with steatosis had significant increases in the percentage of NKT cells. CD3+/CD56+ NKT cells comprised $38.6\% \pm 10.5\%$ of all intrahepatic T cells of subjects with moderate-to-severe steatosis, compared to $21.5\% \pm 14.3\%$ T cells in the liver of subjects without any steatosis (*P* = 0.03). The percentage of CD3+/CD56+ T cells in the liver of subjects with mild steatosis ($24.1\% \pm 12.4\%$) was intermediate between that of normal and moderate-to-severe steatosis, and was significantly lower than that of the subjects with moderate-to-severe steatosis (*P* = 0.05) with a correlation of 0.93. While in all three subject cohorts, the percentage of CD3+/CD56+ cells was significantly lower in the blood compared to the liver, the percentage PBMC CD3+/CD56+ NKT cells of subjects with moderate-to-severe steatosis ($12.3\% \pm 5.6\%$) was significantly greater than both subjects with no steatosis ($5.1\% \pm 5.5\%$, *P* = 0.05) and those with mild steatosis ($2.5\% \pm 1.5\%$, *P* = 0.02).

Percentage of invariant α 24 NKT cells in patients with steatosis and IFN- γ and IL-4 expression

We also analyzed invariant NKT cells; the CD-1d-reactive, glycolipid-activating NKT cells which express α 24^[7]. We found that a minority of CD3+/CD56+ NKT cells express α 24. In addition, we did not find significant differences in expression of α 24 between subjects without steatosis, those with mild or those with moderate-to-severe steatosis in the liver or blood (Table 2). When stimulated by alpha-galactosylceramide, the prototypical stimulant of α 24, invariant NKT cells, hepatic-derived lymphocytes produced greater amounts of IFN- γ , as measured

Table 2 Percentage of CD3+ lymphocyte populations in patients with normal livers, mild hepatic steatosis, and moderate to severe steatosis *n* (%)

Lymphocyte population	N steatosis	M steatosis	MS steatosis	<i>P</i> value (N <i>vs</i> M)	<i>P</i> value (N <i>vs</i> MS)	<i>P</i> value (M <i>vs</i> MS)	Correlation coefficient
CD3+/CD4-/CD8- PBMC	8.13	3.62	22.88	0.22	0.1	0.04 ^a	0.73
CD3+/CD4-/CD8- IHL	12.61	9.12	26.58	0.4	0.1	0.05 ^a	0.76
CD3+/CD56+ PBMC	5.09	2.45	12.32	0.22	0.049 ^a	0.016 ^a	0.71
CD3+/CD56+ IHL	21.49	24.13	38.62	0.7	0.03 ^a	0.048 ^a	0.93
CD3+/CD56+/CD161+ PBMC	2.45	1.15	9.64	0.3	0.027 ^a	0.017 ^a	0.79
CD3+/CD56+/CD161+ IHL	15.50	18.90	35.81	0.6	0.006 ^a	0.017 ^a	0.93
CD3+/Vα24+ PBMC	0.60	0.53	0.57	0.48	0.23	0.14	-0.43
CD3+/Vα24+ IHL	0.43	0.42	0.76	0.9	0.37	0.36	0.85
CD3+/CD8+ IHL	55.59	49.30	26.58	0.51	0.0003 ^a	0.006 ^a	-0.95

Each percentage is the proportion of a specific CD3+ lymphocyte population out of all CD3+ lymphocytes. ^a*P* < 0.05. PBMC: Peripheral blood mononuclear cell; IHL: Intrahepatic lymphocyte. N: Normal; M: Mild; MS: Mod/sev.

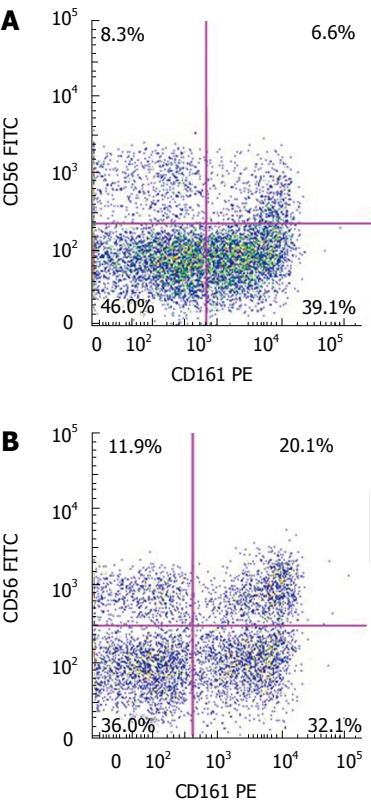


Figure 1 Flow cytometry of CD3+/CD56+/CD161+ intrahepatic lymphocytes in a patient with a normal liver versus a patient with moderate steatosis. Cells were initially selected via CD3+ gating prior to analysis for expression of CD56 and CD161. There are a greater percentage of natural killer T cells (CD56+/CD161+) in patients with moderate steatosis (20.1%) as compared to patients with normal livers (6.6%). A: Normal liver; B: Moderate steatosis.

by ELISA, compared to peripheral lymphocytes, though no differences were noted between patient cohorts (data not shown). In the majority of samples, IL-4 secretion remained undetectable.

Expression of CD161+ on NKT cells is increased in patients with moderate to severe steatosis

CD161 (NKR-P1A) is a receptor that is primarily associ-

ated with NK cells, but is also expressed on NKT cells, and may indicate an effector and memory subset of such cells^[19]. We therefore assessed the expression of CD161 on the CD3+/CD56+ populations in the liver and blood (Figure 1). Again, in each cohort, there were a higher percentage of CD3+/CD56+ cells that expressed CD161 in the liver, compared to the blood (Table 2). Further, the percentage of CD161-expressing CD3+/CD56+ cells in the liver ($35.8\% \pm 9.1\%$) and blood ($9.6\% \pm 4.9\%$) of subjects with moderate-to-severe hepatic steatosis were significantly increased compared to those without steatosis (liver: $15.5\% \pm 12.6\%$, *P* = 0.01, blood: $2.5\% \pm 3.8\%$, *P* = 0.03) and those with mild hepatic steatosis (liver: $18.9\% \pm 12.5\%$, *P* = 0.02, blood: $1.2\% \pm 1.1\%$, *P* = 0.02).

Moderate-to-severe steatosis alters the percentages of non NKT cell lymphocyte population

In addition to increases in the percentages of NKT cells, other minor lymphocyte subsets were significantly affected in patients with moderate-to-severe hepatic steatosis. Intrahepatic percentages of double negative T cells (CD3+, CD4-, CD8-) were increased in the liver of subjects with moderate-severe steatosis ($26.6\% \pm 17.0\%$), compared to those without steatosis [$12.6\% \pm 10.4\%$, *P* = 0.05 (Table 2)].

The CD3+/CD8+ lymphocytes were the only lymphocyte population found to significantly decrease in patients with moderate-to-severe steatosis. In these patients, the percentage of CD3+/CD8+ lymphocytes ($27.3\% \pm 9.6\%$) decreased significantly as compared to patients with mild steatosis ($49.3\% \pm 10.7\%$, *P* < 0.001) or without steatosis ($55.6\% \pm 14.3\%$, *P* < 0.001). CD3+/CD8+ lymphocytes also decreased in the peripheral blood in patients with moderate-to-severe steatosis as compared to normal livers and approached significance ($17.4\% \pm 8.5\%$ *vs* $26.2\% \pm 7.0\%$, *P* = 0.06).

DISCUSSION

NAFLD and NASH are increasing in importance throughout the world. While our immune system plays an important role in the pathogenesis of this disease, our under-

standing of the specifics of the immunopathogenesis of NAFLD is limited. Much of our information regarding NAFLD has come from murine models, and NKT cells have been shown to be a key mediator of murine fatty liver disease^[12]. However, there are very few studies of intrahepatic NKT cells in humans. In this study, we sought to investigate the changes in lymphocyte populations, with a focus on NKT cells, in obese patients with histologically confirmed steatosis or steatohepatitis. We found that NKT cells, defined as CD3+/CD56+ lymphocytes, are significantly increased in patients with moderate to severe steatosis as compared to patients with no steatosis or mild steatosis. These findings differ from the numerous studies performed in mice and suggest a different role of NKT cells in fatty liver disease in humans.

There have been 4 previous studies investigating NKT cells and fatty liver disease in humans, each using different techniques and yielding different results. In a study by Xu and colleagues, the investigators found a decrease in peripheral α 24+ NKT cells as compared to healthy matched non-obese controls^[14]. In that study, the diagnosis of NAFLD was made on a clinical basis, as opposed to our utilization of histology, which is a more specific means of diagnosis, and IHLs were not examined. In a study by Kremer and colleagues, the investigators also found a decrease in NKT cells in patients with moderate to severe steatosis^[15]. However, they defined NKT cells by expression of CD3+/CD57+, and used immunohistochemistry staining instead of flow cytometry for quantification, both of which can account for the differences in their results and ours. Finally, Tajiri and colleagues evaluated liver biopsy specimens of patients with NAFLD and performed flow cytometry on 20 of the specimens. In these 20 specimens, they found that in patients with more severe steatosis there was an increase in CD3+/CD56+ NKT cells^[16], and is in agreement with the results reported here. Finally Syn *et al*^[17] studied 6 liver biopsies, 2 of which had confirmed NASH cirrhosis, and found an increase percentage of NKT cells in the livers with NASH cirrhosis compared to healthy controls and patients with other forms of hepatitis. With 27 patients enrolled in this study, this is the largest sample size to date to evaluate lymphocyte populations in patients with NAFLD. Further, we also quantified the presence of invariant NKT cells, expression of CD161 and other minor T cell populations in our biologic samples, as well as examining cytokine production.

NKT cells may play a number of immunoregulatory roles in the liver and are considered by some to be a bridge between the innate and adaptive immune systems^[20]. NKT cells participate in pro-inflammatory, Th1, and anti-inflammatory Th2 mediated pathways *via* the secretion of IFN- γ and IL-4, respectively. In murine models, it has been proposed that depletion of NKT cells shifts the hepatic immune environment toward a Th1 milieu, leading to immunocyte infiltration and development of steatohepatitis^[9]. Leptin deficient mice develop steatosis and NASH, but they do not develop cirrhosis^[20]. Alternatively, NKT cells, when shifting the immune environment toward a Th2

milieu may be responsible for collagen deposition in the liver. Stimulation and proliferation of NKT cells in leptin deficient mice, through adrenergic stimulation, results in hepatic collagen deposition and fibrosis secondary to IL-4 and IL-13 secretion and activation of Th2 mediated pathways^[12,20]. In our study, we found an increased percentage of intrahepatic CD3+/CD56+ NKT cells in patients with moderate to severe steatosis and a low incidence of steatohepatitis, which could support a protective role of NKT cells against steatohepatitis. In addition we found a decrease in CD3+/CD8+ intrahepatic lymphocytes which may implicate NKT cells in shifting the hepatic immunoregulatory environment towards more Th2 mediated mechanisms. We were unable to identify a difference in the secretion of IFN- γ or IL-4 by NKT cells in patients with various degrees of steatosis, although interferon, but not IL-4 production was elaborated when NKT cells were stimulated in the liver samples studied. Future studies should focus on investigating the functional role of NKT cells in human fatty liver disease.

The multiple definitions of NKT cells can lead to much confusion when discussing their role in the liver. We classified NKT cells in two different ways, both by expression of CD3+/CD56+, as well as by expression of α 24+. Human NKT cells were initially described in liver donor patients by Doherty *et al*^[21] as CD3+/CD56+ cells and were shown to be capable of lysing NK sensitive cells. CD3+/CD56+ lymphocytes have been analyzed for mRNA expression of α 24 and approximately 5% of human hepatic CD3+/CD56+ lymphocytes expressed α 24 mRNA, which encodes the TCR that recognizes CD1d ligands^[21]. Thus, NKT cells are also defined functionally as α 24+ lymphocytes or *via* isolation of CD3+ lymphocytes using CD1d ligands, and are classified as invariant NKT cells. The CD3+/CD56+ lymphocytes, which are also called NKT-like cells, are populations that incorporate many different type of lymphocytes such as invariant T-cells and CD161+ lymphocytes which can potentially create confusion^[22,23]. Thus, although we find that this broader more diverse population (CD3+/CD56+ NKT cells) is significantly increased with greater degrees of steatosis, the more specific subgroup of invariant α 24 NKT cells were unchanged. It is possible that other functional subgroups of CD3+/CD56+ lymphocytes such as CD161+ lymphocytes play a larger role in human NAFLD and NASH. This is in contrast to the murine model where there are higher percentages of invariant NKT cells normally found in the liver^[24]. These findings highlight the importance of investigating the role of invariant NKT and NKT-like lymphocytes in human disease, rather than just using murine models.

The results of the study are limited by the small sample size, and impaired our ability to further characterize the role of NKT cells in NAFLD. Nevertheless the increase in NKT cells in moderate to severe steatosis was significant and correlates with other studies. Absolute lymphocyte numbers were not reported here because the values were affected by the varied size of the liver biopsy samples

taken in each patient. Thus, NKT cell percentages of total lymphocyte were reported for more precise comparison between subjects. Immunohistochemistry has not yet been performed on the liver biopsies, however we hope to conduct future studies to further elucidate the role of NKT cells in NAFLD.

In this study, we examined the change in lymphocyte populations in obese patients with NAFLD, with a focus on intrahepatic NKT cells. We found an increase in NKT cells, defined as CD3+/CD56+ and as well as CD161+ lymphocytes, in obese patients with moderate and severe steatosis. These results differ from previous murine models and some human studies. In addition we reported other changes in lymphocyte populations, such as depletion in CD3+/CD8+ lymphocytes and an increase in CD3+/CD4-/CD8- cells, which have not yet been reported in NAFLD. The results of this study highlight the importance of investigating NKT cells and other lymphocyte populations in humans with NAFLD since the pathophysiology of human NAFLD likely differs from that in murine models. Future studies to investigate the role of NKT cells in NAFLD in humans are warranted in order to elucidate the mechanisms behind the pervasive disease of NAFLD.

COMMENTS

Background

Non alcoholic fatty liver disease (NAFLD) is a common disease where fat infiltrates the liver, which can lead to inflammation and cirrhosis. natural killer T (NKT) cells have been implicated in the pathogenesis of NAFLD. In obese mice, NKT cells are depleted in the liver and are associated with a greater degree of steatosis. When the NKT cells are upregulated in mice, the degree of fatty infiltration diminishes. There is limited data about NKT cells in human NAFLD, and this study adds to our understanding of NKT cells in human NAFLD.

Research frontiers

The data on intrahepatic NKT cells and its role in human steatosis has been mixed. There have been 3 studies investigating NKT cells and NAFLD. One found that NKT cells are depleted with increased steatosis whereas the others found an increase in NKT cells with steatosis. This study contains the largest sample to date which investigates NKT cells in human NAFLD.

Innovations and breakthroughs

The authors found that NKT cells, defined as CD3+/CD56+ lymphocytes are increased in human livers with moderate and severe steatosis. In addition, they reported other changes in lymphocyte populations with steatosis, such as depletion in CD3+/CD8+ lymphocytes and an increase in CD3+/CD4-/CD8- cells, which have not yet been reported in NAFLD.

Applications

These findings further support the role of NKT cells in NAFLD and highlight an important difference between NKT cells in the murine model of fatty liver disease and human NAFLD.

Terminology

NKT cells are a highly conserved subset of lymphocytes with properties of both T cells (CD3+ expression) and NK cells (CD56+ and CD161+ expression). The liver contains a high percentage of these unique lymphocytes, which have been implicated in the pathogenesis of non alcoholic fatty liver disease.

Peer review

The authors showed that patients with moderate or severe steatosis had a higher percentage of intrahepatic CD3+/CD56+ NKT cells than that with mild steatosis or without steatosis. Further, the percentage of CD3+/CD56+CD161+ cells in the liver of subjects with moderate-to-severe hepatic steatosis were significantly increased compared to those with mild hepatic steatosis or without steatosis. This is an interesting finding and may provide more information about the NKT

cells in human NAFLD because the data on NKT cells in human NAFLD is limited at present. However, there are several areas of the manuscript that the authors should expand upon that would enhance the presentation.

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Gastrotomy closure with a new tissue anchoring device: A porcine survival study

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Abstract

AIM: To evaluate the feasibility, reproducibility and efficacy of a new tissue anchoring device in a porcine survival model.

METHODS: Gastrotomies were performed using a needle-knife and balloon dilator in 10 female Yorkshire pigs weighing 30-35 kg. Gastric closure was attempted using a new tissue anchoring device. The tightness of the closure was confirmed by means of air insufflation and the ability to maintain gastric distension with stability in peritoneal pressure measured with a Veress needle. All animals were monitored daily for signs of peritonitis and sepsis over 14 d. During necropsy, the peritoneal cavity and the gastric access site were examined.

RESULTS: Transgastric access, closure and 14 d survival was achieved in all pigs. The mean closure time was 18.1 ± 19.2 min and a mean of 2.1 ± 1 devices were used. Supplementary clips were necessary in 2 cases. The closure time was progressively reduced (24.8 ± 13.9 min in the first 5 pigs vs 11.4 ± 5.9 min in the last 5, $P = NS$). At necropsy, the gastric access site was correctly closed in all cases with all brace-bars present. One device was misplaced in the mesocolon. Minimal adhesions were observed in 3 pigs and signs of mild peritonitis and adhesions in one.

CONCLUSIONS: The use of this new tissue anchoring device in porcine stomachs is feasible, reproducible and effective and requires a short learning curve.

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Key words: Gastrotomy; Closure; Suture; Survival; Porcine model; Notes

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INTRODUCTION

Natural orifice transluminal endoscopic surgery (NOTES) has changed the approach to the peritoneum in the last

few years^[1-5]. This novel technique permits access to the peritoneal organs through the mouth, rectosigmoid or vagina with diagnostic and therapeutic purposes. Numerous hybrid NOTES procedures (combining NOTES with laparoscopy) have been described in the last five years^[6-9], but it was not until 2007 that the first pure NOTES procedures in humans were reported^[10-13]. Although the transluminal approach holds great potential, secure access site closure remains a critical issue^[14]. In recent cases and series, endoscopic closure is substituted by use of rigid instruments, using the transvaginal access in almost all cases. However, this approach excludes the male population.

Considering the safety of laparoscopy, studies are mandatory to evaluate secure and reproducible closure methods in NOTES procedures^[15]. Several closure techniques have been tested^[16], including clips^[11,17-19], septal occluders^[20], T-tags^[21-23], more complex suturing devices^[24-26], and linear endoscopic staplers^[27]. T-tags have been tested recently to treat gastrogastic fistulas in humans^[28]. However, most of these devices are time consuming and often difficult to implement endoscopically and the current data do not allow definitive conclusions regarding the different options^[16,29].

The aim of this study was to assess the feasibility, reproducibility and efficacy of a new tissue anchoring device as a gastric suture system in a porcine survival model.

MATERIALS AND METHODS

Animals

A total of ten female Yorkshire pigs weighing 30-35 kg were included in the study. Animals underwent a 3-d quarantine and acclimation period. During this period of time, veterinary personnel evaluated each animal to ensure baseline health. Animals were fed the same diet and had unlimited access to water. The study was conducted at the University of Barcelona Medical School's animal facilities. The protocol was approved by the University of Barcelona's Animal Ethics Committee.

Preoperative care and anesthesia

Animals fasted from solids 24 h prior to the procedure. All procedures were performed with pigs under general anesthesia with endotracheal intubation and mechanical ventilation.

Procedure

A non sterile endoscope (GIF 160, Olympus Medical Systems, Europe, Hamburg, Germany) was first inserted through the pig's mouth and the esophagus and stomach were inspected. Afterwards, gastric lavage was performed with water until the stomach was free of solid particles. An iodated solution followed by an antibiotic suspension (ceftriaxone 1 g/300 mL saline solution) was instilled and the antibiotic solution was left in the stomach for 10 min. From this point on, all the instruments used were sterile or high level disinfected. With a regular endoscope, an overtube was inserted and a double channel gastroscope (GIF 2T160, Olympus Medical Systems, Europe, Hamburg, Germany)

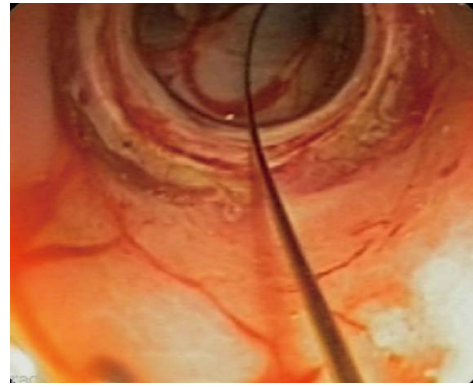


Figure 1 The incision is enlarged with a balloon dilator. Through the balloon we can see peritoneal structures.

was used until the end of the peritoneoscopy. By external palpation, the anterior gastric wall was selected to perform the gastric access. A 5 mm incision was made with a needle-knife (KD-V451M, Olympus Europe, Hamburg, Germany) and it was subsequently dilated with an 18 mm balloon (CRE wire-guided balloon, Boston Scientific Microvasive, Natick, MA) (Figure 1). Then, the scope was passed through the gastric wall for a 30 min peritoneoscopy.

A Veress needle was placed at the lower left quadrant of the abdomen to control intraperitoneal pressure. To avoid respiratory compromise and impaired venous return, intraperitoneal pressures were monitored and maintained below 15 mm H₂O. Pneumoperitoneum was maintained with CO₂ insufflation through the scope.

Tissue-anchoring device

The tissue-anchoring device prototype is called brace-bar (Olympus Medical Systems, Europe, Hamburg, Germany) and is an evolution of a former prototype^[21]. It consists of a single 18-gauge flexible needle catheter (Figure 2A), and a bifurcated nylon thread ("Y" shaped) with 3 small tags (2 regular tags fixed at both bifurcated distal ends and the other tag stopper at the single proximal end, which will be used for tightening) (Figure 2B). The tag stopper is movable and can be slid forward for cinching of the other tissue-anchoring tags. The proximal end of the thread is fixed to the needle with a small metallic guide (Figure 2C). Before deployment, the device has to be extracorporally loaded inside the needle catheter. The two distal tags are consecutively inserted into the needle (Figure 2D) and, finally, the needle is pulled back into the sheath inserting also the stopper tag (Figure 2E). Once inside the gastric cavity, the needle is pushed forward and the device is ready for use. The pusher button (Figure 2F) allows release of one tag at each side of the incision and the suture is tightened by pressing the tag Stopper with the needle sheath. Finally, the suture is released by extracting the metallic guide that fixed it to the needle.

The tightness of the closure was confirmed by means of air insufflation and the ability to maintain gastric distension with stability in peritoneal pressure measured with the Veress needle.

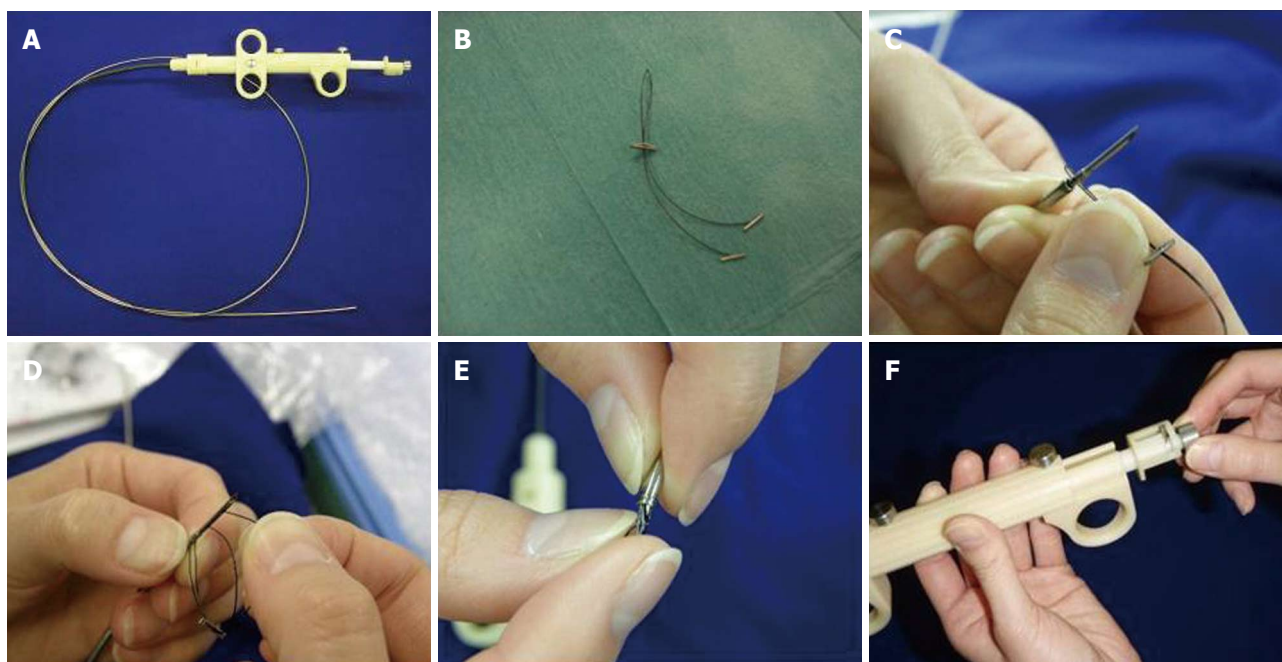


Figure 2 Description of the tissue anchoring device. A: Single 18-gauge flexible needle catheter; B: Bifurcated nylon thread ("Y" shaped) with 3 small tags (2 regular tags fixed at both bifurcated distal ends and the other tag stopper at the single proximal end, which is used for tightening); C: The proximal end of the thread is fixed to the needle with a small metallic guide; D: The two distal tags are consecutively inserted into the needle; E: The needle is pulled back into the sheath inserting also the stopper tag; F: The pusher button allows releasing one tag at each side of the incision.



Figure 3 The incision looks completely sealed after the insertion of one brace-bar and the stomach is able to maintain air distension.

A single channel scope (Olympus GIG 160) was only used for the suture. Endoclips were added when the sutures were not placed at the middle of the incision and one of the sides seemed not completely sealed.

Postoperative care and necropsy

Water was immediately allowed and food was allowed after 24 h. All animals received intravenous ceftriaxone 1 g daily for 3 d and they were monitored daily for signs of peritonitis and sepsis during the next 14 d. Weight was controlled prior to surgery and necropsy. During necropsy, the peritoneal cavity and the gastric access site were examined for signs of peritonitis (exudates, abscesses) or other complications.

Statistical analysis

Data were expressed as mean \pm SD or range. Results were analyzed using the χ^2 test with Yates correction and Fisher exact test for qualitative variables and Mann-Whitney test for quantitative parameters. A P value < 0.05 was considered statistically significant. Statistical analysis was performed with SPSS Statistical Package (version 17.0, SPSS Inc., Chicago, IL).

RESULTS

All transgastric accesses were achieved with no difficulties and a mean time of 5.7 ± 3.6 min (range 2-14). No major complications occurred. Minor complications included an accidental injury to the anterior abdominal wall and 4 minor bleedings during the use of the needle-knife. A 30 min peritoneoscopy was possible in all animals.

The brace-bar was used in all cases and closure was easily achieved in 18.1 ± 19.2 min. This time was reduced to 11.4 ± 5.9 min when considering only the last 5 cases and was less than 9 min in the last 3 cases, whereas it was 24.8 ± 13.9 min in the first 5 ($P = 0.1$). Details of all procedures are described in Table 1. A total of 21 sets of sutures (mean 2.1 ± 1 , range 1-4) were used to achieve closure but only 15 (mean 1.5 ± 0.5 , range 1-2) could be completely tightened. Therefore, in 5 cases, the incision was closed with only 1 suture (Figure 3), but in two of them 1 and 3 clips respectively, were added.

In total, 6 sutures (29%) were ineffectively positioned. In 5 attempts one of the tags did not stay attached to the

Table 1 Summary of procedure details

Case	Number of brace-bars used	Number of brace-bars correctly placed	Cause of Misplaced brace-bar	Adjunctive method	Closure time (min)	Endoscopic view
1	2	2		Omental patch	8	Correct
2	1	1		3 endoclips	42	Correct
3	4	2	1 tag detached 1 brace-bar not tightened	None	36	Correct
4	2	2		None	19	Correct
5	2	1	1 tag detached	1 endoclip	19	Correct
6	3	2	1 tag detached	None	15	Correct
7	3	1	2 tags detached	None	20	Correct
8	2	2		None	9	Correct
9	1	1		None	7	Correct
10	1	1		None	6	Correct

Table 2 Summary of necropsy findings

Case	Weight gain (kg)	Abdominal cavity	Incision	Location of tags
1	6.85	Minimal adhesions	Totally closed	1 tag at mesocolon 3 tags at gastric serosa
2	6.90	Normal	Totally closed	2 tag stoppers at gastric mucosa 2 tags at gastric serosa.
3	5.00	Minimal adhesions	Totally closed	1 tag stopper at gastric mucosa 4 tags at gastric serosa
4	3.70	Normal	Totally closed	2 tag stoppers at gastric mucosa 4 tags inside gastric wall
5	1.90	Normal	Totally closed	1 tag at gastric serosa 1 tag inside gastric wall
6	3.74	Small clot	Totally closed	1 tag stopper at gastric mucosa 3 tags at gastric serosa 1 tag inside gastric wall
7	0.90	Normal	Totally closed	1 tag stopper at gastric mucosa 1 tag at gastric serosa 1 tag at gastric wall
8	0.00	Normal	Totally closed	1 tag stopper at gastric mucosa 1 tag at gastric serosa 3 tags at gastric wall
9	-2.78	Fibrin exudates Minimal adhesions	Totally closed	2 tag stoppers at gastric mucosa 2 tags at gastric wall
10	6.42	Minimal adhesions	Totally closed	1 tag stopper at gastric mucosa 2 tags at gastric wall 1 tag stopper at gastric mucosa

mucosa either immediately after the tag release or when tightening the suture. The remaining failure was caused by a thread rupture after steady tightening.

Immediately after gastrotomy closure, all brace-bars seemed well positioned and gastric distension with air was possible in all cases without changes in intraperitoneal pressure, suggesting the closure was correct (mean peritoneal pressure before and after the closure: 14.3 ± 3.3 mmHg, range 3-15).

The mean procedure time, including gastric access creation, peritoneoscopy and gastrotomy suture, was 63.7 ± 18.2 min.

All the pigs completed the 14 d follow-up period. They had a weight gain of 3.3 ± 3.2 kg. At necropsy, the gastric access site was completely closed in all cases and all brace-bars were present (Table 2). The tags were usually attached

at the gastric serosa ($n = 15$) or inside the gastric wall ($n = 14$). In the first case, 1 tag was misplaced inside the mesocolon. In this case, an omental patch had been added to the suture pulling the omentum inside the stomach through the incision. Minimal adhesions were observed in 3 pigs and signs of mild peritonitis and adhesions in one.

DISCUSSION

NOTES holds great appeal as a less invasive alternative to laparoscopic surgery. As NOTES heads toward human trials, it is essential that the creation and closure of transluminal incisions be performed in a safe, rapid, and reproducible manner^[14-16,28].

In this study, we assessed the feasibility of a new generation tissue anchoring device with relatively good results.

Moreover, it turned out to be easy and intuitive to use and the time for placement was short and progressively reduced. It was not necessary to use complementary clips when we gained experience with the system. One of the advantages of this device (and the main difference with the former prototype used by Sumiyama *et al.*^[22]) is that it can be used with a single channel endoscope. The same needle catheter is used for releasing the tags and tightening them later without need for a different forceps grasper, and this makes the procedure shorter. Furthermore, the depth of the needle insertion is limited to 20 mm and this might decrease the risk of complications.

However, we still found some problems with the device: the needle had to be loaded extracorporally after each set of tissue anchors was applied and this prevented sequential stitching. Moreover, we observed a dysfunction of the needle after several attempts which could explain the high rate of sutures being ineffectively positioned because the tags could not be released deeply enough. We think that pre-charged and non-reusable devices might improve the procedure time and security. On the other hand, we did not drop any tags in the peritoneal cavity and, since each pair of tags is attached to a thread, we think that the possibility of dropping one in the peritoneum is extremely low.

The possibility of an inadvertent injury of organs and structures outside of the gut wall has been described as a possible limitation of T-tag based systems^[30,31]. Sumiyama *et al.*^[22] produced 12 gastric perforations in 6 pigs that were closed with 48 tissue anchor sets and three of the 24 used in the anterior gastric wall (12.5%) penetrated surrounding organs (2 penetrated the liver and 1 the anterior abdominal wall). However, as mentioned above, with the new brace-bar prototype the depth of the needle insertion is limited to 20 mm and this fact was crucial in the low incidence of surrounding structure injuries in our series (1 tag out of 21 sets, 4.8%). This was the first case and we tried to perform an omental patch pulling the omentum through the incision. During this maneuver, the mesocolon was probably unsafely moved towards the gastric wall causing this complication. In the remaining cases in which the omental patch was not attempted no lesions occurred at the adjacent structures. The importance of the depth of the needle to avoid complications has been demonstrated very recently by Park *et al.*^[32] These authors performed needle punctures of 1-1.5 mm using a different anchor-based endoscopic system (the TAS o tissue apposition system) and they did not have any adjacent organ penetration with a 100% of closure effectiveness.

Although the ex-vivo study of Voermans *et al.*^[33] suggested that t-tag based methods do not permit the serosa to serosa approach and leaking pressure is lower than with other devices, the surviving pigs showed a good post-operative course. This fact could be explained because physiological intraluminal pressures are much lower than pressures obtained in acute bursting tests and, therefore, might not be a necessary objective test for viscerotomy closure^[34]. From a clinical standpoint, the critical test for a

gastric closure is animal survival without clinical signs of leakage or complications.

Previous studies have demonstrated that peritoneal contamination occurs when using transgastric access. A conservative interpretation of these findings is that the current "aseptic" technique may require further refinement, as suggested by Rolanda *et al.*^[18] and Ryou *et al.*^[35]. In fact, we had some difficulties in completely cleaning some of the stomachs and it was very common to notice residual liquid near the incision. Only one pig did not have a satisfactory recovery and the necropsy showed the presence of fibrin exudates in the upper abdomen. Although the incision site was seen completely sealed at necropsy, we cannot totally exclude the possibility that an initial suture failure occurred but it could be also related with a potential contamination of the abdominal cavity by the stomach content. Because in this case we used only one brace-bar, we now consider it prudent to use two devices to ensure a safer closure.

The present study has some limitations: first, the number of cases is low and we did not include a control group. Second, the 14 d survival period might be short to evaluate late complications. Finally, the use of complementary clips in two cases might modify the results of the study.

In conclusion, the use of a brace-bar in a gastric porcine model is easy, fast, and reproducible after a short learning curve and permits the use of a single channel endoscope. We believe this tissue anchoring system holds tremendous potential as a suturing method for both iatrogenic and intentional perforations of the gastric wall. Unfortunately, it is still far from a safe application in humans. Further studies and more technological improvements are still mandatory before expanding its use to humans.

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COMMENTS

Background

Natural orifice transluminal endoscopic surgery (NOTES) has changed the approach to the peritoneum in the last few years. Secure closure of the gastrotomy access is one of the most important issues for the development of NOTES. However, most new suturing devices are time consuming and often difficult to implement endoscopically.

Research frontiers

T-tag based systems have already been used with variable results. The possibility of an inadvertent injury of organs and structures outside of the gut wall has been described as a possible limitation of these devices. In this study, the authors demonstrate the usefulness and safety of a new tissue-anchoring-device prototype.

Innovations and breakthroughs

One of the advantages of this device (and the main difference with the former prototype) is that it can be used with a single channel endoscope. On the other hand, the same needle catheter is used for releasing the tags and tightening

them later without need for a different forceps grasper, and this makes the procedure shorter. Furthermore, the depth of the needle insertion is limited to 20 mm and this might decrease the risk of complications.

Applications

Because the use of the brace-bar is easy, fast, and reproducible after a short learning curve, we believe this tissue anchoring system holds tremendous potential as a suturing method for both iatrogenic and intentional perforations of the gastric wall.

Terminology

Natural orifice transluminal endoscopic surgery permits access to the peritoneal organs without the need of skin incisions. Tissue-anchoring devices are endoscopic suturing devices based on a nylon thread and a small tag at the distal end that are deployed within the gastric wall. When two or more of them are tight together, the margins of the incision approach and the incision is sealed.

Peer review

This is well written and succinct with appropriate interpretation and caution in the discussion.

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MR-arteriportography: A new technical approach for detection of liver lesions

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Abstract

AIM: To evaluate the benefit and effectiveness of MR-arteriportography (MR-AP) to achieve the highest sensitivity for detection and evaluation of hepatocellular carcinoma (HCC).

METHODS: Twenty liver cirrhosis patients with suspected HCC were included before transarterial chemoembolization. In all patients double-enhanced Magnetic resonance imaging (MRI) was performed. A bolus of 10 mL Magnevist® was injected through a selectively placed catheter in the superior mesenteric artery and MRI of the liver was performed in arteriportographic phase. Two independent readers evaluated number, size and localization of detected lesions. Diagnostic quality was determined using a 4-point scale. Differences were analyzed for significance using a *t*-test. Interobserver variability was calculated.

RESULTS: In all 20 patients (100%), MR-AP was feasible. Diagnostic quality was, in all cases, between 1 and

2 for both modalities and readers. MR-AP detected significantly more lesions than double-enhanced MRI (102.5 vs 61, respectively, $P < 0.0024$). The inter-observer variability was 0.881 for MRI and 0.903 for MR-AP.

CONCLUSION: Our study confirmed that the MR-AP as an additional modality for detection of HCC is beneficial, as significantly more lesions were detected compared to MRI with liver-specific contrast.

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Key words: MR-arteriportography; Magnetic resonance imaging; Hepatocellular carcinoma; Liver lesions

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and often develops in patients with underlying liver cirrhosis due to excessive alcohol intake, chronic hepatitis or primary biliary cirrhosis.

In the treatment of hepatocellular carcinoma, surgical resection is considered the only potentially curative therapy. However, technical improvements in hepatic surgery have extended the indications for surgery remarkably, and also regional therapeutic procedures such as transcatheter

arterial chemoembolization (TACE)^[1-3] and radiofrequency ablation (RFA)^[1,4,5] have proved to be very successful. A prolonged time of survival following diagnosis is noted. Therefore, the pre-operative or pre-interventional workup of patients with suspected liver malignancy is even more important, especially concerning the evaluation and characterization of focal or diffuse lesions in the cirrhotic liver. Magnetic resonance imaging (MRI) has been used to improve identification of focal hepatic masses in a cirrhotic liver.

Dynamic MRI after a bolus injection of gadopentetate dimeglumine has been accepted as a valuable method for the detection and characterization of liver tumors^[6-9]. Studies have shown that superparamagnetic iron oxide-enhanced magnetic resonance imaging (SPIO-MRI) increases sensitivity^[4,10].

In order to determine the treatment of choice for HCC, studies have shown that examinations by both computed tomography angiography (CTA) and computed tomography arteriportography (CT-AP) are indispensable because of the high sensitivity of CT-AP in detecting hepatic lesions and the capability of CTA to characterize them^[11,12]. However, in contrast to its high sensitivity in detecting lesions, the specificity of CT-AP for characterizing intrahepatic lesions is low. Tumor-mimicking benign perfusion abnormalities and benign lesions (e.g. hemangiomas, arterio-venous shunts) have led to a reported incidence of false-positive lesions between 9% and 63% in primary and secondary liver lesions^[13,14].

Despite advances in CT or MRI, ultrasound (US) with or without application of contrast agents also plays a key role in the diagnostic algorithm of HCC due to its low cost, availability and non-invasiveness.

Until now, there have hardly been any studies comparing the effectiveness of MR-arteriportography (MR-AP) and contrast-enhanced MRI for diagnosis of malignant liver lesions. Thus, the purpose of this study was to combine the advantages of modern contrast-enhanced MRI with the technique of arteriportography to achieve the highest sensitivity for diagnosis of malignant liver lesions in patients suffering from HCC.

MATERIALS AND METHODS

Patients

Approval for this study was obtained from the institutional review board in conformity with the Declaration of Helsinki. Before the procedures were conducted, written informed consent was obtained from each patient for MRI, MR-AP and angiography after the nature of the procedure was fully explained.

During the period from February 2005 to September 2007, 20 patients [18 men, 2 women, age range from 47 to 76 years (mean age, 62 years)] with symptoms suggestive of primary malignant hepatic tumors were referred to our department. As HCC is commonly associated with liver cirrhosis, all patients had deteriorated liver but a tolerable renal function, and the cardiovascular status was stable. Twelve of our 20 patients suffered from alcohol toxic

liver cirrhosis. In 4 out of 20 patients the underlying disease was chronic hepatitis (2 patients with chronic hepatitis B, 2 patients with chronic hepatitis C). In 4 out of 20 patients, the fundamental disease could not be elicited. Concerning the severity of cirrhosis, 8 out of 20 patients were classified as Child Pugh score A, 7 out of 20 patients as Child Pugh score B and 5 out of 20 patients as Child Pugh score C.

The existence of malignant hepatic tumors was confirmed using multislice MRI and CT. MR-AP was performed to evaluate the tumor extent in order to suggest interventional therapy, surgery or chemotherapy.

Diagnosis of HCC was histologically confirmed in 15 out of 19 patients. In one patient (patient No. 16), existence of a malignant hepatic lesion was excluded histologically following liver transplantation.

In 13 out of 20 patients the α -fetoprotein (AFP) level was elevated, ranging from 16.4-2513 ng/mL (mean 488.1 ng/mL). In 7 patients (including patient No. 16) AFP levels were within a normal range.

Imaging procedures

Angiography: Before TACE and for MR-arteriportography, the femoral common artery was punctured under local anesthesia using the Seldinger technique and a 5-French angiographic catheter (Cobra, Cook Medical, USA) was positioned in the proximal superior mesenteric artery. A diagnostic angiography was performed to visualize the portal vein and to exclude shunts which could involve contrasting *via* the portal vein.

MRI: MRI was performed on a 1.5-T whole-body scanner (Magnetom Sonata, Siemens Medical Solutions, Germany) equipped with a high-performance gradient (Quantum) system (maximum gradient strength, 30 mT/m; slew rate, 125 T/ms). A combination of the standard body phased-array coil with spine array coils was used for signal reception.

MR-AP standard protocol (Table 1): For MR-AP, 10 mL gadopentetate dimeglumine was injected through the catheter placed in the superior mesenteric artery at a rate of 2 mL/s with a power injector (Medrad Spectris MR Injector, USA).

MRI standard protocol (Table 2): For MRI, 0.2 mmol/kg body weight gadopentetate dimeglumine was injected intravenously at a rate of 2 mL/s with a power injector (Medrad Spectris MR Injector). T1-weighted VIBE transversal Dynamic scans were acquired 20, 40, and 120 s after application of gadopentetate dimeglumine. T2-star-weighted Flash 2D scans and T2-weighted TSE FS scans were obtained after application of 1.4 mL Ferucarbotran (Resovist®, Bayer Schering Pharma AG, Germany).

Image analysis

In the retrospective reviewing procedure, all images of each technique were interpreted and evaluated independently by two observers with great experience in abdominal MRI.

Table 1 MR-arteriportography standard protocol

Scans	Plane	TE	TR	Flip angle
Unenhanced				
T2-weighted TRUFI	Coronal	1.9	3.8	71°
T2-weighted TRUFI	Transversal	1.88	3.76	71°
T1-weighted VIBE	Transversal	2.02	4.78	10°
Enhanced				
T1-weighted FLASH FS CE	Transversal	4.76	123	70°
T1-weighted VIBE dynamic ¹	Transversal	2.02	4.78	10°

¹Dynamic scans were started immediately following application of 10 mL Gd-DTPA. MRI: Magnetic resonance imaging.

Table 2 Magnetic resonance imaging standard protocol

Scans	Plane	TE	TR	Flip angle
Unenhanced				
T1-weighted VIBE	Transversal	1.59	4.37	10°
T2-star-weighted FLASH 2D	Transversal	10.00	169.00	90°
T2-weighted TSE FS	Transversal	105.00	2740.00	170°
T1-weighted FLASH opp	Transversal	2.71	100.00	70°
T1-weighted FLASH in	Transversal	4.76	87.00	60°
T2-weighted HASTE	Transversal	85.00	1000.00	150°
T2-weighted TRUFI	Coronal	1.83	3.65	71°
T1-weighted VIBE	Transversal	1.55	4.81	10°
Enhanced				
T1-weighted VIBE dynamic ¹	Transversal	1.55	4.81	10°
T1-weighted FLASH FS	Transversal	4.76	123.00	70°
T1-weighted FLASH FS	Coronal	4.76	94.00	70°
T2-star-weighted FLASH 2 ²	Transversal	10.00	169.00	90°
T2-weighted TSH FS ²	Transversal	105.00	2740.00	170°

¹Dynamic scans were started immediately following application of Gd-DTPA (0.2 mmol/kg); ²Following application of 1.4 mL Resovist.

No clinical information or patient diagnosis was given to the observers. The images from each technique were interpreted in separate sessions in a randomized sequence. In the first session, the two observers reviewed a set of images that included both unenhanced and gadopentetate dimeglumine-enhanced, as well as Resovist-enhanced, images.

In the second session, each observer reviewed a set of images (MR-AP set) that included gadopentetate dimeglumine-enhanced images after injection *via* the superior mesenteric artery.

For characterization of liver lesions, all images of each examination were reviewed together using all the sequences available. Each observer recorded the number of suspected lesions noted, their size, and the segmental location. Furthermore, the image quality of MR and MR-arteriportography was documented on a four point scale: 1-excellent, 2-minor diagnostic limitations, 3-major diagnostic limitations, 4-non-diagnostic.

Statistical analysis

Statistical software (SPSS, version 14, Chicago, USA) was used for statistical analysis. We evaluated the differences with regard to number of lesions found using MR-arteriportography and MRI. Furthermore, the inter-observer differences in evaluation of MR-arteriportography and double-enhanced MRI were analyzed. Paired-samples

Table 3 Number of lesions detected in MR-arteriportography and magnetic resonance imaging

Patient	Age (yr)	MR-AP number of lesions Σ 102/103		Double-enhanced MRI number of lesions Σ 60/56	
		Reader 1	Reader 2	Reader 1	Reader 2
1	62	9	9	5	4
2	47	7	8	2	2
3	70	4	5	2	2
4	74	7	8	4	3
5	51	3	3	3	3
6	76	1	2	1	1
7	70	6	6	9	8
8 ¹	63	-	-	(7)	(7)
9	70	4	4	2	2
10	63	9	8	2	2
11	51	9	8	6	5
12	63	10	11	3	2
13	57	6	6	2	2
14	67	5	4	4	5
15	51	4	3	3	2
16	53	0	0	1	1
17	51	2	2	2	2
18	54	5	6	3	3
19	57	9	8	4	4
20	73	2	2	3	3

¹Patient No. 8 was excluded from the evaluation for diffuse infiltration of virtually all liver segments. $P = 0.0024$ (relation of the number of lesions detected in MR-AP and MRI). MR-AP: MR-arteriportography; MRI: Magnetic resonance imaging.

t tests and χ^2 test were used to compare. In paired-samples t tests, $P < 0.05$ indicated a statistically significant difference.

RESULTS

Twenty patients with liver cirrhosis underwent combined MR-arteriportography and SPIO-MRI examinations. No adverse reactions were experienced by any of the patients who received Gd-DTPA and SPIO.

In all 20 patients (100%), MR-arteriportography was feasible. The image quality in both modalities (MR-AP and MRI) was excellent (1-2 in MR-arteriportography, SD: 0.4865; 1-2 in MR, SD: 0.4292). In patient No. 8, a MR-AP evaluation was not suitable due to a diffuse infiltration of virtually all liver segments, thus the patient was excluded. Altogether, 102.5 hepatic lesions were detected using MR-AP, whereas only 61 lesions could be detected using MRI (Table 3). This difference is considered to be statistically significant ($P < 0.0024$).

The kappa analyses of two observers regarding the number of lesions detected by both modalities showed substantial to excellent agreement ($\kappa = 0.903$, 95% CI: 0.844 to 0.962 for MR-AP; and $\kappa = 0.881$, 95% CI: 0.795 to 0.966 for MRI). In particular, κ values with MR-AP imaging indicated excellent agreement.

The lesions found in all patients ranged in size from 7-120 mm (mean 28.24 mm) for MRI and from 4-120 mm (mean 24.62 mm) for MR-AP. This difference is not con-

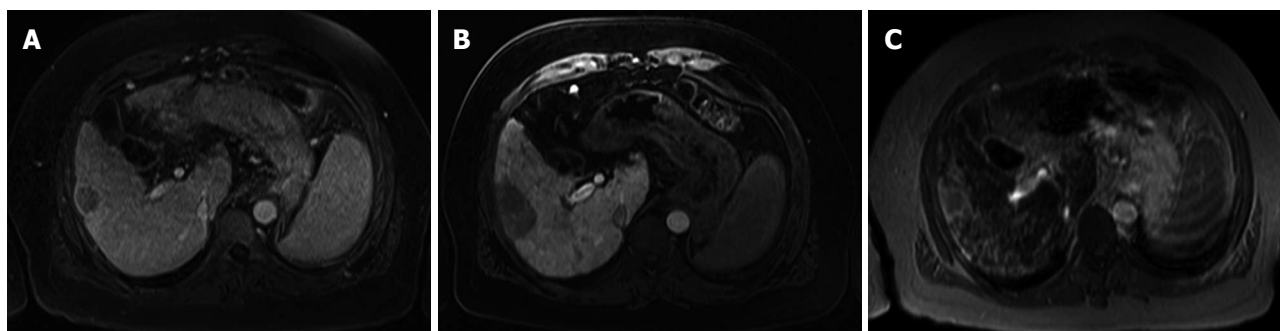


Figure 1 A 49-year-old man with hepatic cirrhosis due to chronic hepatitis B and C infection. Histology obtained following liver transplantation confirmed the diagnosis of a multifocal hepatocellular carcinoma. A: Magnetic resonance imaging (T1-weighted VIBE) during early venous phase shows a low signal intensity nodule with a diameter of approximately 3 cm in segment VIII/V; B: SPIO-enhanced T2-weighted fast image shows an area of increased signal intensity (segment VIII/V) within the otherwise lower but very inhomogenous signal of the liver parenchyma with profound cirrhosis; C: MR-AP (T1-weighted VIBE) during early venous phase displays an area of decreased enhancement (approx. 4.5 cm diameter) in segment VIII/V. Note the multiple smaller hypointense lesions in segments VII/VI. SPIO: Superparamagnetic iron oxide-enhanced.

sidered to be statistically significant. In MRI, 16 lesions (26.2%) were 10 mm or less in diameter. In MR-AP, 30 lesions (29.3%) were 10 mm or less in diameter.

In a total of 15 out of 19 patients, more lesions were detected by MR-AP compared to MRI with liver-specific contrast. According to the characteristic features displayed in MR-AP, all lesions were classified as malignant. Thus, as a consequence of the finding of these additional lesions, further treatment (TACE, RFA or surgery) was performed upon the patients.

In all but one patient (patient No. 16), the lesions found displayed a characteristic MRI signal, including arterial enhancement to a greater or lesser extent, with late wash out and an absent accumulation of Ferucarbotran (Resovist®). Thus, these lesions were classified as malignant. In addition, these lesions also showed characteristic features in MR-AP, such as absent enhancement of gadopentetate dimeglumine with profound demarcation compared to healthy liver tissue. Hence, the lesions were also classified as malignant.

Diagnosis of HCCs was histologically confirmed in 15 out of 19 patients. In patient No. 16, one lesion was found in segment 8 of the liver which showed a portal venous enhancement with accumulation of Ferucarbotran and no traceability in MR-AP. The lesion therefore was classified as benign, i.e. regenerated nodule that was confirmed histologically following liver transplantation (Figures 1 and 2).

DISCUSSION

Before decisions are made as to hepatic resection of hepatocellular carcinoma or interventional treatment such as TACE, percutaneous ethanol injection therapy and radio-frequency ablation (RFA), accurate information regarding the number and localization of lesions is essential.

Because of its high sensitivity for detecting lesions, CT-AP is one of the most reliable tools for detection of liver lesions. The rationale of CT-AP is for contrast material to be delivered directly to the liver through the portal vein before it can return to the hepatic artery from the

systemic circulation, to optimize the detection of tumor lesions that do not have portal vein flow and appear as hypodense nodules. The aim of our study was to combine the advantages of arteriportal contrast and MRI to detect liver lesions in patients with HCC. MRI with liver-specific contrast agents is currently the imaging modality of choice. Most studies that have directly compared MRI with CT-AP in patients with HCC or metastases reported no significant differences in sensitivity^[15-17], but in some studies a higher specificity for MRI is reported^[13].

Preoperative or pre-interventional workup of patients with suspected liver malignancy is even more important, especially concerning the evaluation and characterization of a focal lesion or diffuse infiltration of the cirrhotic liver.

Previous studies have shown the usefulness of ferumoxide-enhanced MR imaging for the detection of hepatic tumors of different histological types^[18,19]. After intravenous injection, the SPIO-particles are cleared by macrophages and can be identified histologically in Kupffer's cells of the liver. Poorly differentiated hepatic tumors lack Kupffer's cells; therefore, the T2 relaxation does not change after the administration of SPIO causing an increased lesion-to-liver contrast.

Studies of patients with hepatic metastases have shown that ferumoxide-enhanced MR imaging is more sensitive than unenhanced MR imaging^[20,21] or contrast-enhanced CT^[22,23], and at least as accurate as CT during arteriportography^[10].

Regarding the detection of HCCs, it has been reported that the sensitivity of combined CT during arteriportography and CT hepatic arteriography is 89%-95%^[24,25] in comparison to 80.4% on contrast-enhanced CT alone^[26]. Furthermore, the sensitivity of MR sequences with ferumoxide enhancement was reported to be 78%-92%^[18,19,24], and that of MR-AP 94%-97%^[27,28].

Diffusion-weighted imaging (DWI) is a MRI technique that provides imaging of diffusion in biological tissues. Recent technical developments have reduced the image deformation associated with this technique and have increased the signal to-noise ratio, thus making DWI of the

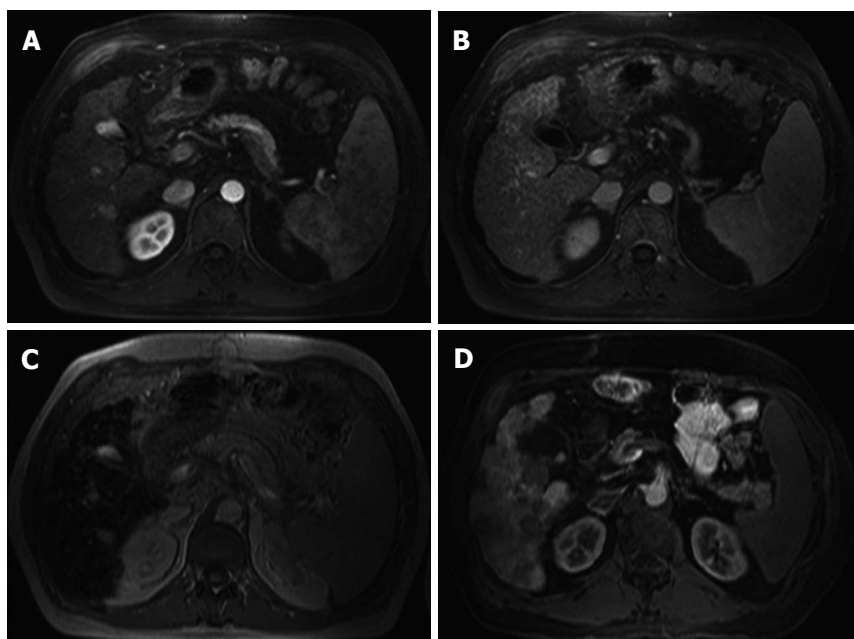


Figure 2 A 63-year-old man with hepatic cirrhosis due to a chronic hepatitis C infection. Histology obtained following liver transplantation confirmed the diagnosis of a well differentiated hepatocellular carcinoma. A: Magnetic resonance imaging (T1-weighted VIBE) during arterial phase shows a singular hyper-vascularized nodule with a diameter of approximately 2 cm in segment VII; B: T1-weighted VIBE during early venous phase shows an inhomogeneous signal of the liver parenchyma with masking of the lesion in segment VII; C: SPIO-enhanced T2-weighted fast image shows an area of increased signal intensity (segment VII) within a low signal of the liver parenchyma; D: MR-AP (T1-weighted VIBE) during early venous phase displays an area of decreased enhancement (approx. 3 cm diameter) in segment VII and various low signal lesions in segments I, IVa, VIII and VII. SPIO: Superparamagnetic iron oxide-enhanced.

body feasible^[29], especially for detection of liver malignancies^[30]. Studies have reported a higher sensitivity of DWI for detection of small hepatic metastases compared to SPIO-enhanced T2-weighted images and breath-hold T2-weighted images^[31,32], particularly due to a profound signal intensity of the HCC in the cirrhotic liver. In comparison, cysts and hemangiomas, the most frequently found benign lesions of the liver, typically show low or absent signal intensities^[33].

Our study demonstrated that MR-arteriportography as an alternative method for detection of HCC is not only feasible but showed a significantly larger number of lesions than ferumoxide-enhanced MR imaging, especially in patients with hepatic cirrhosis.

Also, the lesion's size seems to play a crucial role. Out of the 41 lesions that were exclusively found using MR-AP, 21 were 10 mm or less in diameter. This confirms the high sensitivity of MR-AP, especially regarding smaller lesions. However, a definite differentiation among HCC nodules, regenerative dysplastic nodules or, for example, small arterio-venous shunts that are very common in patients with hepatocellular carcinoma is not possible.

One limitation of the study is that a histological confirmation of HCC or benign lesions could only be obtained in 16 out of 20 patients. In the other 4 patients, further treatment was based upon the combination of typical image morphology and elevated alpha fetoprotein. Another limitation is the relatively small number of patients that were examined in this study. Prospective studies are necessary in order to ascertain the diagnostic reliability of MR-AP compared to established methods.

In summary, our study confirmed that the use of MR-arteriportography as an additional modality for detection of hepatocellular carcinoma is useful. Using this technique, significantly more lesions could be detected in comparison to MRI with liver-specific contrast agent.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and often develops in patients with underlying liver cirrhosis. In the treatment of HCC, surgical resection is considered the only potentially curative therapy; however, other regional therapeutic procedures such as transcatheter arterial embolization or radiofrequency ablations have proved to be very successful. Thus, the pre-interventional evaluation of patients with suspected liver malignancy is even more important, especially concerning the evaluation and characterization of focal or diffuse lesions in the cirrhotic liver.

Research frontiers

MR imaging with liver-specific contrast agents has been used to improve identification of focal hepatic masses in a cirrhotic liver. Also, studies have shown that examinations by computed tomography arteriportography (CT-AP) have a very high sensitivity for detection of hepatic lesions compared to a relatively low specificity. In this study, the benefit and effectiveness of MR-arteriportography (MR-AP) in achieving the highest sensitivity for detection and evaluation of HCC was evaluated.

Innovations and breakthroughs

Until now, there have hardly been any studies comparing the effectiveness of MR-AP and contrast-enhanced MRI for diagnosis of malignant liver lesions. This study confirmed that MR-arteriportography as an additional modality for detection of HCC is truly beneficial and may lead to change in treatment in many patients.

Applications

The results showed that using the MR-AP approach, significantly more lesions could be detected in comparison to MRI with liver-specific contrast agent. Thus, it might play an important role for future strategy of therapeutic interventions.

Terminology

MR-AP is an MRI procedure where the contrast agent is injected through a catheter placed in the superior mesenteric artery.

Peer review

It is well-written and is novel work.

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Carbachol promotes gastrointestinal function during oral resuscitation of burn shock

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Abstract

AIM: To investigate the effect of carbachol on gastrointestinal function in a dog model of oral resuscitation for burn shock.

METHODS: Twenty Beagle dogs with intubation of the carotid artery, jugular vein and jejunum for 24 h were subjected to 35% total body surface area full-thickness burns, and were divided into three groups: no fluid resuscitation (NR, $n = 10$), in which animals did not receive fluid by any means in the first 24 h post-burn; oral fluid resuscitation (OR, $n = 8$), in which dogs were gavaged with glucose-electrolyte solution (GES) with volume and rate consistent with the Parkland formula; and oral fluid with carbachol group (OR/CAR, $n = 8$), in which dogs were gavaged with GES containing carbachol (20 μ g/kg), with the same volume and rate as the OR group. Twenty-four hours after burns, all animals were given intravenous fluid replacement, and 72 h after injury, they received nutritional support. Hemodynamic

and gastrointestinal parameters were measured serially with animals in conscious and cooperative state.

RESULTS: The mean arterial pressure, cardiac output and plasma volume dropped markedly, and gastrointestinal tissue perfusion was reduced obviously after the burn injury in all the three groups. Hemodynamic parameters and gastrointestinal tissue perfusion in the OR and OR/CAR groups were promoted to pre-injury level at 48 and 72 h, respectively, while hemodynamic parameters in the NR group did not return to pre-injury level till 72 h, and gastrointestinal tissue perfusion remained lower than pre-injury level until 120 h post-burn. CO₂ of the gastric mucosa and intestinal mucosa blood flow of OR/CAR groups were 56.4 ± 4.7 mmHg and 157.7 ± 17.7 blood perfusion units (BPU) at 24 h post-burn, respectively, which were significantly superior to those in the OR group (65.8 ± 5.8 mmHg and 127.7 ± 11.9 BPU, respectively, all $P < 0.05$). Gastric emptying and intestinal absorption rates of GES were significantly reduced to the lowest level (52.8% and 23.7% of pre-injury levels) in the OR group at about 2 and 4 h post-burn, and did not return to 80% of pre-injury level until 24 h. In the first 24 h post-burn, the rate of gastric emptying and intestinal water absorption were elevated by a mean 15.7% and 11.5%, respectively, in the OR/CAR group compared with the OR group. At 5 days, the mortality in the NR group was 30% (3/10), 12.5% in the OR group (1/8), and none in the OR/CAR group.

CONCLUSION: Carbachol had a beneficial effect on oral resuscitation of burn shock by promoting gastric emptying and intestinal absorption in our canine model.

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Key words: Burn shock; Fluid therapy; Oral rehydration; Carbachol; Animal model; Gastric emptying; Intestinal absorption

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INTRODUCTION

Rapid intravenous infusion of large quantity of fluids containing electrolytes and colloidal solutions remain the key measure to resuscitate hypovolemic shock as a result of a massive burn injury. This life-saving measure has unanimously been accepted worldwide. It has also been recognized that a delay in such replenishment could sometimes be fatal due to complications subsequent to delayed resuscitation of hypovolemic shock. Unfortunately, in certain cases, such as mass casualties in an incendiary bomb attack in battlefields, or a forest or prairie fire in regions with an austere environment with poor medical support and transportation facilities due to geographical barriers, not only there would be a shortage of medics to introduce an intravenous needle, but also the weight and bulk of the necessary intravenous fluids would make the treatment unrealistic. In such cases, it is our supposition that oral administration of fluids might be more practical, and it might be able to maintain the life of the victims till intravenous fluid replacement was available.

Oral fluid resuscitation has been reported with success in early clinical studies of burn care^[1]. Thomas *et al*^[2] have described oral fluid replacement in burn patients, and have concluded that oral resuscitation may have a slower initial onset of hemodynamic effectiveness, but after 3-4 h, it can be similarly effective. It is unanimously recognized that the main limiting factors for effective oral resuscitation of burn shock are diminution of gastric emptying capacity and intestinal absorption of fluid and electrolytes due to undermined gastrointestinal perfusion. It seems to be necessary to overcome these two hurdles before oral hydration can be successful for treatment of burn shock.

As enough data of oral resuscitation of burn shock could not be accumulated in clinical practice in ordinary situations, most investigations have been done with animals^[3,4]. We found that the previous animal experiments have been limited by their short experimental duration of < 24 h post-burn, and performed under general anesthesia, which might interfere with the observation of gastric emptying and intestinal absorption of fluid and electrolytes. With these in mind, a canine model of burn shock was devised in which oral resuscitation was given in the first 24 h post-burn, followed by delayed intravenous fluid replacement, and the whole course of the experiment lasted for 120 h. Also, in this experiment, the effect of general anesthesia was eliminated, and carbachol, which is a cholinergic receptor agonist, was used in

an attempt to shorten gastric emptying time and restore intestinal peristalsis.

MATERIALS AND METHODS

All the experimental protocols were reviewed and approved by the Committee of Scientific Research of First Affiliated Hospital of General Hospital of PLA (Beijing, China).

Surgical preparation

Pure bred Beagle dogs (purchased from Experimental Animal Center of Academy of Military Medical Sciences of PLA, Beijing, China, License of qualification SCX 2005-0005), aged 16-20 mo, body weight 11-13 kg, were used. They were acclimatized in the animal house of our Research Laboratory for 2 wk before use. They were fasted for 24 h, and water was withheld for 4 h before the surgical preparation. Under anesthesia with 8 mg/kg ketamine (Gu-Tian Pharmacy, Fu Jian Province, China), the right carotid artery and jugular vein were individually cannulated for hemodynamic monitoring, collecting blood samples, and administration of drugs. Both cannula were led out through a subcutaneous passageway and fixed to the skin. A midline incision was made to open the peritoneal cavity to expose the proximal part of the jejunum, and small incisions were made on the jejunum 10, 20, and 50 cm distal to the Treitz ligament. A Silastic tube, 3 mm in diameter and 25 cm in length, was introduced into the jejunal lumen through each of the above openings. They were fixed with purse-string sutures, led out through a subcutaneous tunnel and fixed to the skin. A cystostomy was done for collecting urine.

Burn protocol

Twenty-four hours after the above surgical procedures, an intravenous injection of 0.5 mL/kg propofol was given to all the animals to produce brief anesthesia for 10-15 min, which was long enough to eliminate pain during burn injury. Napalm (3%) was applied to the shaved neck and back of the dogs, and it was ignited for 30 s to produce a full-thickness burn that involved about 35% of the total body surface area (TBSA). The depth of the burn injury was verified by pathological examination.

Experimental groups

The injured dogs were grouped into no fluid replacement (NR, $n = 10$), oral fluid replacement (OR, $n = 8$), and oral replacement of fluid with addition of carbachol (OR/CAR, $n = 8$). For NR, the animals received no fluid replacement or any other treatment. Dogs in the OR group received intragastric, pre-warmed glucose saline solution (each 1 L containing 59.83 mmol/L NaCl, 29.76 mmol/L NaHCO₃, 20.18 mmol/L KCl, and 114.94 mmol/L glucose in distilled water), and the rate of gastric infusion was consistent with that of the Parkland formula^[5] (4 mL/kg for each % TBSA burn, half of the total amount given in the first 8 h). In the OR/CAR group, 20 µg/kg carbachol (Sigma, St Louis, MO, USA) was added to the glucose-saline solution. Twenty-four hours after the burn injury, all the animals in the three groups were given intravenous fluid

replenishment. Seventy-two hours after the burn injury, all the surviving animals were given 10% glucose with a mixture of 17 amino acids (Beijing Pharmaceutical, Beijing, China) and 30% fat emulsion (Hua Rui Pharmaceutical, Wu Xi, China). Also, intravenous Ringer's solution was given to make up 100-150 mL of fluid per kg body weight and maintain blood potassium level > 3.5 mmol/L.

Measurement of hemodynamics

Picco-Plus (Pulsion, Germany) was used to determine mean arterial pressure (MAP) and cardiac output (CO). Plasma volume was determined with the indigo green dilution method^[6]. At each time point for determination, 12.5 mg indocyanine green (ICG) (Dan Don Pharmaceutical, Liaoning, China) was intravenously given, and 3-mL blood samples were obtained at 1, 2 and 3 min after injection. The specimens were centrifuged at 1800 *g* at 4°C, and 1 mL plasma was obtained to determine the OD value of ICG with a 723N spectrophotometer (wave length, 820 nm), and the concentration of ICG was obtained from a plotted standard curve.

Measurement of gastrointestinal mucosal perfusion

To determine CO₂ partial pressure of the gastric mucosa (PgCO₂), a gastric mucosa tonometer (Tonoca, Finland) was used. Intestinal mucosal blood flow (IMBF) was determined by passing a fiberoptic detector of a laser Doppler flow monitor (Peri Flus 5000 Master; Perimed, Jaarnfalla, Sweden) through the intestinal catheters, ensuring that the tips of the detectors were in contact with intestinal mucosa. The signals were transformed into blood perfusion units (BPU), which were input into a computer, and the PEIMED software package PSW 2.0 was used to plot the curves. Thirty seconds were spent for each measurement, and a stable curve that covered 10 s was taken for the calculation of the average.

Gastric emptying rate

The gastric emptying rate was determined by using phenol red (Sigma) as a signal, according to the Scarpignato principle^[7]. Before the experiment, a standard curve was plotted with ODs of different dilutions of phenol red at 500 nm. To determine gastric emptying time, 2 mL phenol red at 100 mg/L was introduced into the stomach through an indwelling gastric tube. Two milliliters of gastric juice was obtained when the dye was well mixed with the gastric juice. The amount of the dye in the gastric content was determined by spectrophotometry. Thirty minutes later, another sample of gastric content was withdrawn and phenol red was again determined. With this process, the gastric emptying time was calculated.

Intestinal absorption rate

Intestinal absorption rate was determined using a modified Cooper method^[8]. Since phenol red is a large molecular dye, it is not absorbed by the intestinal mucosa. With the absorption of fluid, the concentration of the dye increases. Thus, intestinal absorption rate of fluid could be assessed. The method used in our experiment

was as follows. Since three enteral tubes were implanted into the jejunum in equal distance, a known amount of fluid was introduced into the most proximal catheter (A) with a known velocity V_a (mL/h) using an intelligent infusion pump (ZNB-XB; Xu-li Scientific Technology Co. Beijing, China), and then specimens of intestinal fluid (1 mL) were collected from two distal catheters, where fluid velocities were V_b and V_c . By measuring the concentrations of phenol red in various specimens, the intestinal absorption rate was calculated: $V_b = V_a \times C_a / C_b$, $V_c = V_b \times C_v / C_c$, $\Delta V = V_b - V_c$.

ΔV was the absorption rate in the segment of intestine between catheters B and C. By dividing the value by the length of the intestinal segment between B and C, the intestinal absorption rate of the oral feeding fluid could be calculated, and it was expressed as mL/h.m², i.e. the amount of orally fed liquid absorbed by unit length of the intestine during unit time.

Statistical analysis

All data are presented as the mean \pm SD. Statistical analysis was done using SPSS 11.0 statistical software for the *F* test. $P < 0.05$ was considered statistically significant.

RESULTS

Deaths within 5 d

In the NR group, three animals died at 12, 18 and 23 h after burn injury, with a 5 d mortality of 30.0% (3/10). In the OR group, one dog died 18 h after burn injury, with a 5 d mortality of 12.5% (1/8). In the OR/CAR group, no deaths occurred within 5 d of injury, with zero 5 d mortality.

Hemodynamic parameters

As depicted in Table 1, MAP was lowered 2 h after burn injury in all three groups (all $P < 0.05$). It gradually rose after this period, and MAP in the OR and OR/CAR groups was higher than that in the NR group ($P < 0.05$) 4 h post-burn, and returned to pre-injury level 8 h after injury, but it was still lower than the pre-injury level at 72 and 120 h after injury in the NR group. CO and plasma volume (PV) were lowest at 24 h post-burn in the NR group, and increased later, but never to the level before burn injury. However, CO and PV in the OR and OR/CAR groups were higher than in the NR group at 4 and 8 h post-burn (all $P < 0.05$). CO and PV in the OR/CAR group were higher than in the OR group at 8 and 24 h post-burn (all $P < 0.05$). However, MAP showed no significant change between the OR/CAR and OR groups.

Gastro-intestinal perfusion

As shown in Table 2, PgCO₂ was rapidly elevated, and IMBF sharply decreased in all three groups. They were seen to elevate gradually afterwards. In the NR group, these two values were still worsen than that before injury at 120 h post-burn (all $P < 0.05$). However, in the OR and OR/CAR groups, they recovered to pre-burn levels at 72 h post-burn. From 2 h post-burn, PgCO₂ in the OR group was lower than that in the NR group ($P < 0.05$), al-

Table 1 Effects of carbachol on hemodynamic parameters in oral resuscitation of burn shock (mean \pm SD)

Post-burn (h)	MAP (mmHg)			CO (L/min)			PV (mL/kg)		
	NR	OR	OR/CAR	NR	OR	OR/CAR	NR	OR	OR/CAR
0	135 \pm 9.3	131 \pm 18.7	128 \pm 12.6	2.46 \pm 0.13	2.43 \pm 0.17	2.39 \pm 0.19	49.6 \pm 5.6	46.8 \pm 4.2	48.3 \pm 4.0
2	100 \pm 7.2 ^a	94 \pm 15.3 ^a	89 \pm 10.0 ^a	1.28 \pm 0.11 ^a	1.34 \pm 0.1 ^a	1.30 \pm 0.11 ^a	38.2 \pm 3.8 ^a	40.3 \pm 3.0 ^a	41.3 \pm 3.1 ^a
4	108 \pm 8.3 ^a	136 \pm 18.9 ^b	130 \pm 15.1 ^b	1.12 \pm 0.10 ^a	1.54 \pm 0.11 ^{a,b}	1.48 \pm 0.12 ^{a,b}	32.8 \pm 2.3 ^a	39.0 \pm 3.8 ^a	40.8 \pm 3.9 ^a
8	120 \pm 14.3	125 \pm 15.9	127 \pm 14.3	1.20 \pm 0.17 ^a	1.65 \pm 0.12 ^{a,b}	1.86 \pm 0.14 ^{a,b,c}	30.9 \pm 3.4 ^a	36.2 \pm 3.4 ^{a,b}	42.2 \pm 3.4 ^{a,b,c}
24	124 \pm 8.9	126 \pm 8.8	129 \pm 8.9	1.07 \pm 0.17 ^a	1.94 \pm 0.18 ^{a,b}	2.16 \pm 0.15 ^{a,b,c}	30.6 \pm 4.4 ^a	40.4 \pm 3.0 ^{a,b}	45.8 \pm 3.6 ^{b,c}
48	129 \pm 8.7	135 \pm 20.8	123 \pm 15.8	1.88 \pm 0.15 ^a	2.39 \pm 0.23 ^b	2.49 \pm 0.16 ^b	34.5 \pm 2.4 ^a	43.0 \pm 3.8 ^b	45.6 \pm 3.6 ^b
72	115 \pm 13.7 ^a	137 \pm 11.0 ^b	135 \pm 13.1 ^b	2.10 \pm 0.13 ^a	2.34 \pm 0.12 ^b	2.41 \pm 0.20 ^b	35.8 \pm 2.9 ^a	43.8 \pm 3.4 ^b	45.8 \pm 4.0 ^b
120	112 \pm 11.4 ^a	134 \pm 14.6 ^b	131 \pm 14.2 ^b	2.15 \pm 0.15 ^a	2.39 \pm 0.15 ^b	2.41 \pm 0.20 ^b	37.6 \pm 3.5 ^a	44.7 \pm 3.2 ^b	46.9 \pm 3.4 ^b

Compared with that at 0 h, ^a P < 0.05; compared with no fluid resuscitation (NR), ^b P < 0.05; compared with oral fluid resuscitation (OR), ^c P < 0.05. MAP: mean arterial pressure; CO: Cardiac output; PV: Plasma volume; CAR: Carbachol.

Table 2 Effect of carbachol on gastrointestinal perfusion in oral resuscitation of burn shock (mean \pm SD)

Post-burn (h)	PgCO ₂ (mmHg)			IMBF (BPU)		
	NR	OR	OR/CAR	NR	OR	OR/CAR
0	32.2 \pm 3.7	33.2 \pm 6.1	33.8 \pm 6.8	203.8 \pm 17.6	198.3 \pm 11.9	207.3 \pm 13.9
2	73.1 \pm 7.7 ^a	60.8 \pm 8.2 ^{a,b}	57.4 \pm 8.0 ^{a,b}	74.2 \pm 10.8 ^a	101.2 \pm 12.2 ^{a,b}	112.6 \pm 10.2 ^{a,b}
4	83.1 \pm 6.5 ^a	74.0 \pm 6.5 ^{a,b}	70.0 \pm 6.2 ^{a,b}	71.5 \pm 15.3 ^a	108.8 \pm 12.2 ^{a,b}	138.8 \pm 14.1 ^{a,b,c}
8	86.4 \pm 8.6 ^a	69.2 \pm 6.8 ^{a,b}	68.5 \pm 5.8 ^{a,b}	77.8 \pm 10.0 ^a	114.7 \pm 12.0 ^{a,b}	134.7 \pm 13.9 ^{a,b,c}
24	82.5 \pm 7.6 ^a	65.8 \pm 5.8 ^{a,b}	56.4 \pm 4.7 ^{a,b,c}	79.2 \pm 17.3 ^a	127.7 \pm 11.9 ^{a,b}	157.7 \pm 17.7 ^{a,b,c}
48	61.5 \pm 8.2 ^a	56.0 \pm 8.4 ^a	57.0 \pm 6.4 ^a	146.8 \pm 13.8 ^a	159.3 \pm 19.1 ^a	179.3 \pm 19.1 ^{a,b}
72	56.8 \pm 6.6 ^a	39.4 \pm 8.9 ^b	35.4 \pm 5.6 ^b	168.5 \pm 9.7 ^a	180.7 \pm 18.5	198.7 \pm 16.5 ^b
120	45.8 \pm 6.2 ^a	31.2 \pm 5.0 ^b	34.2 \pm 4.0 ^b	178.8 \pm 16.5 ^a	203.5 \pm 23.2 ^b	200.5 \pm 18.2 ^b

Compared with that at 0 h, ^a P < 0.05; compared with no fluid resuscitation (NR), ^b P < 0.05; compared with oral fluid resuscitation (OR), ^c P < 0.05. IMBF: Intestinal mucosal blood flow; CAR: Carbachol.

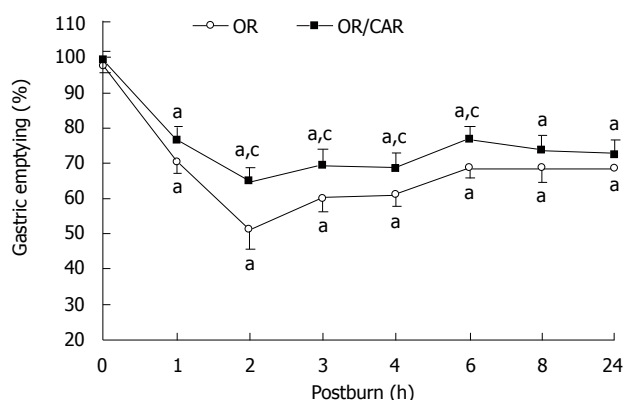


Figure 1 Carbachol promoted gastric emptying rate in oral fluid resuscitation/Carbachol group compared with those of oral fluid resuscitation group at 2, 3, 4 and 6 h after burn injury. ^a P < 0.05 vs 0 h, ^c P < 0.05 vs oral fluid resuscitation (OR) group (one-way ANOVA). Error bars represent mean \pm SD. CAR: Carbachol.

though it was higher than that in the OR/CAR group, but there was no significant difference (P > 0.05). IMBF was always higher in the OR and OR/CAR groups compared with the NR group (all P < 0.05), but the value was lower in the OR group compared with the OR/CAR group at 4, 8 and 24 h post-burn (all P < 0.05).

Gastric emptying rate

Figure 1 shows that, in the OR and OR/CAR groups, gastric emptying rate was lowered, especially in the for-

mer group, with 52.8% of the normal emptying rate at 2 h, and 70.5% at 24 h post-burn. In the OR/CAR group, it was also lowered, but reached 65.2% at 2 h, and 73.0% at 24 h post-burn, and the values were all significantly higher than those in the OR group at 2, 3, 4 and 6 h post-burn (all P < 0.05). Thus, it was estimated that approximately 15.7% more gastric content was expelled from the stomach in 24 h in the OR/CAR group as compared with the OR group.

Intestinal absorption rate

As shown in Figures 2 and 3, the rate of absorption of orally administered water and Na⁺ was lowest at 3 h post-burn in the OR group, and the values were 23.7% and 50.3% of pre-injury levels. These absorption rates were gradually increased to 44.4% and 65.1%, respectively, at 24 h post-burn. In the OR/CAR group, all these values were higher than those in the OR group at 3, 4 and 6 h post-burn (all P < 0.05). In the first 24 h post-burn, the rate of intestinal water absorption was elevated by 11.5% in the OR/CAR group. It was estimated that within 24 h after injury, the water absorption rate at 24 h in the OR and OR/CAR groups was 110.9 \pm 17.1 mL/h.m and 127.8 \pm 17.3 mL/h.m, respectively, and they were actually higher than the pre-requisite of the Parkland formula 82.1 \pm 11.2 mL/h.m (P < 0.05).

DISCUSSION

Mass casualties from burn injury may occur in a region

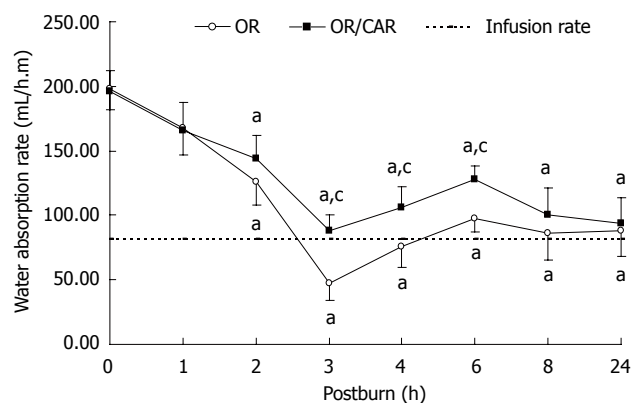


Figure 2 Carbachol significantly improved rate of water absorption of intestine in oral fluid resuscitation/Carbachol group compared with those of oral fluid resuscitation group at 3, 4 and 6 h after burn injury. ^a $P < 0.05$ vs 0 h, ^c $P < 0.05$ vs oral fluid resuscitation (OR) group (one-way ANOVA). Error bars represent mean \pm SD. CAR: Carbachol.

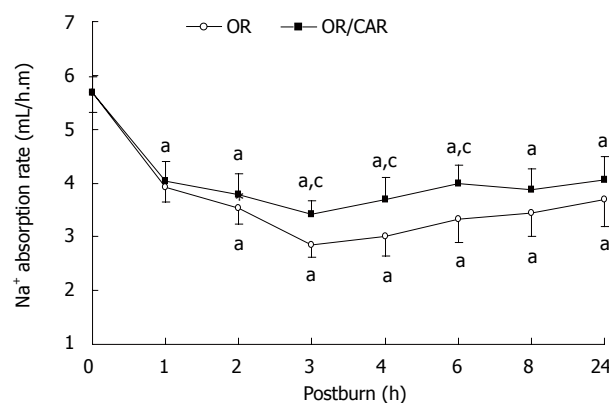


Figure 3 Carbachol significantly improved rate of Na⁺ absorption of intestine in oral fluid resuscitation/Carbachol group compared with those of oral fluid resuscitation group at 3, 4 and 6 h after burn injury. ^a $P < 0.05$ vs 0 h, ^c $P < 0.05$ vs oral fluid resuscitation (OR) group (one-way ANOVA). Error bars represent mean \pm SD. CAR: Carbachol.

where the major problems facing medical personnel are the availability and probability of fluid replacement with sterile intravenous fluids. If we resuscitate a single burn victim weighing > 60 kg with a burn of 30% TBSA it would require > 5 -7 kg of fluid for a medic to carry. In a very harsh environment with lack of decent medical support, resuscitation of burn shock is severely handicapped, and the life of those with extensive burn injury is jeopardized. One strategy for reducing such a hazard is to try to supplement liquid with sufficient electrolytes by mouth until intravenous infusion fluids are available^[9]. Early in 1970, Monaf^[10] reported a clinical trial of oral administration of hypertonic lactated saline solution to patients with burn injury of various extents, and he found that at least partial oral resuscitation of severe burns could be successful. However, oral resuscitation of burn shock has not been popular, because, ordinarily, intravenous resuscitation is almost always available, especially in cities, and even in many rural areas where medical facilities have been established. Nevertheless, in certain scenarios, such as fire disasters in cities after a strong earthquake, forest fires in mountainous terrain, or in combat zones after incendiary devices have exploded, when transportation is seriously lacking or hampered due to geographical barriers, and medical support is lacking, so that sterile intravenous fluids and trained personnel are not available, oral intake of fluids should be considered, in the hope that the victims can be tided over burn shock.

Sufficient data about oral resuscitation of burn shock can not be accumulated in clinical practice under normal circumstances, therefore, most investigations have been done with animals. In these experiments, animals were always under general anesthesia to prevent restlessness of the animals^[1,3,4]. Thus, the results of oral hydration might not reflect the true states of fluid absorption because the anesthesia inevitably inhibits gastrointestinal motility and other functions. The present study was planned to measure all the physiological parameters in a conscious state. All the catheters for measuring physiological indexes were implanted 1 d before burn injury. Burn injury was

produced under brief anesthesia to eliminate mental strain and pain. Thereafter, all the measurements were made with the animals in a fully conscious state. Pure bred Beagle dogs were used because they were tame, docile and cooperative. No restriction of the body was necessary during the whole course of the experiment, which rendered all the measurements complete without any restriction of the animals or general anesthesia. Thus, all the disturbances to gastrointestinal mobility or absorption were greatly alleviated. Therefore, there was no external interference to influence the measurements during the whole course of the experiment, which lasted for 120 h, which increased the reliability of the measurements.

The degree of perfusion of the gastrointestinal tract is at present considered as an important index of circulatory shock and tissue oxygen delivery. The determination of PCO₂ of the gastric mucosa has been used to estimate the pH of the mucosa, and the change in pH of the mucosa reflects the condition of blood perfusion and oxygen delivery to the gastric tissue^[11]. The procedure is untraumatic, and the catheter can also be used to give necessary fluids as well as a convenient tool for measuring gastric emptying rate. To determine blood perfusion of the intestinal mucosa by way of preformed fistulae has already been a standard method in monitoring the circulatory state of transplanted intestine^[12]. In our experiment, a flexible fiberoptic detector of a laser Doppler flow monitor was passed through the preformed fistulae. Each reading took about 1 min only, so that it did not give any discomfort to the animal, and sequential measurements were assured. The experimental results showed that, in injured dogs without the benefit of fluid gavage, blood perfusion was rapidly diminished in the intestinal mucosa. It was also shown that this change was consistent with a decrease gastrointestinal mucosal perfusion, and its recovery lagged behind the improvement in hemodynamic parameters. Up to 120 h after injury, it was still lower than that before injury. These phenomena corroborated that which was found in human patients with hypodynamic shock.

When a solution with electrolytes and glucose is intro-

duced into the stomach, the emptying rate of the liquid from the stomach depends on the pressure gradient between the stomach and duodenum. It also is influenced by the state of blood supply to the stomach and regulatory activity of the vagus nerve (cholinergic nerve) and humoral agents (motilin)^[13]. Absorption of water through the intestinal mucosa depends on translocation of Na^+ ions, while the latter process could only be realized with the presence and activity of Na^+/K^+ -ATPase, which is localized in the basal layer of the intestinal mucosal epithelium. Therefore, it is obvious that the rate of absorption of water by the intestinal mucosa is under the influence of the intestinal blood flow, activity of Na^+/K^+ -ATPase, and aquaporin (AQP)-1 expression, along with consumption of ATP. Thus, it is evident that with ischemia and hypoxia of the intestinal mucosa during hypovolemic shock, water absorption by the intestinal mucosa is hampered.

Heavy leakage of fluid from the circulation results in a sharp decrease in ATP. This shortage in ATP is further amplified by an increased demand created by feeding and absorption of water and electrolytes. Under such conditions, the presence of glucose, which can be metabolized into lactic acid, alanine and CO_2 to produce ATP under anaerobic conditions, is essential. This additional ATP is helpful for the absorption potential of the intestinal mucosa. During the process of absorption, the transport of glucose is coupled with the transport of water molecules and Na^+ ions, and two Na^+ ions and 223 water molecules are absorbed. Therefore, addition of glucose to an electrolyte solution is an ideal liquid for oral replacement during hypovolemic shock^[14,15].

Thomas *et al.*^[2] have described an experiment to study gastric emptying with oral replacement of fluid in hypovolemic shock. They gavaged glucose-electrolyte solution (GES) into the stomach of pigs with 40% TBSA burn injury, according to the Parkland formula. They showed that the gastric emptying volume increased with an increase in the volume of gavaged fluid. However, the volume of fluid passed into the duodenum was one half of the volume required by the Parkland formula, and hemodynamic parameters did not recover to the pre-injury level. Michell *et al.*^[4] have performed a study of duodenal infusion of glucose-electrolyte solution in pigs with 40% TBSA burns, and demonstrated that a total of 93% of infused solution was absorbed during the course of the 4 h experiment. However, these experiments were performed in animals under general anesthesia, and there was unavoidable impairment of gastrointestinal peristalsis and absorption ability. Therefore, the results could not be considered as reflecting the true condition of the gastrointestinal tract.

In our present study, we determined the gastric emptying and intestinal absorption rate of GES without the interfering effects of general anesthesia. We found that gastric emptying and intestinal absorption were significantly reduced to the lowest level (52.8% and 23.7% of pre-injury levels) in the OR group at about 2 and 4 h post-burn, respectively, and did not return to 80% of pre-injury level until 24 h. It was estimated that within 24 h

after injury, the water absorption rate in the OR group was 110.9 ± 17.1 mL/h.m, and it was actually higher than the pre-requisite of the Parkland formula (82.1 ± 11.2 mL/h.m). This intestinal absorptive rate was similar to that of Michael *et al.*^[4]. The above results suggest that, in large animals (e.g. pigs or dogs), with < 40% TBSA burns, almost all GES infused according to the Parkland formula could be fully absorbed, although intestinal absorption was inhibited due to gut hypoperfusion. Therefore, we considered that gastric emptying is the main limitation to effective gastrointestinal resuscitation.

The results of using carbachol in our experiment clearly demonstrated that this drug could improve tissue blood perfusion of the gastrointestinal tract. Thus, it was helpful in expediting gastric emptying of its contents and improving intestinal absorption of water and electrolytes. Carbachol is a cholinergic receptor agonist. It stimulates peristalsis of the gastrointestinal tract by activating M cholinergic receptors, and also activates $\alpha 7$ subunits of cholinergic nicotinic receptors on macrophage and endothelial cells which leads to cellular deactivation and inhibition of cytokine release, thus attenuating the systemic or regional inflammatory response^[16-18]. In addition, it is an antioxidant and inhibitor of apoptosis^[19,20]. In our study, the addition of carbachol to the resuscitation fluid did improve blood perfusion of the gastrointestinal tissues, expedite gastric emptying, and improve intestinal absorption rate of water and electrolytes. These beneficial effects of carbachol may be attributable to the following mechanisms: (1) inhibition of release of proinflammatory cytokines alleviates the inflammatory reaction of the gastrointestinal tissue, thus resulting in reduced loss of AQP1^[21,22]; (2) improvement in intestinal peristalsis and blood perfusion due to stimulation of M receptors facilitates intestinal absorption; and (3) promotion of activity of Na^+/K^+ -ATPase, which is essential for absorption of water and especially Na^+ by intestinal mucosa^[23]. Our experiment showed that the gastric emptying time was promoted by 15.7%, and the intestinal absorption rate was increased by 11.5%.

In conclusion, our experiment has successfully reproduced a canine model of serious burn injury. In this model, we are able to study the effects of oral replenishment of GES for resuscitation of burn shock. The hemodynamic parameters, gastric emptying rate, and intestinal absorption of GES can be relatively accurately determined. The results of the experiment also show that burn shock can be ameliorated to a certain extent by administration of GES, with the addition of carbachol to enhance gastric emptying and intestinal absorption of fluids. Oral resuscitation for burn shock might be a surrogate measure where mass casualties from burn injury occur in an area where medical support is minimal and transportation is difficult. Carbachol may be beneficial because it is a cholinergic receptor agonist, and it possesses the function of enhancing gastric emptying and intestinal absorption of fluid when given by mouth. Therefore, in circumstances when intravenous replacement of fluids for resuscitation of burn shock is not fea-

sible, oral feeding of GES, with addition of carbachol, may be possible.

COMMENTS

Background

Rapid intravenous infusion remains unanimously the key measure to resuscitate hypovolemic shock as a result of massive burn injury. Unfortunately, in armed conflicts or massive disasters (such as forest fire, earthquake, or terrorist attack) mass casualties occur in an austere environment with poor medical support and transportation facilities. In such cases, it is our supposition that oral or gastrointestinal administration of fluids might be more practical, and could have a positive effect in maintaining the life of the victims until intravenous replacement of fluids is available.

Research frontiers

Gastrointestinal tract ischemia and hypoxia due to massive surgical stresses such as severe trauma, extensive burns and major surgery resulting in hypovolemic shock can lead to dysfunction of gastric emptying and intestinal absorption, followed by poor transportation and absorption of oral electrolytes and nutrients in the gastrointestinal tract. Improvement of mucosal blood perfusion, and enhancement of gastrointestinal tolerance to oral rehydration fluid and enteral nutrition are not only the foci of research in the surgical and critical care fields, but they are key factors for facilitation of oral resuscitation of hypovolemic shock.

Innovations and breakthroughs

A large animal model of severe burn injury was reproduced to investigate the feasibility of oral resuscitation of burn shock, without the disturbing effects of general anesthesia. The effects of carbachol, which is a cholinergic receptor agonist, on blood circulation, gastrointestinal perfusion, gastric emptying and intestinal absorption of fluid and electrolytes were investigated. The results indicated that oral resuscitation with the help of such a drug might be an ideal way in lieu of intravenous resuscitation for burn shock, especially in battlefields or other sites of mass casualties.

Applications

Orally administered fluid can be considered to be a simple and effective means of replacement of body fluid that is feasible for resuscitation of hypovolemic shock, especially when there is an extreme shortage of means of medical support in an austere environment such as battlefields and disasters.

Peer review

The manuscript is very well written and the conclusions applicable.

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Gastroesophageal reflux in cirrhotic patients without esophageal varices

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Abstract

AIM: To evaluate the esophageal motility and abnormal acid and bile reflux incidence in cirrhotic patients without esophageal varices (EV).

METHODS: Seventy-eight patients with liver cirrhosis without EV confirmed by upper gastroesophageal endoscopy and 30 healthy control volunteers were prospectively enrolled in this study. All the patients were evaluated using a modified protocol including Child-Pugh score, upper gastrointestinal endoscopy, esophageal manometry, simultaneous ambulatory 24-h esophageal pH and bilirubin monitoring. All the patients and volunteers accepted the manometric study.

RESULTS: In the liver cirrhosis group, lower esophageal sphincter pressure (LESP, 15.32 ± 2.91 mmHg), peristaltic amplitude (PA, 61.41 ± 10.52 mmHg), peristaltic duration (PD, 5.32 ± 1.22 s), and peristaltic velocity (PV, 5.22 ± 1.11 cm/s) were all significantly abnormal in comparison with those in the control group ($P < 0.05$), and LESP was negatively correlated with Child-Pugh score. The incidence of reflux esophagitis (RE) and pathologic reflux was 37.18% and 55.13%, respectively

(vs control, $P < 0.05$). And the incidence of isolated abnormal acid reflux, bile reflux and mixed reflux was 12.82%, 14.10% and 28.21% in patients with liver cirrhosis without EV.

CONCLUSION: Cirrhotic patients without EV presented esophageal motor disorders and mixed acid and bile reflux was the main pattern; the cirrhosis itself was an important causative factor.

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Key words: Gastroesophageal reflux disease; Liver cirrhosis; Esophageal varices; Esophageal manometry; pH; Bilirubin; Monitoring

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common diseases in modern civilization, which greatly affects people's health and quality of life^[1]. GERD is defined as reflux of gastroduodenal content to the esophagus, and includes reflux esophagitis (RE), nonerosive reflux disease (NERD) and Barrett's esophagus (BE). GERD originates from a disturbance in the structure and function of the lower esophageal sphincter (LES) barrier, and dysfunctional esophageal motility coupled with a weak LES can cause uncoordinated propulsion, regurgitation of gastric and/or duodenal contents into the esophagus^[2].

Gastroesophageal reflux consists of a broad mixture

of oral-esophageal, gastric, and duodenal secretions. It is accepted that acid reflux plays an important role in the pathogenesis of GERD^[3]. But the role of non-acid reflux is still a controversy^[4,5], and some recent studies have shown that duodenogastroesophageal reflux (DGER) is another important causative factor in esophageal mucosal damage^[6,7]. So the combination of esophageal pH and bilirubin monitoring is indispensable for a precise diagnostic test in acid and non-acid reflux of GERD.

GERD can be induced or aggravated under many conditions including liver diseases. It has been reported that in patients with liver cirrhosis and portal hypertension, gastroesophageal reflux occurs at a high frequency (64%)^[8]. Moreover, hepatic cirrhosis has a high morbidity and mortality due to the portal hypertension with the development of esophageal varices, and the possibility of a digestive hemorrhage and worsening of hepatic insufficiency^[9-11]. It is important to identify predictive or aggravating factors and if possible, to prevent these factors. Esophageal motor disorders have been found to be associated with acid gastroesophageal reflux in cirrhotic patients with esophageal varices, and functional studies have shown decreased functions of the lower esophageal sphincter with low amplitude of primary peristalsis and acid clearance, which might attribute to a mechanical effect of the presence of varices^[12]. It is still unclear whether the presence of cirrhosis itself presents as a causative factor for the onset of gastroesophageal reflux, and there are few studies on the incidence of acid reflux and DGER in the cirrhotic patients without esophageal varices. Therefore, this study was designed to evaluate the esophageal motility and abnormal acid and bile reflux incidence in cirrhotic patients without esophageal varices.

MATERIALS AND METHODS

Patients

Seventy-eight patients with liver cirrhosis without EV confirmed by upper gastroesophageal endoscopy from March 2008 to November 2010 were prospectively enrolled to this study. All the patients were the inpatients of Beijing Tiantan Hospital, Capital Medical University. Patients with systemic diseases related to esophageal motor disorders and/or gastroesophageal reflux diseases (progressive systemic sclerosis, diabetes mellitus, neuromuscular disorders), alcohol abusers within 6 mo and chronic drug users that influence esophageal motility (such as theophylline, nitrates and calcium channel blockers) were excluded.

All the patients were evaluated by the same physician according to a modified protocol including Child-Pugh score^[13], ascites, and other complications and a reflux disease questionnaire (RDQ; AstraZeneca R and D, Wuxi, China). RDQ is a detailed questionnaire regarding the severity and frequency of four symptoms: heartburn, acid regurgitation, food regurgitation, and retrosternal pain, and each symptom is graded in severity and frequency. The diagnosis of liver cirrhosis was verified by the clinical, laboratory, radiologic and histopathological results according to the criteria of the Chinese Medical

Society for Liver Diseases^[14].

Thirty healthy volunteers (15 women) with a mean age of 33 years served as controls in this study. None of them had a history of reflux disease or of surgery in the upper gastrointestinal tract or thorax. All the volunteers accepted the manometric study without medication.

The written informed consent for the study was approved by the hospital ethics committee, and obtained from all the subjects and the procedure followed the principles of the Declaration of Helsinki.

Methods

Upper gastrointestinal endoscopy. To exclude the EV cases, all patients received the upper gastrointestinal endoscopic examination (OlympusXQ260; Olympus, Japan). Gastric varices and/or related congestive gastropathy were also recorded. Reflux esophagitis if present was classified according to the Los Angeles classification standards. Barrett's esophagus was defined as a columnar-lined esophageal mucosa with intestinal metaplasia.

Esophageal manometry. Manometry was performed using a water-perfused manometric assembly (Medtronic, Deutschland). The manometric probe consisted of a 4.5-mm polyvinyl catheter (Medtronic) with eight measuring sites (0, 1, 2, 3, 5, 10, 15, and 20 cm). The position, length and pressure of the lower esophageal sphincter (LES) were identified by the method of stepwise retraction of the probe through gastroesophageal junction (GEJ). After correct positioning of the catheter in the esophageal lumen, patients were asked to swallow ten 5-mL boluses of water. Manometric signals were recorded on a computer for subsequent display and analysis, and the information included: the length of the LES, antegrade and retrograde peristalses, synchronous and isolated contractile waves, peristaltic amplitude (PA), peristaltic duration (PD), and peristaltic velocity (PV) of primary peristaltic wave in distal esophagus. LES disorder and esophageal body dysmotility were diagnosed according to the criteria in a previous study^[15].

Simultaneous ambulatory 24-h esophageal pH and bilirubin monitoring. After esophageal manometry, an antimony esophageal pH electrode and fiber optic probe for detecting acid and bilirubin were positioned pernasally 5 cm above the upper border of the LES and connected with an ambulatory pH recorder (Digrapper Mk III 2000, Synetice Medical, Sweden) and an ambulatory duodenogastroesophageal reflux (DGER) monitoring system (Bilitic 2000, Synetice Medical, Sweden), respectively. The method was reported previously^[16]. The recorded data were analyzed using the Synectics PM Software.

In brief, the 24-h pH ambulatory recording was carried out with a portable digital system composed of a catheter with an antimony electrode and external reference electrode. Patients were instructed to keep a diary recording the time of meals, position changes, and the time and type of their symptoms, and encouraged to pursue their normal daily activities and maintain their usual diet, avoiding citric fruit and soft drinks. Proton pump inhibitor if in use, were discontinued at least 7-10 d prior to the

Table 1 Results of esophageal manometry in liver cirrhosis patients and controls (mean \pm SD)

Group	LESP (mmHg)	PA (mmHg)	PD (s)	PV (cm/s)
Liver cirrhosis (<i>n</i> = 78)	15.32 \pm 2.91 ^a	61.41 \pm 10.52 ^a	5.32 \pm 1.22 ^a	5.22 \pm 1.11 ^a
Child A (<i>n</i> = 28)	16.18 \pm 2.81	70.52 \pm 8.93 ^a	3.91 \pm 1.03 ^a	4.56 \pm 1.22 ^a
Child B (<i>n</i> = 27)	15.41 \pm 3.13 ^c	67.4 \pm 9.3 ^c	5.11 \pm 1.21 ^c	5.10 \pm 1.02 ^c
Child C (<i>n</i> = 23)	14.52 \pm 2.91 ^e	56.13 \pm 10.06 ^e	6.02 \pm 1.23 ^e	5.91 \pm 1.01 ^e
Control (<i>n</i> = 30)	16.21 \pm 5.33	74.41 \pm 17.53	2.70 \pm 0.81	3.71 \pm 1.82

^aCompared with control, *P* < 0.05; ^cCompared with Child A, *P* < 0.05; ^eCompared with Child B, *P* < 0.05. LESP: Lower esophageal sphincter pressure; PA: Peristaltic amplitude; PD: Peristaltic duration; PV: Peristaltic velocity.

examination, H₂ blockers at least 48–72 h and prokinetics agents 24 h. An esophageal pH of less than 4 for at least 15 s was considered to be a reflux episode. Pathological acid reflux was considered if the percentage of the time with the intraesophageal pH less than 4 was greater than 4%, the number of reflux episodes was larger than 50 or the DeMeester value was higher than 14.72^[17].

The fiber optic spectrophotometer Bilitec 2000 was used to quantify DGER. The system consisted of a miniaturized probe measuring 1.5 mm in diameter that carried light signals into the esophagus and backed *via* a plastic fiberoptic bundle. Before each study, the probe was calibrated in water, and the probe tip was checked for obstruction after completion of the study.

Patients were also encouraged to maintain normal activities, sleep schedule, and to follow a particular low-fat diet containing light food elements, and not to take coffee, tea and fruit juice, in order to prevent any interference with the spectrophotometric recording. Skimmed milk and non-sparkling water were allowed. An episode of DGER was defined as an increase in esophageal bilirubin absorbance 0.14 for more than 10 s^[18,19].

Blood sample detection

Blood samples were drawn for a complete analysis of blood cell count and levels of prothrombin, albumin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase, gamma glutamyl transferase, bilirubin, cholesterol, creatinine.

Statistical analysis

Statistical analysis was performed using the statistical program SPSS 13.0 for Windows (SPSS Inc., Chicago, USA). All data were presented as mean \pm SD, and *P* values lower than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

Seventy-eight patients met the inclusion criteria, 40 males (51.28%) and 38 females (48.72%), with a mean age of 56.41 \pm 9.72 years (range, 18–75 years). Twenty-eight patients were classified as Child A, 27 as Child B and 23 as Child C patients. Typical symptoms of gastroesophageal reflux disease were present in 25 (32.05%) patients. The RDQ scores were significantly higher in liver cirrhosis group (11.32 \pm 3.14) than in control group (6.25 \pm 3.31)

(*P* < 0.01). There were no statistical differences of RDQ scores among the liver cirrhosis subgroups, and no relationship between Child-Pugh score and abnormal reflux (*P* > 0.05).

Esophageal manometry

In the liver cirrhosis group, LESP (15.32 \pm 2.91 mm Hg), PA (61.41 \pm 10.52 mmHg), PD (5.32 \pm 1.22 s), and PV (5.22 \pm 1.11 cm/s) were all significantly abnormal in comparison with those in the control group (*P* < 0.05) (Table 1). The results showed a gradual decrease of LESP and PA, also an extension of PD and PV in the liver cirrhosis group from Child A to Child C. LESP was negatively correlated with Child-Pugh score (*P* < 0.01, *r* = -0.625).

24-h esophageal pH monitoring

The results demonstrated a stepwise increase of pathologic esophageal pH-metry in liver cirrhosis patients, and an increase of acid reflux episodes and percentage of a pH < 4 in the upright, supine and total phases of measurement (*P* < 0.05) (Table 2).

24-h esophageal bilirubin monitoring

The results showed a significant stepwise increase of pathologic esophageal bilirubin-metry in liver cirrhosis patients, along with significant increases of bile reflux episodes and percentage of absorbance > 0.14 in the upright, supine, and total phases of measurement (*P* < 0.05) (Table 3).

Incidence of RE and abnormal reflux

The incidence of RE and pathologic reflux was 37.18% and 55.13% in patients with liver cirrhosis, respectively, which were all higher than those in the control group (*P* < 0.05) (Table 4 and Figure 1). The incidence of isolated abnormal acid reflux, bile reflux and mixed reflux was 12.82%, 14.10% and 28.21% in patients with liver cirrhosis, respectively (Table 5). And the incidence of BE was 5.13% (4/78) in patients with liver cirrhosis, and none was found in the control group.

DISCUSSION

As a complication of chronic liver disease, GERD in cirrhotic patients with EV accounted for about 20%, which mainly belongs to a dyskinetic type^[20]. Previous studies found that esophageal varices played an important role

Table 2 Results of ambulatory 24-h esophageal pH monitoring in liver cirrhosis patients and controls (mean ± SD)

Group	Number of acid reflux episodes	Number of acid reflux episodes lasting ≥ 5 min	Mean time pH < 4 (%)		
			Total	Upright	Supine
Liver cirrhosis (n = 78)	61.17 ± 33.35 ^a	15.25 ± 5.73 ^a	10.34 ± 4.45 ^a	5.22 ± 2.71 ^a	9.56 ± 3.42 ^a
Child A (n = 28)	51.24 ± 20.54 ^a	10.66 ± 7.28 ^a	8.11 ± 2.32 ^a	4.48 ± 1.76 ^a	7.32 ± 5.44 ^a
Child B (n = 27)	60.35 ± 18.66 ^c	12.35 ± 9.83 ^c	10.51 ± 1.62 ^c	5.64 ± 1.31 ^c	9.14 ± 4.37 ^c
Child C (n = 23)	73.52 ± 28.63 ^e	17.34 ± 12.46 ^e	12.34 ± 2.15 ^e	6.79 ± 1.51 ^e	11.56 ± 5.43 ^e
Child D (n = 30)	39.62 ± 29.32	4.81 ± 2.04	2.35 ± 1.53	3.58 ± 1.34	8.69 ± 3.45

^aCompared with control, *P* < 0.05; ^cCompared with Child A, *P* < 0.05; ^eCompared with Child B, *P* < 0.05.

Table 3 Results of ambulatory 24-h esophageal bilirubin monitoring in liver cirrhosis patients and controls (mean ± SD)

Group	Number of bile reflux episodes	Number of bile reflux episodes lasting ≥ 5 min	Mean time Abs > 0.14 (%)		
			Total	Upright	Supine
Liver cirrhosis (n = 78)	36.53 ± 9.31 ^a	4.09 ± 1.15 ^a	6.73 ± 1.15 ^a	3.32 ± 1.05 ^a	4.37 ± 1.44 ^a
Child A (n = 28)	27.32 ± 10.31 ^a	3.85 ± 1.34 ^a	5.12 ± 1.45 ^a	3.15 ± 0.92 ^a	4.12 ± 0.97 ^a
Child B (n = 27)	39.46 ± 18.31 ^c	4.11 ± 1.65 ^c	6.54 ± 1.21 ^c	3.37 ± 1.13 ^c	5.04 ± 1.11 ^c
Child C (n = 23)	48.54 ± 26.41 ^e	4.23 ± 2.14 ^e	7.32 ± 1.34 ^e	4.28 ± 1.22 ^e	5.52 ± 1.12 ^e
Control (n = 30)	12.76 ± 6.97	2.15 ± 1.36	1.98 ± 0.86	1.03 ± 0.23	0.83 ± 0.62

^aCompared with control, *P* < 0.05; ^cCompared with Child A, *P* < 0.05; ^eCompared with Child B, *P* < 0.05. Abs: Absorbance.

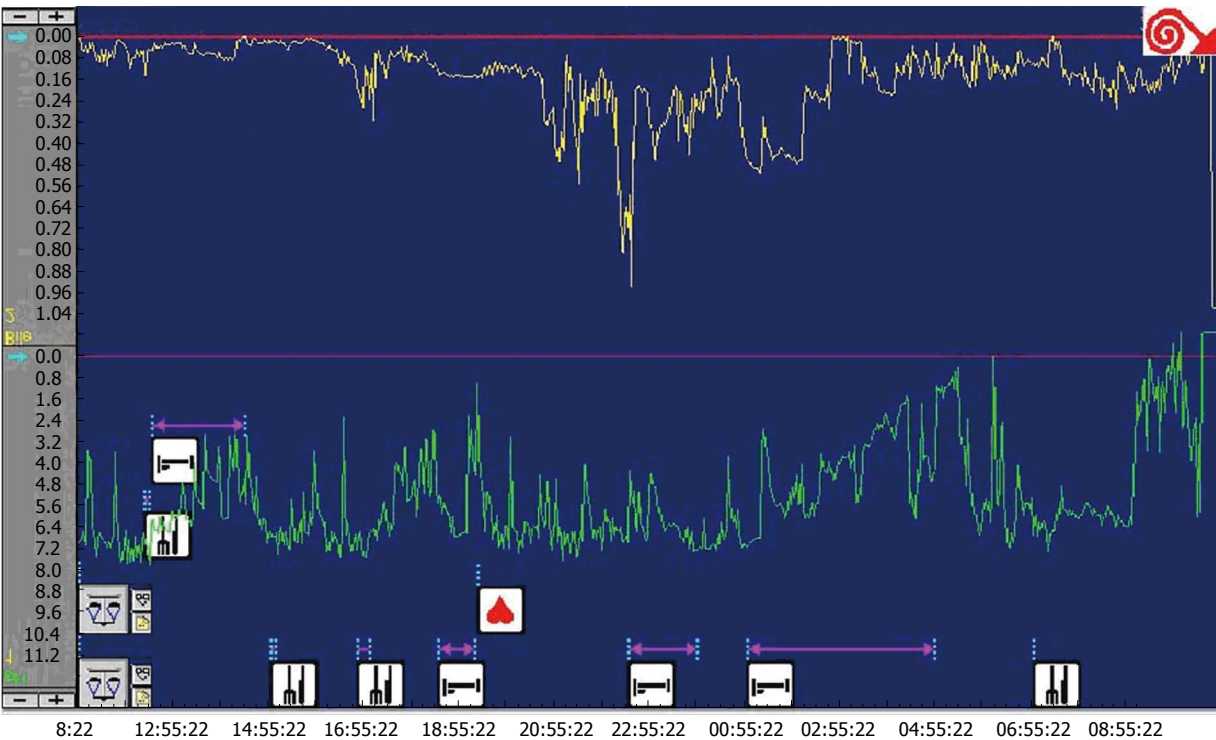


Figure 1 Mixed abnormal acid and bilirubin reflux curves in a typical Child C patient.

Table 4 Relationship between liver function classification of cirrhotic patients and gastroesophageal reflux disease n (%)

Group	RE	Abnormal reflux
Child A (n = 28)	8 (28.57)	12 (42.86)
Child B (n = 27)	11 (40.74)	15 (55.56)
Child C (n = 23)	10 (43.48)	16 (69.57)
Total (n = 78)	29 (37.18)	43 (55.13)

RE: Reflux esophagitis.

in the development of esophageal motor disorders and abnormal gastroesophageal reflux in these patients, who presented obvious esophageal motor and motility disorders^[21,22]. The most prevalent disorder was the inefficient esophageal motility, along with abnormal PA, PD and PV^[12,21-23]. Some studies found that motor disorders existed in the esophageal body in these cirrhotic patients with EV, as compared with the cirrhotic patients without varices and control group^[24]. Thus, it seemed that EV itself, inde-

Table 5 Abnormal reflux in liver cirrhosis patients

Group	Isolated abnormal acid reflux	Isolated abnormal bile reflux	Mixed abnormal reflux	No abnormal reflux
Child A (<i>n</i> = 28)	4	3	5	16
Child B (<i>n</i> = 27)	3	4	8	12
Child C (<i>n</i> = 23)	3	4	9	7
Total (<i>n</i> = 78)	10 (12.82%)	11 (14.10%)	22 (28.21%)	35 (44.87%)

pendent of the cirrhosis, delayed esophageal clearance and increased the contact time between acid and mucosa.

In this study, LESP, PA, PD and PV in cirrhotic patients without esophageal varices were significantly abnormal as compared with those in the control group. LESP was markedly lower in patients with severe liver function damage, and negatively correlated with Child-Pugh score ($P < 0.01$, $r = -0.625$). The results showed that cirrhosis itself was another important factor for the esophageal motor disorder.

The incidence of esophageal acid reflux among cirrhotic patients with EV has also been studied in the last decades using pH-metry recording. It has been postulated that acid reflux may contribute to esophagitis and variceal bleeding in cirrhotic patients, and it occurs at a high frequency (64%) in patients with liver cirrhosis and portal hypertension, irrespective of the etiology of cirrhosis and the grade of esophageal varices^[8]. The results indicated that there was a correlation between typical gastroesophageal reflux disease and abnormal reflux, but no relationship between ascites, variceal size, congestive gastropathy and Child-pugh score and abnormal reflux.

The high incidence of RE in patients with severe chronic liver disease was also demonstrated, and asymptomatic RE was more common in cirrhotic and liver failure patients^[25,26]. In the present study, abnormal reflux and RE were demonstrated in 55.13% and 37.18% of the cirrhotic patients without esophageal varices, and the more severe liver function damage, the more abnormal parameters of acid and bilirubin reflux. In the mean time, typical symptoms of gastroesophageal reflux disease were presented in only 32.05% of the cirrhotic patients in this study, and abnormal reflux was found in 62% of the patients in the night possibly due to the lowered esophageal defenses during this period, with reduction of saliva production, swallowing and esophageal clearance.

GERD may occur in acid, bile or a mixed form, and DGER is considered as an independent risk factor for complicated GERD. However, few studies have reported the incidence of DGER among cirrhotic patients. Patients with Barrett's esophagus had significantly higher levels of DGER than patients with uncomplicated GERD, and bile reflux either alone or mixed with acid reflux contributed obviously to the severity of erosive and non-erosive reflux disease^[6]. Moreover, DGER in acid medium was more injurious to the esophagus than DGER in alkaline pH^[7]. We studied for the first time the incidence of BE and DGER in cirrhotic patients without esophageal varices. We found that the mixed acid and bile reflux was the predominant pattern of reflux in GERD patients, and the reflux incidence was also higher in Child B or C group than in Child

A group. A stepwise increase of mixed reflux was demonstrated along with the severity of liver function damage. Four BE patients (2 with mixed abnormal reflux, 2 with DGER) were found in Child C group.

The causes and the mechanism of liver cirrhosis in patients with abnormal GERD have not been fully elucidated. In this study, we demonstrated an obvious esophageal motility disorder and abnormal gastroesophageal reflux in cirrhotic patients without esophageal varices, and abnormalities of esophageal motility and reflux parameter were correlated with the severity of liver function damage. It seemed that not only mechanical effect (EV), but also neural and humoral factor are related to the high incidence of GERD in patients with liver cirrhosis. The progress of liver dysfunction decreased the incidence of LESP, worsened the esophageal motility and the reflux in the cirrhotic patients. In some studies, the levels of plasma vasoactive peptides and neurotensin were markedly higher in patients with liver cirrhosis than in the normal population, which were also known to lower the pressure of the LES, facilitating the reflux of the stomach content^[21,27].

The importance of nitrous oxide (NO) in the exacerbation of portal hypertension in liver cirrhosis was also reported^[28,29]. This substance can be found in large amounts in the systemic circulation of cirrhotic patients, and NO concentration increased significantly in patients with liver disease, which was closely related to the transient LES relaxation, suggesting that NO played an important role in the process of GERD. Whether the excessive NO in cirrhotic patients could exacerbate these manifestations, needs to be further confirmed.

We found that gastric half-emptying of liquid food was delayed in patients with liver cirrhosis, and the function of gastric emptying was also influenced by the damaged liver function^[30]. Ascites induced an increase in intra-abdominal pressure, compressing the stomach and the stomach content reflux^[31].

In summary, the majority of cirrhotic patients without EV presented esophageal motor disorders; mixed acid and bile reflux was the main pattern of reflux in GERD patients; and the presence of cirrhosis itself was an important causative factor for the onset of gastroesophageal reflux. Further researches on the functional and humoral factors and mechanism of GERD in liver diseases will gain a broad attention and interest in this field.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is one of the most common diseases

in modern civilization, and it has been reported that gastroesophageal reflux disease occurs at a high frequency in patients with liver cirrhosis.

Research frontiers

The relationship between esophageal motor disorders and acid gastroesophageal reflux in cirrhotic patients with esophageal varices has been reported. This study was designed to evaluate the esophageal motility and abnormal acid and bile reflux incidence in cirrhotic patients without esophageal varices.

Innovations and breakthroughs

This study showed that the presence of cirrhosis itself was an important causative factor for the onset of gastroesophageal reflux in patients with liver cirrhosis without varices. It is the first research on the incidence of Barrett's esophagus and DGER in cirrhotic patients without esophageal varices.

Applications

This study helped better understand the mechanism of GERD in patients with liver cirrhosis, and contributed to the diagnosis and treatment of liver cirrhosis and its complications in clinical practice.

Peer review

This is an interesting study on GERD in patients with liver cirrhosis, but without esophageal varices. Since it has before been thought that esophageal varices somehow have something to do with the increased frequency of GERD in patients with liver disease, the authors have made an interesting contribution to the literature by showing that reflux symptoms and pathologic esophageal motility changes are more common in patients with cirrhosis but without varices.

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Association between polymorphism rs6983267 and gastric cancer risk in Chinese population

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Abstract

AIM: To explore the association between single nucleotide polymorphisms (SNPs) at 8q24 and gastric cancer risk.

METHODS: A case-control investigation including 212 gastric cancer patients and 377 healthy controls was conducted. The genotypes of SNPs (rs6983267, rs7008482 and rs10808555) were examined and established through polymerase chain reaction-restriction

fragment length polymorphism (PCR-RFLP). Multivariate logistic regression models were used to evaluate the association between SNPs and gastric cancer.

RESULTS: The genotype frequencies of rs6983267 in gastric cancer patients were obviously different from those in the control ($P = 0.005$). GT genotype of rs6983267 was associated with an increased risk of gastric cancer compared with GG genotype (adjusted odds ratio = 2.01, 95% confidence interval: 1.28-3.14). Further stratified analysis indicated that rs6983267 GT genotype facilitated the risk of gastric cancer of non-cardiac and intestinal type (OR: 2.638, 95% CI: 1.464-4.753; OR: 1.916, 95% CI: 1.166-3.150, respectively).

CONCLUSION: This study demonstrates for the first time that rs6983267 is involved in susceptibility to gastric cancer, although further large-sample investigations are still needed.

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Key words: Gastric cancer; Genetic susceptibility; Single nucleotide polymorphism; MYC; 8q24

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INTRODUCTION

Gastric cancer is the second most common cause of death

from cancer in the world^[1] and the incidence rate was 16.2 per 100 000^[2]. Despite of a marked decrease in gastric cancer mortality rate in many countries, there is a higher prevalence of gastric cancer in the Chinese population than in other races. No doubt, either a high absolute number or a high mortality of gastric cancer has become a key public health issue in China.

Although numerous biological and epidemiological studies have shown risk factors for gastric cancer, the available knowledge is still insufficient to reveal the exact mechanism of gastric cancer. Current researches have shown that both genetic and environmental factors play an important role in gastric carcinogenesis^[3,4] and genetic susceptibility accounts for 35% of disease etiology^[5]. Recently, the association between variants at 8q24 and breast, prostate and colorectal cancers has been discovered and confirmed by several research groups^[6-15], which suggested a complex contribution of polymorphisms at 8q24 to the formation of multiple adenomas. However, whether these common variants in 8q24 are also associated with the risk of gastric cancer has so far not been published.

In the present study, we conducted a case-control association study to evaluate the effect of rs6983267, rs7008482 and rs10808555 in 8q24 in the risk of gastric cancer in the Chinese population.

MATERIALS AND METHODS

Subjects

A total number of 216 cases and 400 controls were enrolled from January 2009 to January 2010 in Tongji Hospital, Shanghai. Among the 1360 subjects who were invited to take part in this study, only 45% individuals agreed to participate and donated 3 mL venous blood sample. All the gastric cancer cases had been checked by the gastroscopy and diagnosed by the specialized physician. The exclusion criteria of cases included: (1) Having a history of any other cancers or any metastasized cancer (carcinomas were not originally from stomach); and (2) Having undergone radiotherapy or chemotherapy. Controls were randomly selected among the first-visit outpatients who were confirmed to have no cancer or a prior history of neoplasm. Available baseline characteristics, including age, gender, race, tumor location, histological type, were recorded. All the subjects were genetically unrelated ethnic Han Chinese. This study was approved by the institutional review board of Tongji University School of Medicine. Written informed consent was obtained from all participants.

Genotyping

According to the manufacturer's protocol, we used Flexi Gene DNA Kit (Qiagen, Hilden, Germany) to extract genomic DNA from peripheral blood leukocytes of the subjects and stored extracted DNA at -20°C. Unique primer sequences were designed in the website of primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>) and primer sequences for rs6983267, rs7008482 and rs10808555 were as follows: 5'-ATGAAGGCGTTCGTCCTCAAATGA-3'

(forward) and 5'-TTGGCTGGCACTGTCTGTATA-3' (reverse); 5'-CCAAGCAGAGAGGAACCAACT-3' (forward) and 5'-GCCACCTTTTATTCTCCAACC-3' (reverse); 5'-ATATGGTCCCTGCCCTCAAG-3' (forward) and 5'-CACTGTGCTAAAGGAATCAGCAA-3' (reverse), respectively. Polymorphisms genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Amplification reactions were carried out in a total volume of 15 µL containing 0.3 mmol/L of each deoxynucleoside triphosphate, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 2 mmol/L MgCl₂, 20% Q solution (Qiagen, Hilden, Germany), 0.16 µmol/L of each primer, 10 ng genomic DNA, and 1 U Taq (TaKaRa, Otsu, Shiga, Japan). Cycling conditions were: 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 64°C for 30 s with a 0.5°C decrement of the annealing temperature per cycle and 72°C for 30-45 s, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s, followed by 72°C for 8 min. PCR products were digested overnight at 37°C with a predicted restriction enzyme, Ts-p45I (Fermentas, Vilnius, Lithuania) for rs6983267, CviQI (Fermentas, Vilnius, Lithuania) for rs7008482, Eco130I (Fermentas, Vilnius, Lithuania) for rs10808555 and were analyzed on 3% agarose with ethidium bromide staining. Three sorts of PCR products were digested into 3 different types of fragments. For rs6983267, the G allele resulted in two fragments of 198-bp and 344-bp, and the C allele produced one fragment of 498-bp. For rs7008482, the G allele resulted in two fragments of 270-bp and 131-bp, while the T allele produced one fragment of 401-bp. For rs10808555, the G allele digested into two fragments of 193-bp and 110-bp, and the A allele generated one fragment of 303-bp.

All the samples were assayed blindly without knowing the case or control status. After genotyping was performed, two research assistants read the gel pictures independently. When they failed to reach a consensus on the tested genotypes (< 1%), they would repeat the genotyping again so as to achieve a final consensus. To ensure the genotyping accuracy, randomly selected PCR products were reevaluated by DNA sequencing^[16]. In addition, 5% of all samples were randomly selected and genotyped in duplicate, and the results were 100% concordant.

Statistical analysis

Hardy-Weinberg equilibrium was tested using the two-sided χ^2 test. χ^2 test was used to compare genotype frequency and demographic distributions between cases and controls. Multivariate unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CIs) for the association between genotypes and gastric cancer, adjusting for age and gender. The co-dominant and the dominant models were used for the analysis. In the co-dominant model, each SNP was separated into three categories, 1 for each genotype, with one genotype chosen as the reference group. For the dominant model, each SNP was modeled as a dichotomous variable with

Table 1 Characteristics of cases and control

Variables	Gastric cancer <i>n</i> (%)	Control <i>n</i> (%)	<i>P</i> value ²
Overall	212	377	
Sex			0.063
Male	152 (71.7)	242 (64.2)	
Female	60 (28.3)	135 (35.8)	
Age			0.670
Mean ± SD (yr)	62.47 ± 11.6	62.89 ± 11.3	
Histological types			
Intestinal	155 (73.1)		
Diffuse	44 (20.8)		
Mixed	13 (6.1)		
Tumor location ¹			
Cardia	74 (38.5)		
Noncardia	118 (61.5)		

¹The number of subjects in cases for tumor location (*n* = 192) was less than the total number (*n* = 212) because some information was not obtained;

²Two-sided χ^2 test for the frequency distribution of variants between gastric cancer cases and controls.

1 genotype chosen as the reference group, and the other two genotypes combined into one category. All tests were two-sided and *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS 16.0 software package (SPSS, Chicago, IL)

RESULTS

Demography

Among 216 gastric cancer cases and 400 controls, 4 cases and 23 controls were dropped out due to poor-quality genomic DNA. Of the 212 cases of gastric cancer, 155 were intestinal type, 44 diffuse type and 13 mixed-type. The mean age of cases was 62 years and the mean age of controls was 63 years. The characteristics of the cases and controls are summarized in Table 1. There was no significant difference between the groups with respect to the age and gender distributions.

SNPs and gastric cancer risk

Among the controls, the genotype distributions of rs6983267 and rs7008482 were in Hardy-Weinberg equilibrium (*P* > 0.1 and *P* > 0.9, respectively), but rs10808555 did not fit Hardy-Weinberg equilibrium (*P* < 0.05). The genotype frequencies of rs6983267 were obviously different between gastric cancer patients and control ($\chi^2 = 10.8$, *P* = 0.005). Analysis under both co-dominant model and dominant model showed that only rs6983267 was significantly associated with gastric cancer risk, after adjustment for age and gender (Table 2). In the co-dominant model, rs6983267 GT genotype was associated with approximately 2 times higher odds of gastric cancer risk (OR: 2.01, 95% CI: 1.28-3.15) compared with the GG genotype. In the dominant model, combined genotypes (GT + TT) of rs6983267 were significantly associated with increased risk of gastric cancer in comparison with GG genotype (OR: 1.82, 95% CI: 1.18-2.81). However, the genotype frequencies of rs7008482 were similar between gastric cancer

Table 2 Association between variation in single nucleotide polymorphisms rs6983267 and rs7008482 and risk of gastric cancer

Genotype	Control <i>n</i> (%)	Cases <i>n</i> (%)	<i>P</i> value
rs6983267			
GT/TT	268 (72.8)	166 (83.0)	0.007
TT	72 (19.6)	32 (16.0)	0.369
GT	196 (53.3)	134 (67.0)	0.002
GG	100 (27.2)	34 (17.0)	
rs7008482			
GT/GG	224 (61.0)	142 (67.0)	0.138
GG	52 (14.2)	38 (17.9)	0.108
GT	172 (46.9)	104 (49.1)	0.250
TT	143 (39.0)	70 (33.0)	

patients and controls (*P* > 0.05).

Furthermore, we evaluated the contributions of SNPs to subgroups according to age, gender, different histological types and tumor locations (Table 3). In the subgroup aged ≤ 60 years, rs6983267 GT genotype markedly increased the risk of gastric cancer referring to GG genotype (OR: 3.21, 95% CI: 1.52-6.68), but in the subgroup aged > 60 years, no significant difference was found (*P* = 0.137). In addition, rs6983267 GT genotype was significantly associated with augmentation of gastric cancer risk in both male and female. As to the histological types and tumor sites, rs6983267 GT heterozygote had a significantly increased risk for non-cardiac gastric cancer (OR: 2.64, 95% CI: 1.46-4.75) and intestinal-type gastric cancer (OR: 1.92, 95% CI: 1.17-3.15) in contrast with GG genotype. Further analysis in Table 4 demonstrated that rs6983267 GT genotype increased the risk of an intestinal-type gastric adenocarcinoma from non-cardiac region. For rs7008482, only GG genotype was associated with significantly increasing risk of gastric cancer compared with TT genotype in male subgroup (OR: 1.88, 95% CI: 1.01-3.47) (Table 3).

DISCUSSION

This is the first study to discover the association between rs6983267 at 8q24 and the susceptibility of gastric cancer, although other previous studies had reported that rs6983267 was associated with the risk of colorectal cancer and prostate cancer^[13,17]. Our observation and analysis indicated that compared with GG genotype of rs6983267, GT genotype and combined genotypes (GT + TT) were both markedly associated with the increasing risk for gastric cancer. And further stratified investigation confirmed that rs6983267 GT genotype facilitated the risk of non-cardiac and intestinal-type gastric cancer. Therefore, rs6983267 is a novel gastric cancer associated polymorphism in 8q24 in Chinese Han population.

Rs6983267 resides at 8q24, proximal to a processed pseudogene, *POU5F1P1*, which is a retrotransposed copy of the POU-domain transcription factor Oct4^[18]. At least one mouse *Oct4* pseudogene has been shown to mediate stem cell regulatory function^[19], suggesting that *Oct4*

Table 3 Association between rs6983267 and rs7008482 polymorphism and clinicopathological features of gastric cancer

	rs6983267							rs7008482						
	GG		GT		TT			TT		GT		GG		
	HC/GC	HC/GC	OR (95% CI)	P value	HC/GC	OR (95% CI)		HC/GC	HC/GC	OR (95% CI)	P value	HC/GC	OR (95% CI)	P value
Age (yr)														
≤ 60	46/11	76/59	3.21 (1.52-6.77)	0.002	29/13	1.86 (0.73-4.72)	0.191	66/30	71/46	1.42 (0.80-2.51)	0.230	15/11	1.61 (0.66-3.92)	0.295
> 60	54/23	120/75	1.54 (0.87-2.74)	0.137	43/19	1.05 (0.50-2.16)	0.901	77/40	101/58	1.15 (0.69-1.90)	0.592	37/27	1.44 (0.77-2.70)	0.258
Sex														
Male	60/26	125/94	1.77 (1.04-3.02)	0.036	48/23	1.10 (0.56-2.17)	0.783	98/46	107/77	1.52 (0.96-2.41)	0.072	32/29	1.88 (1.01-3.47)	0.045
Female	40/8	71/40	3.68 (1.49-9.06)	0.005	24/9	2.22 (0.73-6.74)	0.160	45/24	65/27	0.83 (0.42-1.65)	0.599	20/9	0.98 (0.38-2.54)	0.967
Histological types														
Intestinal	100/26	196/98	1.92 (1.17-3.15)	0.010	72/23	1.20 (0.63-2.28)	0.571	143/52	172/73	1.17 (0.77-1.78)	0.047	52/30	1.56 (0.89-2.72)	0.118
Diffuse	100/7	196/25	1.93 (0.80-4.66)	0.144	72/8	1.74 (0.60-5.09)	0.309	143/15	172/23	1.36 (0.68-2.71)	0.390	52/6	1.26 (0.46-3.47)	0.651
Mixed	100/1	196/11	5.56 (0.71-44.52)	0.103	72/1	1.37 (0.08-21.06)	0.827	143/3	172/8	2.29 (0.63-9.43)	0.228	52/2	1.95 (0.34-13.47)	0.474
Location														
Cardia	100/16	196/40	1.26 (0.67-2.38)	0.469	72/14	1.18 (0.54-2.58)	0.681	143/25	172/35	1.18 (0.67-2.07)	0.574	52/14	1.52 (0.73-3.18)	0.263
Noncardia	100/16	196/82	2.64 (1.46-4.75)	0.001	72/12	1.06 (0.47-2.38)	0.888	14/39	172/59	1.28 (0.81-2.04)	0.295	52/20	1.47 (0.78-2.77)	0.228

Multivariate unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CIs) for the association between genotypes and gastric cancer, adjusting for age and gender. HC: Health control; GC: Gastric cancer.

Table 4 Stratified analysis of rs6983267 genotypes and gastric cancer

	Control n (%)	Intestinal case		Diffuse case	
		n (%)	OR (95% CI)	n (%)	OR (95% CI)
Cardia					
TT	72 (19.6)	12 (22.2)	1.243 (0.53-2.90)	1 (8.3)	0.44 (0.04-4.31)
GT	196 (53.3)	29 (53.7)	1.131 (0.56-2.28)	8 (66.7)	1.36 (0.35-5.28)
GG	100 (27.1)	13 (24.1)		3 (25)	
Noncardia					
TT	72 (19.6)	8 (9.6)	0.99 (0.38-2.59)	4 (16.7)	1.91 (0.44-8.25)
GT	196 (53.3)	64 (77.1)	2.95 (1.49-5.86)	16 (66.6)	2.56 (0.80-8.17)
GG	100 (27.1)	11 (13.3)		4 (16.7)	

pseudogene may exert influence in regulating stem cell proliferation^[7]. *Oci4* also plays a critical role in maintaining stem cell pluripotency^[20], self-renewal, and lineage commitment^[21]. *Oci4* has been found to promote tumor growth in a dose-dependent manner^[22] and epithelial dysplasia by interfering with progenitor cell differentiation^[23]. Although the expression of many *Oci4* pseudogenes in poorly differentiated tumors^[24] has been observed, the related molecular mechanism in cancer is unknown.

On the other hand, rs6983267 is located in the region which is 335 kb away from the nearest gene, *MYC*. *MYC* is able to increase the growth and proliferation of normal gastric cells^[25], and may enhance the canceration of gastric epithelial cells by regulating a variety of genes related to proliferation, differentiation^[26], and apoptosis^[27].

MYC overexpression has been described in over 40% of gastric cancer (in both intestinal- and diffuse-type gastric adenocarcinoma)^[28]. Overexpression of *MYC* gene can influence some biological characteristics of normal gastric cells, directly regulate the genes involved in cell cycle regulation^[29], such as *cyclin A*, *cyclin B* and *cdk4*^[30], and accelerate cancerous growth ultimately. The promotion of the growth and proliferation of these cells helps tumor cells maintain malignant phenotype. Moreover, the therapeutic medicine inhibits gastric cancer cell growth by suppressing *MYC* gene expressions, which consistently confirms the crucial function of *MYC* in gastric cancer cell growth^[31]. The region harboring rs6983267 is a transcriptional enhancer and differentially binds transcription factor 7-like 2 (TCF7L2) due to rs6983267, leading to a different physi-

cal interaction with *MYC*^[32]. Given that the cancer risk-associated SNP enhances the expression of *MYC* through increased distal enhancer activity^[32,33], it is reasonable to speculate that rs6983267 may alter expression of *MYC* through modifying regulatory sequences in this region. Despite the research progress, further studies are needed about the concrete molecular mechanisms of the joint effect between *MYC* and rs6983267 polymorphism.

Previous studies have demonstrated that rs6983267 is possibly related to some kinds of malignant tumor. In the present study we found that rs6983267 is a novel gastric cancer related polymorphism. Stratified analysis indicated that the associations between rs6983267 GT genotype and gastric cancer tended to vary with tumor sites and histological types. Rs6983267 GT genotype was associated with both intestinal and non-cardiac type of gastric cancer but not associated with the diffuse and cardiac type, and increased the risk of intestinal type among the non-cardiac gastric cancer, which suggested that rs6983267 GT genotype is more important in modulating the intestinal and non-cardiac type of gastric cancer. However, TT genotype in rs6983267 tended to be a protective factor in intestinal type among the non-cardiac gastric cancer although this was not significant in the association analysis. This phenomenon could be explained, because distinct clinical, epidemiological and molecular features have been noted among tumors arising from cardia or non-cardia, and among intestinal or diffuse histological subtypes^[34]. For instance, the loss of p16 and smad4 protein expression and the positive *EBstein-Barr virus* (EBV) status are more frequent in cardiac carcinomas than that in non-cardiac carcinomas reported by Kim *et al*^[35]. Lu *et al*^[36] reported that intestinal-type gastric cancer predominates in high-risk geographic areas, especially in Japan, Korea and China, whereas the diffuse-type gastric cancer has a uniform geographic distribution. The observed differences between gastric cancers in tumor location and histological types suggest that they are distinct diseases with different etiologies^[37]. Thus, various genetic factors, including rs6983267, may be involved in different subtypes of gastric cancer (cardiac or non-cardiac; intestinal or diffuse). Another plausible explanation for this situation may be the genetic heterogeneity which may limit the ability to detect an association between TT genotype and gastric cancer. Other variants, which have a strong association with risk of gastric cancer, including as yet undiscovered susceptibility genes, may affect the outcome of this research. Moreover, the limited sample size may be not sufficient to generate this association. Overall, the genetic susceptibility and environmental factors have been proposed to play an important role in the etiology of gastric cancer, and different subtypes of gastric cancer may have diverse biological mechanisms.

Apart from the discovery in rs6983267, this study failed to demonstrate the association between rs7008482 and the risk of gastric cancer, although rs7008482 was reported to be associated with prostate and colorectal cancer^[10,11]. Nevertheless, positive association between rs7008482 GG genotype and risk of gastric cancer in male subgroup has been

shown. Rs7008482 lies within an intronic region of the *NSMCE2* (also called *MMS21*) gene, and MMS21 protein is a SUMO ligase which is required for DNA replication, recombination and repair^[11]. Considering the function of *NSMCE2* gene and MMS21 protein, we wonder whether the limited sample size hampered the detection of association between rs7008482 and gastric cancer risk. Therefore, further studies are still needed to confirm it.

In conclusion, our data demonstrated for the first time that rs6983267 may predispose to the susceptibility of gastric cancer, especially the intestinal and non-cardiac type. However, as the sample size of the present study is relatively small, additional tests of variant at 8q24 for its association with gastric cancer in a larger population, and functional studies of *MYC* and other nearby genes will be required to fully understand the mechanisms of the cancer-specific risk at 8q24.

COMMENTS

Background

Gastric cancer (GC) is one of the most common cancers, and the second most frequent cause of cancer-related deaths in the world. Epidemiological studies have shown that genetic factors play a crucial role in gastric carcinogenesis. Recently, common polymorphisms located at chromosome 8q24 have been identified to increase the tumor risk. The authors investigated the associations between rs6983267 polymorphisms and GC risk.

Research frontiers

Chromosome 8q24 is an established risk locus for many common epithelial cancers. Polymorphism rs6983267 is a susceptibility marker for prostate and colon cancers, and perhaps also ovarian and other cancers. The relationship between rs6983267 polymorphism and GC needs to be addressed.

Innovations and breakthroughs

To our knowledge, this is the first study of GC risk variant at 8q24 in a Chinese population. Polymorphism rs6983267 was found to be associated with an increased risk of GC, which had been not reported before. The result of stratified analysis according to histological types confirms the contribution of rs6983267 in non-cardiac and intestinal type of gastric carcinogenesis.

Applications

These findings might be of value in the explanation of gastric carcinogenesis. They could be used for further investigations about the association between genetic predisposition and the risk of GC at 8q24.

Terminology

MYC is an oncogene, the protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and proliferations. Single nucleotide polymorphism is a DNA sequence variation occurring when a single nucleotide-A, T, C, or G-in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual.

Peer review

The authors investigated the association of the 3 single nucleotide polymorphisms (SNPs) (rs6983267, rs7008482 and rs10808555) with the risk of gastric cancer by a case-control study, and found that GT genotype of rs6983267 was associated with an increased risk of gastric cancer compared with GG genotype (AOR = 2.01, 95% CI: 1.28-3.14). After stratification, rs6983267 GT genotype was associated the risk of non-cardiac and intestinal type of gastric cancer. This study provides some new SNP for the evaluation of gastric cancer risk.

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EUS for choosing best endoscopic treatment of mesenchymal tumors of upper gastrointestinal tract

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were treated by endoloop, 10 stromal tumors were treated by endoscopic mucosal resection and 8 stromal tumors were treated by endoscopic submucosal dissection. Complete resection of the lesion was achieved in all cases. Of the mesenchymal tumors, 90.38% diagnosed by EUS were also identified by pathohistology. All wounds were closed up nicely and no recurrence was found in the follow-up after 2 mo.

CONCLUSION: EUS is an effective means of diagnosis for upper GIMTs and is an important tool in choosing the endoscopic therapy for GIMTs, by which the lesions can be treated safely and effectively.

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Key words: Leiomyoma; Stromal tumor; Endoscopic ultrasonography; Endoscopic therapy

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Abstract

AIM: To evaluate the value of endoscopic ultrasonography (EUS) in the choice of endoscopic therapy strategies for mesenchymal tumors of the upper gastrointestinal tract.

METHODS: From July 2004 to September 2010, 1050 patients with upper gastrointestinal mesenchymal tumors (GIMTs) were diagnosed using EUS. Among them, 201 patients underwent different endoscopic therapies based on the deriving layers, growth patterns and lesion sizes.

RESULTS: Using EUS, we found 543 leiomyomas and 507 stromal tumors. One hundred and thirty-three leiomyomas and 24 stromal tumors were treated by snare electrosection, 6 leiomyomas and 20 stromal tumors

INTRODUCTION

Gastrointestinal mesenchymal tumors (GIMTs) originate from mesenchymal cells other than epithelial cells or lymphocytes. They are further classified as stromal tumors, leiomyomas, leiomyosarcomas, neural tumors, fibroblast tumors or liparomphalus. Clinically, mesenchymal tumors are usually incidentally discovered as subepithelial bulges during routine endoscopic examinations for unrelated conditions. The classification and management of these

lesions can be challenging. In recent years, with the wide use of endoscopic ultrasound (EUS) to clarify the nature and origin of the subepithelial tumor, great progress has been made in diagnosis and treatment of GIMTs^[1,2]. Importantly, under the guidance of the EUS, GIMTs can be removed by appropriate endoscopic treatment without severe complications^[2-4].

From July 2004 to September 2010, we analyzed 1050 patients with GIMTs diagnosed by EUS in our hospital. Of these patients, 201 underwent different endoscopic therapies based on the EUS results. Our aim in this retrospective study was to evaluate the value of EUS in the choice of endoscopic therapy strategies for mesenchymal tumors of the upper gastrointestinal tract.

MATERIALS AND METHODS

Patients

The medical records of 1050 patients with upper GIMTs diagnosed by EUS examination in the First Affiliated Hospital of Zhejiang University were retrospectively reviewed. All these patients with submucosal protruding lesions in the upper gastrointestinal tract by routine endoscopy were examined by EUS. There were 499 men and 551 women, with a mean age of 52.6 years (range, 19-86 years). Of these patients, 201 patients underwent endoscopic therapy in the First Affiliated Hospital of Zhejiang University, Beilun Zongrui Hospital, the Traditional Chinese Medical Hospital of Ninghai and Jinhua Wenrong Hospital, respectively.

Methods

A two-channel endoscope (GIF-2T240, Olympus, Tokyo, Japan) and a 12 MHz probe (GF-UM 2R, Olympus, Tokyo, Japan) were used for the ultrasonographic study. Scanning of the tumor was performed after filling the upper gastrointestinal tract with 100-500 mL of deaerated water. Diagnosis was made according to the layer of origin, size, nature, internal echo pattern, outer margin and grow pattern of the lesion. Following the EUS procedure, if the lesion was identified as an intramural lesion ≤ 2.5 cm, endoscopic treatment was performed. A lesion > 2.5 cm in size and suspected to be malignant was suggested for surgery. A large proportion of patients were followed up with EUS, because their poor conditions were unsuited for the therapy or the lesion was too small.

An Olympus GIF-XQ240/260 gastroscope (Tokyo, Japan) was used for the resection when it was indicated. Informed consent was given by each patient before the endoscopic therapy. Four different resection techniques were used: snare electrosection, endoloop, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD).

EMR procedure: Epinephrine (0.001%) was injected into the submucosal layer to lift the lesion, and then a conventional electrosurgical snare (FD-IU, Olympus, Tokyo, Japan) and an electrosurgical unit (VIO 200D,

ERBE, Tübingen, Germany) were used for removal of the overlying mucosa and resection of the tumor.

ESD procedure: the surrounding area of the lesion was marked with argon plasma coagulation (APC 300A, ERBE, Tübingen, Germany). Normal saline solution with 0.002% indigo carmine and 0.001% epinephrine was injected into the submucosal layer to lift the lesion. An initial incision was made outside the marking dots with a hook-knife (KD-620LR, Olympus, Tokyo, Japan). The submucosal resection under the lesion was done with insulation-tipped (IT) electrosurgical knife (KD-610L, Olympus, Tokyo, Japan). Finally, a snare was used to remove the surrounding tissues. Bleeding and visible vessels in the resection area were closed using hemoclips (HX-201YR-135, Olympus, Tokyo, Japan).

Postoperative EUS examination was made to check whether the lesions were completely removed except those with endoloop ligation. The specimens were sent for pathologic study, some of which were assayed by immunohistochemistry. All 201 patients were examined two months later with EUS.

RESULTS

Using EUS, we identified 1050 patients with GIMTs, 543 of them had leiomyomas. Five hundred and twenty lesions were in the esophagus, 22 in the stomach, and 1 in the duodenum. The mean maximal tumor diameter was 3.3 cm. Five hundred and seven cases were stromal tumors. Forty-nine lesions were in the esophagus, 428 were in the stomach, and 30 were in the duodenum. The mean maximal tumor diameter was 6.6 cm. EUS features of leiomyomas and stromal tumors were characteristic with regular borders, a hypoechoic mass with homogeneous or heterogeneous echo patterns (Figures 1-4). The echogenicity of leiomyomas was slightly lower than the normal proper muscle layer, while that of stromal tumors was slightly higher. Malignant stromal tumors often appeared as a heterogeneous mass with irregular borders.

One hundred and thirty-nine leiomyomas and 62 stromal tumors underwent endoscopic therapy after EUS examination. No obvious malignant signs were seen in these lesions. The location, origin level and removal methods of the lesions are shown in Table 1. The mean maximal tumor diameter was 2.5 cm. For a tumor protruding into the cavity, if it originated from the muscularis mucosa or from submucosa ≤ 1 cm, snare electrosection was directly used. If the lesion originating from submucosa was flat and > 1 cm, electrosection would be expected to fail, and other treatments, such as endoloop, EMR or ESD, were used. For a lesion originating from muscularis propria but not growing outward, endoloop or ESD was used. Among these patients, 133 leiomyomas and 24 stromal tumors were treated by snare electrosection, 6 leiomyomas and 20 stromal tumors were treated by endoloop, 10 stromal tumors were treated by EMR and 8 stromal tumors were treated by ESD (Figures 1-4). Complete resection of

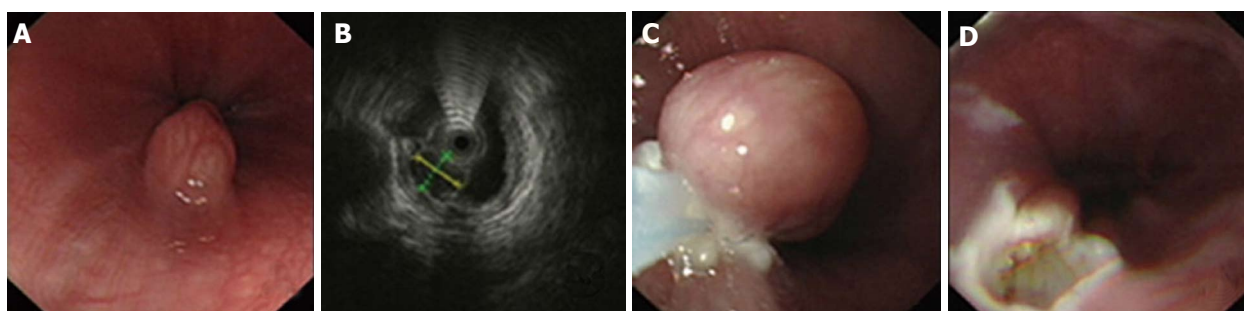


Figure 1 An esophageal leiomyoma treated by snare electrosection. A: An elevated lesion in the lower esophagus; B: A homogeneous, hypoechoic mass ($1.0 \times 0.7 \text{ cm}^2$) with a regular border originated from muscularis mucosa, which was diagnosed as a leiomyoma by endoscopic ultrasonography; C: The tumor was snared at the base, and then it was resected by snare electrosection; D: Postoperative wounds.

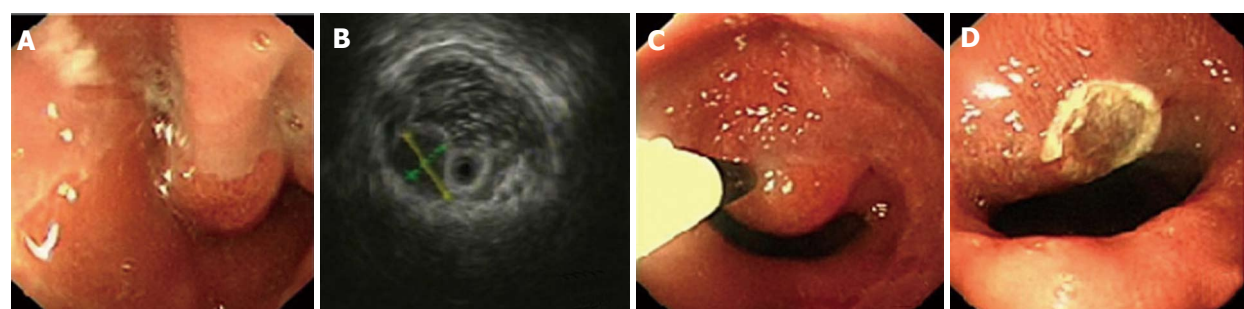


Figure 2 A gastric stromal tumor treated by endoscopic mucosal resection. A: An elevated lesion in the cardia; B: A homogeneous, hypoechoic mass ($1.4 \times 0.8 \text{ cm}^2$) with a regular border originating from submucosa, which was diagnosed as a stromal tumor by endoscopic ultrasonography; C: Epinephrine (0.001%) was injected into the submucosa to lift the lesion; D: Postoperative wounds.

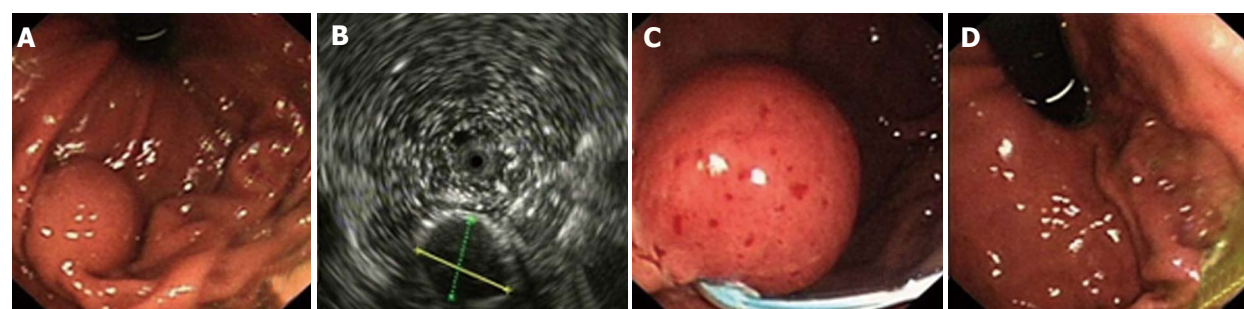


Figure 3 A gastric stromal tumor treated by endoloop. A: An elevated lesion in gastric fundus; B: A homogeneous, hypoechoic mass ($2.0 \times 1.5 \text{ cm}^2$) with a regular border originating from muscularis propria, which was diagnosed as a stromal tumor by endoscopic ultrasonography; C: Endoscopic ligation with an endoloop; D: Endoscopic view of an ulcer scar without tumor recurrence at the ligation site 2 mo later.

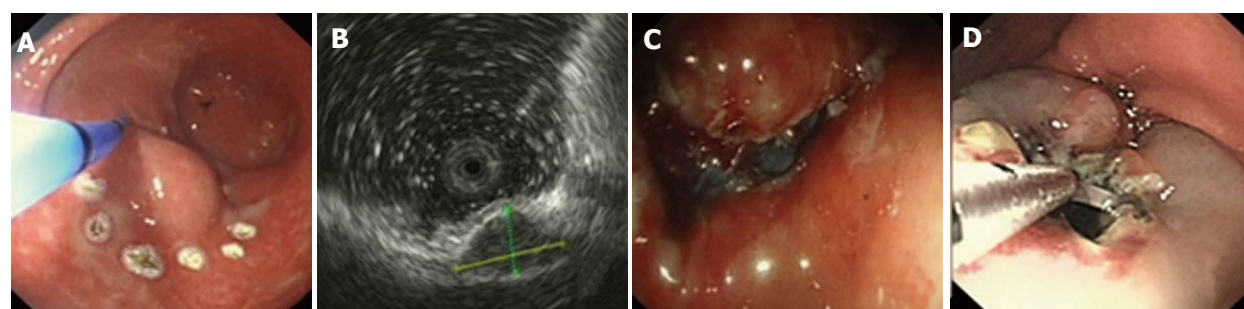


Figure 4 A gastric stromal tumor treated by endoscopic submucosal dissection. A: An elevated lesion in the gastric antrum; B: A homogeneous, hypoechoic mass ($2.0 \times 1.2 \text{ cm}^2$) with a regular border originating from muscularis propria, which was diagnosed as a stromal tumor by endoscopic ultrasonography; C: The surrounding area of the lesion was marked with argon plasma coagulation. After normal saline solution with 0.002% indigo carmine and 0.001% epinephrine was injected into the submucosal layer to lift the lesion, an initial incision was made outside the marking dots with hook-knife. Submucosal dissection under the lesion was performed with an IT knife; D: The tumor was dissected and the postoperative wounds were closed using hemoclip.

Table 1 Location, origin and treatment of 201 gastrointestinal mesenchymal tumors

Diagnosis by EUS	Location		Layer of origin			Treatment			
	Esophagus	Stomach	Muscularis mucosa	Submucosa	Muscularis propria	Snare electrosection	Endoloop	EMR	ESD
Leiomyoma	134	5	121	15	3	133	6	0	0
Stromal tumor	22	40	18	19	25	24	20	10	8
Total	156	45	139	34	28	157	26	10	8

EUS: Endoscopic ultrasonography; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

the lesion was achieved in all cases. No residual lesion was detected by postoperative EUS examination except for those by endoloop ligation. None of the patients suffered from severe hemorrhage or resection-related perforation. Postoperative histological results showed that 141 of 156 patients were in agreement with the preoperative diagnosis of EUS. All the specimens tested had complete envelope and negative resection margin in pathology. Wounds were closed up nicely in all patients when rechecked after two months. No residual lesion was detected by EUS examination and pathology demonstrated negative results at the same time.

DISCUSSION

Leiomyomas and stromal tumors are the most common GIMTs of the upper gastrointestinal tract. Many lesions are subepithelial, and they are often difficult to diagnose by general endoscopy. Some also need to be identified with extrinsic compression. EUS can reliably characterize the nature, size, and layer of origin of lesions, and accurately differentiate intramural from extramural, leading to a diagnosis^[5]. Features of leiomyomas and stromal tumors seen with EUS often include: a round shape, and a homogeneous, hypoechoic mass with regular borders^[6]. A marginal halo, hyperechoic spots and higher echogenicity as compared with the normal muscle layer is seen more frequently in stromal tumors than in the leiomyomas^[7]. Malignant stromal tumors are characterized by large size (> 5 cm), irregular borders, and echogenic foci^[8,9].

In this study, we identified 1050 patients with GIMTs using EUS. There were 543 leiomyomas and 507 stromal tumors. The majority of leiomyomas were located in the esophagus while most stromal tumors were located in the stomach, which is in accordance with other studies^[6-10]. For these mesenchymal tumors, 90.38% diagnosed by EUS were also identified by pathohistology. Among these, 5 retention cysts, 4 stromal tumors with leiomyoma differentiation, and 1 hyperplastic polyp were diagnosed as leiomyoma, and 3 leiomyoma and 2 hyperplastic polyps were diagnosed as stromal tumors by EUS. Submucosal retention cysts are small and often filled with thick fluid, and thus the ultrasonographic image is of a hypoechoic mass that may be confused with mesenchymal tumors. Stromal tumors with leiomyoma differentiation are also difficult to discriminate by routine pathology and should be identified by immuno-

histochemistry. Samples from EUS-guided fine-needle aspiration biopsies can be sent for cytological, pathological and immunohistochemical assays which may enable clinicians to make more accurate diagnoses than using EUS examination alone^[6-11].

In the past, conventional endoscopy could not accurately determine the location and categorization of subepithelial lesions. Therefore, GIMTs were usually treated by surgery. The introduction of EUS has solved these problems and it has played an important role in the choice of endoscopic therapy for mesenchymal tumors. Based on EUS images, we treated 201 GIMTs with different endoscopic therapies, including snare electrosection, endoloop, EMR and ESD. Complete resection of the lesions was achieved in all cases. None of the patients suffered from severe hemorrhage or resection-related perforation. All wounds were closed up nicely and no recurrence was found in the follow-up after 2 mo.

Electrosection is the most common endoscopic treatment, and its value for the treatment of gastrointestinal submucosal tumors has been recognized^[12,13]. It is mainly used for protuberant lesions (especially the pedunculated ones). In this study, 157 GIMTs arising from non-muscularis propria, with a diameter of ≤ 1 cm, were treated by snare electrosection after EUS examination. It is reported that serious complications rarely occurred when electrosection is used to cut non-muscularis propria tumors with a diameter ≤ 3 cm^[3]. Tumors originating from muscularis propria are associated with an increased risk of perforation and hemorrhage complications during endoscopic treatment, and snare electrosection was not used in these cases.

Compared with ordinary snare removal, EMR is more suitable for the treatment of flat lesions generally confined to < 2 cm^[14]. In this study, 10 flat lesions were treated by EMR. We injected 0.001% epinephrine into the submucosal layer to lift the lesion and made it easy to snare. Furthermore, this may provide a buffer to protect the inherent muscle function, which could reduce the bleeding and perforation risk during the process of muscle removal. Examination by EUS before surgery to determine the size and depth of lesions could help determine the injection site and the resection scope.

Endoloop ligation of tumors at the base, blocking blood supply and causing tumor necrosis, could significantly reduce the risk of hemorrhage and perforation^[15,16]. But, the procedure is not suitable for large lesions. Incomplete ligation might leave residual tumors,

while ligation could increase the risk of hemorrhage and perforation. Therefore, the range and depth of ligation should be strictly controlled according to the results of EUS during surgery. In the past, the majority of tumors studied have been only the muscularis mucosa and submucosa^[3]. Recently, it was reported that endoloop could remove tumors arising from muscularis propria safely and effectively^[15,17]. In this study, we also used endoloop removal of lesions arising from muscularis propria, without hemorrhage or perforation. The tumor from the muscularis propria can grow inside or outside the cavity, therefore, preoperative EUS for defining the tumor growth pattern is very important to determine whether the lesion can be safely and completely removed.

ESD should be performed using a high-frequency electric knife to dissect the subepithelial tumor, which is more suitable for treatment of large and flat lesions. Tumors derived from the muscularis mucosa and submucosa can be completely dissected^[18,19]. It is difficult to dissect lesions from the muscularis propria because of the increased risk of hemorrhage and perforation. In Lee *et al.*'s^[20] study, among 12 cases of gastrointestinal submucosal muscle tumors arising from muscularis propria treated by ESD, 9 tumors were completely dissected. The size of these tumors ranged from 0.6 to 4 cm (average, 2 cm). In this study, 8 stromal tumors arising from submucosa or muscularis propria were treated safely by ESD. All of them were dissected once and clipping was used to close deep wounds to reduce hemorrhage and perforation risk.

Our clinical practice demonstrates that endoscopic treatment can be applied to GIMTs arising from muscularis mucosa, submucosa and muscularis propria. Based on the results of the EUS procedure, lesions > 2.5 cm in size and suspected to be malignant should be considered for surgery. Moreover, if the tumor grew outside the cavity, endoscopic treatment should be aborted as well. We also suggested a follow-up with EUS for the few patients who are not indicated for the endoscopic therapy or whose tumor is too small.

In conclusion, EUS can help determine the origin, size, shape, nature and growth pattern of lesions, with a high diagnostic accuracy for upper GIMTs. Preoperative EUS examination is important for choosing the type of endoscopic therapy for mesenchymal tumors, by which the lesions can be treated safely and effectively.

COMMENTS

Background

Clinically, gastrointestinal mesenchymal tumors (GIMTs) are usually incidentally discovered as subepithelial bulges during routine endoscopy for unrelated conditions. The classification and management of these lesions can be challenging.

Research frontiers

With the wide use of endoscopic ultrasonography (EUS) to clarify the nature and origin of the subepithelial tumor, great progress has been made in diagnosis and treatment of GIMTs. However, the value of EUS in the choice of endoscopic treatment strategies for GIMTs has not been well established.

Innovations and breakthroughs

This study indicated that EUS could help determine the origin, size, shape,

nature and growth pattern of lesions, with a high diagnostic accuracy for upper GIMTs. Under the guidance of the EUS, GIMTs could be removed by appropriate endoscopic treatment, such as snare electrosection, endoloop, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) without severe complications.

Applications

The results of this study demonstrate that EUS is an effective means of diagnosis for upper GIMTs. Preoperative EUS examination is important for choosing the type of endoscopic therapy for mesenchymal tumors. The study will guide the clinical application of EUS in the endoscopic therapy for upper GIMTs.

Terminology

GIMTs are tumors which originate from mesenchymal cells other than epithelial cells or lymphocytes. They are further classified as stromal tumors, leiomyomas, leiomyosarcomas, neural tumors, fibroblast tumors or liparomphalus. EMR is a minimally invasive technique for resection of a lesion that requires the separation of the submucosa by injecting a fluid agent. ESD is a new endoscopic method using special knife for complete en bloc resection of early gastrointestinal neoplasms.

Peer review

This is a well written paper which describes the experience of the authors in the EUS diagnosis and subsequent endoscopic treatment of gastrointestinal mesenchymal tumors. The pictures well support the authors' findings and conclusions.

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β -catenin accumulation in nuclei of hepatocellular carcinoma cells up-regulates glutathione-s-transferase M3 mRNA

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RESULTS: Two up-regulated genes, glutamine synthetase and glutathione-s-transferase M3 (GSTM3), were identified in radiation-induced mouse HCC cells. Influence of β -catenin accumulation in nuclei of HCC cells on up-regulation of GSTM3 mRNA was investigated. The nearby upstream domain of GSTM3 contained the β -catenin/Tcf-Lef consensus binding site sequences [5'-(A/T)(A/T)CAAAG-3'], and the total GST activity ratio was considerably higher in B6C3F1 mouse HCC cells with β -catenin accumulation in nuclei of HCC cells than in those without β -catenin accumulation (0.353 ± 0.117 vs 0.071 ± 0.064 , $P < 0.001$). The TWS119 (a distinct GSK-3 β inhibitor)-induced total GST activity was significantly higher in HepG2 cells with β -catenin accumulation than in those without β -catenin accumulation in nuclei of HCC cells. Additionally, the GSTM3 mRNA level was significantly higher at 24 h than at 12 h in TWS119-treated HepG2 cells.

CONCLUSION: β -catenin accumulation increases GST activity in nuclei of HCC cells, and GSTM3 may be a novel target gene of the β -catenin/Tcf-Lef complex.

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Key words: β -catenin accumulation; Differential display analysis; Glutathione-s-transferase M3; Hepatocellular carcinoma; Radiation

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Abstract

AIM: To identify the differentially over-expressed genes associated with β -catenin accumulation in nuclei of hepatocellular carcinoma (HCC) cells.

METHODS: Differentially expressed genes were identified in radiation-induced B6C3 F1 mouse HCC cells by mRNA differential display, Northern blot and RT-PCR, respectively. Total glutathione-s-transferase (GST) activity was measured by GST activity assay and β -catenin localization was detected with immunostaining in radiation-induced mouse HCC cells and in HepG2 cell lines.

Li YS, Liu M, Nakata Y, Tang HB. β -catenin accumulation in nuclei of hepatocellular carcinoma cells up-regulates glutathione-s-transferase M3 mRNA. *World J Gastroenterol* 2011; 17(13): 1772-1778 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i13/1772.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i13.1772>

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary cancer of the liver chronically injured by infection, metabolic disease or various drugs^[1]. As generally observed in other carcinomas, HCC is attributed to accumulated genetic alterations, including (1) Activation of oncogenes N-ras, H-ras, and K-ras, c-erbA, c-met, RB and c-myc^[2-5]; (2) Transcriptional activation of c-jun and nuclear factor κ B by hepatitis B virus factors^[6]; (3) Repression or mutation of the p53 anti-oncogene^[7]; and (4) Accumulation of β -catenin^[8]. Although the genetic events responsible for either HCC initiation or progression are not clear, they involve at least three carcinogenesis pathways: the p53, RB and Wnt/ β -catenin signaling pathways^[11-10].

β -catenin is an essential downstream effector of the canonical Wnt signaling pathway^[11,12]. Approximately 20% of HCC cells display β -catenin aberrant activation^[9,13]. In the normal steady state, β -catenin is continuously phosphorylated at serine and threonine residues by glycogen synthase kinase 3 β (GSK-3 β) in a complex with adenomatous polyposis coli (APC)-axin/conductin and is quickly degraded through the ubiquitin/proteasome pathway. In mice, liver-specific deletion of APC induces β -catenin stabilization and increases the number of HCC cells. Although the activation of β -catenin is likely an initiating or contributory factor for HCC, more fundamental information is required for a better understanding of the detailed genetic mechanism underlying HCC associated with β -catenin.

To uncover the detailed genetic mechanisms underlying HCC in the present study, several genes in mouse cancerous liver tissue samples were identified to disclose more of the genes that play a very important role in the regulation of cell proliferation and the development of HCC. We identified several cDNA fragments that were differentially expressed in radiation-induced mouse HCC and compared with those in matched nontumorous liver tissue. Samples using a differential display technique^[14]. We determined whether the nearby upstream domain of those genes contain the β -catenin/Tcf-Lef consensus binding site sequences. The influence of β -catenin accumulation in nuclei of HCC cells on activation of protein encoded by the gene containing β -catenin/Tcf-Lef consensus binding site sequences was further investigated.

MATERIALS AND METHODS

Sample preparation

Surgically resected HCC and adjacent nontumorous tissue samples were taken from the livers of 18-mo-old mice irradiated by 3.5 Gy 60Co γ -ray for 1 wk immediately after they were born. B6C3F1 mouse HCC and matched nontumorous liver tissue samples were obtained immediately under the same conditions for measurement of total GST activity^[15], isolation of total RNA^[16], and immunohistochemical expression of β -catenin and hematoxylin-eosin (HE) staining. Histological analysis of HCC tissue samples from mice was carried out according to the general rules for clinical and pathological study of primary liver cancer.

Table 1 Primer sets used in mRNA differential display analysis

Anchor primers	Arbitrary primers
T12MG (10 μ mol/L)	AP-10 (2 μ mol/L), 5'-TAGCAAGTGC-3'
T12MA (10 μ mol/L)	AP-11 (2 μ mol/L), 5'-CAGACCGTTC-3'
T12MT (10 μ mol/L)	AP-12 (2 μ mol/L), 5'-TGCTGACCTG-3'
T12MC (10 μ mol/L)	AP-14 (2 μ mol/L), 5'-AATGGGCTGA-3'

M represents a degenerated mixture of dA, dG and dC. The 16 different primer sets used for PCR amplification were randomly combined from the four arbitrary primers and the four anchor primers.

On the other hand, HepG2 cells obtained from China Center for Type Culture Collection were maintained in DMEM supplemented with 10% fetal calf serum (Sigma, Louis, MO), 200 mmol/L L-glutamine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a water-saturated atmosphere containing 5% CO₂. Cell cultures were allowed to reach 90% confluence. The cells were then treated with or without 1 μ mol/L of 4, 6-disubstituted pyrrolopyrimidine (TWS119, Cayman Chemical, Ann Arbor, MI) and incubated at 37°C for 24 h. Finally, total RNA or cytoplasmic proteins, including total GST proteins, were extracted from surgically resected frozen tissue samples or HepG2 cells by homogenization with a Vibra cell sonicator (Sonics and Materials, Inc., Danbury, CT) under a regularity condition.

mRNA differential display analysis and DNA sequencing

mRNA differential display analysis was performed as previously described^[14] with a RNAmapping kit A (GenHunter, Nashville, TN) (Table 1). Total RNA (0.4 μ g) extracted from radiation-induced mouse HCC and matched nontumorous liver tissue samples was reverse-transcribed with different combinations of arbitrary and anchor primers (Table 1) for initial cDNA synthesis. The thermal cycler parameters were as follows: 1 cycle at 94°C for 4 min, followed by 40 cycles at 94°C for 30 s, at 40°C for 2 min and at 72°C for 30 s. Amplified subpopulations were distributed on a 6% DNA sequencing gel. The bands of interest were cut out from the polyacrylamide gel, and cDNA fragments were re-amplified using the same pair of primers and the same cycle parameters as described above. The re-amplified cDNA fragments were purified from 2% agarose gels and subcloned into pCRII-TOPO vectors using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones were selected by the same size of bands cut from the polyacrylamide gel as described above, followed by inverse hybridization and DNA sequencing. DNA sequences were compared with those in GenBank by the Blast Service provided by NIH (Bethesda, MD).

Northern blot analysis

Total RNA extraction (10 μ g) was denatured and electrophoresed in a 1.2% agarose gel containing 0.66 mol/L formaldehyde and then transferred onto a Hybond-nylon membrane (Amersham Biosciences, Buckingham, England). The membranes were UV cross-linked, pre-hybrid-

ized and hybridized. The respective cDNA fragments obtained from the differential display reaction were used as a probe for Northern blot analysis. Probes were [α - 32 P] dCTP (110 TBq/mmol) labeled using the Megaprime™ DNA labeling kit (Amersham, UK), pre-hybridized to filters at 42°C for 30 min, and then hybridized to the filters overnight at 42°C. The filters were washed twice at 55°C in 1 × SSC, 0.1% SDS for 15 min, and then exposed to X-ray film for 24–72 h at -80°C.

Immune staining

Immunofluorescence and immunocytochemistry analyses of β -catenin localization were performed, respectively, with mouse monoclonal anti- β -catenin antibody diluted at 1:500 for immunofluorescence, and diluted at 1:100 for immunocytochemistry (Sigma, Louis, MO) on 5- μ m paraffin-embedded sections and paraformaldehyde-fixed HepG2 cell sections that were differentially resected from 24 radiation-induced B6C3F1 mouse HCC and adjacent nontumorous liver tissue samples^[17,18]. The tissue sections were then incubated for 1 h at room temperature with Alexa Fluor 546 goat anti-mouse IgG diluted at 1:1000 (Molecular Probes, Eugene, OR, USA), washed three times with PBS and visualized under a Nikon Eclipse Ti fluorescent microscope (Japan). The HepG2 cell sections were further treated with a Histofine simple stain rat MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan), stained brown with a DAB substrate kit (Nichirei, Tokyo, Japan) and blue with hematoxylin. Negative controls were stained with the omitted primary antibody. Omission of the primary antibody resulted in no staining of the cells.

GST activity assay

Total GST activity was assayed as previously described^[15]. Total GST activity with aromatic substrates was determined by monitoring changes in absorbance with a microplate reader (Infinite M200, Tecan, Switzerland). A complete assay mixture without total GST was used as a control. HepG2 cells, HCC cells and matched nontumorous liver tissue cells were broken for 20 s at 4°C, respectively, in 1 mL 0.1 mol/L potassium phosphate buffer (pH 6.5) using a sonicator. Samples were collected into different tubes containing EDTA, centrifuged at 10000 r/min for 1 h at 4°C, and the supernatant was stored at -20°C. GST activity was assayed with 5 μ g protein in duplicate with 1 mmol/L 1-chloro-2, 4-dinitrobenzene and glutathione (Sigma, Louis, MO) and used without further purification in a total volume of 1 mL. Optical density of GST was measured within at least 3 min after incubation at 25°C for 15 min at a wavelength of 340 nm (ϵ = 9.6 mmol/L per cm). The activity of GST was expressed as a unity of nmol mg per min.

Real-time PCR for detection of glutathione-s-transferase M3 mRNA expression

Total RNA harvested from HepG2 cells was subjected to reverse transcription into cDNA using a Superscript kit (Life Technologies, Gaithersburg, MD) according to

its manufacturer's protocol. Thereafter, 2 μ g of cDNA samples was used immediately in measurement of GSTM3 mRNA level by real-time PCR with iQ SYBR Green Supermix (Bio-Rad, Tokyo, Japan), forward primer (5'-GCTCCTGGAGTTCACGGATA-3'), and reverse primer (5'-GCTCCTGGAGTTCACGGATA-3') on a DNA engine Opticon 2 real-time PCR detection system (Bio-Rad, Tokyo, Japan). The thermal cycler parameters were as follows: 1 cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, at 60°C for 30 s and at 72°C for 30 s^[19]. A β -actin control was run simultaneously with the same reaction recipe listed in the instruction manual for the iQ SYBR Green Supermix (Bio-Rad, Tokyo, Japan). All data were normalized to β -actin mRNA levels to account for any variation in RNA concentrations between the samples obtained from three separate experiments.

Statistical analysis

The data are presented as mean \pm SE. Statistical analyses were performed among the three groups by a one-way analysis of variance followed by Bonferroni's test, and between two groups by the unpaired Student's *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

Identification of differentially expressed genes in mouse HCC and matched nontumorous liver tissue samples

Differential display analysis is a powerful tool for the comparison of differential gene expressions between two or more mRNA populations^[14]. Using this method, we compared the mRNA expression patterns of B6C3F1 mouse HCC and matched nontumorous liver tissue samples. Representative differentially displayed autoradiographs of “I and II” cDNAs are shown in Figure 1A. The interesting “I and II” cDNA fragments were successfully recovered from the dried DNA sequencing gel, re-amplified (Figure 1B), purified, and subcloned into the TA cloning site of pCRII-TOPO vector. Its nucleotide sequences were compared with those in GenBank (Figure 1C). Concurrently, the purified products of “I and II” fragments from 2% agarose gels were used as probes for reverse Northern blotting. The Northern blotting patterns of “I and II” fragments differentially expressed in mouse HCC cells are shown in Figure 2A. In comparison with a nucleotide sequence in GenBank, “I and II” nucleotide sequences were identified as the up-regulated gene coding products, such as glutathione-s-transferase M3 (GSTM3) and glutamine synthetase (GLNS) in radiation-induced B6C3 F1 mouse HCC cells.

Dependence of mouse GSTM3 activity on β -catenin in B6C3F1 mouse HCC

It has been reported that nuclear translocation of β -catenin may represent an early event in liver carcinogenesis^[20]. Therefore, we are interested in the relation between β -catenin and the discovered gene described above. Analysis of mRNA expression showed that the expression level

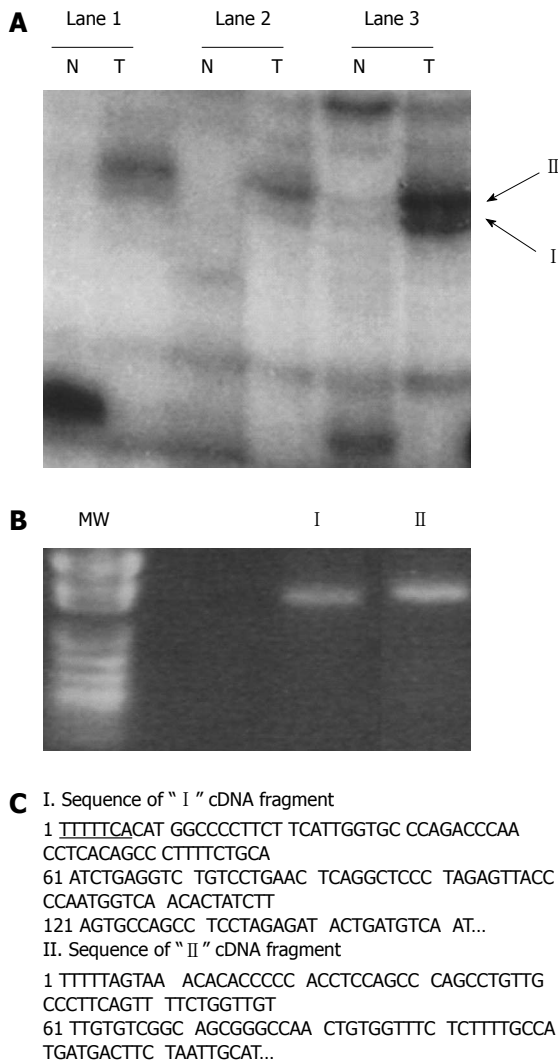


Figure 1 Identification of differentially displayed cDNA fragments from paired hepatocellular carcinoma cells (T) and nontumorous liver tissues (N). A: Differentially displayed PCR products ("I" and "II") amplified with AP-10 primer and T_{12} MA. Lanes 1-3 denote the three B6C3 F1 mouse samples, respectively; B: Recovered "I" and "II" from the dried DNA sequencing gel reamplified by PCR. MW lane shows the pUC118 DNA fragments cut by HapII (a restriction enzyme) as molecular weight markers; C: Nucleotide sequences of the bands shown in A. Flanking sequences of T_{12} MA primers are underlined. The two insert-containing fragments were sequenced and identified as gene fragments of GLNS and GSTM3 in comparison with those of nucleotide in GenBank.

of GSTM3 mRNA was significantly higher in cell nuclei of B6C3F1 mouse HCC cells with β -catenin accumulation than in those without β -catenin accumulation (Figure 2B), suggesting that β -catenin can increase the GSTM3 activity. To confirm whether β -catenin increases the GSTM3 activity, the gene near the upstream domain of individual mouse GSTM3 containing the β -catenin/Tcf-Lef consensus binding site sequence [5'-(A/T)(A/T) CAAAG-3'^[21]] was detected by searching it in GenBank. On the other hand, whether the increased GSTM3 activity in mouse HCC cells is correlated with β -catenin accumulation in nuclei of HCC cells was also assayed (Figure 3), showing that the nearby upstream domain of mouse GSTM3 contains β -catenin/Tcf-Lef consensus binding site sequences [5'-(A/T)(A/T) CAAAG-3']. Because the GSTM3 activity

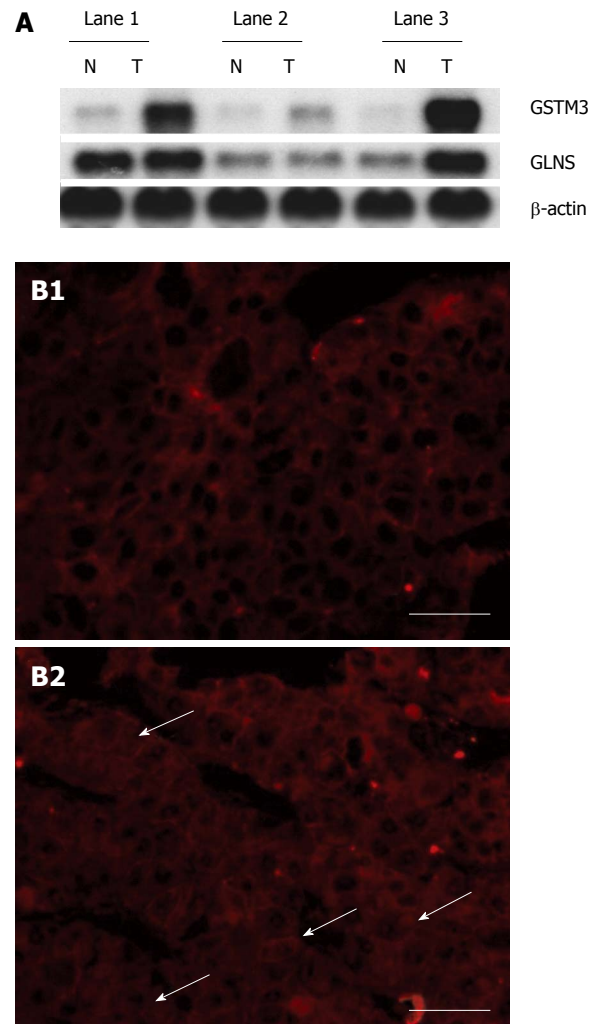


Figure 2 Levels of glutamine synthetase and glutathione-s-transferase M3 mRNA and expression of β -catenin. A: T denotes surgically resected B6C3F1 mouse hepatocellular carcinoma (HCC) tissue samples and N denotes matched nontumorous liver tissue samples; B: Representative immunofluorescence photomicrographs for β -catenin (red photomicrographs) in HCC tissue samples. B1 denotes negative β -catenin staining in nuclei of HCC cells, B2 denotes positive β -catenin staining in nuclei of HCC cells, and white arrows indicate β -catenin detected in nuclei of HCC cells. Bars: 50 μ m. GLNS: Glutamine synthetase; GSTM3: Glutathione-s-transferase M3.

level was rather variable in normal mouse tissue samples, it was difficult to estimate the β -catenin dependence on GSTM3 activity using the absolute GST activity level. Therefore, we analyzed the increased total GST activity in HCC tissue samples relative to that of normal tissue samples by the ratio of (T-N)/N, where T and N denote the total GST activity in HCC and normal tissue samples, respectively. On the other hand, to see the directly significant relation between total GST activity and β -catenin accumulation in nuclei of HCC cells, the average total GST activity level was also measured in B6C3F1 mouse HCC and matched nontumorous liver tissue samples with or without β -catenin accumulation (Table 2). It can be clearly seen from Table 2 that the total GST activity ratio was considerably higher in B6C3F1 mouse HCC tissue samples with β -catenin accumulation than in those without β -catenin accumulation in nuclei of HCC cells. The

Samples	Total GST activity (nmol/mg per min)		Values of GST activity ratio (T-N)/N
	T	N	
(+)	3122 ± 189 (n = 13)	2447 ± 180 (n = 13)	0.353 ± 0.117 ^a
(-)	2644 ± 199 (n = 11)	2523 ± 205 (n = 11)	0.071 ± 0.064

To further elucidate the above findings, we mimicked the canonical Wnt pathway in cultured HepG2 cells using TWS119 (an inhibitor of GSK-3), which led to phosphorylation, nuclear translocation, and abnormal accumulation of β -catenin in nuclei of HepG2 cells. We treated the cultured HepG2 cells with or without 1 $\mu\text{mol/L}$ TWS119 at 37°C for 24 h, measured the total GST activity in these cells, and then analyzed the relation^[15] between GST-GSH protein complex concentration and time (at least 15 min) in “control” and “TWS119” groups using a microplate reader at a wavelength of 340 nm. Concurrently, the β -catenin accumulation in HepG2 cells was detected with immunocytochemical staining with a rabbit polyclonal antibody against β -catenin as previously described^[17]. Thereafter, we comprised the linear function of total GST-GSH protein complex concentration between the two groups. It can be clearly seen from Figure 4A that the total GST activity ratio was considerably higher in the “TWS119” group with abnormal nuclear accumulation of β -catenin in

It was reported that β -catenin plays an important role in cell-cell adhesion^[27] and in Wnt signaling pathway^[28,29]. β -catenin can enter the nuclei of HCC cells by binding the

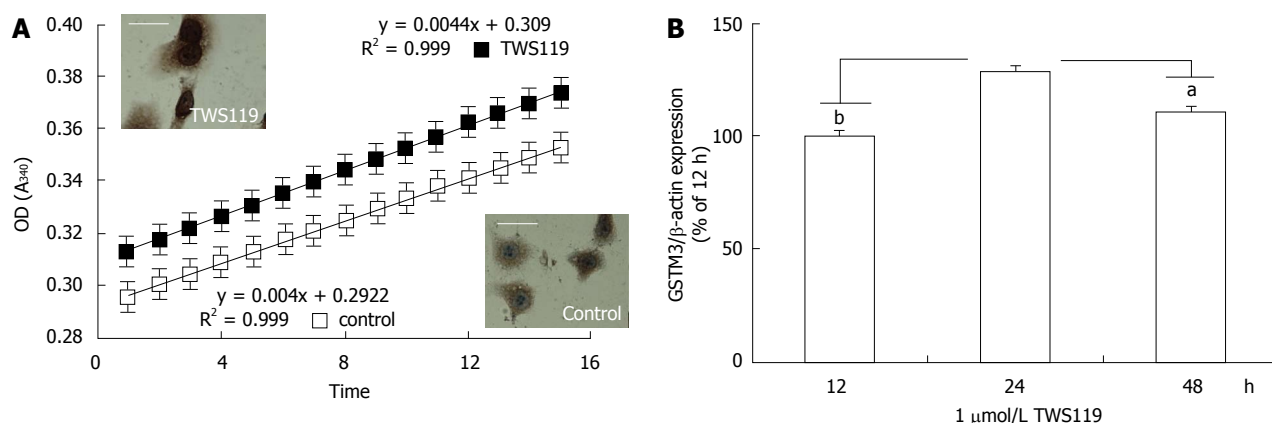


Figure 4 Total glutathione-s-transferase activity (A) and glutathione-s-transferase M3 mRNA expression (B) in HepG2 cells. ^a $P < 0.05$, ^b $P < 0.01$ vs TWS119 at 24 h by one-way analysis of variance followed by Bonferroni's test.

Tcf-Lef family of DNA binding proteins, and regulate the transcription of target genes (for example, c-myc, gastrin, cyclin D1 and PPAR are identified as target genes of the β -catenin/Tcf-Lef complex)^[30-33]. In the present study, the GSTM3 mRNA level was higher in B6C3F1 mouse HCC cells with β -catenin accumulation than in those without β -catenin accumulation. To our knowledge, no similar observation has been reported. The total GST activity was much higher in B6C3F1 mouse HCC cells with β -catenin accumulation than in normal tissue samples without β -catenin accumulation (Table 2). The averaged GST activity ratio was significantly higher in HCC cells with β -catenin accumulation than in those without β -catenin accumulation (0.747 ± 0.360 vs 0.071 ± 0.213 , $P < 0.001$), suggesting that the GST activity ratio is significantly different (Table 2). Furthermore, by searching the GenBank, we found that the upstream region of the GSTM3 gene contained three β -catenin/Tcf-Lef consensus binding site sequences in mouse GST polymorphisms. The canonical Wnt pathway in cultured HepG2 cells was further mimicked using TWS119, a GSK-3 β inhibitor, which caused abnormal β -catenin accumulation. As a result, TWS119-induced β -catenin accumulation enhanced the GST activity and the GSTM3 mRNA expression in HepG2 cells, suggesting that β -catenin accumulation in nuclei of HCC cells can increase the activity of mouse GSTM3, one of the enzymes responsible for the metabolism of a variety of xenobiotics and carcinogens, and that mouse GSTM3 may be a novel downstream target gene of the β -catenin/Tcf-Lef complex in mouse HCC.

It was reported that GLNS can catalyze the synthesis of glutamine^[34], a major energy source of cells (an important ATP source), and is a precursor for the synthesis of nucleotides and numerous amino acids, and up-regulated in a subset of human HCC^[35]. In this study, at 3 wk after tumor implantation, the glutamine synthetase activity in rats increased by 34%, which is consistent with the reported findings^[36]. However, further study is needed to observe the possible pharmacological action (s) of β -catenin in the up-regulated expression of GLNS mRNA.

In conclusion, GSTM3 and GLNS genes are differen-

tially expressed in mouse HCC cells. The expression level of GSTM3 mRNA and total GST activity are higher in B6C3F1 mouse HCC cells with β -catenin accumulation than in those without β -catenin accumulation, indicating that GSTM3 may be a novel target gene for the β -catenin/Tcf-Lef complex in mouse HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a primary cancer of the liver. However, the genetic events responsible for HCC initiation and progression are not clear. Since approximately 20% of HCC display β -catenin aberrant activation, Wnt/ β -catenin signaling pathways may be involved in HCC occurrence.

Research frontiers

Recent data show that β -catenin may be an initiating or contributory factor for HCC. In this study, the authors demonstrated that glutathione-s-transferase M3 (GSTM3) might be a novel target gene of the β -catenin/Tcf-Lef complex in mouse HCC.

Innovations and breakthroughs

The authors identified two up-regulated genes, glutamine synthetase (GLNS) and GSTM3, in nuclei of radiation-induced mouse HCC cells with β -catenin accumulation. Three β -catenin/Tcf-Lef consensus binding site sequences were observed in mouse glutathione-s-transferase (GST) polymorphisms. GST activity and GSTM3 mRNA levels were induced in cultured HepG2 cells by TWS119 (an inhibitor of GSK-3 β). To our knowledge, no similar observation has been reported.

Applications

By demonstrating that GSTM3 may be a novel target gene of the β -catenin/Tcf-Lef complex in mouse HCC, this study may represent a future strategy for therapeutic intervention in patients with HCC.

Terminology

β -catenin plays an important role in cell-cell adhesion and in Wnt signaling pathway, can enter nuclei by binding to the Tcf-Lef family of DNA binding proteins and regulate the transcription of target genes.

Peer review

This paper reports the results of investigations on some differentially over-expressed genes associated with β -catenin accumulation in nuclei of HCC cells, showing that GSTM3 may be a novel target gene of the β -catenin/Tcf-Lef complex in mouse HCC using mRNA differential display, Northern blot analysis, immunostaining and RT-PCR techniques, respectively. It is worthy of publication.

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Nutrition support in surgical patients with colorectal cancer

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Abstract

AIM: To review the application of nutrition support in patients after surgery for colorectal cancer, and to propose appropriate nutrition strategies.

METHODS: A total of 202 consecutive surgical patients admitted to our hospital with a diagnosis of colon cancer or rectal cancer from January 2010 to July 2010, meeting the requirements of Nutrition Risk Screening 2002, were enrolled in our study. Laboratory tests were performed to analyze the nutrition status of each patient, and the clinical outcome variables, including postoperative complications, hospital stay, cost of hospitalization and postoperative outcome, were analyzed.

RESULTS: The "non-risk" patients who did not receive postoperative nutrition support had a higher rate of postoperative complications than patients who received postoperative nutrition support (2.40 ± 1.51 vs 1.23 ± 0.60 , $P = 0.000$), and had a longer postoperative hospital stay (23.00 ± 15.84 d vs 15.27 ± 5.89 d, $P = 0.009$). There was higher cost of hospitalization for patients who received preoperative total parenteral nutrition (TPN)

than for patients who did not receive preoperative TPN ($62\,713.50 \pm 5070.66$ RMB Yuan vs $43\,178.00 \pm 3596.68$ RMB Yuan, $P = 0.014$). Applying postoperative enteral nutrition significantly shortened postoperative fasting time (5.16 ± 1.21 d vs 6.40 ± 1.84 d, $P = 0.001$) and postoperative hospital stay (11.92 ± 4.34 d vs 15.77 ± 6.03 d, $P = 0.002$). The patients who received postoperative TPN for no less than 7 d had increased serum glucose levels (7.59 ± 3.57 mmol/L vs 6.48 ± 1.32 mmol/L, $P = 0.006$) and cost of hospitalization ($47\,724.14 \pm 16\,945.17$ Yuan vs $38\,598.73 \pm 8349.79$ Yuan, $P = 0.000$). The patients who received postoperative omega-3 fatty acids had a higher rate of postoperative complications than the patients who did not (1.33 ± 0.64 vs 1.13 ± 0.49 , $P = 0.041$). High level of serum glucose was associated with a high risk of postoperative complications of infection.

CONCLUSION: Appropriate and moderate nutritional intervention can improve the postoperative outcome of colorectal cancer patients.

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Key words: Nutritional support; Nutrition assessment; Colorectal cancer; Surgery; Prognosis

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INTRODUCTION

Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women

worldwide^[1]. It is also a significant cause of morbidity and mortality throughout the world^[2]. Malnutrition is common in patients presenting for surgical management of colorectal cancer, and multiple factors, such as tumor location, tumor type, tumor stage, and preoperative radiation or chemotherapy, may predispose the patients to malnutrition^[3]. Postoperative outcomes, including incidence of complications, morbidity and survival, are usually better in the patients who are in a good nutritional condition^[4]. Comprehensive clinical application of nutrition support in colorectal cancer patients appears to be necessary.

Unfortunately, malnutrition has remained a troublesome problem because of lack of nutrition support routines and a discrepancy between clinical practice and guidelines regarding nutrition support^[5].

Currently, international guidelines on nutrition support have been established, such as the European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines and the American Society for Parenteral and Enteral Nutrition (ASPEN) guidelines. Both are the authoritative guidelines at present, and should be followed and used in clinical practice as appropriate to the specific medical condition. However, since fewer than one sixth of the recommendations in the current guidelines are Grade A, and more than 50% are Grade C^[6], more and better controlled trials are needed in the specific fields.

We carried out a retrospective study to evaluate the nutritional risk of colorectal cancer patients who underwent elective surgery, and assessed the nutrition support process by analyzing the postoperative clinical outcomes and comparing with the international recommendations or guidelines. In particular, we investigated the current status of nutrition support for patients undergoing surgery for colorectal cancer, and determined the requirements of feasible and appropriate nutrition support strategies for such patients.

MATERIALS AND METHODS

Case selection

We reviewed a total of 220 consecutive patients admitted to our hospital with a diagnosis of colon cancer or rectal cancer from January 2010 to July 2010, and excluded 18 patients, including one with hydroperitoneum according to the exclusion criteria of the Nutrition Risk Screening (NRS) 2002^[7], and 17 who had received non-surgical treatment. The remaining 202 patients were enrolled in this study.

Methods

In order to evaluate the clinical effect of different nutritional strategies in colorectal cancer patients with different nutritional status, we stratified the patients into five groups.

In Group A, to evaluate the effect of NRS score, we excluded the patients who received preoperative nutritional support, and divided the remaining 199 patients into a “non-risk” group ($n = 148$) whose NRS score was 0-2, and an “at-risk” group ($n = 51$) whose NRS score was ≥ 3 . We further divided the two groups into two subgroups, a

nutrition support group (NS) who received postoperative nutrition support and a non-nutrition support group (NNS) who did not receive postoperative nutrition support (Table 1). Diagnosis and tumor stage were used to illustrate preoperative health status. The tumor stage was determined according to the 7th edition of the AJCC cancer staging manual (American Joint Committee on Cancer)^[8]. Complications, postoperative hospital stay, cost of hospitalization and postoperative outcomes were used to assess the clinical effect of postoperative nutritional intervention. In addition, we graded complications as none = 1, infection = 2, fistula = 3, others = 4, and postoperative outcome as recovery = 1, no recovery = 2, death = 3, for statistical evaluation of the results.

Group B consisted of five patients whose NRS score was > 4 , the clinical effect of preoperative TPN in patients with severe malnutrition was evaluated. They were divided into two groups: group 1 ($n = 2$) who received preoperative TPN and group 2 ($n = 3$) who did not (Table 2). Diagnosis, tumor stage, and preoperative albumin, potassium, and sodium levels reflected the preoperative nutrition status. Postoperative enteral nutrition (EN), postoperative TPN and postoperative TPN duration were indicative of the postoperative nutritional intervention. Postoperative serum glucose level was fluctuated according to the proportion of insulin in TPN. Postoperative day 1 (POD1) albumin, potassium and sodium, and POD5 albumin, potassium and sodium levels reflected the postoperative nutritional status. Complications, postoperative hospital stay, cost of hospitalization, and postoperative outcomes were used to assess clinical outcome.

In Group C, the application of postoperative EN in colorectal cancer patients was assessed. Patients who received preoperative nutrition support were excluded, and the remaining 199 patients were divided into two groups: group 1 ($n = 25$) who received postoperative EN and group 2 ($n = 174$) who did not (Table 3). Diagnosis, tumor stage and NRS 2002 score reflected the preoperative nutrition status. As an interferential factor in this group, postoperative TPN was used in the statistical analysis to identify the effect of postoperative EN. Postoperative fasting time, occurrence of complications, postoperative hospital stay, cost of hospitalization, and postoperative outcome indicated clinical outcome.

In Group D, to determine the effect of postoperative TPN duration, we excluded the patients who received preoperative nutrition support and postoperative EN, and included the remaining 174 patients who received postoperative TPN only, dividing them into two groups: group 1 ($n = 66$) with postoperative TPN duration of no less than 7 d, and group 2 ($n = 108$) with a duration of less than 7 d (Table 4). Diagnosis, tumor stage, NRS 2002 score, preoperative albumin, potassium and sodium levels gave an indication of preoperative nutrition status. POD1 albumin, potassium and sodium, and POD5 albumin, potassium and sodium reflected postoperative nutritional status. Complications, postoperative hospital stay, cost of hospitalization and postoperative outcome

Table 1 Nutrition risk screening

	Non-risk		<i>P</i>	At-risk		<i>P</i>
	NS	NNS		NS	NNS	
Patient number	143	5		49	2	
Diagnosis (colon / rectal cancer)	50/93	1/4		24/25	2/0	
Gender (male/female)	63/80	2/3	0.893	30/19	2/0	0.088
Tumor stage ¹			0.066			0.358
Complications ²	1.23 ± 0.60	2.40 ± 1.51	0.000	1.20 ± 0.45	1.50 ± 0.70	0.348
None (= 1)						
Infection (= 2)						
Fistula (= 3)						
Others (= 4)						
Postoperative hospital stay (d)	15.27 ± 5.89	23.00 ± 15.84	0.009	14.55 ± 4.11	14.50 ± 2.12	0.986
Cost of hospitalization (RMB Yuan)	43469.88 ± 9961.67	35825.00 ± 16271.94	0.301	41802.97 ± 13300.99	33845.80 ± 8374.80	0.187
Postoperative outcome ²	1.02 ± 0.16	1.00 ± 0.00	0.707	1.10 ± 0.30	1.50 ± 0.70	0.090

¹The tumor stage of the patients was judged according to the 7th edition of American Joint Committee on Cancer staging manual^[8]; ²Complications are defined as none = 1, infection = 2, fistula = 3, others = 4, and postoperative outcome as recovery = 1, no recovery = 2, death = 3, for easier statistical presentation of the results. NS: Nutrition support; NNS: Non-nutrition.

Table 2 Preoperative total parenteral nutrition in malnourished patients

	Group 1	Group 2	<i>P</i>
Patient number	2	3	
Diagnosis (colon/rectal cancer)	2/0	1/2	
Gender (male/female)	2/0	1/2	0.219
Tumor stage	1	2	
Preoperative albumin (g/L)	36.30 ± 5.65	32.83 ± 9.00	0.669
Preoperative potassium (mmol/L)	4.53 ± 0.36	3.75 ± 0.92	0.352
Preoperative sodium (mmol/L)	138.45 ± 2.05	142.13 ± 3.40	0.274
Postoperative EN	2	0	
Postoperative TPN	2	2	
Postoperative TPN duration (d)	7.00 ± 1.41	3.66 ± 3.2	0.276
POD1 ^a serum glucose (mmol/L)	6.91 ± 1.11	9.05 ± 3.65	0.498
POD1 albumin (g/L)	27.80 ± 3.11	26.13 ± 3.19	0.605
POD1 potassium (mmol/L)	4.49 ± 0.36	4.45 ± 0.78	0.953
POD1 sodium (mmol/L)	135.8 ± 3.11	137.66 ± 2.51	0.508
POD5 ^a serum glucose (mmol/L)	6.29 ± 0.24	6.39 ± 2.88	0.968
POD5 albumin (g/L)	28.65 ± 5.16	32.33 ± 4.67	0.466
POD5 potassium (mmol/L)	4.67 ± 0.11	4.42 ± 0.12	0.115
POD5 sodium (mmol/L)	135.95 ± 1.34	135.93 ± 2.72	0.994
Complications			0.445
Postoperative hospital stay(d)	10.50 ± 4.94	13.00 ± 2.64	0.500
Cost of hospitalization (RMB)	62713.50 ± 5070.66	43178.00 ± 3596.68	0.014
Postoperative outcome			0.495

^aPOD1: Postoperative day 1; POD5: Postoperative day 5; EN: Enteral nutrition; TPN: Total parenteral nutrition.

were used to assess the clinical outcome. In addition, the comparison of preoperative serum glucose, POD1 serum glucose, and POD5 serum glucose reflected the contribution of postoperative TPN duration to postoperative serum glucose.

Group E excluded the patients who received preoperative nutrition support or postoperative EN, and included the remaining 167 patients who received postoperative TPN. This group was subdivided into two groups: group 1 (*n* = 102), those who received postoperative application of omega-3 fatty acids, and group 2 (*n* = 65), those who

Table 3 Postoperative enteral nutrition and clinical outcome

	Group 1	Group 2	<i>P</i>
Patient number	25	174	
Diagnosis (colon / rectal cancer)	8/17	66/108	0.568
Gender (male/female)	9/16	91/83	0.129
Tumor stage			0.777
NRS 2002 score	1.88 ± 0.88	1.96 ± 1.01	0.689
Postoperative fasting time (d)	5.16 ± 1.21	6.40 ± 1.84	0.001
Postoperative TPN	27	167	0.996
Complications	1.04 ± 0.20	1.29 ± 0.66	0.060
None (= 1)			
Infection (= 2)			
Fistula (= 3)			
Others (= 4)			
Postoperative hospital stay (d)	11.92 ± 4.34	15.77 ± 6.03	0.002
Cost of hospitalization (RMB)	44210.88 ± 7635.85	42060.09 ± 13066.15	0.752
Postoperative outcome	1.00 ± 0.00	1.06 ± 0.27	0.214
Recovery (= 1)			
Unrecovery (= 2)			
Dead (= 3)			

Group 1: Patients who received enteral nutrition postoperatively; Group 2: Patients who did not receive enteral nutrition postoperatively. TPN: Total parenteral nutrition; NRS: Nutrition risk screening.

did not, as shown in Table 5. Diagnosis, tumor stage, and NRS 2002 score were indicative of preoperative nutrition status. The total lymphocyte count reflected the immune status. Complications, postoperative hospital stay, cost of hospitalization, and postoperative outcome were used to assess the clinical outcome.

We also analyzed the relationship between postoperative day 5 serum glucose levels and postoperative complications of infection (Figure 1).

Statistical analysis

Analyses were performed using SPSS statistical software

Table 4 Postoperative total parenteral nutrition duration

	Group 1	Group 2	P
Patient number	66	108	
Diagnosis (colon/rectal cancer)	31/35	35/73	
Gender (male/female)	32/34	59/49	0.434
Tumor stage			0.493
NRS 2002 score	1.92 ± 0.94	1.99 ± 1.05	0.676
Preoperative serum glucose (mmol/L)	6.10 ± 1.86	5.75 ± 1.17	0.134
Preoperative albumin (g/L)	38.47 ± 4.44	39.51 ± 6.37	0.249
Preoperative potassium (mmol/L)	3.98 ± 0.38	6.57 ± 1.84	0.270
Preoperative sodium (mmol/L)	140.98 ± 3.23	139.58 ± 14.1	0.435
POD1 serum glucose (mmol/L)	8.59 ± 3.39	7.37 ± 2.06	0.100
POD1 albumin (g/L)	32.24 ± 3.65	35.49 ± 4.11	0.725
POD1 potassium (mmol/L)	4.05 ± 0.44	3.99 ± 0.45	0.424
POD1 sodium (mmol/L)	136.35 ± 3.59	135.72 ± 14.50	0.763
POD5 serum glucose (mmol/L)	7.59 ± 3.57	6.48 ± 1.32	0.006
POD5 albumin (g/L)	31.79 ± 3.53	39.91 ± 3.66	0.063
POD5 potassium (mmol/L)	4.21 ± 0.50	4.16 ± 0.53	0.553
POD5 sodium (mmol/L)	136.5 ± 18.60	137.84 ± 2.81	0.348
Complications			0.533
Postoperative hospital stay (d)	15.59 ± 5.32	15.87 ± 6.45	0.761
Cost of hospitalization (RMB)	47724.14 ± 16945.17	38598.73 ± 8349.79	0.000
Postoperative outcome			0.166

Group 1: The duration of postoperative total parenteral nutrition (TPN) was not less than 7 d; Group 2: The duration of postoperative TPN was less than 7 d. NRS: Nutrition Risk Screening.

(SPSS for Windows Ver. 11.5). Results of different groups were compared using descriptive statistics (mean ± SD). $P \leq 0.05$ was considered statistically significant.

RESULTS

Nutrition risk screening is a necessary and effective tool to identify the nutritional status of colorectal cancer patients, and to aid in providing the appropriate nutrition intervention. As Table 1 shows, the “non-risk” patients who did not receive postoperative nutrition support had a higher rate of postoperative complications than those who received postoperative nutrition support (2.40 ± 1.51 vs 1.23 ± 0.60 , $P = 0.000$), and also had a longer postoperative hospital stay (23.00 ± 15.84 vs 15.27 ± 5.89 , $P = 0.009$), which indicated that postoperative nutrition support may be necessary for “non-risk” patients. Postoperative nutrition support or not did not show a significant difference in the outcome of “at-risk” patients, though postoperative nutrition support tended to improve the postoperative outcome (1.10 ± 0.30 vs 1.50 ± 0.70 , $P = 0.090$), thus moderate nutrition support is allowable for “at-risk” patients.

Table 2 shows that the cost of hospitalization for malnourished patients who received preoperative TPN was significantly higher than in patients who did not ($62\,713.50 \pm 5070.66$ RMB Yuan vs $43\,178.00 \pm 3596.68$ RMB Yuan, $P = 0.014$) with no significant difference in the outcome.

Postoperative EN markedly improved postoperative recovery course, including a reduction in postoperative fasting time (5.16 ± 1.21 d vs 6.40 ± 1.84 d, $P = 0.001$) and postoperative hospital stay (11.92 ± 4.34 d vs 15.77

Table 5 Postoperative administration of omega-3 fatty acids

	Group 1	Group 2	P
Patient number	102	65	
Diagnosis (colon / rectal cancer)	42/60	40/25	
Gender (male/female)	49/53	45/38	0.089
Tumor stage			0.317
NRS 2002 score	1.94 ± 0.87	2.00 ± 1.15	0.710
Preoperative total lymphocyte count	1.80 ± 0.63	1.93 ± 0.59	0.186
POD1 total lymphocyte count	0.99 ± 0.34	1.11 ± 0.40	0.067
POD5 total lymphocyte count	1.26 ± 0.59	1.29 ± 0.35	0.660
Complications	1.33 ± 0.64	1.13 ± 0.49	0.041
None (= 1)			
Infection (= 2)			
Fistula (= 3)			
Others (= 4)			
Postoperative hospital stay (d)	16.04 ± 5.81	14.81 ± 4.29	0.159
Cost of hospitalization (RMB)	43936.75 ± 14260.31	39938.89 ± 10741.40	0.055
Postoperative outcome	1.09 ± 0.33	1.06 ± 0.27	0.055
Recovery (= 1)			
Unrecovery (= 2)			
Dead (= 3)			

Group 1: Patients who received omega-3 fatty acids; Group 2: Patients who did not receive omega-3 fatty acids.

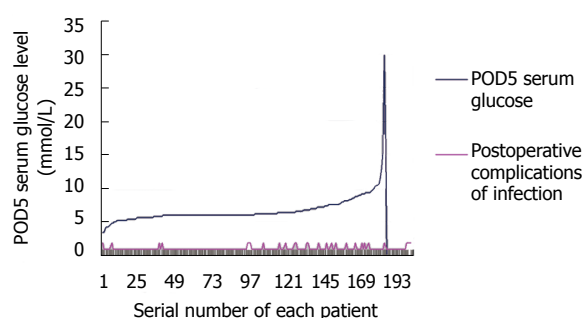


Figure 1 Relationship between postoperative serum glucose level and complications of infection. Abscissa: the serial number of each patient, arranged according to the Postoperative day 5 serum glucose level; Ordinate: numerical value; The red line shows the incidence of postoperative complications of infection.

± 6.03 d, $P = 0.002$), as shown in Table 3.

Longer postoperative TPN was not associated with better clinical outcome (Table 4). The patients who received postoperative TPN for no less than 7 d had increased POD5 serum glucose (7.59 ± 3.57 mmol/L vs 6.48 ± 1.32 mmol/L, $P = 0.006$) and cost of hospitalization ($47\,724.14 \pm 16\,945.17$ Yuan vs $38\,598.73 \pm 8\,349.79$ Yuan, $P = 0.000$), compared to those with less than 7 d postoperative TPN, suggesting that less than 7 d nutrition support for postoperative colorectal cancer patients is adequate.

More postoperative complications occurred in the patients with postoperative administration of omega-3 fatty acid (1.33 ± 0.64 vs 1.13 ± 0.49 , $P = 0.041$) than in patients who did not receive the fatty acid (Table 5).

Postoperative complications were positively correlated with the postoperative serum glucose level, a high postoperative serum glucose level being associated with a higher risk of complications of infection (Figure 1).

DISCUSSION

Malnutrition is common in patients with colorectal cancer, and in our study, 52 (25.7%) of the 202 cases had a NRS score of more than 3, and had a high nutrition risk^[7]. Poor nutrition status impacts on the recovery of physical performance status in cancer patients after treatment^[9]. It was reported that about 20% of cancer patients died of malnutrition or related complications rather than the malignant disease itself^[10]. Malnutrition is often neglected in our daily clinical practice, and can also induce many clinical problems, including impaired wound-healing, immunocompromization, diminished cardiac and respiratory function, and a host of other complications that can lead to longer hospitalization and a higher mortality rate^[11]. Although provision of nutrition support to cancer patients may cause tumors to grow more quickly, nutrition support is recommended when the nutrition status is so compromised that patients are at a high risk of complications, or cannot comply with the oncologic therapy as reported in the clinical practice ESPEN guidelines^[12]. Thus perioperative nutrition support is beneficial for moderately or severely malnourished gastrointestinal cancer patients^[13]. The implementation of nutrition support guidelines has facilitated many appropriate nutritional support procedures for colorectal cancer patients^[4,6,14-16].

Preoperative nutrition risk screening can identify nutritional risks

Patients with cancer are at a risk of malnutrition, and nutrition screening should be performed to identify those who require nutrition support^[17]. When a patient is admitted to our ward, knowledge of the nutritional status, which is a clinical predictor of postoperative mortality and morbidity in surgery for colorectal cancer^[18-20], is essential, not only for screening malnourished or non-malnourished patients, but also for multimodal oncological treatment^[21]. There are various kinds of screening methods, including NRS 2002, which is a rapid screening tool recommended by ESPEN^[7], and has been proven to be an appropriate scoring system for predicting unfavorable clinical outcomes^[22]. ASPEN suggested using a subjective global assessment (SGA) as a screening tool^[23], and was shown to be a reliable assessment tool which could predict hospital stay and medical expenditure of surgical gastrointestinal cancer patients^[24,25]. We believe that the assessment of nutritional status requires a multidimensional approach, which includes different clinical indices and various nutritional parameters, so it is better to use both SGA and NRS 2002 to predict the clinical outcome^[26]. Our study indicated that, for “non-risk” colorectal patients, postoperative nutrition support is necessary to avoid postoperative complications and shorten postoperative hospital stay. Although postoperative nutrition support to “at-risk” colorectal patients showed no significant advantage, in our opinion, moderate nutrition support is allowable, as no harm or economic burden was incurred. Further prospective studies are necessary to confirm this.

Preoperative TPN is not always necessary

The goal of preoperative nutrition support is to minimize negative protein balance by avoiding starvation, to maintain muscle, immune and cognitive functions, and to enhance postoperative recovery, as the ESPEN guidelines indicated^[27]. Preoperative parenteral nutrition is indicated in severely undernourished patients in whom enteral nutrition cannot be adequately administered either orally or enterally. Conversely, its use in well-nourished patients has no benefit but increased morbidity. In our study, preoperative nutrition support in severely malnourished colorectal cancer patients only increased the economic burden, with little beneficial effect. This is in agreement with the ASPEN guidelines, which recently recommended that nutrition support should not be used routinely in patients undergoing major cancer surgeries^[17]. Because of the limited sample size, further prospective studies with a larger sample size should be carried out.

Gunerhan's study^[28] recently showed that preoperative immunonutrition resulted in a significant increase in serum prealbumin levels, but it did not significantly alter the T lymphocyte subpopulation count, the rate of postoperative complications and the hospitalization duration, thus preoperative immunonutrition should not be provided routinely. None of our patients received preoperative immunonutrition.

Postoperative EN can shorten the fasting time and hospital stay

Previously, many colorectal doctors believed that nutrients in the gut disrupted anastomoses, so they preferred delaying the EN postoperatively, and administered TPN instead to avoid anastomotic leak, which requires substantial use of hospital resources^[29]. However, Seidner^[11] emphasized that there were no significant differences in morbidity and mortality between patients who received EN or TPN, and recommended the guideline: if the gut works, use it. The available evidence lends support to the use of enteral over parenteral feeding in inpatients with functioning gastrointestinal tracts^[30]. The application of EN can reverse the loss of gut mucosal integrity resulting from surgical trauma^[31], and early nutrition support (EEN) is associated with a decreased infection risk, a decreased mortality, a reduced hospital stay, an increase in collagen deposition at anastomosis and wound strength, and a clear trend of a reduction in anastomotic breakdown^[32,33]. In addition, EEN can reduce the use of nasogastric tubes, which may delay the return of bowel function and increase pulmonary complications^[34,35]. Osland^[3] even suggested adopting EEN as a standard of care in cancer patients undergoing gastrointestinal resections. As Table 3 shows, postoperative EN in colorectal cancer patients can significantly shorten the postoperative fasting time and postoperative hospital stay, and there is a tendency to reduce postoperative complications ($P = 0.060$). Although it remains to be determined how much should be provided initially, underfeeding with a small amount of nutrients, which “bathe” the gut mucosa, makes EEN necessary or desirable.

The risk of overfeeding should not be neglected, as it can overwhelm the digestive and absorptive capacity of the gastrointestinal tract, and lead to occurrence of some clinical complications, such as gastric distention, nausea, and diarrhea^[32].

Postoperative TPN can offer a smooth postoperative recovery

Parenteral nutrition (PN) has been widely used in clinical practice, and a safe PN system must be developed which minimizes procedural incidents and maximizes the ability to meet individual patient requirements^[36]. Thus, it is desirable to provide, devise, or make available customized PN formulations for individuals who have complex requirements secondary to disease or underlying illness, or when otherwise warranted by routine monitoring of electrolytes, organ function, growth, and development. Not only fat and carbohydrates, but also a full range of vitamins and trace elements should be important components of the TPN bag, and optimal nitrogen-sparing can be achieved when all components of the PN mix are administered simultaneously over 24 h. However, when early oral food intake or EN is combined with PN, intravenous supplementation with vitamins appears to be unnecessary^[27].

Should colorectal cancer patients be administered postoperative TPN? Planas^[4] recommended that such patients having elective surgery should not be given postoperative PN routinely. Seidner^[11] implied that administering PN in disregard of the patient's nutritional status could do more harm than good, and suggested that postoperative TPN should be reserved for patients who have a prolonged postoperative ileus, generally more than 7-10 d, and for those who are severely malnourished and whose feeding cannot be started within 3-5 d. According to the ESPEN guidelines^[27], postoperative PN is recommended in patients who cannot meet their caloric requirements within 7-10 d both orally or enterally, and in patients who require postoperative artificial nutrition, enteral feeding or a combination of enteral and supplementary parenteral feeding.

In our study, most colorectal cancer patients could resume feeding 5-8 d postoperatively (Table 3), so postoperative TPN may be beneficial during the period of postoperative fasting. Should we give the patients TPN for 7 d or more, or is less than 7 d adequate? The results in Table 4 indicate that a longer duration of TPN incurs high hospitalization costs and induces hyperglycemia, which is associated with a higher rate of postoperative complications (Figure 1), thus less than 7 d' postoperative TPN appears to be appropriate.

PN can be delivered through short-term, non-tunneled central venous catheters, and the appropriate choice, insertion, and monitoring of the venous access are of paramount importance to avoid a catheter-related bloodstream infection, an important and still very common complication of PN^[37]. Such infections can be reduced by adopting cost-effective, evidence-based interventions, including specific training of staff, an adequate handwashing, the correct type

of device and site of insertion, the use of maximal barrier protection during insertion, and removal of central lines as soon as they are no longer necessary.

Postoperative application of omega-3 fatty acids

There is controversy as to whether visceral proteins should be used to assess nutrient status in hospitalized patients. Seidner^[11] suggested that visceral proteins can be used in the hospital setting, because they can identify patients at risk of a poor outcome who may benefit from nutrition support. In addition, the total lymphocyte count can be used to assess a patient's immune function, which has been shown to correlate with the degree of visceral protein depletion and clinical outcome. Therefore, total lymphocyte counts were used in our study to assess the effect of the omega-3 fatty acids.

Postoperative supplementation of omega-3 fatty acids by TPN has been reported to have a favorable effect in the outcomes of colorectal cancer patients undergoing radical resection, by lowering the magnitude of the inflammatory response and modulating the immune response^[38,39]. In contrast, the application of omega-3 fatty acids showed no significant benefit in our study, and indeed there was a trend of an increased risk of postoperative complications, an increased economic burden, and a poorer postoperative outcome. Further prospective research is necessary with a larger sample to assess the functional benefit or otherwise of omega-3 fatty acids in the postoperative setting.

Currently, many barriers, including low priority of nutritional support, no routine or established procedures in many medical centers, insufficient knowledge of nutritional support, lack of qualified and optional nutritional menus for the patients, and lack of leadership support from the medical team, make the nutritional therapy difficult to carry out in many hospitals^[40]. A greater effort should be made in the nutritional assessment of patients.

In conclusion, nutrition support is an important therapy for colorectal cancer patients, and appropriate and moderate nutritional intervention can significantly improve the postoperative recovery course, relieve the patient's suffering, and reduce the medical cost of the patients. Clinicians must be aware of nutrition support principles and methods in order to administer appropriate nutrition support and avoid blind nutrition administration.

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COMMENTS

Backgrounds

Nutrition support has been widely used in the area of surgery, where the benefit on patients' prognosis is evident. Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women worldwide, and is also a significant cause of morbidity and mortality throughout the world, thus an appropriate and feasible nutrition support strategy is necessary and beneficial for patients' prognosis.

Research frontiers

Nutritional support is widely used in postoperative colorectal cancer patients, but the role of nutrients has not been clearly defined. This study investigated the effect of nutrition support on the outcomes of patients with different nutritional status.

Innovations and breakthroughs

The authors found that appropriate and moderate nutritional intervention can significantly improve the postoperative outcome of the patients with colorectal cancer.

Applications

The study provides a reference for daily clinical practice and future research. A prospective, multicenter, randomized, controlled trial with a larger sample is necessary to validate the statistical results and diminish bias.

Peer review

Although this is a retrospective review, I believe it will be of interest to the readers. And, it does add something to the literature.

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Application of a wire-guided side-viewing duodenoscope in total esophagectomy with colonic interposition

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Abstract

Therapeutic endoscopic retrograde cholangiopancreatography (ERCP) is the mainstay treatment for bile duct disease. The procedure is difficult per se, especially when a side-viewing duodenoscope is used, and when the patient has altered anatomical features, such as colonic interposition. Currently, there is no consensus on the standard approach for therapeutic ERCP in patients with total esophagectomy and colonic interposition. We describe a novel treatment design that involves the use of a side-viewing duodenoscope to perform therapeutic ERCP in patients with total esophagectomy and colonic interposition. A gastroscope was initially introduced into the interposed colon and a radio-opaque standard guidewire was advanced to a distance beyond the papilla of Vater, before the gastroscope was withdrawn. A side-viewing duodenoscope was then introduced along the guidewire under fluoroscopic guidance. After cannulation into the papilla of Vater, endoscopic retrograde chol-

angiography (ERC) revealed a filling defect (maximum diameter: 15 cm) at the distal portion of the common bile duct (CBD). This defect was determined to be a stone, which was successfully retrieved by a Dormia basket after complete sphincterotomy. With this treatment design, it is possible to perform therapeutic ERCP in patients with colonic interposition, thereby precluding the need for percutaneous drainage or surgery.

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Key words: Wire-guided; Duodenoscope; Endoscopic retrograde cholangiopancreatography; Esophagectomy; Interposition of colon

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INTRODUCTION

The application of a side-viewing duodenoscope in total esophagectomy with colonic interposition is technically difficult, because of the altered structure of the colon and the redundancy of the endoscopic route. We report a wire-guided treatment designed to overcome this pitfall by introducing a side-viewing duodenoscope along a radio-opaque standard guidewire to facilitate therapeutic ERCP in patients undergoing esophagectomy with colonic interposition. The use of this treatment method ensured the safety of wire-guided therapeutic ERCP in patients undergoing total esophagectomy with colonic interposition.

CASE REPORT

An 87-year-old man was referred to our hospital, a tertiary referral medical center, for the management of episodic fever, chills, and right upper quadrant abdominal pain, which had been occurring intermittently for two months. He had undergone total esophagectomy with colonic interposition 17 years ago for the treatment of intractable esophageal ulcers with massive bleeding (Figure 1). He denied having passed tea-colored urine or clay-colored stool. Abdominal ultrasonography revealed dilatation of the common hepatic duct (CHD) and common bile duct (CBD; diameter: 1.45 cm). Magnetic resonance cholangiopancreatography (MRCP) showed the presence of a stone impacted at the distal portion of the CBD (Figure 2). The patient was intravenously administered midazolam (3 mg), pethidine (50 mg), and butylscopolamine (20 mg), and ERCP was performed with the patient in the left lateral position. A forward-viewing gastroscope (GIF-Q260, Olympus) was initially introduced; it was advanced through the interposed colonic segment, gastric remnant, and duodenum to reach the papilla of Vater. A radio-opaque standard guidewire (THSF-35-480, Wilson-Cook) was inserted deep into the small intestine, up to a distance beyond the papilla of Vater, via the accessory channel (Figure 3). The gastroscope was then withdrawn over-the-wire. Under fluoroscopic guidance, and with the patient in the left-lateral position, a side-viewing duodenoscope (IJF-240, Olympus) was introduced carefully along the guidewire until it reached the papilla of Vater. After cannulation with an ERCP catheter (StarTip cannula, PR-106Q-1, Olympus) as usual, cholangiography showed a filling defect (diameter, 1.5 cm) in the distal portion of the CBD; the lesion was determined to be a CBD stone (Figure 4). Complete sphincterotomy with a traction sphincterotome was performed (Figure 5). The pigmented stone was successfully retrieved using a Dormia basket (Figure 6). Subsequent balloon-occlusion cholangiography showed complete clearance of the CBD. The patient was followed up in the outpatient department and remains well.

DISCUSSION

The colon has been used as an esophageal substitute since 1911. It has been proven to be superior to other substitutes, such as the stomach and small intestine, because of its length, acid resistance, and richness of vascular supply. It affords good overall satisfaction and allows maintenance of a wider surgical resection margin in patients with cancers of the gastroesophageal junction. The disadvantages of its application include prolonged operation time, extensive preoperative preparation, and the late redundancy of colonic grafts^[1,2].

Therapeutic ERCP with the application of a side-viewing duodenoscope is widely used in the management of pancreatic or hepatobiliary diseases, such as biliary stones^[3]. Technically, it is difficult to advance a side-viewing duodenoscope through the colon because the duodenoscope affords visualization of only areas to the sides of the scope, and because of the presence of colonic interhaustral folds,

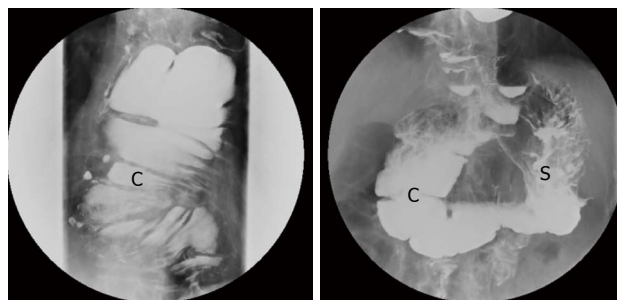


Figure 1 Esophagography showing the interposition of the colon (C) and the gastric remnant (S).

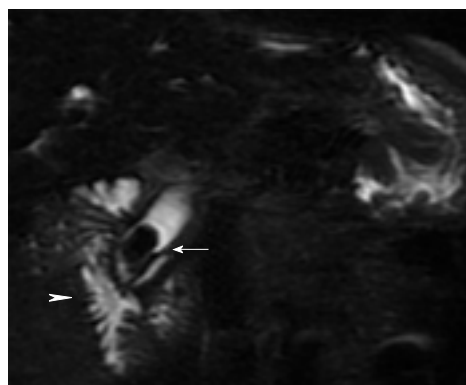


Figure 2 Magnetic resonance cholangiopancreatography showing a stone in the distal common bile duct (arrow). The arrowhead shows the second portion of the duodenum.



Figure 3 The radio-opaque standard guidewire (arrowhead) was inserted through the working channel of the gastroscope.

the angulation of the colon, and the redundancy of the colonic graft^[4]. To date, several techniques have been described for using the side-viewing duodenoscope to visualize the colon. Dafnis reported the successful application of a unique technique for approaching an inaccessible colonic polyp at the splenic flexure using an overtube to advance the side-viewing duodenoscope^[5]. Another report of a case series on the management of inaccessible colonic polyps, advocated the technique of slightly bending the tip of the side-viewing duodenoscope, thereby providing a sloped-forward view for performing polypectomy^[6]. We believe that the use of a wire-guided side-viewing duodenoscope

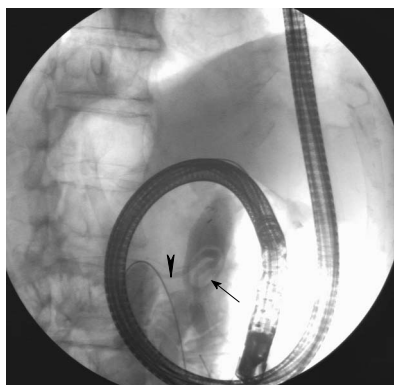


Figure 4 With the patient in the left-lateral position, endoscopic retrograde cholangiopancreatography showed a filling defect in the distal part of the common bile duct (arrow). The arrowhead shows the pancreatic duct.

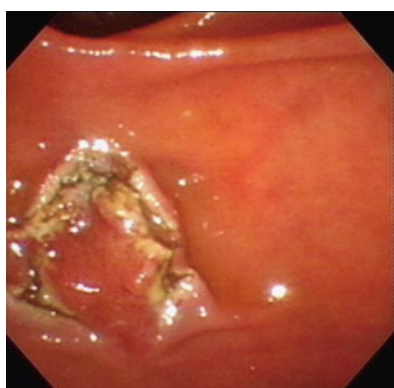


Figure 5 Complete sphincterotomy.

might represent a safe technique for approaching inaccessible colonic polyps.

In the present case, our most important concern was the smooth advancement of the duodenoscope through the colonic graft. To address this concern, we inserted a radio-opaque guidewire to serve as a roadmap. Fry *et al.*^[7] reported an over-the-wire method by using a Super-Stiff Amplatz guidewire, which was actually designed for cardiac catheterization, to intubate the duodenum with a side-viewing duodenoscope in a patient with large paraesophageal hernia. The reason we chose the standard guidewire, instead of a Super-Stiff Amplatz guidewire, was because it is entirely radio-opaque. It facilitated the localization and visualization of the tip of the duodenoscope under close fluoroscopic guidance. Despite this, the duodenoscope did, at one point, move away from the appropriate path in the gastrointestinal tract, during the procedure. When the graft lumen could not be visualized on the endoscopic screen, we pushed the duodenoscope forward once its axis was the same as that of the wire, as determined by fluoroscopy; the scope was advanced in this manner until the graft lumen could be seen (Figure 7). The duodenoscope was advanced through the graft, and the CBD stone was eventually retrieved.

Manipulation of the guidewire is an art. One of its principles is to avoid looping, especially in a spacious cavity, such as the stomach. In our experience, we have observed

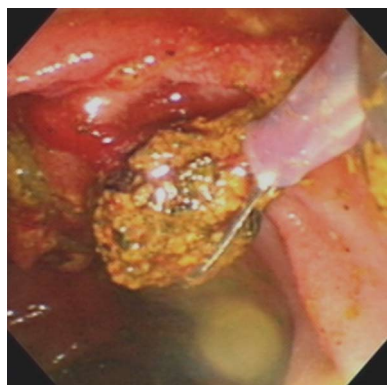


Figure 6 The pigment stone retrieved by a Dormia basket.

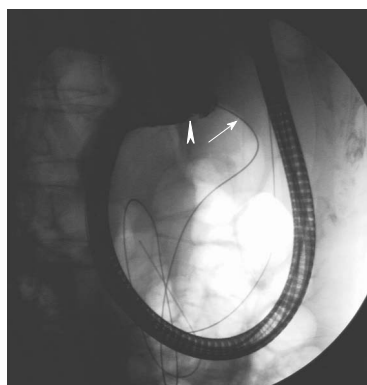


Figure 7 The duodenoscope (arrowhead) was pushed along the guidewire (arrow) at the same axis under fluoroscopic guidance.

that the looping of the guidewire may cause the failure of esophageal or duodenal metallic stent implantation in patients with malignant obstruction. The looping of the guidewire could render it difficult to introduce the scope further. To avoid this looping, we advanced the tip of the guidewire to a distance beyond the papilla of Vater, instead of stopping within the stomach.

Some experienced endoscopists prefer to backload the guidewire through the working channel of the duodenoscope. However, we think that this is not feasible because the side-viewing characteristic, with its acute angle of elevation. Backloading would render it difficult to insert the duodenoscope and would increase the number of loops formed. Furthermore, the double-balloon enteroscope could not be applied in our case because it is a forward-viewing scope and lacks the angle of elevation required to support the use of ERCP accessories.

Another technique that could have been considered in the present case would be the direct introduction of the side-viewing duodenoscope without the initial use of the forward-viewing gastroscope; however, this would have made it difficult to clearly visualize the lumen, especially as this patient had undergone colonic interposition. Such an approach would be accompanied by a high risk of perforation. The successful application of our technique for performing therapeutic ERCP is proof of the feasibility of this technique. To the best of our knowledge, this is the

first report on the use of this novel technique for treating a CBD stone in a patient with esophagectomy and colonic interposition.

In conclusion, in cases with rare clinical presentations, it is necessary to carefully and accurately estimate possible hindrances and develop appropriate solutions to successfully overcome them.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYs, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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>

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- 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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Contents

Weekly Volume 17 Number 14 April 14, 2011

EDITORIAL

- 1791 Inguinodynia following Lichtenstein tension-free hernia repair: A review
Hakeem A, Shanmugam V

REVIEW

- 1797 Role of conventional therapies in the era of biological treatment in Crohn's disease
Gionchetti P, Calabrese C, Tambasco R, Brugnera R, Straforini G, Liguori G, Fornarini GS, Riso D, Campieri M, Rizzello F

ORIGINAL ARTICLE

- 1807 Hepatitis B and C infection and liver disease trends among human immunodeficiency virus-infected individuals
Buskin SE, Barash EA, Scott JD, Aboulafia DM, Wood RW
- 1817 X-ray diagnosis of synchronous multiple primary carcinoma in the upper gastrointestinal tract
Yang ZH, Gao JB, Yue SW, Guo H, Yang XH
- 1825 Breviscapine attenuates acute pancreatitis by inhibiting expression of PKC α and NF- κ B in pancreas
Zhang H, Cai CZ, Zhang XQ, Li T, Jia XY, Li BL, Song L, Ma XJ

BRIEF ARTICLE

- 1831 Predictive factors of clinical response in steroid-refractory ulcerative colitis treated with granulocyte-monocyte apheresis
D'Ovidio V, Meo D, Viscido A, Bresci G, Vernia P, Caprilli R
- 1836 Gastrointestinal stromal tumors: Thirty years experience of an Institution
Arolfo S, Mello Teggia P, Nano M
- 1840 Feasibility of a finger prick-based self-testing kit in first- and second-degree relatives of children with coeliac disease
Pichler J, Zilbauer M, Torrente F, Heuschkel R, Philips A, Salvestrini C
- 1844 Role of ERCP in the era of laparoscopic cholecystectomy for the evaluation of choledocholithiasis in sickle cell anemia
Issa H, Al-Salem AH
- 1848 Thrombotic microangiopathy-like disorder after living-donor liver transplantation: A single-center experience in Japan
Hori T, Kaido T, Oike F, Ogura Y, Ogawa K, Yonekawa Y, Hata K, Kawaguchi Y, Ueda M, Mori A, Segawa H, Yurugi K, Takada Y, Egawa H, Yoshizawa A, Kato T, Saito K, Wang L, Torii M, Chen F, Baine AMT, Gardner LB, Uemoto S

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Sugimoto M, Nishino M, Kodaira C, Yamade M, Uotani T, Ikuma M, Umemura K, Furuta T
- 1866 Clinicopathologic significance of GLUT1 expression and its correlation with Apaf-1 in colorectal adenocarcinomas
Jun YJ, Jang SM, Han HL, Lee KH, Jang KS, Paik SS
- 1874 Acoustic radiation force impulse elastography for hepatocellular carcinoma-associated radiofrequency ablation
Kwon HJ, Kang MJ, Cho JH, Oh JY, Nam KJ, Han SY, Lee SW
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Lim R, Sun Y, Im SA, Hsieh RK, Yau TK, Bonaventura A, Cheirsilpa A, Esser R, Mueser M, Advani S
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Chen XH, Zhang BH, Xin Y, Ren ZG, Fan J, Qiu SJ, Zhou J
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Guo XB, Jing CQ, Li LP, Zhang L, Shi YL, Wang JS, Liu JL, Li CS
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Yang DH, Ye ZY, Jin B, He XJ, Zhang Q, Zhou WM, Xu WJ, Lu HX
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Wang GQ, Zhou FF, Olman V, Su YY, Xu Y, Li F
- 1915 Oxidative stress and hypoxia-induced factor 1 α expression in gastric ischemia
Wang T, Leng YF, Zhang Y, Xue X, Kang YQ, Zhang Y

CASE REPORT

- 1923 Intraductal papillary neoplasm of the bile duct in liver cirrhosis with hepatocellular carcinoma
Xu J, Sato Y, Harada K, Yoneda N, Ueda T, Kawashima A, Ooi A, Nakanuma Y

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APPENDIX I Meetings
I-VI Instructions to authors

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Inguinodynia following Lichtenstein tension-free hernia repair: A review

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Abstract

Chronic Groin Pain (Inguinodynia) following inguinal hernia repair is a significant, though under-reported problem. Mild pain lasting for a few days is common following mesh inguinal hernia repair. However, moderate to severe pain persisting more than 3 mo after inguinal herniorrhaphy should be considered as pathological. The major reasons for chronic groin pain have been identified as neuropathic cause due to inguinal nerve(s) damage or non-neuropathic cause due to mesh or other related factors. The symptom complex of chronic groin pain varies from a dull ache to sharp shooting pain along the distribution of inguinal nerves. Thorough history and meticulous clinical examination should be performed to identify the exact cause of chronic groin pain, as there is no single test to confirm the aetiology behind the pain or to point out the exact nerve involved. Various studies have been performed to look at the difference in chronic groin pain rates with the use of mesh *vs* non-mesh repair, use of heavyweight *vs* lightweight mesh and mesh fixation with sutures *vs* glue. Though there is no convincing evidence favouring

one over the other, lightweight meshes are generally preferred because of their lesser foreign body reaction and better tolerance by the patients. Identification of all three nerves has been shown to be an important factor in reducing chronic groin pain, though there are no well conducted randomised studies to recommend the benefits of nerve excision *vs* preservation. Both non-surgical and surgical options have been tried for chronic groin pain, with their consequent risks of analgesic side-effects, recurrent pain, recurrent hernia and significant sensory loss. By far the best treatment for chronic groin pain is to avoid bestowing this on the patient by careful intra-operative handling of inguinal structures and better patient counselling pre- and post-herniorrhaphy.

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INTRODUCTION

Chronic Groin Pain (Inguinodynia) is a potential complication following inguinal hernia mesh repair and has significant impact on the quality of life^[1]. The incidence varies among studies, ranging between 0% and 62.9%, with 10% of patients fitting in the moderate to severe pain group^[2-6]. However, only 2%-4% of the patients are adversely affected by chronic groin pain in their everyday

life. This is significant, considering the volume of the operations performed worldwide^[7]. Management of chronic groin pain constitutes challenging issues for the clinician. Additionally, it has an impact on the health system and economy. In this review, we highlight various aspects of chronic groin pain (inguinodynia) following Lichtenstein's open inguinal herniorrhaphy.

LICHTENSTEIN TENSION-FREE HERNIA REPAIR

Lichtenstein *et al.*^[8] described the tension-free hernioplasty in 1989. By using prosthetic mesh, Lichtenstein showed that inguinal hernias could be repaired without distortion of the anatomy and, most importantly, without any tension along the suture line. In spite of various modifications over the last two decades, Lichtenstein hernia repair (LHR) is still considered the gold standard in the management of inguinal hernia by open technique^[9]. With significant reduction in recurrence with LHR, the most common morbidity has been chronic groin pain.

CHRONIC GROIN PAIN (INGUINODYNIA)

It is vital to differentiate early post-operative pain from chronic groin pain. The post-operative pain is usually relieved with analgesics, whereas chronic groin pain would need further assessment and medical or surgical intervention^[10,11]. Different studies have quoted various time scales for chronic groin pain. These range from first postoperative day to any empirical time period after surgery. However, the International Association for the Study of Pain (IASP) described chronic groin pain as "groin pain reported by the patient at or beyond 3-mo following inguinal hernia repair"^[12]. Major consensus currently has been to take 3 mo as a cut-off point to differentiate between patients with post-operative pain and chronic groin pain due to various causes^[13].

REASONS FOR INGUINODYNIA

The main reasons hypothesised for chronic groin pain are peri-operative nerve damage, post-operative fibrosis, or mesh-related fibrosis. They have been classified as either neuropathic or non-neuropathic pain. The three nerves potentially involved are the Ilioinguinal Nerve (IIN), Iliohypogastric Nerve (IHN) and genital branch of the Genitofemoral Nerve (GFN). These nerves can be damaged either by trauma during dissection or retraction of tissues, or nerve entrapment from post-operative fibrosis, mesh-related fibrosis or sutures used to fix the mesh. Smeds *et al.*^[14] suggested that the injury is mainly due to inadequate dissection, failure to visualise and protect the nerves, and failure to recognise the aberrant location and anatomic variations of the nerves. Any partial or complete transection of the nerve leads to neuroma formation and consequent pain along the distribution of that nerve.

The explanations for non-neuropathic causes are ex-

cessive scar formation resulting from prosthetic mesh reaction, periosteal reaction from sutures or staples inserted into the pubic tubercle or due to rolled-up bulky mesh leading to mechanical pressure. Another group of patients may have diffuse pain situated in the proximity of the spermatic cord without nerve entrapment, which may be due to venous congestion or mesh-related inflammation of the spermatic cord^[15].

Fränneby *et al.*^[16] predicted the possible factors contributing to inguinodynia. It has been shown that age below median, absence of a visible bulge before the operation, recurrent hernia repair and history of moderate to severe pre-operative groin pain are some of the common factors that influence the post-operative inguinodynia.

Sexual dysfunction secondary to chronic groin pain

Ducic *et al.*^[17] and Aasvang *et al.*^[18] have shown that chronic groin pain contributes to sexual dysfunction with symptoms of chronic genital pain, erectile dysfunction and dysejaculation. A nationwide survey in Denmark by Aasvang *et al.*^[19] showed pain during sexual activity in 22.1% of patients, of whom 6.7% had moderate or severe pain occurring every third time or more. Ejaculatory pain was noted in 12.3% of post-herniorrhaphy patients, with a quarter of them describing that the pain impaired their sexual activity significantly resulting in the avoidance of sexual activity. The ejaculatory pain was usually secondary to compression and dilatation of the vas deferens resulting from post-operative fibrosis or direct contact between the mesh and the vas deferens causing inflammation and fibrosis. The study also pointed out that only 1.8% of the patients who reported pain during sexual activity reported this to the physician, thereby showing under-reporting of this problem.

A prospective follow-up study was conducted by Zieren *et al.*^[20] to assess post-herniorrhaphy sexual function by preserving IIN in the control group and elective division in the intervention group. This study showed that prophylactic IIN excision led to reduced sexual symptoms post-operatively in comparison with those who had preserved nerve.

ASSESSMENT TOOLS USED FOR DIAGNOSING INGUINODYNIA

The symptom complex of chronic groin pain varies from a dull ache to sharp shooting pain along the distribution of inguinal nerves. Walking, twisting or hyperextension of the hip often triggers the symptoms. They can be relieved by bed rest, sedentary life style or flexion of the thigh. The complex nature of chronic groin pain has led researchers to use diverse measurement tools, thereby leading to difficulty in comparison of the studies. The most frequently used self-rating pain tools that assess multidimensional nature of the pain are the Visual Analogue Scale (VAS) and McGill Pain Questionnaire, both of which have been shown to be reliable, valid and consistent^[21]. The McGill Pain Questionnaire assesses the

multidimensional nature of the pain using 78 different pain descriptors^[22]. The most commonly used simple assessment tool has been VAS and this uses a scale 10 cm in length, with no pain at 0 to severe pain at 10.

Neuropathic pain can be reproduced by tapping the skin medial to the antero-superior iliac spine or over an area of tenderness. It is extremely difficult to identify the exact nerve involved in causing the pain because of the overlapping nature of their sensory innervations and peripheral communication between the nerves. All three nerves arise from T12-L1 nerve roots. One, two or all three of them can be involved in the aetiology of chronic groin pain, thus making it difficult to pinpoint the entrapped nerve. Clinicians have tried peripheral nerve block or paravertebral block to differentiate the neuropathic pain. Beldi *et al*^[23] showed that the objective assessment of pain and hypoesthesia by von Frey monofilament prior to and after surgery is a good clinical tool. Ultrasound and computed tomography scans have helped in the diagnosis of non-neuropathic chronic groin pain by identifying excess fibrosis or mesh-related factors.

MESH VS NO-MESH

In an attempt to reduce chronic groin pain, researchers have tried tension-free repairs without mesh. A Cochrane review showed that the recurrence rate is reduced by 50%-75% when mesh is used for inguinal hernia repair compared to repairs without mesh^[24]. There is also some evidence of earlier return to work and of lower rates of persisting pain following mesh repair. A meta-analysis of RCTs comparing hernia repair with or without synthetic mesh showed a significant reduction in chronic groin pain when a mesh was applied, by the simple principle of reducing tension between suture lines^[25]. As a general consensus, mesh repair is considered to be more effective in reducing recurrence and chronic groin pain, in comparison with no-mesh repair.

TYPE OF MESH AND PAIN

The majority of patients who present with chronic groin pain also suffer from foreign body sensation and stiffness in the groin area. Post *et al*^[26] and O'Dwyer *et al*^[27] suggested that the pain might be caused by the weight and composition of implanted prosthetic material itself. Heavy-weight (HW) polypropylene meshes such as Prolene® (Ethicon) and polymer meshes with both polypropylene and polyglactin fibres such as Vypro I® and Vypro II® increase the surface area of the mesh, thereby causing extensive fibrosis and greater risk of infection and pain. An implant knitted from monofilament fibres, such as Ultrapro® (Ethicon) which is composed of polypropylene and poliglecaprone absorbable fibres, causes less tissue reaction^[28,29]. Alternatively, light-weight (LW) meshes have shown promise in reducing the groin pain rate. However, because of their lesser tensile strength, there have been recent reports of increases in early and mid-term recurrence rates^[30,31].

A randomised controlled trial comparing HW with LW mesh showed higher incidence of groin pain for HW mesh at 6 mo follow-up (6.3% *vs* 0%, respectively). This was statistically significant^[32]. Randomised controlled trials have shown that the feeling of foreign body sensation is higher in HW mesh groups compared to LW mesh: 43.8% *vs* 17.2% by Post *et al*^[26] and 32.8% *vs* 20.9% by Nikkolo *et al*^[32]. However, the follow-up in both these RCTs was only for 6 mo, thereby they did not account for higher recurrence rates associated with LW meshes. O'Dwyer *et al*^[27] randomised 162 patients in a LW group and 159 in a HW group and showed that the recurrence rate was higher in the former group (5.6% *vs* 0.4%) at 12 mo follow-up, which was statistically significant.

There are very few reports of well controlled RCTs on LW meshes with long-term follow-up. In a RCT of 590 patients with 3 year follow-up, Bringman *et al*^[33] showed no differences in neuralgic pain, hypoaesthesia or hyperaesthesia between the HW and LW mesh groups. There were no major differences in response to the pain questionnaire, except that fewer men with LW mesh had pain when rising from lying down to a sitting position. Significantly more men in the standard mesh group could feel the mesh in the groin: 22.6% *vs* 14.7%. More importantly, this study with longer follow-up showed no difference in recurrence rates with either HW or LW meshes^[33]. Similar views were shared by a single centre RCT on three different composite meshes with a 2 year follow-up^[34].

European hernia guidelines for open hernia repair emphasise a Grade A recommendation for the use of synthetic non-absorbable flat mesh or composite mesh with a non-absorbable component. Though the use of lightweight/material-reduced/large pore (1000 µm) meshes in open inguinal hernia repair can be considered to decrease long-term discomfort, this is possibly at the cost of increased recurrence rate (possibly due to inadequate fixation and/or overlap). Large randomised studies with longer follow-up are needed to justify the routine use of LW meshes.

MESH FIXATION VS PAIN

Complications associated with sutured fixation of the mesh have prompted surgeons to use atraumatic fixation using substances such as human fibrin glue^[35]. These adhesives have shown reduced incidence of chronic groin pain, foreign body sensation and groin numbness in both randomised trials and observational studies^[36-39].

Randomised controlled trials regarding skin staples to fix the mesh have shown reduced intra-operative times and early return to normal activity. However, there was no difference in complications or post-operative pain rates^[40]. A randomised trial in which bilateral hernias received sutured fixation of the mesh on one side and glue fixation on the opposite side showed less inflammatory reaction and therefore post-operative pain on the glue fixed side, with no increased recurrence rates^[37]. Similar results were shown in a RCT with 3 mo of follow-up^[36].

A randomised study comparing sutures, fibrin glue and

N-butyl-2-cyanoacrylate for mesh fixation showed higher post-operative pain, numbness and haematoma formation both in the short term and 12 mo following hernia repair, with an increased rates of foreign body sensation and chronic groin pain, in the sutured fixation group^[39]. There was no recurrence in any group, confirming the fact that tissue adhesives form enough fibrotic reaction to give the much needed tensile strength and at the same time negate the nerve or tissue damaging effects of suture repair. However, the lack of long-term follow-up reports on recurrence rates with glue fixation and increased cost of these glues have made their routine use uncommon.

IMPORTANCE OF NERVE IDENTIFICATION

Lange *et al*^[41] and Alfieri *et al*^[42] showed there was less incidence of chronic groin pain with identification of all 3 nerves during open inguinal hernia repair compared to no nerve identification. A large prospective multicentre study conducted at 11 Italian institutions involving 955 patients showed that the overall pain rate was 5.5% and moderate to severe pain rate was 1.3% when all three nerves were identified. If no nerves were identified the rates of overall pain and moderate to severe pain were 21.6% and 4.7%, respectively. This was statistically significant. Alfieri *et al*^[42] showed that relative risk of chronic groin pain increases from 2.2 to 19.2 if one or three nerves have not been recognised during the inguinal hernia repair.

Smeds *et al*^[43] showed that non-identification of nerves leads to worse pain rates and that non-identification of IIN is worse than actual identification of both IHN and GFN. Amid and Wijsmuller suggested that identification of inguinal nerves helps avoid damage to them by mesh or sutures and also that it is beneficial to cut clean if already damaged during dissection in order to avoid neuroma formation^[44,45]. This is from the understanding that neurectomy causes only numbness, whereas nerve injury causes pain.

The practice of identification of all 3 nerves is quite poor. Ravindran *et al*^[46] conducted a survey in the United Kingdom regarding the handling of inguinal nerves during open hernia repair and showed that IIN was routinely identified by 88% of surgeons, IHN by 58% and GFN by 54%. The individual nerves were routinely divided by 7%, 5% and 6% of surgeons, respectively. There was no definite consensus available on routine identification of inguinal nerves and preservation or division. The survey also pointed out that those surgeons who performed more than 50 hernias per year were more likely to preserve the nerve and others were more likely to ignore it^[46].

The difficulty in nerve identification has been shown to be due to variation in the anatomy and absence of one or more nerves, which is not uncommon in the inguinal area. An anatomical study by Wijsmuller *et al*^[47] defined identification zones which make all three nerve identifications feasible. Lange *et al*^[41], in a prospective anatomical study, showed that identifying all three ingui-

nal nerves should only add 3-4 min of operating time. Overall, the general consensus has been to identify all 3 nerves during open inguinal hernia repairs to avoid iatrogenic injury and consequent chronic groin pain.

NERVE EXCISION VS NERVE PRESERVATION

Traditional teaching has always been to preserve the nerve, but recent studies have looked into the intentional severance based on the concept of “no nerve, no pain”^[48]. RCTs comparing deliberate IIN neurectomy *vs* preservation have shown conflicting results. Two RCTs have shown significant reduction in chronic groin pain post-neurectomy^[49,50], whereas two other studies concluded there was no influence of neurectomy on pain rates^[46,51]. The diverse results may be due to different assessment tools used and poorly conducted and underpowered studies. All of these studies have taken only IIN into consideration, leaving the other two nerves unaccounted for.

A recent RCT by Karakayali *et al*^[52] has shown significant reduction in chronic groin pain with IIN and IHN neurectomy in comparison with all 3 nerves preserved. Nevertheless, this study does not address the fact that all 3 nerves traverse the inguinal area, and that any of the 3 nerves can be involved in causing chronic groin pain. Though studies have shown high incidence of groin numbness and sensory loss following deliberate neurectomy of the inguinal nerves, no significant differences have been shown in the quality of life with such neurosensory changes^[52].

A previous systematic review by Wijsmuller *et al*^[47] in 2007 showed no significant difference in pooled mean percentage of patients with chronic groin pain following either IIN preservation or division. A Cochrane systematic review is currently being undertaken to address this issue.

SOLUTION FOR CHRONIC GROIN PAIN

Avoiding chronic groin pain should be a prime goal for any hernia surgeon, considering that 5-7% of patients with post-herniorrhaphy groin pain will sue their surgeons^[53] amidst proposed measures to avoid chronic groin pain. These important steps are: leaving the cremasteric layer to safeguard IIN; not to recreate too small an external ring to prevent constriction of the IIN during external oblique closure; not to lift the IIN from its bed; careful adequate dissection to prevent injury to the prematurely surfaced branches of the IIN or IHN; and also, avoid suturing the lower edge of the internal oblique muscle to the inguinal ligament because passing sutures can lead to injury to the intramuscular portion of the IIN. One way to avoid nerve scarring in the operative field is to transect the nerve under tension so that it retracts behind the peritoneum or else to implant the transected nerve within the fibres of internal oblique muscle, to prevent it adhering to the inguinal ligament or external oblique aponeurosis.

Even if conventional thinking dictates that every effort should be made for preservation of the nerves from trauma, this is often impossible. Detailed discussion about various treatment options is beyond the remit of this article.

CONCLUSION

There has been increasing evidence in the literature over the last decade regarding the growing incidence of chronic groin pain. The exact cause for the pain is still unclear and various aetiologies have been suggested, including the type of mesh, suture materials and tissue handling techniques. It is important to understand the definition of chronic groin pain occurring after 3 post-operative months following herniorrhaphy and this should not be confused with immediate post-operative pain. This will give the opportunity for better reporting of this complex problem and proper understanding of its aetiology. By far the best treatment for chronic groin pain is to avoid bestowing this on the patient by careful intra-operative handling of inguinal structures and better patient counselling pre- and post-herniorrhaphy.

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Role of conventional therapies in the era of biological treatment in Crohn's disease

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Abstract

Outstanding progress regarding the pathophysiology of Crohn's disease (CD) has led to the development of innovative therapeutic concepts. Numerous controlled trials have been performed in CD. This review concentrates on the results of randomized, placebo-controlled trials, and meta-analyses when available, that provide the highest degree of evidence. Current guidelines on the management of CD recommend a step-up approach to treatment involving the addition of more powerful therapies as the severity of disease and refractoriness to therapy increase. The advent of biological drugs has opened new therapeutic horizons for treating CD, modifying the treatment goals. However, the large majority of patients with CD will be managed through conventional therapy, even if they are a prelude to biological therapy.

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Key words: Crohn's disease; Sulfasalazine; 5-Aminosalicylic acid; Azathioprine; 6-Mercaptopurine; Biological therapies; Anti-tumor necrosis factor- α

INTRODUCTION

Medical treatment of the acute exacerbation of Crohn's disease (CD) is successful in most patients, but some of the most difficult tasks in medicine are to modify the pattern of chronic inflammatory disease, preventing complications such as strictures, abscesses, fistulae and chronic disease activity, and maintaining remission^[1]. The location of CD changes infrequently over time, in contrast to disease behavior, which changes in most patients with increasing disease duration. Whereas the great majority of patients have inflammatory, non-penetrating and non-stricturing disease behavior at diagnosis, the risk of intestinal complications ultimately affects the majority over time^[2]. Although medical treatment of CD is the focus of this review, non-pharmacological factors, including changes in lifestyle, should not be neglected. Probably the most important recommendation is smoking cessation. Smoking has been shown to be a risk factor for CD relapse after medically- or surgically-induced remission^[3] and is associated with the need for higher doses of corticosteroids and immunosuppressants^[4]. Furthermore, a prospective trial has shown that only one

year of smoking cessation leads to a more benign course of disease with a lower rate of relapse^[5]. This trial also showed that the ability to quit smoking clearly depended on the physician's role. Consequently, conventional treatment of CD should start, if you will, with convincing the patient to stop smoking when appropriate.

ACTIVE DISEASE

The general principles for treating active disease are to consider activity, site and behavior (inflammatory, stricturing, fistulating) of disease^[1]. The choice of treatment is also influenced by the previous response to treatment, side effect profile of medication, presence of extraintestinal complications and the course of disease.

The activity of CD can be assessed clinically and endoscopically, or more formally by different indices^[6]. The most established way for clinical trials is through the Crohn's disease activity index (CDAI), which includes symptoms and objective criteria such as anemia and body weight^[7]. Index values of 150 and below are associated with quiescent disease; values above that indicate active disease, and values above 450 indicate extremely severe disease. Other markers of activity such as erythrocyte sedimentation rate, C-reactive protein (CRP), or platelet count should also be taken into account. Fecal lactoferrin and calprotectin are highly sensitive and specific markers for detecting intestinal inflammation. These markers are similarly useful for both CD and ulcerative colitis to monitor the therapeutic response to treatments^[8-10].

Endoscopic evaluation is unnecessary in every exacerbation, but helps when there is a disparity between symptoms and objective markers of inflammation, or when it is necessary to re-evaluate disease localization. The American College of Gastroenterology has characterized the different disease activities in clinical practice as follows^[11]: mild to moderately active disease as "ambulatory patients able to tolerate oral alimentation without manifestations of dehydration, toxicity (high fevers, rigors, and prostration), abdominal tenderness, painful mass, obstruction or > 10% weight loss". In contrast, moderate to severe disease can be recognized in "patients who have failed to respond to treatment for mild to moderate disease, or those with more prominent symptoms such as fever, significant weight loss, abdominal pain or tenderness, intermittent nausea or vomiting (without obstructive findings), or significant anemia". Severe disease refers to "patients with persisting symptoms despite the introduction of steroids as outpatients, or individuals presenting with high fever, persistent vomiting, evidence of intestinal obstruction, rebound tenderness, cachexia, or evidence of an abscess".

Aminosalicylates

No debate regarding the therapy for CD has been as long-standing and controversial as whether 5-aminosalicylic acid (5-ASA)-containing drugs in CD are justified or not. Numerous studies have been performed over the last 25 years, but the data from those currently available do not unequivocally support either point of view. This is partly because

differences in study design and dosage render comparison of the outcomes rather difficult. Sulfasalazine is the parent compound, consisting of 5-ASA linked by an azo-bond to sulfapyridine which is split off by bacterial azo-reductase in the colon. The efficacy of sulfasalazine can therefore be expected to be limited to colonic disease. Furthermore, up to 50% of patients are unable to tolerate sulfasalazine at a dose of 4 g/d, due to nausea, headache, vomiting, or epigastric pain. These side effects are usually caused by the sulfapyridine moiety. Therefore, other 5-ASA formulations (mesalazine formulations and the pro-drugs olsalazine and balsalazide) without sulfapyridine have been introduced with different pharmacodynamic and pharmacokinetic profiles. These different preparations are best considered to be different and not interchangeable.

Sulfasalazine is significantly better than placebo in randomized clinical trials for inducing remission in active CD^[12-14]. As anticipated, subgroup analyses suggest that patients with isolated colonic disease benefit most from sulfasalazine therapy^[11,12], whereas patients treated previously with prednisone fail to respond^[11]. Treatment with prednisolone is likely to be a marker of disease severity, rather than a generic modification of the response to sulfasalazine. Sulfasalazine has not been shown to have steroid-sparing properties^[13-15]. The European Crohn's and Colitis Organisation (ECCO) consensus on the management of CD states that active colonic CD can be treated with sulfasalazine if only mildly active, but that it cannot be recommended as first-line therapy because of the high incidence of side effects^[1]. It may, however, be appropriate in selected patients, such as those with arthropathy. Once 5-ASA was identified as the active moiety in sulfasalazine, it is not surprising that other 5-ASA-containing formulations (such as mesalazine) were tested in CD.

Different pharmacological preparations allow release of the active drug in different parts of the intestine. Therefore, mesalazine, in contrast to sulfasalazine, might conceivably be used in CD affecting the small bowel. The studies on induction of remission in active CD with mesalazine, however, have yielded conflicting data. Six placebo-controlled trials with different dosages of mesalazine for treating active CD have so far been published. Two studies did not detect a benefit of mesalazine over placebo for inducing remission^[16,17]. Tremaine observed a significantly greater number of patients with a response (defined as a decrease in CDAI ≥ 70 and/or CDAI < 150), but this benefit was minute (9 patients with mesalazine treatment *vs* 4 patients in the placebo group). However, there was no significant difference for clinical remission alone^[18]. Singleton *et al*^[15] conducted three separate trials with mesalazine (Pentasa) that have been combined in a meta-analysis, even though two of the three trials were never published in full^[19]. This analysis observed a statistically significant benefit of mesalazine over placebo, but the benefit was technical (a greater reduction in CDAI of 18 points compared to placebo in the intention-to-treat-analysis), and of debatable clinical significance. Consequently, if mesalazine is used to treat active CD, the physician must be aware that it is little more effective than

placebo. On the other hand, treatment with placebo is not the same as no treatment at all!

Budesonide

The introduction of the topically acting steroid budesonide has become an attractive option for treating patients with CD located in the terminal ileum or right colon. Due to rapid metabolism by cytochrome P-450 enzymes in the liver, budesonide has less systemic bioavailability than conventional corticosteroids. Budesonide has been shown to be effective in inducing remission of active CD in several controlled studies^[20-26], with remission rates ranging from 51% to 69% of patients over a period of 8 to 12 wk. A Cochrane Systematic Review combined data from 12 published studies investigating budesonide in comparison to placebo, 5-ASA and systemic corticosteroids^[27], and showed that budesonide is more effective at inducing short-term remission, within 8 wk of treatment, in moderately active CD than placebo [relative risk (RR): 1.96, 95% confidence interval (CI): 1.19 to 3.23] or mesalamine (RR: 1.63; 95% CI: 1.23 to 2.16). Budesonide was significantly less effective than conventional steroids for induction of remission (RR: 0.86, 95% CI: 0.76 to 0.98), particularly among patients with severe disease (CDAI > 300) (RR: 0.52, 95% CI: 0.28 to 0.95). Fewer adverse events occurred in those treated with budesonide compared to conventional steroids (RR: 0.64, 95% CI: 0.54 to 0.76) and budesonide was better able to preserve adrenal function (RR for abnormal ACTH test 0.65, 95% CI: 0.55 to 0.78). The recommended dose of budesonide is 9 mg/d, usually for 6 wk, and then tapered 3 mg every 2-4 wk unless continued therapy with budesonide is considered (see below). The ECCO Consensus recommends budesonide 9 mg daily as the preferred treatment for mildly active localized ileocecal CD^[1].

Systemic corticosteroids

The effect of systemic steroids for remission induction in CD has been studied in several uncontrolled^[2-4] and controlled trials^[5,6]. Overall, the clinical response rate achieved varies from 60% to 97% over a period of 1 to 5 mo. In active CD, corticosteroids have been shown to be superior to sulfasalazine, azathioprine and placebo^[11,12]. Remarkably, no dose-finding studies have been performed. Reported doses range from 30 mg/d up to 1 mg/kg per day, but most clinicians start with prednisolone 40-60 mg/d, although some vary this according to body weight (1 mg/kg), especially in children. A Cochrane Systematic Review by Benchimol *et al*^[7] included data from two placebo-controlled and six 5-ASA-controlled studies. Systemic steroids were significantly more effective (RR: 1.99, 95% CI 1.51-2.64, $P < 0.00001$) than placebo and significantly more effective than 5-ASA (RR: 1.65, 95% CI: 1.33-2.03, $P < 0.00001$). Across the different studies, systemic steroids have been shown to be effective in mild to severely active CD of any localization.

However, the short-term (i.e. 7 to 18 wk) high remission rate induced by systemic corticosteroids does not last, as 16% to 36% of patients become steroid-dependent, and less than half of the initially responding patients will still

be in remission 1 year after the initial treatment with steroids^[2,12]. In a multi-center, randomized, open-label study comparing a conventional treatment algorithm (steroids followed by immunomodulators in case of relapse after tapering off steroids) *vs* early combined immunosuppression [three infusions of infliximab (IFX) and concomitant immunomodulators], 74% of patients in the conventional arm were receiving immunomodulators at week 104^[13], again underlining the poor long-term outcome of remission induced with systemic steroids. Comparing the two arms with respect to the primary end-point, steroid-free remission, 40/65 (61.5%) in the early combined immunosuppression arm *vs* 27/64 (42.2%) in the conventional arm were in remission at week 54 (absolute difference 19.3%, 95% CI: 2.4-36.3, $P = 0.0278$). However, beyond 1 year of follow-up, there was no difference in the steroid-free remission rate between the two groups. It is thus currently unclear whether replacing steroids by anti-tumour necrosis factor (TNF) agents in first-line therapy for active CD will lead to better long-term outcomes. There are no data available offering a direct comparison of treatments other than IFX (e.g. immunomodulators) with corticosteroids as first-line therapy for remission induction.

The short-term outcome of a first course of steroids shows that approximately 50%-60% of patients have a complete response, 30% a partial response, and about 10%-20% have no response. However, at 1 year, only a third of patients will have a durable response, while another third become corticosteroid-dependent and cannot be withdrawn from treatment without a relapse of symptoms, and another third develop steroid resistance^[27,28]. Corticosteroid-dependence is a particular concern due to well-established systemic and metabolic toxicities associated with long-term corticosteroid use. Recent data from a referral centre in France identified the need for corticosteroids during the first flare as a predictor of a disabling disease course over the subsequent 5 years^[29].

Tapering should be performed according to improvement of clinical symptoms and is usually done in steps of 5-10 mg/wk. At lower dosages, tapering might be reduced to 2.5-5 mg/wk. Intravenous steroids are frequently used when oral treatment has not been effective, but whether this has an advantage over oral therapy in acute severe flares is unclear. The ECCO Consensus on CD management recommends that systemic corticosteroids should be used initially in patients with moderate to severe disease^[1].

Corticosteroids are undoubtedly effective in relieving symptoms in CD^[12,13]; however, high-dose corticosteroids induce complete mucosal healing in only 13% of patients and a proportion of patients who achieve clinical remission on corticosteroids may in fact have worsened disease at the mucosal level^[30].

Azathioprine/mercaptopurine

The most commonly used immunomodulators are the thiopurines, mercaptopurine (MP) and its prodrug azathioprine (AZA). A number of clinical trials have studied the efficacy of these immunomodulators in active CD. The most convincing data were obtained in the early trial

by Present where 67% patients in the MP group achieved remission compared to 8% given placebo^[31]. Other trials have not observed a significant difference of AZA compared to placebo^[11,32], but this partly reflects trial design, dose and duration of therapy for this drug, which takes up to three months to be effective.

Despite these conflicting data, a recent Cochrane analysis evaluated 8 randomized placebo-controlled trials of AZA and 6-MP therapy in adult patients: five dealt with active disease and three had multiple therapeutic arms. The odds ratio (OR) of a response to AZA or 6-MP therapy compared with placebo in active CD was 2.43 (95% CI: 1.62 to 3.64). This corresponded to a number needed to treat (NNT) of about 5 to observe an effect of therapy in one patient. When the two trials using 6-MP in active disease were excluded from the analysis, the OR was 2.06 (95% CI: 1.25 to 3.39). Treatment > 17 wk increased the OR to 2.61 (95% CI: 1.69 to 4.03). A steroid-sparing effect was seen with an OR of 3.69 (95% CI: 2.12 to 6.42), corresponding to a NNT of about 3 to observe steroid-sparing in one patient. Adverse events requiring withdrawal from a trial, principally allergy, leukopenia, pancreatitis and nausea, were increased with active therapy with an OR of 3.44 (95% CI: 1.52 to 7.77). The NNT to observe one adverse event in one patient treated with azathioprine or 6-mercaptopurine was 14^[33].

Because thiopurines are slow-acting drugs they are used less frequently to induce remission and more commonly to maintain remission. A combination of prednisolone and AZA was superior to prednisolone monotherapy in one study^[34]. Consequently, the main role for thiopurines is as a steroid-sparing therapy and they should be started for corticosteroid-dependent or corticosteroid-refractory patients.

Most evidence is available to support the dose-escalating method: AZA may be started at 50 mg daily and the dose increased by 25 mg every 1-2 wk to a target dose of 2.0-3.0 mg/kg along with monitoring for leukopenia and other potential adverse events. Similarly, 6-MP may be started at 50 mg daily and the dose increased by 25 mg every 1-2 wk to a target dose of 1.0-1.5 mg/kg along with similar monitoring for leukopenia and other potential adverse events. In a recent survey study, most gastroenterologists escalated the dose of AZA or 6-MP relatively rapidly, generally within 4 wk, reporting weight-based target dosing^[35].

However, benefits of this therapy are offset by higher treatment-related risk of lymphoproliferative disorders. In particular, it has been shown recently^[36] that a multivariate-adjusted hazard ratio of lymphoproliferative disorder between patients receiving thiopurines and those who had never received the drugs was 5.28 (95% CI: 2.01 to 13.9, $P = 0.0007$).

The ECCO Consensus recommends that AZA/MP be added to corticosteroids for severe CD in the event of relapse^[1]. Thiopurines are capable of achieving mucosal healing. AZA heals the mucosa in up to 58% of patients at 1 year and 70% at 2 years, and is superior to budesonide (mucosal healing rate 15%)^[37,38]. For those who had been on AZA for longer than 3.5 years and who were in clinical remission, complete mucosal healing was seen in 36% and absence of ulcers in 53%^[12].

In the same study, endoscopic scores correlated well with clinical activity indicators. In the postoperative setting, AZA can achieve complete mucosal healing of recurrent ileitis in 40% of patients and improvement in 93% after at least 6 mo of therapy^[12]; however, endoscopy at 6 mo may be too early to assess the effect of AZA in *de novo* disease, as seen in the Study of Biologic and Immunomodulator-Naïve Patients in CD (SONIC)^[39].

AZA/6-MP treatment should be maintained for several years due to the high relapse rate of patients when these drugs are discontinued. Many studies have investigated the duration of maintenance of remission after AZA/6MP withdrawal in CD patients who were in long-term remission while on this therapy. Withdrawal of AZA/6-MP after up to a median of 6 years under treatment and long-standing remission was associated with a high risk of relapse, whatever the duration of remission under this treatment. Thus, AZA/6-MP withdrawal is not equivalent to continued therapy for maintenance of remission in patients with CD who have been in remission on this therapy. These data suggest that if AZA/6-MP is well tolerated, it should not be interrupted^[40,41]. Younger age and a higher daily dose of 6-MP were associated with a higher rate of relapse^[40].

Methotrexate

In a pivotal trial conducted by Feagan, methotrexate (MTX) given intramuscularly 25 mg once a week was more likely to induce remission than placebo. Steroid-sparing properties were noted^[41]. However, side effects were more common with MTX therapy than with placebo. Other studies using low-dose MTX have not shown a significant benefit^[42,43] and no benefit was observed when high-dose intravenous MTX was compared to oral AZA^[43]. Like AZA/MP, MTX is only rarely used to treat acute exacerbations of CD, but much more frequently for persistently active CD^[44]. Side effects of MTX (notably liver dysfunction and myelotoxicity) need to be monitored and it is contradicted during pregnancy. Consequently, it should be used very cautiously in women of child-bearing potential. MTX has the same indications for treating CD as the thiopurines (although neither are licensed for treating CD in most countries), but because of greater familiarity with thiopurines, most gastroenterologists reserve MTX for active or relapsing CD in patients refractory to, or intolerant of, AZA/MP.

Antibiotics

Although antibiotics are frequently used to treat CD, there is little substantive evidence from randomized trials. Nevertheless, increasing awareness of the importance of mucosal bacteria in the pathogenesis of CD provides a rationale for exploring antibiotic therapy^[45]. Metronidazole (20 mg/kg per day) was superior to placebo at reducing the CDAI, but not at inducing remission, in one of the few randomized trials^[46]. Furthermore, this benefit was only seen in patients with colonic or ileocolonic disease and no benefits were found with disease limited to the ileum. Similar findings were reported in another trial where a few patients with Crohn's colitis showed an improve-

ment^[47]. On the other hand, another study reported no benefit of metronidazole compared to placebo^[48], nor to sulfasalazine, in a 4 mo cross-over study. However, in the cross-over study, patients switched to metronidazole showed CDAI response, although there was no change in CDAI in those switched from metronidazole to sulfasalazine^[49,50].

Ciprofloxacin is the other antibiotic used in clinical practice, commonly in combination with metronidazole. Ciprofloxacin was significantly better than placebo at inducing remission in a small trial^[51] and similarly effective to mesalazine^[52]. In contrast, corticosteroids resulted in higher rates of clinical remission compared to ciprofloxacin and metronidazole^[53]. In patients with persistent disease activity given budesonide, the addition of metronidazole and ciprofloxacin was not superior over budesonide monotherapy, despite a trend towards benefit in patients with colonic CD^[54]. Further studies are warranted to establish the role of antibiotics in the treatment of CD, but for the time being they cannot be recommended as standard therapy.

As stated by the ECCO, at present, antibiotics are only considered appropriate for septic complications, symptoms attributable to bacterial overgrowth, or perineal disease. Anti-mycobacterial therapy cannot be recommended on the evidence from controlled trials^[1].

MAINTENANCE OF REMISSION

Maintaining medically- or surgically-induced remission of disease is one of the most important, but most difficult, therapeutic goals in the treatment of CD. Maintenance therapy in CD is hampered by few available drugs, moderate rates of efficacy and frequent side effects. In total, 40%-70% of CD patients will experience a symptomatic relapse within a year of medically- or surgically-induced remission^[11,12]. Silverstein reported that a surgically-induced remission lasted a mean 766 d, whereas a non-surgical induced remission lasted only 120 d, suggesting that a surgically-induced remission might be more stable^[55]. However, surgery is generally used for obstructive symptoms and not for inflammatory disease, so like was not being compared with like, although physicians should not forget the relatively extended period of medication- or symptom-free remission that surgery can offer. It is generally recommended to base decisions regarding a relapse-preventing therapy on the likelihood of relapse in an individual patient. Estimates of risk remain controversial, but well-established risk factors such as continuing to smoke, frequent relapses in the past, perianal or penetrating disease are widely accepted^[56]. To stop smoking is an important therapeutic goal^[2,57]. Systemic corticosteroids should not be used for maintaining remission due to lack of efficacy and severe long-term side effects.

MEDICALLY-INDUCED REMISSION

5-ASA

Numerous randomized, placebo-controlled studies, including four meta-analyses, have tried to establish a role for

5-ASA in the maintenance of remission. Different study regimens, dosages and durations of therapy have been performed, while a substantial number of trials included small numbers of patients. The two most recent meta-analyses failed to show a benefit for mesalazine over placebo in the maintenance of medically-induced remission^[57,58].

Azathioprine/mercaptopurine

Azathioprine or mercaptopurine is the treatment of choice for patients with a high risk of relapse. The effectiveness of AZA has been confirmed in the most recent meta-analysis which included 7 trials of AZA therapy and one of 6-MP. Azathioprine and 6-mercaptopurine both had a positive effect on maintaining remission. The Peto OR for maintenance of remission with AZA was 2.32 (95% CI: 1.55 to 3.49) with a NNT of 6. The Peto OR for maintenance of remission with 6-MP was 3.32 (95% CI: 1.40 to 7.87) with an NNT of 4. Higher doses of AZA improved response. A steroid-sparing effect with AZA was noted, with a Peto OR of 5.22 (95% CI: 1.06 to 25.68) and NNT of 3 for quiescent disease. Withdrawals due to adverse events were more common in patients treated with AZA (Peto OR 3.74; 95% CI: 1.48 to 9.45, NNT = 20) than with placebo^[59].

A steroid-sparing effect has also been confirmed^[60]. The following indications for starting thiopurine maintenance therapy are generally accepted: frequent flares (two or more per year), persistent disease activity, and steroid dependence. The thiopurines are slow-acting drugs and an effect is usually observed after 2-3 mo, with approximately 90% of patients who are going to respond doing so within the first 4 mo.

An early AZA maintenance study suggested that the drug might no longer be effective after 3.5 years^[61]. However, a subsequent randomized, placebo-controlled withdrawal study showed that AZA remained effective at 3.5 years and beyond^[62]. Treton *et al*^[63] showed that even after a long duration of clinical remission under AZA, withdrawal of this drug is associated with a high risk of relapse. Interruption of AZA can be reasonably considered, at least temporarily, in a selected group of patients having no predictive factor of relapse. Debate continues about the potential for a small increase in the risk of lymphoma and this cannot be excluded in long-term treatment with AZA/MP^[64,65]. This must be weighed against the improved quality of life from thiopurine therapy for patients with CD and discussed with individual patients.

Methotrexate

In a follow-up to the induction study, patients who had achieved remission after 25 mg intramuscular MTX/week were randomized to 15 mg/wk MTX or placebo. Methotrexate was found to be significantly better than placebo at maintaining remission^[66]. However, side effects were significantly higher than with placebo. Methotrexate has not been studied after remission has been induced surgically or by other drugs (such as corticosteroids). In general, MTX is considered an alternative to AZA/MP for the maintenance of remission. It also has steroid-sparing properties and the mean time to respond is about 2 mo.

With regard to the ECCO consensus on CD management, patients receiving azathioprine or mercaptopurine who relapse should be evaluated for adherence to therapy and have their dose optimized. Change of their maintenance therapy to methotrexate or anti-TNF therapy should be considered^[1].

Budesonide

Low doses of budesonide (3 or 6 mg) have been studied for their potential to maintain remission. The maintenance of remission with budesonide has been studied in several controlled trials. These have been reviewed by Benchimol *et al*^[38] in a Cochrane Systematic Review, based on 10 controlled trials. Eight studies used a controlled ileal release form of budesonide, while three used a pH-modified release formulation. Budesonide is not more effective than placebo or weaning prednisolone for maintenance of remission in CD. Some modest benefits are noted in patients receiving budesonide compared with placebo in terms of lower CDAI scores and longer time to relapse of disease. However, these benefits are offset by higher treatment-related adverse event rates and more frequent adrenocorticoid suppression in patients receiving budesonide.

The ECCO Consensus on CD management states that corticosteroids are not effective for maintenance of medically-induced remission in CD. Budesonide may delay relapse after medically-induced remission, but is not effective at maintaining remission for 12 mo. Budesonide can replace prednisolone in steroid-dependent patients to improve tolerability^[1].

POSTOPERATIVE CD (SURGICALLY-INDUCED REMISSION)

About 75% of CD patients with ileal or ileocolonic disease will require surgery within the first 20 years of diagnosis^[66,67]. Recurrence rates after surgical resection are high, but are influenced by the definition of recurrence^[68]. After the first resection, up to 80% of patients show an endoscopic recurrence within the first year, although most patients are not symptomatic^[66,67,69]. Up to 20% have clinical symptoms and 5% require further surgical intervention within the first year. After 5 years, about half have had a clinical relapse. Neither systemic corticosteroids nor budesonide are effective at preventing postoperative relapse^[69,73]. Risk factors for postoperative recurrence have rarely been studied in a prospective manner. Continued smoking is the most consistently described risk factor for postoperative relapse^[57,74]. Rutgeerts has shown that preoperative disease activity and endoscopic lesions in the neoterminal ileum within the first year after surgery are also associated with a higher risk of postoperative recurrence^[69]. A more recent study has suggested that CD patients with CARD15 mutations have a higher risk of postoperative relapse compared to patients without mutated CARD15^[74].

5-ASA

Despite the controversies about 5-ASA in the medical treatment of active or quiescent CD, data on the preven-

tion of postoperative recurrence are relatively solid. A meta-analysis by Cammà described a risk reduction of 13.1% in clinical relapse during mesalazine treatment compared to placebo^[58]. A subsequent placebo-controlled trial reported no effect of mesalazine after surgical resection for CD, except in patients with isolated small bowel resection^[75]. 5-ASA is well tolerated and generally recommended after resection^[74]. The ECCO Consensus on CD management suggested that high dose mesalazine is an option for patients with an isolated ileal resection^[1].

Azathioprine/Mercaptopurine

A recent meta-analysis evaluated 4 controlled trials comparing azathioprine ($n = 3$) or 6-MP ($n = 1$) with control arms (placebo with or without antibiotic induction therapy or mesalamine). In the overall analysis, purine analogs were more effective than control arms in preventing clinical recurrence at 1 year (mean difference, 95% CI: 8, 1%-15%, $P = 0.021$, NNT = 13) and at 2 years (mean difference, 95% CI: 13, 2%-24%, $P = 0.018$, NNT = 8). In sensitivity analyses, the efficacy of purine analogs was superior to that of placebo for the prevention of clinical and endoscopic recurrence at 1 year (mean differences, 95% CI: 13, 1.8%-25%, $P = 0.025$, NNT = 7, and 23, 9%-37%, $P = 0.0016$, NNT = 4, respectively). At 1 year, in the overall analysis, purine analogs were more effective than control arms were in preventing severe (Rutgeerts score ≥ 2 -4) endoscopic recurrence (mean difference, CI 95%: 15, 1.8%-29%, $P = 0.026$, NNT = 7), but they were not effective in the prevention of very severe (≥ 3 -4) recurrence. The rate of adverse events leading to drug withdrawal was higher in thiopurine-treated patients than in control arms (17.2% *vs* 9.8%, respectively, $P = 0.021$)^[76].

Although there are no robust data to support the use of AZA/MP for preventing postoperative relapse, many clinicians use these drugs for this indication^[77].

The ECCO Consensus on CD management recommends prophylactic treatment after small intestinal resection. Thiopurines are more effective than mesalazine or imidazole antibiotics alone for preventing both clinical and endoscopic recurrences. Azathioprine/6-mercaptopurine are the drug of choice in patients with a risk factor for early postoperative recurrence^[1].

Antibiotics

In a randomized, placebo-controlled trial of metronidazole, a significant decrease in the incidence of severe endoscopic recurrence was observed after ileal resection^[78]. Metronidazole therapy significantly reduced clinical recurrence rates at 1 year, but it is rarely used for this indication because of poor tolerability. Another nitroimidazole antibiotic, ornidazole, has been studied by the same group. Ornidazole significantly reduced the clinical and endoscopic recurrence rate at 1 year compared to placebo, but still more patients in the ornidazole group dropped out because of side effects^[79].

Imidazole antibiotics, as suggested by the ECCO Consensus on CD management, may be a therapeutic option after ileocolic resection but are poorly tolerated^[1].

CONCLUSION

Based on currently available data from randomized, placebo-controlled trials and meta-analyses we have described the conventional therapy of CD. Biological therapy has opened new therapeutic horizons and novel treatment goals; however, current guidelines advocate a step-up approach to treatment, with the addition of more powerful therapies as the severity of disease or refractoriness to therapy increases. In contrast to the cautious, conventional step-up approach, a proactive top-down approach to treatment has been proposed. This regimen advocates biological and immunomodulator therapy at an early stage, shortly after diagnosis of CD.

However, it is important to keep in mind that the CD course in the majority of patients is relatively mild. About three-quarters of patients suffering from CD present at diagnosis with inflammatory disease and one-quarter with either stricturing and/or penetrating disease^[80,81]. After the first year of diagnosis, 55% of patients are in remission and 15% have only mild disease, leaving around 30% suffering from frequently active disease. Generally, 20% display active disease during each of the first 7 years^[82]. In a Markov model, a representative patient with CD spends 65% of lifetime disease course in medical or surgical remission, 27% with mild disease, 7% with severe disease, and 1% in surgery^[55]. Disease behavior tends to alter over time towards a more aggressive phenotype characterized by the development of disabling complications such as abscesses, internal fistulae and strictures^[2]. Most of these complications need surgical interventions that lead to more disabling disease. In a Norwegian cohort, the probability of surgery was 14%, 27% and 38% at 1, 5 and 10 years, respectively^[83].

Specific clinical, serological and/or genetic predictors are needed to help identify patients with the highest risk of developing a disabling disease. At present, no predictors which have been fully validated and replicated in adequately powered studies are available. Active smoking, age less than 40 years, extensive length of affected digestive tract, perianal lesions and steroid therapy during the first flare have been proposed as predictors of a worse prognosis in medically-treated CD patients^[29]. Furthermore, in an Olmsted County cohort, patients with ileal or ileocolonic extent at baseline were five to seven times more likely to experience an evolution in disease behavior from non-penetrating, non-fistulizing to fistula, abscesses or strictures than those with isolated colonic extent^[84]. The only biological index that has been identified as a predictor of more severe clinical course of adult CD is C-reactive protein (CRP)^[85]. Obviously, we don't need predictors at diagnosis when the disease is already considered as severe (stricturing or perforating lesions, multifocal or extensive lesions, severe systemic damage not reversible with treatment). Initiation of immunosuppressives or biologicals early in the disease course in patients at risk of, or already with, complicated disease seems reasonable since this may induce long-term deep remission. The goal should be the induction of mucosal healing and the achievement of symptom-free everyday life, both with

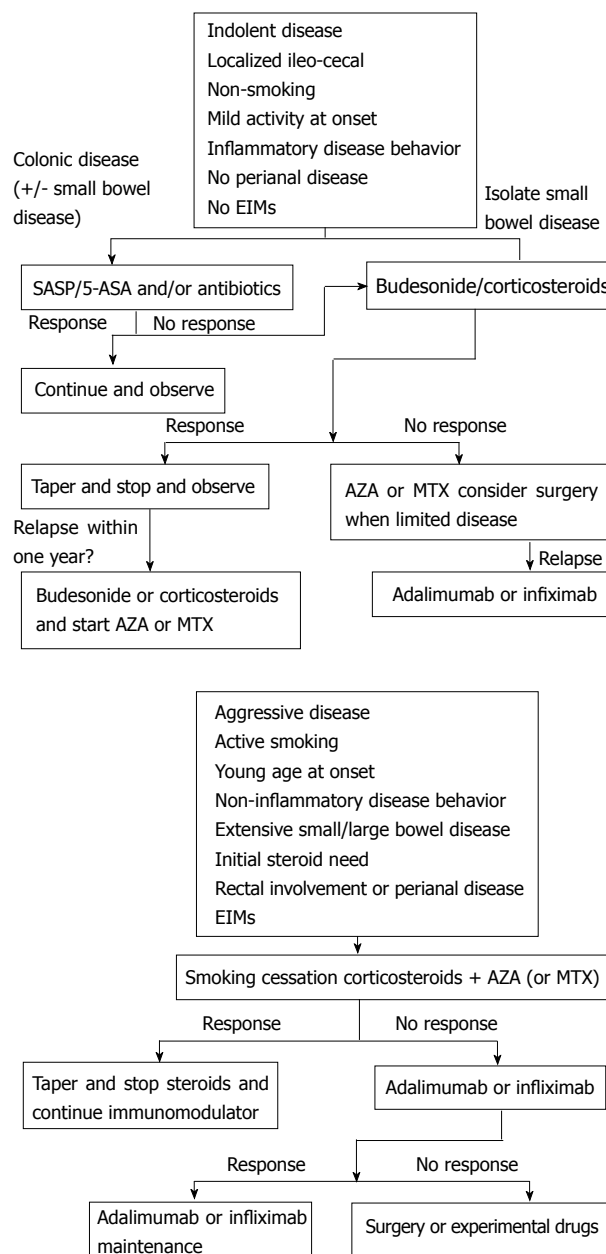


Figure 1 Treatment Algorithms. EIMs: Extraintestinal manifestations; SASP: Sulfasalazine; 5-ASA: Five-aminosalicylic acid; AZA: Azathioprine; MTX: Methotrexate.

minimal use of steroids. Clearly, the potential of early immunosuppressive or biological initiation must be weighed against the possibility of increased risk of treatment side effects, such as more frequent infections or a higher rate of malignancies. Overtreating patients who would have a benign course of the disease is the wrong choice, because of the risk of drug-induced complications. To obtain a sustained remission in CD it is important to optimize conventional therapy, to strictly monitor patients, to identify patients for biological therapy with full consideration of the individual risk/benefit profile, and to introduce biologics in a timely manner, identifying patients who would most benefit from early use (Figure 1).

In conclusion, conventional treatments still remain an important option for management of patients with CD.

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Hepatitis B and C infection and liver disease trends among human immunodeficiency virus-infected individuals

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occurred in 57% of those diagnosed with liver disease relative to 15% overall ($P < 0.001$). Overall 10% of deaths occurred among individuals with a diagnosis of liver disease. Despite care guidelines promoting screening and vaccination for HBV and screening for HCV, screening and vaccination were not universally conducted or, if conducted, not documented.

CONCLUSION: Due to high rates of incident liver disease, viral hepatitis screening, vaccination, and treatment among HIV-infected individuals should be a priority.

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Key words: Human immunodeficiency virus; Hepatitis B; Hepatitis C; Liver disease

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Abstract

AIM: To examine trends in and correlates of liver disease and viral hepatitis in an human immunodeficiency virus (HIV)-infected cohort.

METHODS: The multi-site adult/adolescent spectrum of HIV-related diseases (ASD) followed 29490 HIV-infected individuals receiving medical care in 11 U.S. metropolitan areas for an average of 2.4 years, and a total of 69487 person-years, between 1998 and 2004. ASD collected data on the presentation, treatment, and outcomes of HIV, including liver disease, hepatitis screening, and hepatitis diagnoses.

RESULTS: Incident liver disease, chronic hepatitis B virus (HBV), and hepatitis C virus (HCV) were diagnosed in 0.9, 1.8, and 4.7 per 100 person-years. HBV and HCV screening increased from fewer than 20% to over 60% during this period of observation ($P < 0.001$). Deaths

INTRODUCTION

As highly active antiretroviral therapy (HAART) has improved the health of people living with human immunodeficiency virus (HIV), acquired immune deficiency syndrome (AIDS)-related morbidity and mortality have decreased and mortality from other illnesses, including hepatitis and liver disease, has grown^[1-4]. Among HIV-infected individuals, chronic co-infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) are associated with excess morbidity and mortality^[1,4,5]. Liver dis-

case, from HBV/HCV or other etiologies, is now one of the most frequent causes of death among HIV-infected people^[6,7].

Current care treatment guidelines for people living with HIV include HBV screening, plus vaccination as indicated, and HCV screening^[8]. However, it is not known how widely these guidelines are followed, nor are there clear recommendations regarding the frequency of repeat screening following a negative test for HBV and HCV when risk of infection remains present. Screening for HBV is particularly important before initiating antiviral therapy for HIV, because several therapeutic agents are active against both viruses and use of these agents may cause HBV resistance and subsequent hepatitis flares, if the provider is unaware of HBV infection. Similarly, initiation of HBV treatment without the protection of HAART can lead to HIV antiretroviral resistance.

It is also important to follow trends in HBV, HCV, and hepatic disease diagnoses among people living with HIV for care planning purposes, prevention, education, and treatment of preventable and modifiable factors contributing to liver disease. Although most serious liver morbidity among people living with HIV is attributed to HBV and HCV^[9,10], other factors contributing to liver morbidity may include the HIV virus itself, HIV-induced immunosuppression and/or inflammation, cirrhosis, hepatic carcinoma, alcohol use, aging, injection drug use, and hepatotoxic therapies-including some antiretroviral therapies.

To examine trends in, and factors associated with, both incident and prevalent liver disease and hepatitis diagnoses and screening in a modern HAART-era cohort, we analyzed data from a longitudinal medical record review cohort study, the national adult/adolescent spectrum of HIV-related diseases project 1998-2004.

MATERIALS AND METHODS

The adult/adolescent spectrum of HIV related diseases (ASD) project was a multicenter medical record review surveillance project funded by the centers for disease control and prevention (CDC) and conducted in Atlanta, Detroit, Seattle, Denver, Houston, Dallas, San Antonio, Puerto Rico, New Orleans, Los Angeles, and New York City^[11]. HIV-infected persons of at least 13 years of age who attended participating clinics were eligible for inclusion. ASD began data collection in 1990 and continued until 2004. Data for this manuscript were left truncated to start in 1998 due to protocol revisions at that time-including the addition of hepatitis screening-which remained in place through the project end in 2004. Medical records of ASD enrollees were retrospectively reviewed for the 12-mo period prior to their enrollment date, and at subsequent six month intervals until death, relocation, or loss to follow-up (no contact for 18 mon). Information collected included basic demographic data, mode of exposure to HIV, prescription of antiretrovirals and other medications, CD4+ T-lymphocyte (CD4+) counts, HIV RNA viral

load, complete history of AIDS-defining opportunistic illnesses (OIs)^[12], other infections, selected medical and psychosocial conditions, and behaviors of medical importance. Over 100 medical facilities participated in ASD, including HIV specialty clinics, private practitioners' offices, and community clinics, both hospital-based and freestanding. Men of color and women were oversampled at selected sites. All data were collected by trained abstractors; sites conducted a variety of quality assurance activities, including duplicate record abstraction audits and double data entry.

To describe the mode of HIV infection, we used four categories: men who have sex with men (MSM), injection drug users (IDU), individuals with both exposures (MSM-IDU), and other, including heterosexual exposure to an individual with HIV infection or with a known HIV risk and blood/blood product exposure (transfusion, blood products to treat hemophilia, organ transplant recipient, health care workers with needle stick exposure). For these analyses, substance use included those with a history of IDU as an HIV risk factor, and those with ongoing IDU, non-injection use of illicit drugs (including cocaine and marijuana), and problem alcohol use (defined as a diagnosis of alcoholism or treatment for past or current alcoholism). Cigarette smoking and other tobacco use was not collected in the national ASD project.

HBV and HCV screening for each year was defined as screening conducted up to and including each year individuals contributed follow-time to the project. A prior diagnosis of HCV or HBV was also considered indicative of screening for each infection. To examine trends in prevalence over time and overall death rates, individuals with a prior diagnosis of chronic HBV or any diagnosis of HCV or liver disease were considered to have these conditions in their diagnosis year and all subsequent years of follow time. Acute and chronic HBV and HCV infection were defined by provider diagnosis or laboratory evidence. IgM anti-HBc was considered diagnostic of acute HBV and IgG anti-HBc, or other characteristic antigen and antibody combinations, distinguished chronic HBV. Age at entry was coded as 14-29, 30-39, 40-49, 50-59, and 60 years and greater. Nadir CD4+ was aggregated to < 200, 200-499, and 500+ CD4+ cells/ μ L. Indicator (dummy) variables were used for mode, race/ethnicity, and nadir CD4+ cell count with MSM, Blacks, and CD4+ \geq 500 cells/ μ L as reference groups. Intensity of health services utilization was measured with a grouped continuous variable of the total number of outpatient visits (in 10 visit increments).

An aggregate variable, "liver disease", was created and included cirrhosis (both alcoholic and non-alcoholic), necrosis, abscess, primary liver cancers and liver failure. Liver disease included International Classification of Diseases 9th Revision (ICD-9) codes 570 (acute and subacute necrosis of liver), 571 (chronic liver disease and cirrhosis), 572 (liver abscess and sequelae of chronic liver disease), 573 (other disorders of liver), 572.3 (portal hypertension), 277.1 (α 1 antitrypsin deficiency), 456.0-456.2 (esopha-

geal varices), 789.5 (ascites), and 275.1 (Wilson's disease). Esophageal varices and ascites may not be specific for liver disease; therefore, we compared characteristics of individuals with liver disease including and excluding these additional diagnoses, and found the populations were nearly identical before making the decision to include these with liver disease. HBV and/or HCV alone, when we were able to distinguish these, were not sufficient to define liver disease. A dichotomous variable used only through 1999 indicating liver failure was also included with liver disease. Liver failure was defined by medical practitioner diagnoses recorded in the medical record and supported by abnormal liver function tests, jaundice, and/or special hepatic dietary management. Liver neoplasms and morphology codes of malignant and not-otherwise-specified (NOS) neoplasms were collected.

Death rates

Follow time from 1/1/98 or start of observation until death or censor was calculated overall and for individuals diagnosed with chronic HBV, HCV, or liver disease—starting from the follow-up interval of diagnosis if after 12/31/1997. Death rates were calculated due to any cause and separately for deaths that may have been due to liver disease. Deaths that may have been due to liver disease were defined as those listing liver disease as a contributing factor on a death certificate or individuals with a diagnosis of liver disease during follow-up interval during in which death occurred (thus including individuals with a new or ongoing diagnosis of liver disease at the time of death or within six months of death).

Statistical analyses

Demographic characteristics of individuals with chronic HBV, any diagnosis of HCV, and liver disease were compared to individuals without these diagnoses by χ^2 testing (SAS version 9.1, Cary NC). Multivariate Cox proportional hazards analyses were performed to calculate estimates of the relative risks (proportional hazards ratio) of HCV, chronic HBV, and liver disease (excluding those with prevalent diagnoses at baseline) and simultaneously adjusting for HIV risk mode (specifically, IDU, MSM, and hemophilia), race/ethnicity, nadir CD4+ group (nadirs of < 200 cells/ μ L and 200–499 cells/ μ L), age, gender, alcohol use, IDU, antiretroviral use, intensity of health services utilization, and year entered cohort. Ninety-five percent confidence intervals are given for the proportional hazard ratios; intervals that do not include the value “one” are considered statistically significant. Individuals included in the multivariate analyses were required to have two or more outpatient visits documented in follow-up intervals ending in 1998 or later to minimize the contributions of persons unlikely to be assessed for any of the potential risk factors for liver disease (hepatitis, alcohol use, *etc.*). In the multivariate analyses examining liver disease as an outcome, all exposures were limited to those occurring prior to the diagnosis of liver disease. For chronic HBV and HCV, co-variables could be simultaneous with infection.

RESULTS

From January 1, 1998 to June 30, 2004, 29 490 individuals were followed for an average of 2.4 years per person, and for a total of 69 487 person-years. The proportion of the 29 490 observed each year from 1998 to 2004 was 56% in 1998, gradually declining to 42% in 2003; in 2004 (due to project end) 5% were followed. Liver disease was diagnosed in 3% of the cohort, HCV in 19%, and chronic HBV in 8% (Table 1). Individuals diagnosed with HBV, HCV, and liver disease were more likely to be male ($P < 0.001$), have a history of IDU ($P < 0.001$), use other illicit drugs ($P < 0.001$), be diagnosed with problem alcohol use ($P < 0.001$), and have more advanced HIV disease as measured by diagnoses of AIDS, including low nadir CD4 counts ($P < 0.001$). Sixty-five percent of individuals diagnosed with HCV had a history of IDU, relative to 25% overall. Twenty-five percent of HCV infected individuals were current IDU (medical records documented ongoing IDU over the course of observation) relative to 8% overall. Over half (57%) of individuals diagnosed with liver disease died over the period of observation, compared to a 15% overall mortality rate ($P < 0.001$).

Trends in hepatitis screening, HBV vaccination, and diagnoses of HBV and HCV

Cumulatively, 38% of the cohort was documented to have been screened for HBV and 37% had been screened for HCV. Screening increased significantly over the seven years ($P < 0.0001$ χ^2 for trend test for HBV and HCV, Figure 1). Repeat screenings were uncommon, even in the presence of ongoing risk. For example, for individuals without any documented HBV vaccination and with ongoing injection drug use noted in their medical record, only 12% of those followed for two or more years had two or more HBV screenings documented.

The proportion of the cohort diagnosed with chronic HBV (at any time up to and including the observation year) increased from 7% in 1998 to 8.5% in 2004 ($P < 0.0001$). Similarly 9% were diagnosed with HCV in 1998, increasing to 24% in 2004 ($P < 0.0001$). Over the course of follow-up, chronic HBV was diagnosed at a rate of 1.8 per 100 person-years, and HCV was diagnosed in 4.7 per 100 person-years (Table 2). Between 1998 and 2003, vaccination for HBV among those without a prior diagnosis of HBV increased from 10% to 28%.

Liver disease

Among the 832 individuals diagnosed with liver disease, 262 (31%) were diagnosed with non-alcoholic (or alcohol not specified) cirrhosis, 165 (20%) with alcoholic cirrhosis, 3% with a primary liver cancer, 3% with liver failure not otherwise specified, (these numbers and percents include 7% with two or more of these conditions diagnosed), and 244 (29%) with other liver diseases (Table 3). Of the 832 with liver disease, 212 individuals (25% of those with liver disease, 1% of the cohort) had a liver disease diagnosis

Table 1 Characteristics of human immunodeficiency virus-infected individuals with chronic hepatitis B, hepatitis C, liver disease, and overall; Adult/adolescent spectrum of human immunodeficiency virus-related diseases project, 11 U.S. metropolitan areas; 1998-2004 *n* (%)

Characteristic	Total = 29 490 (100%)	Chronic hepatitis B = 2332 ¹ (8%)	Hepatitis C = 5463 ² (19%)	Liver disease = 832 ¹ (3%)
Gender				
Male	72	84	74	78
Female	28	16	26	22
Race/Ethnicity				
White non-Hispanic	25	31	24	34
Black non-Hispanic	43	49	42	34
Hispanic	19	11	22	29
Asian/Pacific Islander non-Hispanic	1	1	< 1	1
Native American non-Hispanic	< 1	< 1	10	1
Other/unknown	11	8	< 1	2
Age (yr)				
13-29	24	22	11	13
30-39	43	46	41	40
40-49	25	24	37	34
50+	8	7	11	13
HIV transmission mode:				
MSM	39	48	17	30
IDU	18	18	49	39
IDU and MSM	7	12	15	12
Other (i.e. heterosexual or blood product exposure)	16	9	9	9
Unknown	20	13	10	10
Ever prescribed HAART				
Yes	66	75	67	72
No	34	25	33	28
Nadir CD4 count in cells/ μ L				
\geq 500	11	6	10	5
200-499	34	30	32	22
< 200	55	64	58	74
Highest viral load in copies per mL				
Undetectable to 9999	28	23	25	19
10 000-99 999	33	33	32	32
100 000-999 999	33	38	35	42
1 000 000 and higher	7	7	8	7
Vital status				
Alive	85	80	80	43
Died	15	20	20	57
AIDS diagnosis				
Never diagnosed with AIDS	37	26	32	19
AIDS	63	74	68	81
Substance use				
Alcohol use/problem drinking	23	31	40	49
Non-IDU	29	39	44	37
Ongoing IDU	8	10	25	17

¹All comparisons of clinical and demographic characteristics presented in this table between individuals with and without chronic Hepatitis B virus and with and without liver disease are highly statistically significant (P values ≤ 0.001); ²All except two comparisons between individuals with and without chronic hepatitis C virus (HCV) are highly statistically significant (P values ≤ 0.001). These exceptions are gender and HCV ($P = 0.004$) and highly active antiretroviral (HAART) and HCV ($P = 0.05$). MSM: Male-male sex; IDU: Injection drug use.

present at baseline (prevalent disease) and 621 individuals (75% with liver disease, 2% of the cohort) had new or incident diagnoses over follow-up (Table 2). Between 1998 and 2004, the proportion of individuals followed each year with a diagnosis of liver disease increased from 1.8% to 2.2% (χ^2 for trend P value = 0.03, Figure 1). Overall, 496 of the 5005 people who died (9.9%) had a diagnosis of liver disease. This ranged from 8.6% to 11.0% over the seven years of the study, with no significant trend (data not shown).

Death rates

Table 4 presents the death rates per 1000 person-years observed among the entire ASD cohort from 1998-2004, and separately for people with diagnoses of liver disease, chronic HBV, HCV, and none of these diagnoses. Death rates ranged from 61/1000 for individuals without liver disease, HCV, and chronic HBV diagnoses to 74/1000 for people diagnosed with HCV. About 1% of overall deaths may have been due to liver disease among the entire co-

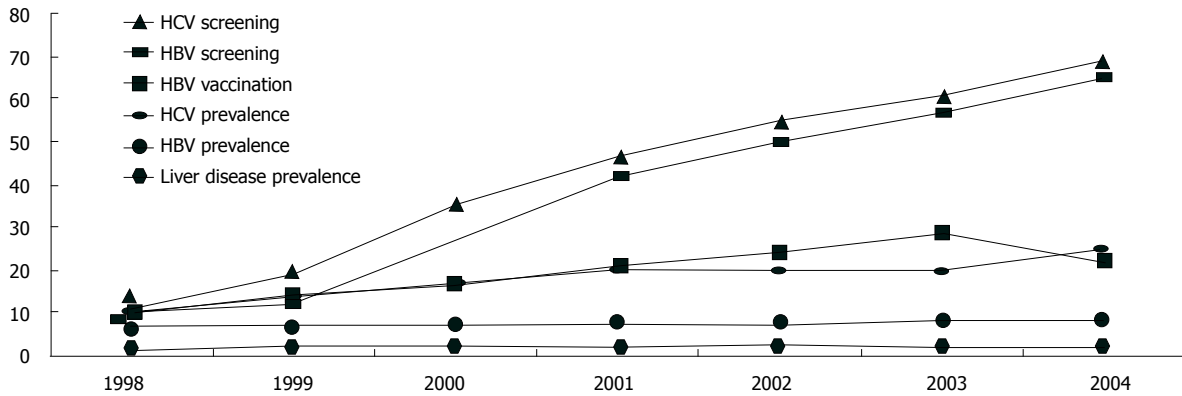


Figure 1 Screening and prevalence rates for hepatitis B and hepatitis C, liver disease prevalence, and hepatitis B vaccinations among human immunodeficiency virus-infected individuals; Adult/adolescent Spectrum of human immunodeficiency virus disease project, 11 U.S. metropolitan areas; 1998-2004. HCV: Hepatitis C virus; HBV: Hepatitis B virus.

Table 2 Chronic and acute hepatitis B, hepatitis C, and liver disease prevalence *versus* incidence (and rates of disease occurrence) among human immunodeficiency virus-infected individuals: Adult/adolescent spectrum of human immunodeficiency virus-related diseases project, 11 U.S. metropolitan areas; 1998-2004 (*n*)

Diagnoses	Total (% of 29 490)	Incident [rate per 100 person-years (py)]	Present at baseline (% of 29 490)
All liver disease	832 (3)	621 (0.9/100 py)	212 (1)
Chronic hepatitis B	2332 (8)	1212 (1.8/100 py)	1120 (4)
Hepatitis C	5463 (19)	3129 (4.7/100 py)	2334 (8)
Acute hepatitis B	862 (3)	575 (0.8/100 py)	287 (1)

Table 4 Overall death rates and deaths potentially due to liver disease among individuals with chronic hepatitis B, hepatitis C, and liver disease diagnoses among human immunodeficiency virus-infected individuals followed by the Adult/adolescent spectrum of human immunodeficiency virus-related diseases project, 11 U.S. metropolitan areas; 1998-2004

Diagnoses	Number of deaths	Overall death rate [Per 1000 person-years (py)]	Potential liver disease death rate [(Per 1000 person-years py)] ¹
Entire cohort	4461	64.2	2.9
Liver disease	475	73.0	31.0
HCV	1073	74.4	7.0
Chronic hepatitis B	455	68.9	6.4
No HCV, no chronic HBV	3099	61.4	1.5

¹ A potential liver disease death was a death where liver disease was included as a contributing factor on a death certificate or a death occurring within approximately six months of a new or ongoing diagnosis of liver disease. HCV: Hepatitis C virus; HBV: Hepatitis B virus.

hort. Among those with HCV or chronic HBV, about 2% of deaths may have been due to liver disease (defined as liver disease as a contributing illness on a death certificate or as a new or ongoing diagnosis within six months of death). Death rates potentially attributable to liver disease ranged from 3/1000 for the entire cohort to 31/1000 among those with a prior diagnosis of liver disease.

Table 3 Types of liver disease diagnosed among human immunodeficiency virus-infected individuals. Adult/adolescent spectrum of human immunodeficiency virus-related diseases project, 11 U.S. metropolitan areas; 1998-2004 (*n* (%))

Liver diseases diagnosed	Not exclusive categories
All liver disease	832 (100)
All cirrhosis	389 (47)
Non-alcoholic cirrhosis	262 (31)
Alcoholic cirrhosis	165 (20)
Both	38 (5)
Other alcoholic liver disease excluding cirrhosis	39 (5)
Liver cancer	25 (3)
Liver failure (not otherwise specified)	22 (3)
Other liver diseases	244 (29)

Overlapping diagnoses and death rates

Figure 2 presents the cumulative number and proportions of individuals with HCV, HBV, liver disease, overlapping diagnoses, and none of these three conditions. Cumulative death rate per 100 persons and 95% confidence intervals for HCV, HBV, liver disease, and overlapping diagnoses are presented in Figure 3. Death rates were similar for individuals with only HBV and only HCV, but increased with dual HBV & HCV infection. The highest death rates occurred among individuals with liver disease regardless of HBV, HCV, and dual HBV/HCV infection status.

Multivariate analyses

Correlates of incident liver disease were examined by proportional hazards regression among 29 279 individuals (excluding the 212 with prevalent disease; Table 2). All demographic and clinical characteristics from Table 1 were included as covariates with the exception of AIDS as it was redundant with AIDS-defining CD4+ cell count categories. We also adjusted for intensity of health services utilization and year of enrollment. IDU and hemophiliacs had more than double the risk of liver disease relative to those without these risk factors (Table 5). Relative to Blacks, all other racial/ethnic groups had a two to three-fold higher risks of a liver disease diagnosis. Risk of liver disease also increased

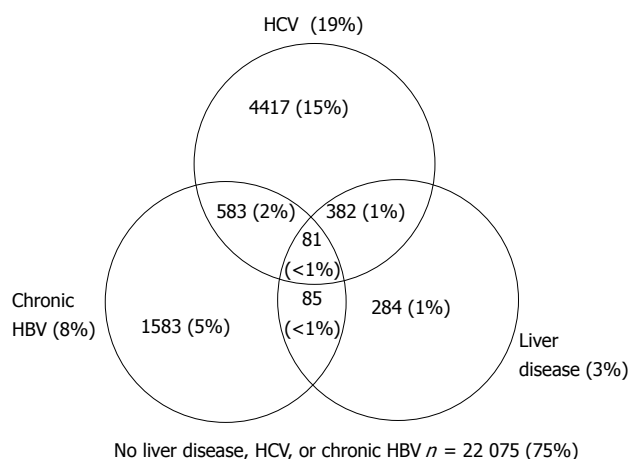


Figure 2 Cumulative prevalence of hepatitis C virus, chronic hepatitis B virus, liver disease, and overlapping diagnoses among 29 490 individuals; Adult/adolescent Spectrum of human immunodeficiency virus-related Diseases Project, 11 U.S. metropolitan areas; 1998-2004. HCV: Hepatitis C virus; HBV: Hepatitis B virus.

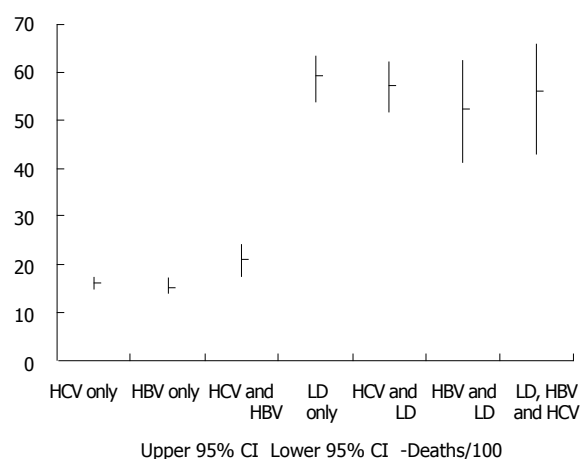


Figure 3 Cumulative death rate per 100 persons and 95% CI for hepatitis C virus, chronic hepatitis B virus, liver disease, and overlapping diagnoses among 29 490 individuals; Adult/adolescent spectrum of human immunodeficiency virus-related diseases project, 11 U.S. HCV: Hepatitis C virus; HBV: Hepatitis B virus; LD: Liver disease.

Table 5 Correlates of liver disease, chronic hepatitis B virus, and hepatitis C virus by multivariate (proportional hazards) analyses. Adult/adolescent spectrum of human immunodeficiency virus-related diseases project, 11 U.S. metropolitan areas; 1998-2004

Characteristic	Proportional hazards ratio ¹ -an estimate of relative risk (95% CI)		
	Liver disease	Chronic HBV	HCV
Male gender	1.2 (1.0-1.5)	1.7 (1.4-2.0)	1.3 (1.1-1.4)
White non-hispanic	1.9 (1.6-2.3)	1.0 (0.9-1.1)	1.3 (1.1-1.4)
Hispanic	2.2 (1.8-2.7)	0.7 (0.6-0.8)	1.4 (1.3-1.4)
Asian/pacific islander non-hispanic	2.3 (1.1-5.0)	1.0 (0.5-1.8)	1.0 (0.6-1.7)
Native American non-Hispanic	3.1 (1.5-6.3)	0.3 (0.1-1.3)	1.4 (0.9-2.1)
Each increase in decade of age relative to those 13-29 yr	1.4 (1.3-1.5)	1.0 (1.9-1.1)	1.3 (1.2-1.3)
MSM	0.9 (0.7-1.1)	1.4 (1.2-1.7)	0.7 (0.7-0.8)
IDU	2.2 (1.8-2.6)	1.2 (1.1-1.4)	4.7 (4.4-5.1)
Hemophiliac	2.6 (1.0-6.5)	0.9 (0.3-2.8)	7.0 (4.8-10.2)
HAART	1.0 (0.8-1.2)	0.1 (0.1-0.2)	0.4 (0.4-0.4)
CD4 < 200 cells/microliter	1.8 (1.2-2.6)	3.9 (2.9-5.2)	1.6 (1.4-1.9)
CD4 200-499 cells/microliter	1.0 (0.7-1.5)	2.0 (1.5-2.7)	1.3 (1.1-1.5)
Alcohol use/problem drinking	1.4 (1.1-1.6)	0.7 (0.6-0.8)	1.1 (1.0-1.2)
Chronic HBV	1.4 (1.1-1.7)	N/A	1.3 (1.1-1.4)
HCV	1.6 (1.3-1.9)	1.6 (1.4-1.8)	N/A

¹Adjusted for each factor in the model plus intensity of health services utilization (number outpatient visits in a grouped linear variable) and study entry year. HAART: Highly active antiretroviral; CD4: CD4+ lymphocyte; N/A indicates not applicable; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MSM: Male-male sex; IDU: Injection drug use.

with increasing age, with AIDS-defining nadir CD4+ cell counts (< 200 cells/ μ L), alcohol use, and a prior diagnosis of HBV or HCV. Once these other factors were adjusted for, neither HAART in aggregate nor any specific antiretrovirals were associated with excess risk of liver disease.

Correlates of chronic HBV and HCV diagnoses were also examined by multivariate proportional hazards regression analyses, excluding those with prevalent chronic HBV ($n = 1120$) and HCV ($n = 2334$), respectively. IDU and hemophiliacs were 4.7-fold and seven-fold more likely to be diagnosed with HCV relative to those with other HIV risk factors. Men, Whites, Latinos, and those co-infected with HBV were all more likely to be diagnosed with HCV.

HCV risk also increased with age among individuals 30 years of age and older relative to those less than 30 years of age. Factors associated with chronic HBV diagnosis include male gender, MSM, IDU, lower CD4+ cell count nadir, alcohol use, and co-infection with HCV.

Liver cancers

There were 25 diagnoses of liver cancer in the cohort. All of these patients were documented to have died, excluding three individuals lost to follow-up. Fifteen of the 25 had hepatocellular carcinoma, four had other specified morphologies (one diagnosis each of sarcoma, hepatoblastoma, lymphoma, and giant cell type), and six were

unspecified. Of the 25, 13 were diagnosed with HCV and 10 with chronic HBV (this includes two diagnosed with both). Of the four diagnosed with neither virus, none had evidence of HBV or HCV screening documented in their medical chart.

DISCUSSION

We found that chronic HBV, HCV, and liver disease were frequent diagnoses among HIV-infected people followed in the ASD project from 1998 to 2004. Roughly one person out of a hundred people followed for a year was diagnosed with liver disease, two per 100 followed for a year were diagnosed with chronic HBV, and five out of a hundred were diagnosed with HCV. We observed relatively low rates of HBV and HCV screening, especially in earlier years of observation, despite consistent recommendations that all HIV-infected patients be screened for both infections in the guidelines for the treatment of HIV and prevention of opportunistic illness (including screening of HBV prior to administration of vaccine) covering 1998-2004^[13]. Fortunately, over the observation period, increasing proportions of individuals were screened for HBV and HCV. Diagnoses of HBV and HCV also increased over the seven years of the study. Among individuals who died, liver disease was present at any point in nearly 10%, and liver disease was listed as a contributing factor or as a new or ongoing diagnosis within six months of death in 1% of deaths. The overall prevalence of liver disease was about 1% each year-this increased only slightly over the seven years. As expected, IDU was associated with all three outcomes examined by multivariate analyses (chronic HBV, HCV, and liver disease). Hemophilia, and thus an exposure to unscreened blood products, was most strongly associated with HCV, and alcohol use was associated with liver disease.

Other researchers have found high rates of liver diseases and viral hepatitis among HIV-infected persons, especially HIV-infected hemophiliacs and injection drug users. The prevalence of HBV and HCV infection varies within HIV-infected cohorts largely due to the underlying prevalence of these infections in the general populations from which the cohorts are drawn and the proportion of IDU included. Relative to the 8% with chronic HBV and the 19% with HCV in our cohort, a New York City HIV clinic following 5639 people living with HIV from 1999 until 2007, found that 4% were HBV-infected and 25% were HCV infected^[14]. The HIV Outpatient Survey (HOPS) conducted at 10 facilities in eight U.S. cities followed 7618 HIV-infected individuals 1996-2007 and found a 24% prevalence of HCV^[15]. In a serological study of incarcerated individuals in three U.S. cities, of those who were HIV-infected, 38% were co-infected with HCV and 8% were HBsAg positive^[16].

The D:A:D Study followed 23441 individuals on three continents between 1999 and 2004, finding a 23% prevalence of HCV and a 7% prevalence of active HBV^[17]. In this study, HCV was associated with a seven-fold excess

risk of liver-related deaths (e.g. hepatocellular carcinoma, end stage liver disease, or hepatic failure) and active HBV was associated with a four-fold excess risk of liver-related deaths. This compares to our similar finding of liver related deaths occurring over four times more frequently among individuals infected with HCV or chronic HBV relative to uninfected individuals. In the Swiss HIV Cohort Study, 37% of 3111 people living with HIV were HCV-infected; this included 92% of IDU and 7% of those with other HIV risks^[18]. In this study, 9% of HCV-co-infected individuals died relative to 4% of those without HCV. Of the deaths among HCV-infected individuals, 16% were possibly to definitely associated with end-stage liver disease relative to 5% of deaths among HIV-infected individuals without HCV^[18]. In the Multicenter Cohort Study of MSM, about 8% of HIV-infected men followed between 1984 and 2000 were HBsAg positive, and liver-related mortality was about seven times higher (14 deaths per 1000 person-years) among HBsAg positive men relative to HBsAg negative men (two deaths per 1000 person-years)^[19].

Of 755 people living with HIV evaluated following initiation of antiretrovirals at one Italian HIV clinic, 3% developed severe hepatotoxicity (4/100 person-years). Nearly all (96%) of these patients had evidence of HCV infection (relative to 67% without hepatotoxicity), 19% had evidence of HBV, and 19% had a history of alcohol abuse (relative to 7% and 13% prevalence of these diagnoses among those without hepatotoxicity, respectively)^[20]. This is consistent with our finding of no excess risk of liver disease for individuals prescribed antiretrovirals once these other factors (HCV, HBV, alcohol use, *etc.*) were controlled for by multivariate analyses. In a Veteran's Affairs cohort with comprehensive evaluations of 299 HIV-infected individuals over 6 mo, 27% had abnormal liver functions and for 51% of these, no underlying cause was established. Among the remainder, 30% had non-alcoholic fatty liver disease as a diagnosis, alcohol was attributed to 13%, chronic HBV to 9% and chronic active HCV to 5%^[21].

Studies of HIV-infected hemophiliacs include a cohort study of 158 HIV-infected hemophiliacs followed in the pre- and post-HAART eras^[22]. The predominant cause of non-AIDS mortality in both periods was end-stage liver disease (ESLD). Of 223 HIV-infected hemophiliacs without clinical AIDS, 9% of those co-infected with HCV developed liver failure after 10-20 years^[23]. In Germany, 144 HIV and HCV co-infected hemophiliacs were examined, and the authors concluded that declining immune function may be associated with progression of liver failure^[24].

Relative to the increase in HCV screening that we observed (from 11% to 69%), the HOPS study documented a very similar trend-HCV screening increasing from 11% to 77% from 1996 to 2007^[15]. However, even if patients are screened, few receive ongoing screening despite ongoing risk, fewer than half are evaluated, and a minority are treated, reflecting the many hurdles that HIV and HCV and HIV and HBV co-infected patients must surmount to

be appropriately managed and treated for HBV and HCV when indicated^[25,26].

Although the number of liver cancers we observed was small ($n = 25$), all 21 with hepatitis screening documented were HBV or HCV-infected. Our study was not designed to compare the occurrence of liver cancer among people living with HIV relative to the general population, but other researchers have found three to six fold excess risk of liver cancer associated with HIV infection^[27,28].

Limitations of our analyses included that the data were collected solely by medical record review. Collecting data by chart review rather than targeted medical examinations, may have introduced errors. Exposures and diagnoses may have been missed, especially if an individual sought medical care at facilities other than those included in ASD. Other diseases recorded may have been in error, such as mistaking a “rule out” diagnosis with a true diagnosis. Further, the use of ICD-9 codes to define liver disease likely resulted in some losses of sensitivity and specificity (for example, including a code such as 571.4-chronic hepatitis-which might have included viral hepatitis infections without any known liver damage). Similarly ICD-9 code 571 includes both fatty liver and nonalcoholic steatohepatitis (NASH), which could not be distinguished from each other without additional information. Information on performance of liver biopsy was not collected, and biopsies were not likely to have been frequently performed, leaving us to rely upon broad clinical diagnoses rather than histological diagnoses. HBV and HCV were particularly difficult diagnoses to ascertain through chart review because of under-diagnosis-especially of asymptomatic infections, false negative screening tests due to low or transient antibody production^[29-31], and abstractor (and practitioner) confusion regarding distinguishing markers of acute, chronic, and chronic active or chronically persistent infection. Observed trends in HBV and HCV diagnoses could have been caused by increases in screening, not increases in incidence and prevalence. Some known predisposing conditions of certain liver disease (such as obesity) were not routinely collected. Deaths and causes of death may have been under-ascertained as well, as some ASD sites linked to core surveillance and national death index data and others did not. Finally, although ASD data collection ended in 2004, we believe there have been no major changes in hepatitis screening, HBV vaccination, or liver disease trends among HIV-infected individuals and that our findings remain highly relevant today.

Despite its limitations, ASD provided a large single cohort in a contemporary HAART era, collected with a consistent protocol and including a wealth of data on the screening and diagnosis HBV and HCV and liver disease and mortality as outcomes. Although HIV case reporting was not required throughout the U.S. during the period of our study, the socio-demographic characteristics (age, gender, race-ethnicity, and HIV risk category) of 38398 newly diagnosed HIV cases in 2004 for the 34 states and five dependent areas, with name-based reporting at that

time, were highly similar to the characteristics of ASD, suggesting ASD was largely representative of national HIV cases at this time^[32].

In conclusion, patients with HIV and liver disease had a much higher mortality compared to those with HIV without a liver disease diagnosis. Although HBV vaccination rates have improved and screening rates for HBV and HCV have climbed steadily, they are still inadequate, and efforts are needed to improve vaccination and screening rates. The high rates of incident HCV (5/100 person-years) indicate that individuals at risk should be screened and while remaining at risk, re-screened on a regular basis. Similarly, a sizable HBV incidence (2/100 person-years) supports improved screening and vaccination^[33]. Further studies are needed to determine the optimal frequency of repeated HCV screening among those with ongoing risk, as well as a frequency to re-visit HBV vaccination among unvaccinated individuals. Until better data are available, annual screenings for HCV and HBV vaccination discussions are suggested. Treatment of HBV and HCV should be considered for all HIV co-infected individuals.

COMMENTS

Background

Despite widespread availability of antiretroviral treatment in the U.S., over 17 000 people living with human immunodeficiency virus (HIV) are estimated to die each year. Although increasing, the median age at death among HIV-infected people was 46 years in 2006, indicating most HIV deaths occur among relatively young individuals. Hepatitis and liver disease are major causes of premature death among people living with HIV. The authors conducted this review of liver disease and hepatitis screening and diagnoses to describe preventive care and the impact of these conditions among HIV-infected individuals receiving medical care in the U.S.

Research frontiers

In this report, the authors examined hepatitis and other hepatic diseases in a dynamic medical record review cohort of over 29 000 HIV-infected people. The authors found, over the study period of 1998 to 2004, improvements in hepatitis screening and hepatitis B vaccination, but the authors also saw there was much room for improvement in screening and vaccination coverage. On average, among 100 HIV-infected people followed for a year one person had a diagnosis of acute hepatitis B infection, two were diagnosed with chronic hepatitis B, five were diagnosed with hepatitis C, and one person with liver disease. Fewer than one-third (28% in 2003) of those without a documented diagnosis of hepatitis B had evidence of any hepatitis B vaccination.

Innovations and breakthroughs

The authors examined hematological diagnoses and risk factors in nearly 30 000 HIV infected individuals from a broad range of HIV risk groups and geographic locations within the U.S. Among other findings, the authors saw elevated death rates among people with chronic hepatitis B, hepatitis C, and liver disease, but mortality rates were elevated the most among individuals with liver disease, with or without chronic hepatitis B and hepatitis C. When the authors conducted a multivariate analysis, the authors saw that Blacks were at lower risk of liver disease than individuals with other racial/ethnic identities, older individuals, injection drug users, alcoholics/problem drinkers, and individuals with AIDS all had increased risks of liver disease. Antiretroviral use was not associated with liver disease.

Applications

The authors' results highlight a need for improved hepatitis screening and vaccination. All HIV-infected individuals should be screened for hepatitis and, if at risk, vaccinated for hepatitis B. Uninfected individuals should be counseled in hepatitis risk reduction strategies, and individuals with chronic hepatitis infections should be assessed for treatment. It is likely that the authors' estimates

for hepatitis screening and vaccination are low, and that documentation of prior screening and vaccinations may have been missed, for example if they occurred at medical facilities not participating in ASD.

Peer review

A series of experiments are well-planned and well-performed and this manuscript is well written.

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X-ray diagnosis of synchronous multiple primary carcinoma in the upper gastrointestinal tract

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Abstract

AIM: To analyze the radiological features of multiple primary carcinoma (MPC) in the upper gastrointestinal (GI) tract, study its biological characteristics and evaluate X-ray examination in its diagnosis.

METHODS: Hypotonic double-contrast GI radiography was performed in 59 multiple primary carcinoma cases, pathologically proved by surgery or endoscopy biopsy. Radiological findings were analyzed.

RESULTS: Of the 59 cases, esophageal MPC (EMPC) was seen in 24, esophageal and gastric MPC (EGMPC) in 27 and gastric MPC (GMPC) in 8. Of the 49 lesions found in 24 EMPC, hyperplastic type was seen in 23, medullary type in 9. The lesions were located at the upper ($n = 17$), middle ($n = 19$) or lower ($n = 13$) segment of the esophagus. In 27 EGMPC, the esophageal lesions were located at the middle ($n = 16$) or lower ($n = 11$) segment of the esophagus, while the gastric le-

sions were located at the gastric cardia ($n = 16$), fundus ($n = 1$), body ($n = 3$) and antrum ($n = 7$). The esophageal lesions were mainly of the hyperplastic type ($n = 12$) or medullary type ($n = 7$), while the gastric lesions were mainly of the hyperplastic type ($n = 18$). A total of 119 lesions in the 59 patients with synchronous multiple carcinoma were proved by surgery or endoscopy biopsy, and preoperative upper radiographic examination detected 100 of them (84.03% sensitivity). Eighteen (52.94%) of the T₁ lesions were found during preoperative diagnosis by radiographic examination. Moreover, only 3 (3.53%) of the T₂₋₄ lesions were misdiagnosed.

CONCLUSION: Hypotonic double-contrast upper gastrointestinal examination, providing accurate information about lesion morphology, location and size, can serve as a sensitive technique for the preoperative diagnosis of MPC.

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Key words: Multiple primary carcinoma; Upper gastrointestinal tract; Radiography

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INTRODUCTION

Although malignant tumors are currently detected more often than in the past due to continued and vast improvements in medical modalities, the incidence of synchronous multiple primary carcinoma (MPC), defined as two

or more primary carcinomas occurring in an individual simultaneously, is low^[1]. Most of these synchronous cancers are in the head and neck region. Other frequently reported sites of synchronous cancer associated with esophageal cancer are the stomach, lung, and urinary bladder^[2,3]. The mucous epithelium of the head and neck, lung, and esophagus is exposed to common carcinogenic agents, leading to multiple carcinomas in these regions. The etiology of synchronous multiple primary carcinomas is still unclear, strong epidemiologic evidence implicates tobacco as the main carcinogen and alcohol as a promoter of carcinogenesis^[4]. Other potential risk factors include hot beverages^[5], nutritional deficiencies^[6], pickled vegetables, nitrosamine-rich food^[7], and some genetic factors^[8,9]. Radiation therapy is also associated with esophageal cancer^[8,10]. Their coexistence can be problematic for surgeons, oncologists and pathologists, as regards diagnosis, treatment and follow-up.

Multiple primary malignant neoplasms in a single patient have been well documented with regard to the frequency of occurrence of primary multiple carcinomas, identification of high-risk groups, early diagnosis, treatment methods, and prognostic factors over the past hundred years^[11]. For some reason, synchronous multiple primary carcinomas of the upper gastrointestinal (GI) tract can often be overlooked at the time of diagnosis, and so far, there has not been a consensus on how best to diagnose mucosal lesions of multiple primary carcinomas in the upper GI tract: by endoscopy or barium radiography? The double-contrast upper GI examination is a safe, noninvasive, inexpensive and cost-effective test for the work-up of a host of GI diseases. However, reports exclusively on evaluating X-ray examination in the diagnosis of synchronous MPC of the upper gastrointestinal tract have been few. In this study, we retrospectively analyzed 59 synchronous MPC cases proved by surgery or endoscopy biopsy: each of the patients underwent radiographic examination. The aim was to analyze the radiological features of synchronous MPC in the upper GI tract, study its biological characteristics, evaluate X-ray examination in its diagnosis, and seek ways to improve its preoperative diagnosis.

MATERIALS AND METHODS

Patients

Patients with MPC were selected from January 1988 to December 2008 at the First Affiliated Hospital of Zhengzhou University. Of the 59 patients aged 42-85 years (mean: 61.20 years), 44 were men and 15 were women. Main clinical manifestations were choking or swallowing difficulties, some cases were associated with anemia, weight loss, abdominal pain and other upper GI signs or symptoms. Specimens for pathologic confirmation of the 59 cases were obtained by endoscopy biopsy alone in 13 patients, by endoscopy biopsy and surgery in 27, and by surgery alone in 19. Thirteen patients did not have surgery, either because they were not considered surgical candidates or because they chose to be treated elsewhere.

X-ray examinations

After 6-8 h of fasting, patients first received 20 mg hypotonic drugs as well as anisodamine (654-2) intravenous injection. Ten minutes later, the patient swallowed 3 g aerogenic agents, and then, as fast as possible, 150 mL of resuspended barium sulfate at 200% weight for volume (w/v). Three to four spot radiographs were obtained in sequence at different levels of the esophagus, and double-contrast esophagograms were obtained with the patient in the upright LPO position. Multi-position dynamic observation of the stomach and duodenum was performed for all cases, and all patients were pathologically confirmed by endoscopic biopsy or surgery.

Imaging criteria

According to the principle of Warren and Gates, multiple primary carcinomas are defined as follows^[12]: (1) Each lesion is histopathologically malignant; (2) Each lesion is separated by the normal mucosa; and (3) Each lesion is not the result of local extension or metastasis of another lesion. Multiple primary cancers may be synchronous or metachronous depending on the interval between their diagnosis. Synchronous carcinomas were diagnosed simultaneously or within an interval of about 6 mo, and metachronous carcinomas were secondary cancers that developed more than 6 mo after the diagnosis of primary cancers usually after treatment of primary lesions. In our series of 59 cases, all were synchronous multiple primary carcinoma.

The radiographs were reviewed retrospectively by two authors to determine the location, size, morphology and interval distance of these tumors. Lesions were classified morphologically as hyperplastic, medullary, infiltrative, ulcerative or mixed type. Pathologic records were also reviewed to determine the postoperative histological classification, depth of invasion and number of lesions being misdiagnosed in this group.

Statistical analysis

We evaluated the clinicopathological differences of esophageal lesions between esophageal MPC (EMPC) and esophageal-gastric MPC (EGMPC), and of gastric lesions between gastric MPC (GMPC) and EGMPC. Statistical analyses were performed using software from SPSS for Windows 17.0. The statistical methods used included χ^2 test for categorical variables and *t* test for continuous variables. *P* < 0.05 was taken to indicate statistical significance.

RESULTS

Characteristics of MPC

In our series of 59 cases, all were synchronous multiple primary carcinoma, and mostly occurred in males: male/female ratio = 2.93/1, 71.19% of the patients were older than 55 years, and average age was 61.2 years old. Table 1 shows the age and sex distribution of these 59 patients.

Multiple esophageal carcinoma was seen in 24, and 49 lesions were found. One case was a triple lesion (Figure 1), and the remaining 23 cases were double lesions (Figures

Table 1 Age and sex distribution of the 59 patients with multiple primary carcinoma included in this study

	Gender			Age(yr)		
	<i>n</i>	Male	Female	Range	Mean	≥ 55
Esophageal MPC	24	13	11	44-85	61.63	15/24 (62.5%)
Esophageal and gastric MPC	27	24	3	42-72	59.89	20/27 (74.07%)
Gastric MPC	8	7	1	48-76	64.38	7/8(87.5%)

MPC: Multiple primary carcinoma.

Table 2 Distance between every two esophageal lesions

Esophageal MPC	<i>n</i> (%)	Interval distance (cm)	
		Range	Mean
Upper-middle	12 (50.00)	5-12.5	8.36
Upper-lower	5 (20.83)	8-13	11.20
Middle-lower	7 (29.17)	5-9	5.83

MPC: Multiple primary carcinoma.

Table 3 Clinicopathological differences of esophageal lesions between esophageal multiple primary carcinoma and esophageal-gastric multiple primary carcinoma

	EMPC			EGMPC (<i>n</i> = 27)
	Total (<i>n</i> = 49)	First lesion	Second or third lesion	
Location				
Upper	17	17	0	0
Middle	19	7	12	16
Lower	13	0	13	11
Largest dimension of lesions (cm)				
mean ± SD ¹	4.33 ± 2.35	3.63 ± 1.96	5.00 ± 2.67	5.56 ± 2.34
95% CI	3.63-5.02	2.80-4.45	3.90-6.10	4.63-6.48
Morphology				
Hyperplastic	23	11	12	11
Medullary	9	4	5	7
Ulcerative	7	5	2	5
Infiltrative	7	3	4	2
Mixed	3	0	3	2
Histology				
Squamous cell carcinoma	49	24	25	26
Sarcoma	0	0	0	1
PT stage ²				
T1	12	9	3	13
T2-4	37	15	22	14

¹Largest dimension of esophageal lesions (cm) between first lesion and second lesion, $t = 2.047$, $P = 0.046$. Largest dimension of esophageal lesions (cm) between esophageal multiple primary carcinoma (EMPC) and esophageal-gastric multiple primary carcinoma (EGMPC), $t = 2.139$, $P = 0.036$; ²PT stage of esophageal lesions between first lesion and second lesion, $\chi^2 = 4.306$, $P = 0.038$; PT stage of esophageal lesions between EMPC and EGMPC, $\chi^2 = 4.414$, $P = 0.036$.



Figure 1 Sixty four-year-old male with three esophageal lesions. The first was a medullary lesion (↑) located at the upper, the other two were small ulcerative lesions (↑) located at the middle-lower segment, and all were squamous cell carcinoma, pathologically proved.



Figure 2 Fifty eight-year-old male with two esophageal lesions. The first was a medullary lesion located at the upper segment, with stenosis and rough rigid margins (↑); the other was a proliferative nodule located at the middle segment, with disruptive mucosa (↑).

2-4). The distance between every two esophageal lesions ranged from 5 to 13 cm, and was 8.21 cm on average (Table 2). Of the 49 lesions, proliferative lesions made up a total of 23, followed by medullary type ($n = 9$). The size of the proliferative lesions ranged from 1.1 to 5.4 cm (average, 2.7 cm), medullary lesions ranged from 5.2 to 9.6 cm (average, 7.6 cm). There were 17 lesions located at the upper, 19 at

the middle and 13 at the lower esophagus. Table 3 shows the largest dimension of the first lesions (3.63 ± 1.96 cm), which were significantly smaller than second ones (5.00 ± 2.67 cm). Regarding the depth of invasion, T₁ lesions were more frequent in first lesions, especially in the upper region. The X-ray features of esophageal multiple primary carcinomas were the same as those of generally solitary ones. All

Table 4 Clinicopathological differences of gastric lesions between gastric multiple primary carcinoma and esophageal-gastric multiple primary carcinoma

	Total (<i>n</i> = 16)	GMPC		EGMPC (<i>n</i> = 27)
		Main lesion	Additional lesion	
Location				
Cardia	8	1	7	16
Fundus	0	0	0	1
Body	3	3	0	3
Antrum	5	4	1	7
Largest dimension of lesions (cm)				
Mean ± SD ¹	2.94 ± 1.39	3.93 ± 1.21	1.94 ± 0.62	4.94 ± 2.63
95% CI	2.20-3.68	2.93-4.95	1.42-2.46	3.90-5.98
Morphology				
Hyperplastic	13	5	8	17
Ulcerative	1	1	0	6
Infiltrative	2	2	0	4
Histology				
Squamous cell carcinoma	0	0	0	4
Adenocarcinoma	16	8	8	23
PT stage ²				
T ₁	6	1	5	3
T ₂₋₄	10	7	3	24

¹Largest dimension of gastric lesions (cm) between main lesion and additional lesion, *t* = 4.161, *P* = 0.001. Largest dimension of gastric lesions (cm) between gastric multiple primary carcinoma (GMPC) and esophageal-gastric multiple primary carcinoma (EGMPC), *t* = 2.820, *P* = 0.007. ²PT stage of gastric lesions between main lesion and additional lesion, χ^2 = 4.267, *P* = 0.039, PT stage of gastric lesions between GMPC and EGMPC, χ^2 = 4.227, *P* = 0.040.

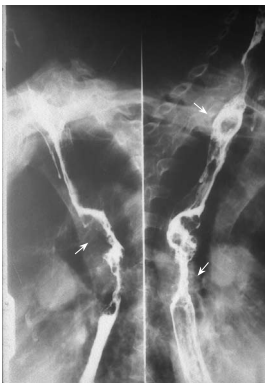


Figure 3 Sixty three-year-old male with two esophageal lesions. The first was a proliferative nodule located at the upper (↑), the other was a medullary lesion located at the middle segment (↑), and all were squamous cell carcinoma, pathologically proved.

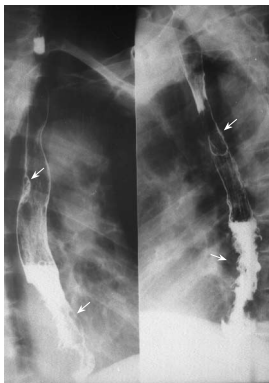


Figure 4 Fifty six-year-old female with two esophageal lesions. The first was a small proliferative nodule located at the middle (↑), the other was a medullary lesion located at the lower segment (↑), and all were squamous cell carcinoma, pathologically proved.

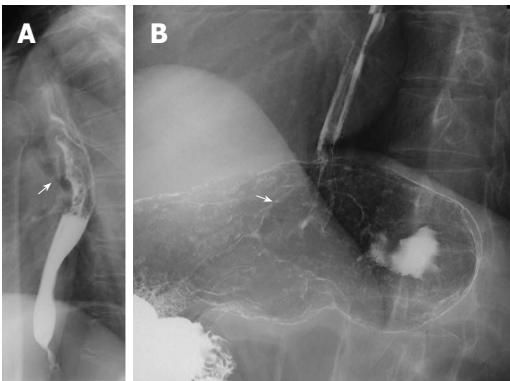


Figure 5 Forty nine-year-old male with esophageal-gastric multiple primary carcinoma. The esophageal lesion was ulcerative, located at the middle segment (↑); the gastric lesion was proliferative, located at the cardia (↑), with distal esophageal infiltration.

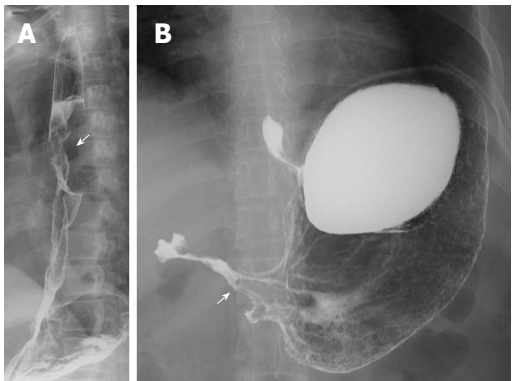


Figure 6 Fifty seven-year-old male with esophageal-gastric multiple primary carcinoma. The esophageal lesion was proliferative located at the middle segment (↑); the gastric lesion was infiltrative located at the antrum (↑).

esophageal-gastric carcinoma, and all were double lesions. Esophageal lesions concentrated in the middle (*n* = 16)

(Figure 5A) and lower segment (*n* = 11). Of them, 11 were proliferative lesions (Figures 6A and 7A), 7 were medullary lesions. The gastric lesions were mainly located at the gas-

Table 5 Clinicopathological characteristics of 19 misdiagnosed synchronous multiple primary carcinomas

Type	Age	Gender	Largest dimension (cm)		Location of lesion		PT stage
			Diagnosed	Missed	Diagnosed	Missed	
1 EMPC	49	Female	3.3	2.2	Middle	Lower	T ₄ /T ₁
2 EMPC	58	Male	3.4	1	Lower	Upper	T ₂ /T ₁
3 EMPC	67	Male	4.7	2.5	Middle	Lower	T ₃ /T ₁
4 EMPC	73	Female	5.5	1.8	Middle	Upper	T ₃ /T ₁
5 EMPC	64	Male	6.2	2	Lower	Upper	T ₂ /T ₁
6 EMPC	57	Male	4.5	1.5	Lower	Middle	T ₂ /T ₁
7 EGMPC	65	Female	6.5	2.2	Lower	Body	T ₄ /T ₂
8 EGMPC	54	Male	5.5	2.5	Lower	Fundus	T ₃ /T ₁
9 EGMPC	58	Male	4.5	1.8	Cardia	Middle	T ₃ /T ₁
10 EGMPC	62	Male	3.2	2.8	Cardia	Middle	T ₃ /T ₁
11 EGMPC	72	Male	3.5	5.7	Cardia	Lower	T ₄ /T ₃
12 EGMPC	63	Male	4.2	3.2	Lower	Antrum	T ₂ /T ₁
13 EGMPC	70	Male	2.8	2.5	Cardia	Middle	T ₂ /T ₁
14 EGMPC	60	Male	4.2	2	Antrum	Middle	T ₃ /T ₁
15 EGMPC	60	Male	4.2	2.2	Cardia	Middle	T ₃ /T ₁
16 EGMPC	57	Male	4.5	3.5	Lower	Cardia	T ₃ /T ₃
17 GMPC	52	Male	2.2	2.8	Cardia	Antrum	T ₂ /T ₁
18 GMPC	57	Male	4.5	1.2	Antrum	Cardia	T ₃ /T ₁
19 GMPC	64	Male	5.5	2.2	Antrum	Cardia	T ₃ /T ₁

GMPC: Gastric multiple primary carcinoma; EGMPC: Esophageal-gastric multiple primary carcinoma; EMPC: Esophageal multiple primary carcinoma.

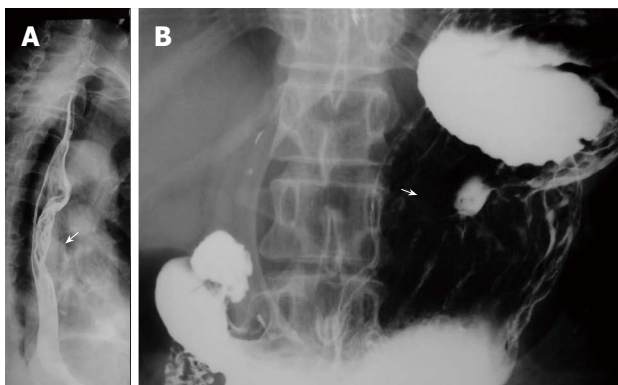


Figure 7 Forty six-year-old female with esophageal-gastric multiple primary carcinoma. The esophageal lesion was proliferative located at the middle (↑); the gastric lesion was ulcerative located at the body (↑).

tric cardia ($n = 16$) (Figure 5B), followed by antrum ($n = 7$) (Figure 6B), body ($n = 3$) (Figure 7B) and fundus ($n = 1$). Seventeen proliferative lesions were mainly located at the gastric cardia and the fundus, numbers of ulcerative type and infiltrating type were 6 and 4 respectively, found mainly at the antrum. In this series, one case of esophageal lesion was pathologically confirmed as sarcoma with a size of 9.1 cm × 5.3 cm × 5.2 cm, the others were squamous cell carcinoma. Twenty-three cases of gastric lesions were adenocarcinoma, and the other four cases were squamous cell carcinoma located at the cardia. From Table 3, we see that the largest dimension of esophageal lesions in EGMPC (5.56 ± 2.34 cm) was significantly larger than that of EMPC (4.33 ± 2.35 cm), and T₁ esophageal lesions were more frequent in EGMPC, especially in the middle region.

Eight patients (13.56%) were found to have synchronous multiple gastric carcinoma, all were double lesions and divided into 2 categories: main lesions (larger or

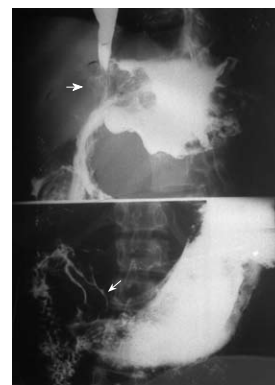


Figure 8 Fifty nine-year-old male with two gastric lesions. The main lesion was proliferative (↑) located at the cardia, with fundus infiltration; the additional one was also a proliferative lesion located at the antrum, with disruptive mucosa (↑). All were adenocarcinoma, pathologically proved.

advanced lesion) and additional lesions. The additional lesions were mostly located in the cardia; of them, two cases had evidence of invasion of the gastric fundus (Figure 8). Four main lesions were located in the antrum, and three at the gastric body. Thirteen (81.25%) cases were proliferative lesions, of which five cases were early gastric protruded lesions. All lesions were adenocarcinoma, pathologically proved. Table 4 shows that the main lesions were significantly larger than the accessory lesions ($P = 0.001$). Regarding the depth of invasion, T₁ lesions were more frequent in accessory lesions, especially in the cardia. Moreover, the largest dimensions of gastric lesions in EGMPC were significantly larger than those of GMPC ($P = 0.007$), and also showed significant differences in the depth of invasion.

Accuracy of pre-operative diagnosis

A total of 119 lesions in the 59 patients with synchronous multiple carcinoma were proved by surgery or endoscopy biopsy, and preoperative upper radiographic examination detected 100 of them (84.03% sensitivity). Thus, 19

(15.97%) lesions were missed out of a total of 119 lesions. Regarding the depth of invasion, 18 (52.94%) of the T₁ lesions were accurately found out of a total of 119 lesions during preoperative diagnosis by radiographic examination. Moreover, only 3 (3.53%) of the T₂₋₄ lesions were misdetected. Table 5 summarizes the clinicopathological characteristics of 19 misdetected lesions in the 19 patients with MPC. By retrospectively reviewing the missed radiographs, we found four lesions mistaken for bubbles, uneven coating of barium appearance, and two lesions in the gastric cardia and lower esophagus were misdiagnosed as invasion. Of the 19 missed lesions, 16 lesions were microscopic early stage and T₁ lesions. Moreover, missed gastric lesions showed a tendency to be of the flat type. The mean size of the missed lesions was 2.40 ± 1.01 cm.

DISCUSSION

X-ray features and clinicopathological characteristics of MPC

Interest in multiple primary malignancies is long-standing, since Warren and Gates description in 1932, and several reports have indicated that the incidence of MPCs has been increasing in recent years. The incidence of synchronous cancers in patients with esophageal cancer varies from 3.6% to 27.1%^[2,13]. In this series, we found that synchronous MPCs in the upper gastrointestinal tract mostly occurred in males: ratio of males/females = 3.21/1, 86.21% of the patients were older than 55 years, and average age was 62.7 years old. As shown in Table 1, 87.5% of patients with multiple gastric carcinomas were men older than 55 years. These findings are comparable to previous studies, and we conclude that elderly individuals aged 65 years or more are at significantly greater risk for multiple gastric carcinomas^[14-16].

In our findings, X-ray characteristics of MPC in general were as follows: (1) Each lesion had a familiar X-ray appearance; (2) Normal X-ray appearance, namely continuous mucous membrane and soft margins, was seen between two lesions; (3) The main morphological feature of multiple primary carcinoma lesions, regardless of whether in the esophagus or stomach, was proliferative in 34 and 30, respectively, total 64 (53.78%); (4) The size of proliferative esophageal MPC lesions was limited, with an average of 2.7 cm; while the size of medullary lesions was relatively extensive, with an average of 7.6 cm; and (5) The distance between every two esophageal lesions ranged from 5 to 13, 8.21 cm on average.

Lesions of esophageal MPC were more commonly located at the upper (17/49) segment close to their predilection sites, which suggests we should pay more attention to observing lesions of the upper segment rather than the middle and lower segments. As to synchronous esophageal-gastric carcinoma, we found that the esophageal lesions were concentrated in the lower segment, while the gastric lesions were concentrated in the cardia, and this finding indicates that the occurrence of the tumor had a concentric trend. Concerning the depth of invasion,

there were significant differences between the first lesion and the second lesion in esophageal MPC; T₁ lesions were more frequent in first lesions (75%), especially in the upper segment. Furthermore, from Table 3 we see that esophageal lesions of synchronous esophageal-gastric carcinoma tend to be less invasive than esophageal MPC ($P = 0.036$). These findings are in accordance with Nagasawa *et al.*^[3], and support the concept that the second malignancy occurred late after carcinogenesis of the main carcinoma lesion or was less aggressive to invade^[17]. In addition, we found that in EMPC the sizes of the first lesions were significantly smaller than second ones; the mean size of esophageal lesions in EMPC was 4.33 ± 2.35 cm, significantly smaller than that of EGMPC (5.56 ± 2.34 cm).

Only 8 patients (13.56%) were found to have synchronous multiple gastric carcinoma, the additional lesions mostly located in the cardia. Of them, 2 cases had evidence of invasion of the gastric fundus. Four main lesions were located in the antrum, and 3 at the gastric body. Lee *et al.*^[18], however, believed that the distribution of location was not significantly different between the main and accessory lesions, and the fact that our sample size was very small should be taken into account. Nevertheless, all assessments indicated the main lesions were significantly larger than the accessory lesions. Regarding the depth of invasion, T₁ lesions were more frequent in accessory lesions, especially in the cardia. Thus, more attention should be paid to this multi-positional location, and detailed and thorough examination is particularly critical. In addition, the largest dimensions of gastric lesions in EGMPC were significantly larger than those of GMPC ($P = 0.007$), and also showed significant differences in the depth of invasion. These findings are in accordance with Lee *et al.*^[18], and support the “collision theory”; that is, that early multiple gastric lesions “fuse” together to form single, advanced gastric cancer lesions^[15].

Diagnostic value of X-ray in MPC compared with endoscopy

So far, there has not been a consensus on how best to diagnose mucosal lesions of the upper GI tract. Despite the diagnostic advantages of upper endoscopy, it is more expensive and requires more staff and technological expertise than upper GI X-ray. In financial terms, the test is not as effective if the cost is high. The cost of upper endoscopy is 3 to 4-fold more expensive than that of upper GI X-ray in Japanese gastric cancer screening programs^[19,20]. Furthermore, it is unlikely that upper endoscopy would be feasible as a mass screening program, even in highly developed countries such as Japan, because of a lack of experienced endoscopists^[19,21]. It is also a more invasive procedure than the barium examination, and associated with a small but measurable risk of complications related to sedation on perforation of the upper gastrointestinal tract^[22,23]. In addition, it is difficult to find lesions located in the upper and middle thirds of the stomach because of technical difficulties of forward-viewing endoscopy.

In a review of a large series of gastric cancers, ma-

lignant tumor was diagnosed or suspected by the use of double-contrast technique with a sensitivity of 96%^[24]. This is comparable with the reported sensitivity of endoscopy and biopsy of 94% and 99%, respectively^[25]. In the same study, only 4% of all patients had been recommended for endoscopy because of the unequivocal findings in double-contrast studies^[24]. In our investigation, preoperative upper radiographic examination detected 100 out of 119 lesions (84.03% sensitivity). Regarding the depth of invasion, 18 (52.94%) of the T₁ lesions were accurately detected during preoperative diagnosis by radiographic examination; moreover, only 3 (3.53%) of the T₂₋₄ lesions were misdetected. As a result, double-contrast studies have a high sensitivity in the diagnosis of upper gastrointestinal carcinoma. As the double-contrast study is safer and less expensive than endoscopy, and also has high diagnostic sensitivity (84.03%), we believe that it is an excellent technique for the detection of synchronous multiple primary carcinomas of the upper GI tract. On the other hand, application of hypotonic measures to restrict the motility of the esophageal or stomach wall, which can better display the fine structure of the stomach, greatly improve the detection rate of smaller lesions and reduce the rate of misdiagnosis.

It is also important to recognize that barium studies are also operator dependent, and our experiences are as follows: (1) Radiologists should recognize the characteristics of multiple primary carcinomas of the upper GI tract, and special attention should be given to elderly male patients to avoid missing synchronous lesions; (2) In order to avoid misdiagnosis, when one lesion is found on barium images, the entire upper GI tract should be carefully evaluated for other synchronous lesions; (3) When esophagus or gastric cardia are seriously obstructed, measures such as hypotonic drugs, diluted barium, multi-position and delayed check should be taken; and (4) It should also be noted that adjacent multiple foci should be carefully observed to judge whether normal tissue exists between two lesions, in order not to automatically make a diagnosis of local extension or metastasis of another lesion.

As to early gastric cancer, the following points should be noted during double-contrast barium examination: (1) Good filling is essential for displaying abnormal gastric margins, 300-350 mL is an appropriate amount to extend the gastric body; (2) Middle or small volumes would be suitable for depressed lesions; (3) Mobile technology and thin-layer technology is necessary. After the lesion is found, local fine structure around the stained lesions should be repeatedly observed under flowing conditions; (4) For depression lesions of the greater curvature, the check bed can first be level and then gradually become erect. In the process, observe the barium slowly down from the upper stomach along the greater curvature, so the positive outlook of depressed lesions can be fully displayed; and (5) For lesions of the anterior, compression methods can best show the lesions.

The incidence of multiple synchronous upper gastrointestinal cancers is increasing gradually; early diagnosis, and thus screening of patients at risk, is key. We believe

that hypotonic double-contrast upper gastrointestinal examination is a sensitive, safe, noninvasive and relatively inexpensive global examination, which can serve as the first choice for the screening and diagnosis of synchronous upper gastrointestinal MPC, especially in China. Furthermore, careful radiographic follow-up is also required to detect metachronous lesions at the earliest possible stage, which will substantially increase patient survival.

COMMENTS

Background

The incidence of multiple synchronous upper gastrointestinal (GI) cancers is increasing gradually, but for some reason, some synchronous primary lesions can often be overlooked at the time of diagnosis. Accurate diagnosis of these synchronous primary lesions before operation is crucial because it can significantly alter clinical management. However, so far, there has not been a consensus on how best to diagnose mucosal lesions of multiple primary carcinomas in the upper GI tract: endoscopy or barium radiography?

Research frontiers

Compared with endoscopy, double-contrast upper GI examination is a safe, non-invasive, inexpensive and cost-effective test for providing accurate information about lesion morphology, location, size, and interval distance of these tumors. In addition, because of improved imaging modalities and application of hypotonic measures to restrict the motility of esophageal or stomach wall which can better display the fine structure of the GI tract, the detection rate of smaller lesions is greatly improved with a reduced rate of misdiagnosis.

Innovations and breakthroughs

Synchronous multiple primary carcinomas (MPC) in the upper GI tract mostly occurred in males, 86.21% of the patients were older than 55 years, and average age was 62.7 years old; the fact that elderly individuals aged 65 years or more are at greater risk for multiple gastric carcinomas is clinically significant. Esophageal MPC (EMPC) and esophageal-gastric MPC (EGMPC) are the main types of MPC in the upper GI tract. In esophageal lesions of EMPC/EGMPC, hyperplastic and medullary types are commonly seen; the lesions are mainly located at the middle and lower segment of the esophagus. Gastric lesions are usually located at the gastric cardia, and hyperplastic type is mostly seen. This series supports the concept that double-contrast upper GI examination is safer and less expensive than endoscopy, and also has high diagnostic sensitivity (84.03%); it can serve as an excellent technique for the detection of synchronous MPC of the upper GI tract.

Applications

Hypotonic double-contrast upper GI examination is a sensitive, safe, noninvasive and relatively inexpensive global examination, which can serve as the first choice for the screening and diagnosis of synchronous upper gastrointestinal MPC, especially in China.

Terminology

Synchronous MPC is defined as two or more primary carcinomas occurring in an individual simultaneously. Most of these synchronous cancers are in the head and neck region; other frequently reported sites of synchronous cancer associated with esophageal cancer are the stomach, lung, and urinary bladder. Their coexistence can be problematic for surgeons, oncologists and pathologists with regard to diagnosis, treatment, and follow-up.

Peer review

It is very interesting for the readers. It is well written, the data is very valuable and the conclusions applicable. It should be accepted for publication in the journal.

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Breviscapine attenuates acute pancreatitis by inhibiting expression of PKC α and NF- κ B in pancreas

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Abstract

AIM: To study the effect of breviscapine (Bre) on activity of protein kinase C α (PKC α) and nuclear factor (NF)- κ B in pancreas, and the mechanism of Bre attenuating acute pancreatitis (AP).

METHODS: One hundred and eight rats were randomly divided into acute necrotizing pancreatitis (ANP) group, Bre group (ANP + Bre group) and sham operation (SO) group, 36 rats in each group. ANP model was induced by a retrograde injection of 4% sodium deoxycholate into the bilio-pancreatic duct. Fifteen minutes after the ANP model was induced, the rats in Bre group were intraperitoneally injected with Bre (0.4 mg/100 g body weight or 0.1 mL/100 g body weight). Survival time and mortality of rats were calculated. Serum amylase and malondialdehyde levels were measured, volume of ascites was recorded and morphology of pancreas

and lung was evaluated at 1, 5 and 10 h, after the ANP model was induced, respectively. Expressions of PKC α and subunit p65 of NF- κ B in pancreas were detected by immunohistochemistry and Western blotting.

RESULTS: The life span of rats was longer and the mortality was lower in Bre group than in ANP group 13.51 ± 5.46 vs 25.36 ± 8.11 ($P < 0.05$). The amylase and MDA levels as well as the volume of ascites were lower and the pathological changes in pancreas and lung were less in Bre group than ANP group ($P < 0.05$), indicating that the pancreatitis is less severe in Bre group than ANP group. The activation of PKC α and NF- κ B p65 in pancreas was induced rapidly and reached their peak at 1 h or 5 h after ANP, but their activity in Bre group was significantly inhibited.

CONCLUSION: Bre exerts its therapeutic effect on AP by inhibiting the activation of PKC α and NF- κ B p65 in pancreas.

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Key words: Breviscapine; Acute pancreatitis; Protein kinase C α ; Nuclear factor- κ B; Rat

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INTRODUCTION

The complete mechanism of acute pancreatitis (AP) has not been established so far. The initial events in this disorder occur in pancreatic acinar cells, including activation of zymogens in acinar cells and release of inflammatory cytokines. It has been shown that nuclear factor (NF)- κ B is a key regulator of the expression of many inflammatory molecules^[1,2]. In experimental pancreatitis, NF- κ B activation in acinar cells is one of the early events^[3], and the inhibition of NF- κ B activation attenuates inflammatory response and the severity of AP^[4,5]. However, the signaling mechanisms mediating NF- κ B activation are unclear.

One candidate for mediating NF- κ B activation in pancreatic acinar cells is protein kinase C^[6,7]. Protein kinase C (PKC) is a member of serine/threonine kinases comprising 10 isoforms that differ in their structures and regulations^[8]. These isoforms can be subdivided into conventional PKC, novel PKC, and atypical PKC isoforms based on their molecular structure and mode of activation. PKC α belongs to conventional PKC isoforms which can mediate pathological secretory processes in experimental pancreatitis and regulate expression of inflammatory mediators^[9]. PKC inhibitors significantly reduce pancreatic and pulmonary levels of myeloperoxidase (MPO) and pancreatic protease activity, and further ameliorate inflammatory injury of organs^[10].

Breviscapine (Bre), a commercially available plant extract from the herb *erigeron breviscapus*, can inhibit PKC activation^[11,12]. Bre possesses comprehensive pharmacological effects and has been widely used in treatment of disorders in blood supply to the heart and brain, as well as ischemic diseases^[12,13]. Bre can elevate the activity of ATPase and superoxide dismutase SOD and reduce the malondialdehyde (MDA) level in brain mitochondria of rats after trauma^[14]. MDA as a product of thiobarbituric acid reactant, is considered a good indicator of lipid peroxidation and one of the useful makers for AP^[15]. It was reported Bre is effective against AP^[16]. However, its therapeutic mechanism is still unclear^[17]. In an attempt to further explore its mechanism in treatment of AP, a rat model of acute necrotizing pancreatitis (ANP) was induced to observe whether Bre exerts its protective effect against ANP by inhibiting the activation of PKC α and NF- κ B.

MATERIALS AND METHODS

Animals and materials

One hundred and eight Sprague-Dawley rats weighing 200-250 g, obtained from Experimental Animal Center of Fourth Military Medical University (Grade SPF Certificate No.2005-005), were fasted for 12 h with free access to water. Antibodies against PKC α and NF- κ B P65 were purchased from Santa Cruz Biotechnology Company (CA, USA). Secondary antibody and kits for immunohistochemistry were provided by Zhongshan Company (Beijing, China). Bre injection was purchased from Feixia Pharmacology Company (Harbin, China). Kits for amylase and

MDA were obtained from Nanjing Jiancheng Biotechnology Company (Nanjing, China). All other chemicals were those of the highest purity.

Induction of rat AP model

The rats were randomly divided into acute necrotizing pancreatitis (ANP) group, Bre group (ANP + Bre group) and sham operation (SO) group, 36 rats in each group. A rat model of ANP was induced by retrograde injection of 4% sodium deoxycholate (0.1 μ L/100 g body weight) into the bilio-pancreatic duct (BPD) as previously described^[18]. Briefly, a small median laparotomy was performed to exposed the pancreas, the BPD was temporarily closed at the hilum of liver with a small soft bulldog clamp to prevent reflux of the infused material into the liver, and then 4% sodium deoxycholate (100 μ L/100 g body weight) was injected into the distal BPD at a pressure of 50 cmH₂O. The clamp was removed 5 min after the injection of sodium deoxycholate. The rats in SO group only underwent laparotomy. Finally, the abdomen was closed with a silk suture. Fifteen minutes after the model was induced, the rats in Bre group were intraperitoneally injected with Bre (0.4 mg/100 g body weight or 0.1 mL/100 g body weight) and those in the other two groups were given the same volume of normal saline.

Preparation of serum and tissue samples

Twelve rats in each group were observed during 72 h, their survival time and mortality in 24 h were recorded. The rats were sacrificed at 1, 5 and 10 h, respectively, after the rat AP model was induced. Whole blood samples were centrifuged at 4°C, and serum was stored at -80°C for amylase and MDA analysis. Ascites volume was recorded, and pancreas and lung morphology was evaluated. Expressions of PKC α and subunit p65 of NF- κ B in pancreas were detected by immunohistochemistry or Western blotting.

Histopathologic score

Pancreas and lung were removed at 1, 5 or 10 h for morphological analyses after the model was induced, immediately immersed in 4% neutral phosphate-buffered paraformaldehyde for 12 h, embedded in paraffin, and cut into 5- μ m thick sections which were stained with H&E to observe the morphological changes under a light microscope. The severity of AP was blindly graded by a semi-quantitative assessment of vacuolization, edema, inflammatory cell infiltration and acinar cell necrosis as previously described^[19]. Ten microscopic fields were randomly chosen to observe them in each rat. Histological scoring of pancreatic tissue was performed to grade the extent of acinar cell vacuolization (0: none, 1: < 20% acini with vacuoles, 2: < 50% acini, 3: > 50% acini), edema (0: no edema, 1: interlobular edema, 2: intralobular edema 3: interacinar edema), inflammation (0: no inflammation, 1: inflammatory cells present at intralobular, 2: inflammatory cells present at intralobular 3: inflammatory cells present at interacini) and acinar cell necrosis (0: no necrosis, 1: < 10% necrosis, 2: < 40% necrosis, 3: > 40% necrosis).

Immunohistochemistry

Immunohistochemical analysis was performed. In brief, frozen pancreas was cut into 8- μ m sections which were air-dried and treated sequentially with acetone at 4°C for 10 min, three times of phosphate-buffered saline (PBS) at pH 7.4, 5% bovine serum album (BSA) in PBS at pH 7.4 for 30 min, PKC α (Santa Cruz Biotechnology Inc. sc-8393) diluted at 1:100 or NF- κ B P65 (Santa Cruz Biotechnology Inc. sc-8008) diluted at 1:80 in a humid chamber overnight, three times of 0.02% Tween 20 in PBS for 10 min each time, biotinylated secondary antibody diluted at 1/500 for 40 min. The bound peroxidase was visualized by reaction for 2-5 min in a solution containing 50 mg of 3, 3-diaminobenzidine (DAB), counterstained with hematoxylin, dehydrated and embedded. For PKC α in common status, the cytoplasm of positive cells was stained, and translocation of positive cells to membrane from cytoplasm meant activation of PKC α . For NF- κ B P65 in common status, the cytoplasm of positive cells was stained, and translocation of positive cells to nuclei from cytoplasm meant activation of NF- κ B P65. The slides were observed under a light microscope.

Western blotting

For Western blotting analysis, cellular proteins were prepared from pancreas with standard methods. Protein concentration was measured (Bio-Rad protein assay) and adjusted to 4 μ g/ μ L with loading buffer. The samples were boiled at 95°C for 5 min before they were loaded 10 μ L, subjected to SDS-polyacrylamide gel electrophoresis, and blotted to PVDF membranes. The membranes were probed with anti-PKC α diluted at 1/1000 (Santa Cruz Biotechnology Inc. sc-8393) and anti-NF- κ B P65 diluted at 1/1000 (Santa Cruz Biotechnology Inc. sc-8008). A low molecular weight protein marker was used to determine the size of bands detected by Western blotting.

Statistical analysis

Data were expressed as mean \pm SD, compared by non paired Student *t* test and one-way analysis of variance (ANOVA). *P* < 0.05 was considered statistically significant.

RESULTS

Bre reduced the mortality and prolonged the survival time of rats with AP

No rat in SO group died in 72 h. The mortality of rats in ANP group was 100% in 24 h with a life span of 13.51 ± 5.46 h, while the mortality of rats in Bre group was 50% with a life span of 25.36 ± 8.11 h (*P* < 0.01, Figure 1A).

Bre decreased the production of ascites and the serum amylase level in rats with AP

Ascites was not produced in SO group at any time points. The volume of ascites was 4.46 ± 1.62 mL at 1 h and 8.57 ± 2.38 mL at 5 h after the model was induced in ANP group. The volume of ascites was smaller in Bre group than in ANP group (*P* < 0.01, Figure 1B).

The serum amylase level was significantly higher at 1, 5, and 10 h after the model was induced in ANP group than in SO group (*P* < 0.01). The amylase activity was lower in Bre group at each time point than in ANP group (*P* < 0.05, Figure 1C).

Bre improved the pancreas and lung morphology in rats with AP

The pancreas and lung morphology was significantly different in rats with AP. Sodium deoxycholate caused mild interlobular edema at 1 h, and inflammatory cell infiltration, hemorrhage and acinar cell necrosis at 5 and 10 h after the model was induced. The morphological change of pancreatic tissue was milder (Figure 1D) and the morphological score was lower in Bre group than in ANP group (*P* < 0.01, Figure 1E). In contrast to the rats in SO group, those in ANP group exhibited severe inflammation of the lungs as indicated by alveolar fluid accumulation and progressive thickening of the interalveolar tissue, while those in Bre group showed milder morphological changes in lungs.

Bre inhibited the production of MDA in rats with AP

The MDA increased significantly with the development of AP in ANP group. The MDA level was significantly lower in Bre group than in ANP group at 1, 5, and 10 h after the model was induced (*P* < 0.05, Figure 1F).

Bre suppressed the expression of PKC α and NF- κ B P65 in pancreas of rats with AP

Immunohistochemical analysis showed no PKC α and NF- κ B P65 expression in acinar cells of rats in SO group at any time points. PKC α was detected at 1 h after the model was induced and localized in cytoplasm of acini (Figure 2A). However, the PKC α expression increased in cytoplasm at 5 h after the model was induced and could be detected on the membrane of acini. Only little staining of acini was observed at 10 h after the model was induced. The expression of NF- κ B P65 was detected at 1 h after the model was induced and localized in cytoplasm or nuclei of acini and inflammatory cells (Figure 2B). The NF- κ B P65 expression level in pancreas was lower at 5 and 10 h than at 1 h after the model was induced AP (*P* < 0.05). Western blotting showed that the expression level of PKC α and NF- κ B P65 in pancreatic tissue was significantly lower in Bre group than in ANP group (*P* < 0.05, Figure 2C).

DISCUSSION

PKC can mediate NF- κ B activation in pancreatic acinar cells^[6,7] and pathological secretory processes in experimental pancreatitis, and regulate the expression of inflammatory mediators^[9]. In our study, ANP was induced with sodium deoxycholate and the expression of PKC α and NF- κ B P65 in frozen sections of pancreas tissue was observed by immunohistochemical analysis. PKC α was detected only in acini, while NF- κ B P65 was localized in acini and inflammatory cells. The expression of PKC α and NF- κ B P65 increased

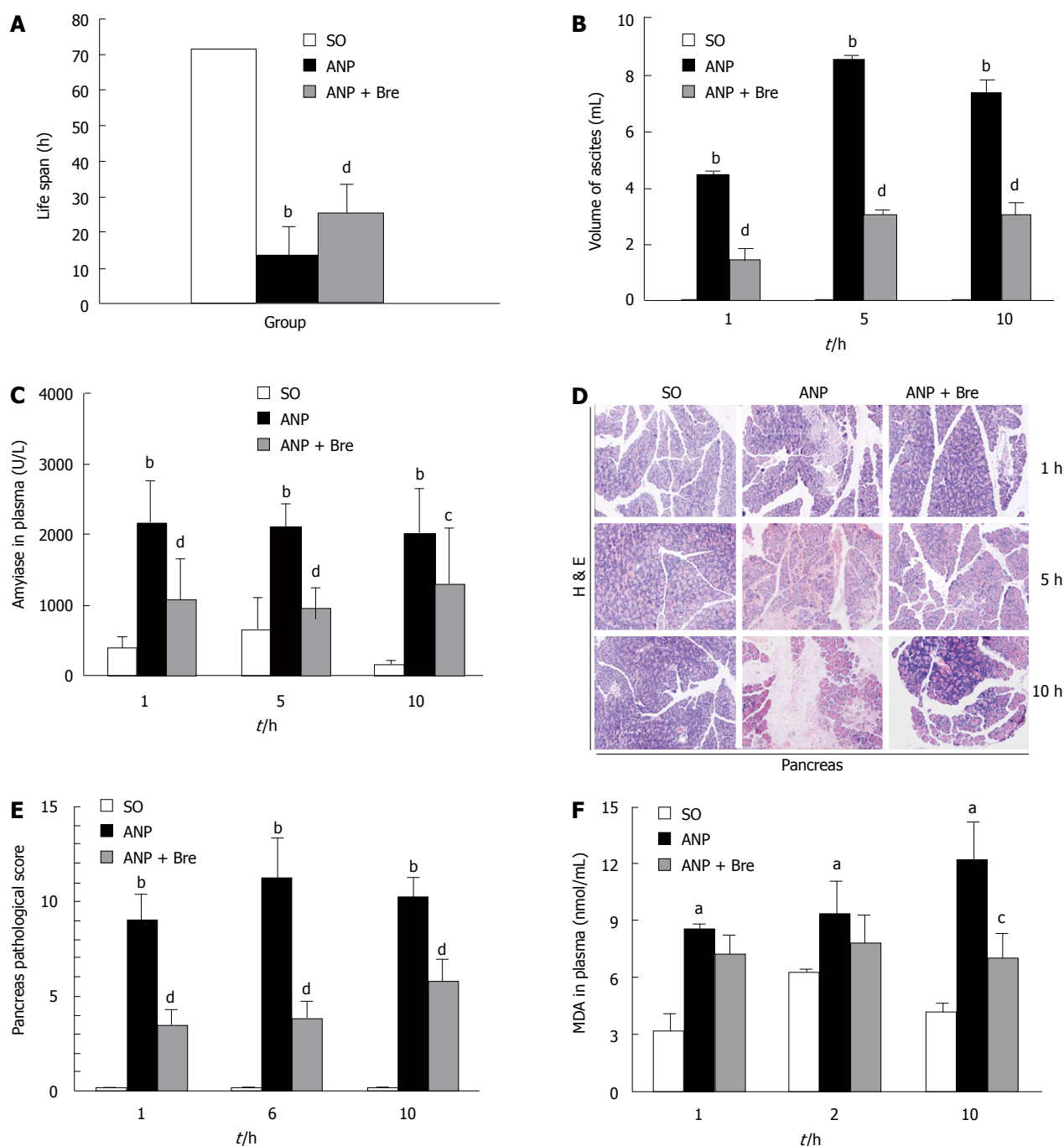


Figure 1 Effect of breviscapine on mortality (A), ascites volume (B) and amylase activity (C), morphological change (D), morphological score (E), and malondialdehyde level (F) in rats with acute pancreatitis. ^a*P* < 0.05, ^b*P* < 0.01 vs SO group; ^c*P* < 0.05, ^d*P* < 0.01 vs ANP group. Bre: Breviscapine; SO: Sham operation; ANP: Acute necrotizing pancreatitis; MDA: Malondialdehyde.

in the early stage of ANP, and both PKC α and NF- κ B P65 played an important role in the development of ANP.

It was reported that NF- κ B can be activated by protein kinase C and intracellular Ca²⁺[6,7], suggesting that PKC is an upstream regulator of NF- κ B activation in pancreatic acinar cells. In the present study, the effect of Bre, a PKC inhibitor, on ANP and activation of PKC α and NF- κ B was observed, showing that Bre could decrease the mortality, the severity of AP, the ascites production, the serum amylase level in rats with ANP, and prolonged their survival time. The morphological changes in pancreas and lungs of rats with ANP after treated with Bre were milder, sug-

gesting that PKC is related to AP and can alleviate the pathological injury by inhibiting PKC. Furthermore, the serum MDA level was lower in Bre group than in ANP group, suggesting that Bre can eliminate the production of oxygen-free radicals and further attenuate the harmful influence of peroxidation on pancreas and lungs^[20,21]. The expression of PKC α and NF- κ B P65 was significantly lower in Bre group than in ANP group, indicating that Bre can inhibit the activation of PKC α and NF- κ B which are important for the release of many inflammatory molecules and inflammatory response^[3,22,23].

In conclusion, PKC activation may contribute to in-

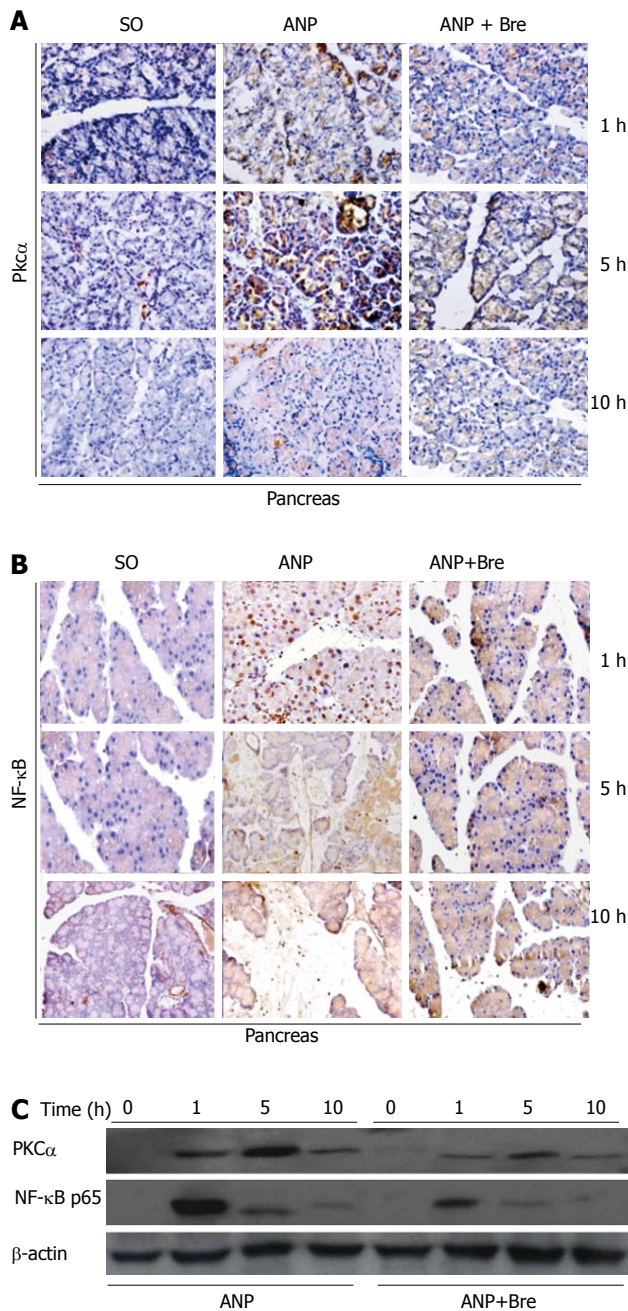


Figure 2 Immunohistochemical analysis showing expression of protein kinase C alpha (PKC α) (A) and nuclear factor (NF)- κ B P65 (B) in cytoplasm, membrane or nuclei of acini and inflammatory cells in pancreas, and western blotting showing the expression level of PKC α and NF- κ B P65 in breviscapine and acute necrotizing pancreatitis groups at different time points (C). Bre: Breviscapine; SO: Sham operation; ANP: Acute necrotizing pancreatitis.

flammatory response and cell injury in pancreas and lungs. Bre exerts its protective effect against ANP by inhibiting PKC and NF- κ B activation, which can explain the therapeutic mechanism of Bre for AP at molecular level.

COMMENTS

Background

Inflammatory response is important in the development of acute necrotizing pancreatitis (ANP). Nuclear factor (NF)- κ B can regulate the expression of many

inflammatory molecules. One candidate for mediating NF- κ B activation in pancreatic acinar cells is protein kinase C (PKC). Breviscapine (Bre) extracted from erigeron can inhibit the activation of PKC.

Research frontiers

Some studies demonstrated that PKC activation plays an important role in the development of acute pancreatitis (AP). PKC inhibitor can significantly reduce pancreatic protease activity and myeloperoxidase (MPO) level in pancreas or lungs, and further alleviate the inflammatory injury of organs.

Innovations and breakthroughs

As an inhibitor of PKC activation, Bre is effective against AP. However, its therapeutic mechanism is still unclear. In the authors' present study, a rat model of ANP was induced to observe the effect and mechanism of Bre on ANP, showing that Bre exerts its protective effect against ANP by inhibiting PKC and NF- κ B activation.

Applications

The results of this study suggest that PKC activation may contribute to inflammatory response and cause cell injury in pancreas and lungs, which may explain the therapeutic mechanism of Bre for AP at biological level and accelerate its applications in treatment of AP.

Terminology

PKCs are a family of serine/threonine kinases comprising 10 isoforms that differ in their structures and regulations. These isoforms can be subdivided into three classes based on their molecular structure and mode of activation, namely conventional PKC isoforms, novel PKC isoforms, and atypical PKC isoforms. PKC α belongs to conventional PKC isoforms which can mediate pathological secretory processes in experimental pancreatitis and regulate the expression of inflammatory mediators.

Peer review

In this study, the authors showed that Bre exerts its protective effect against ANP by inhibiting PKC and NF- κ B activation. As a PKC inhibitor, Bre can significantly reduce pancreatic protease activity and MPO level in pancreas or lungs, and further alleviate the inflammatory injury of organs, and can thus be applied in treatment of AP.

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Predictive factors of clinical response in steroid-refractory ulcerative colitis treated with granulocyte-monocyte apheresis

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7) (OR = 0.770, 95% CI: 0.425-1.394, Wald 3.747, $P = 0.028$) proved to be effective as factors predicting clinical response.

CONCLUSION: GMA may be a valid therapeutic option for steroid-dependent ulcerative colitis patients with mild-moderate disease and its clinical efficacy seems to persist for 12 mo.

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Key words: Granulocyte-monocyte apheresis; Ulcerative colitis; Steroid therapy; Long-term follow-up; Predictive factors

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Abstract

AIM: To identify factors predicting the clinical response of ulcerative colitis patients to granulocyte-monocyte apheresis (GMA).

METHODS: Sixty-nine ulcerative colitis patients (39 F, 30 M) dependent upon/refractory to steroids were treated with GMA. Steroid dependency, clinical activity index (CAI), C reactive protein (CRP) level, erythrocyte sedimentation rate (ESR), values at baseline, use of immunosuppressant, duration of disease, and age and extent of disease were considered for statistical analysis as predictive factors of clinical response. Univariate and multivariate logistic regression models were used.

RESULTS: In the univariate analysis, CAI ($P = 0.039$) and ESR ($P = 0.017$) levels at baseline were singled out as predictive of clinical remission. In the multivariate analysis steroid dependency [Odds ratio (OR) = 0.390, 95% Confidence interval (CI): 0.176-0.865, Wald 5.361, $P = 0.0160$] and low CAI levels at baseline ($4 < \text{CAI} <$

D'Ovidio V, Meo D, Viscido A, Bresci G, Vernia P, Caprilli R. Predictive factors of clinical response in steroid-refractory ulcerative colitis treated with granulocyte-monocyte apheresis. *World J Gastroenterol* 2011; 17(14): 1831-1835 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i14/1831.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i14.1831>

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease characterized by periods of remission and clinical relapses most likely related to a multifactorial dysfunction of the mucosal immune system^[1].

The available pharmacological therapies are aimed at inducing remission and preventing relapses. In active UC, depending on the site of disease and on disease activity, the principle therapy is represented by oral and topical steroids with maintained remission in half of the examined patients (49%) at a 1 year follow-up. Steroids, which

offer a good initial response rate, are however ineffective as maintenance therapy and well-known side-effects are observed in 22% of the patients who develop steroid dependency/resistance^[2].

In those patients with chronic active disease, unresponsive to steroids, immunomodulators are largely used even though these treatments are limited by side-effects occurring in 6%-30% of cases as well as concerns over long-term toxicity^[3]. In severe disease, as azathioprine has a delayed onset of efficacy, cyclosporine or tacrolimus may offer an alternative. The encouraging development of biologicals, such as infliximab (an anti-TNF α antibody showing effect in moderate to severe UC), is counterbalanced by the need for long-term efficacy and safety profile evaluations^[4,5].

Granulocyte-monocyte apheresis (GMA), a selective apheresis technique, represents a recent alternative therapeutic option. Several open trials have been performed so far, showing efficacy rates up to 65%-75% associated with an excellent safety profile^[6-13].

While its safety has been largely proven in the treatment of steroid refractory/dependent UC patients, few and conflicting results are available concerning the long-term follow-up and factors predicting clinical response, therefore current data do not help in identifying UC patients eligible for GMA treatment. The aim of the present study has thus been to single out factors predicting the clinical response to GMA.

MATERIALS AND METHODS

Patients

Between April 2005 and January 2008, a total of 69 patients with mild/moderate UC either steroid-dependent or resistant were selected for GMA treatment, from patients admitted to the GI Units of the Department of Clinical Sciences, Policlinico Umberto I and of Pisa Hospital.

The characteristics of the patient population (39 females, 30 males, mean age 42.36 years; range 27-75 years) are shown in Table 1. Mean disease duration was 41.34 mo (range 12-252 mo). Disease activity was mild/moderate in all the patients as established by a clinical activity index (CAI) value > 4; The mean clinical activity index (CAI) value was 9.10 (range 6-12; lower quartile = 8, median value = 9, upper quartile = 10, interquartile range = 2). Forty-seven patients presented left colitis and 22 pancolitis. Arthralgia was present in 15 patients.

All patients considered for GMA had failed to respond to mesalazine or sulphasalazine, and were under steroid treatment. Forty-nine patients had been found to be dependent on, and 20 resistant to, steroid treatment. The steroid-dependence/resistance have been respectively defined: relapse within 30 d after a complete tapering or at dose reduction impeding discontinuation of prednisolone treatment for more than one year; No response within 30 d at standard maximum daily dosage of prednisone^[14].

The 20 patients resistant to steroids had started azathioprine (at a dose of 1.5-2.5 mg/kg per day) from 6 mo before enrolment.

Major exclusion criteria were severe cardiovascular

Table 1 Characteristics of granulocyte-monocyte apheresis treated patients: Demographic and clinical data

Sex	F/M	39/30
Age (yr)	mean (range)	42.36 (27-75)
Duration of disease (mo)	mean (range)	41.34 (12-262)
Location of disease	Left side/pancolitis	47/22
Steroid course	Dependent/resistant	49/20
CAI	mean (range)	9.10 (6-13)
ESR (mm/h)	mean (range)	33.09 (2-20)
CRP (mg/dL)	mean (range)	2.04 (0.3-7.3)
WBC 10 ³	mean (range)	10.69 (5.7-14)

CAI: Clinical activity index; ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; WBC: White blood cell.

disease (acute myocardial infarction, brain hemorrhage, severe arrhythmia or heart failure) within the past 6 mo, severe renal failure, hypotension (< 80 mmHg systolic pressure), body weight < 35 kg, age < 12 years, pregnancy.

GMA treatment

After giving informed consent, all selected patients were submitted to GMA with Adacolumn (Otzuka Production, Milan, Italy). GMA was performed with the help of the Transfusional Unit of the two Institutions.

Each patient was submitted to 5-10 GMA sessions (1 session/wk), as a basic treatment course, according to the standard protocols^[15]. Each GMA session lasted 60 min.

In particular, enrolled patients were divided into two groups: Presenting mild and moderate disease, respectively, according to their baseline CAI levels (4 < CAI < 7, 7 < CAI < 12 respectively). Out of 69, 29 patients with mild disease were submitted to a 5 session treatment, whereas 40 patients with moderate disease were treated with a further cycle of GMA.

During the GMA sessions, the treatment with oral and topical mesalazine and/or azathioprine was continued, at a stable dosage. Steroid tapering was started after the first session, according to the response to therapy. At the end of the treatment period, all concomitant therapies were carried on (oral and topical mesalazine, azathioprine).

Assessment of clinical response

Clinical efficacy was assessed at enrolment, within the first week after the last GMA session (short-term follow-up) and at 12 mo (long-term follow-up).

All patients were evaluated by clinical and laboratory assessment, including full blood count, erythrocyte sedimentation rate (ESR), C reactive protein (CRP).

Disease activity was evaluated by measuring the CAI according to the Rachmilewitz's criteria^[16]. Clinical remission was defined as a final CAI value equal or < 4. A partial response was defined as a reduction of CAI score from baseline. The increasing of CAI value was classified as no response.

To complete the clinical assessment, QoL was also evaluated by a questionnaire adapted to the Italian population^[17], with 29 questions grouped into 4 domains: Intestinal symptoms, systemic symptoms, emotional and social

functions. Mucosal lesions were measured using the endoscopic score of Rachmilewitz, which evaluates the granular pattern (no = 0, yes = 2), the characteristics of the vascular pattern (normal = 0, impaired = 1, absent = 2), fragility of the mucosa (no = 0, upon touch = 2, spontaneously = 4) and mucosal lesions (no = 0, minor = 2, severe = 4). Endoscopic remission was defined by a score < 2 ^[16].

All responder patients underwent a clinical evaluation at 12 mo of follow-up. All the parameters collected were inserted in a standard database.

Predictive factors of clinical response

Demographic and clinical characteristics of enrolled patients were considered for statistical analysis as predictive factors of clinical response in the short-term follow-up. Steroid dependency, CAI, CRP and ESR values at baseline, use of immunosuppressant, duration of disease (mo), age (year) and extent of disease (left side, pancolitis), were analyzed. No complete laboratory and clinical data were available in the long-term follow-up, so that no data were analyzed to identify factors predicting a sustained response.

Statistical analysis

Univariate analysis of parameters considered at baseline was performed by using the *t* test and the McNemar test for paired data, whereas comparing the same parameters between responders and non-responders, univariate analysis was performed using the *t* test for unpaired data. The parameters proving significance ($P < 0.05$) on univariate analysis were entered into a multivariate logistic regression model with the calculation of relative risk as odds ratios (OR) with 95% confidence intervals (CI), in order to determine the independent contributors to remission.

RESULTS

Assessment of clinical response to GMA treatment

Twenty-nine mild UC patients (CAI < 7) were treated with 5 GMA sessions, and 40 moderate UC patients (CAI < 12) were treated with 10 GMA sessions, following standard protocols. All patients completed the GMA sessions. During the overall 545 sessions of apheresis no major side-effects were registered. Two patients experienced dizziness and headache and one patient experienced a transient episode of arrhythmia.

At 11 wk of follow-up, 40 patients showed a complete clinical remission associated with endoscopic response. The end of treatment corresponded to the complete tapering of steroids in all cases. A partial response was evidenced in 10, and no variation in 19 patients.

As far as the CAI was concerned, the mean value dropped from 9.1 (range 6-13) at enrolment to 4.2 (range 0-10) at the end of GMA treatment ($P < 0.01$). Indeed, a final CAI value < 4 was observed in 40 patients.

In consideration of the individual parameters contributing to the CAI evaluation, complete disappearance of urgency and tenesmus was observed in 38 out of 40 responder patients. In 5 out of 15 patients, peripheral arthralgia

Table 2 Univariate analysis of predictive factors of clinical response to granulocyte-monocyte apheresis: Age, White blood cells, erythrocyte sedimentation rate and clinical activity index score, C reactive protein, location duration of disease at baseline and use of immunosuppressant (mean \pm SD)

	Responders (n = 40)	Non responders (n = 29)	P-value
Age (yr)	42.7 \pm 13.8	41.9 \pm 6.6	0.774
WBC 10 ³	10.2 \pm 2.7	11.2 \pm 2.2	0.106
ESR (mm/h)	30.6 \pm 13.5	38.4 \pm 12.5	0.017
CAI	8.6 \pm 2.1	9.7 \pm 2.2	0.039
CRP (mg/dL)	1.2 \pm 1.2	1.4 \pm 1.4	0.526
Location of disease (left side/pancolitis)	28/12	19/10	0.795
Duration of disease (yr)	11	6	0.581
Use of immunosuppressant	5	15	0.601

CAI: Clinical activity index; ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; WBC: White blood cells.

disappeared. The mean QoL score significantly decreased from 37.9 (range 18-67) to 24.4 (range 7-42) ($P < 0.01$).

ESR, CRP and WBC decreased at the end of the GMA sessions as follows: mean ESR decreased from 33.9 mm/h (range 7-60 mm/h) to 20.1 mm/h (range 2-20 mm/h) ($P < 0.01$); Mean CRP decreased from 2.04 mg/dL (range 0.3-7.3 mg/dL) to 0.29 mg/dL (range 0.15-1.5 mg/dL) ($P = \text{NS}$); Mean WBC count decreased from 10.59 k/ μ L to 7.7 k/ μ L (range 5.7-14 k/ μ L) ($P < 0.01$).

The endoscopic evaluation improved greatly in 40/69 patients with a score ranging from a mean value of 8.08 (range 4-10) to 3.9 (range 1-8). Thirteen patients presented an improvement with the reduction of at least 1 grade of inflammation before the end of treatment. In 16 patients, the endoscopic aspect showed no change.

At 12 mo of follow-up, 46 out of 50 responder patients were evaluated; Thirty-two of them were still in clinical remission (CAI < 4). Twelve patients presented an early moderate relapse (CAI < 12) and started azathioprine (at a dose of 1.5-2.5 mg/kg per day). The remaining 2 patients showed a severe relapse (CAI > 13) and underwent surgery and biological therapy with infliximab, respectively.

Assessment of predictive factors of clinical response

At univariate analysis the differences between responders and non-responders was analyzed. Out of the considered parameters, only CAI ($P = 0.039$) and ESR ($P = 0.017$) levels at baseline were singled out as independent contributors to predict remission in the short-term follow-up, with low rates in the responders group (Table 2).

The logistic regression identified steroid dependency as an independent predictor of clinical response (OR = 3.71, 95% CI: 1.239-11.129, $P = 0.016$) (Figure 1).

Multivariate analysis revealed that steroid dependency (OR = 0.390, 95% CI: 0.176-0.865, Wald 5.361, $P = 0.016$) and low CAI levels at baseline ($4 < \text{CAI} < 8$) (OR = 0.770; 95% CI: 0.425-1.394, Wald 3.747, $P = 0.028$) were independent predictors of favorable clinical response in the short-term follow-up (Table 3).

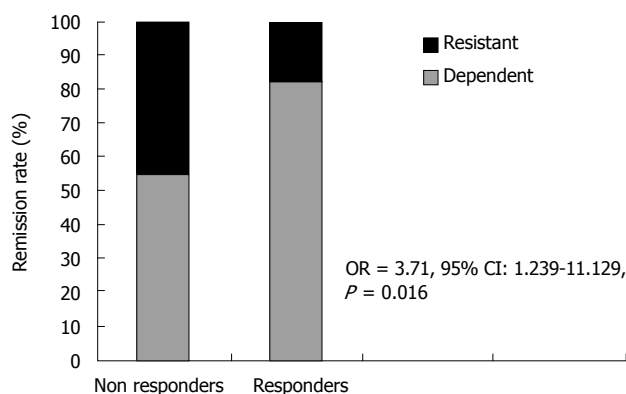


Figure 1 Correlation between clinical remission rate and steroid course. OR: Odds ratio; CI: Confidence interval.

DISCUSSION

GMA induced a complete response in 40 out of 69 patients with mild-moderate UC, with a significant reduction of CAI score levels, ESR, CRP, numbers of circulating neutrophils, as well as a significant improvement in QoL in accordance to the literature data^[18-25]. Sustained remission was also observed in the follow-up in half of the treated patients.

A few heterogeneous studies have been carried out for identifying predictive factors of clinical response to GMA, but to our knowledge predictive factors of sustained remission have not been so far reported.

Moreover, small studies using Adacolumn (GMA) have reported that high CAI level at baseline represented a significant risk factor for GMA failure and that cumulative doses of steroids, previously used for UC treatment, were inversely related to clinical efficacy^[21,24]. Conversely, a relatively large study, performed using Cellsorba device (leukocytapheresis), demonstrated that a steroid-dependent course and a high CRP concentration were independent predictors of favorable response^[26].

The present data confirm that a steroid-dependent course is a predictor of clinical efficacy in GMA-treated patients. Thus, the present study is not in accordance with the previously reported negative association between remission and high cumulative dose of prednisone. Furthermore, these data support the use of standard treatment protocols in steroid-resistant patients, such as cyclosporine/tacrolimus, biological agents, immunosuppressant therapy, or surgery, rather than GMA, with regard to cost-effectiveness and time-saving.

Low baseline CAI scores were predictive of GMA treatment efficacy, thus confirming previously reported data and indicating GMA treatment only for mild-moderate disease.

Albeit a large multicenter, sham-controlled trial has been performed using GMA which did not support its effectiveness (response rate: 44% *vs* 39%, $P = \text{NS}$)^[27], in that protocol duration of the disease was not indicated as well as the duration of concomitant medication. As suggested by the authors, unlike other studies, the study group did not include mainly steroid dependent/refractory patients,

Table 3 Multivariate analysis of predictive factors of clinical response to granulocyte-monocyte apheresis: Steroid course and clinical activity index score at baseline

Independent variables	OR	95% CI	Wald	P-value
Steroid course	0.390	0.176-0.865	5.361	0.016
4 < CAI < 8	0.770	0.425-1.394	3.747	0.028
CRP (mg/dL)	0.908	0.381-2.166	0.047	0.828
ESR (mm/h)	0.965	0.491-1.896	0.011	0.917
Location of disease (Left side/pancolitis)	0.822	0.462-1.462	0.446	0.504
Duration of disease > 120 mo	1.089	0.485-2.466	0.043	0.836

CAI: Clinical activity index; ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; OR: Odds ratio; CI: Confidence interval.

thus the need for further studies for better defining the issue is strongly suggested.

A recent meta-analysis, drawn from 7 randomized controlled trials, has shown homogeneous evidence that confirm GMA is much more effective in UC as compared to conventional therapy^[28].

Despite limitations deriving from the retrospective nature of data analysis and the lack of long-term follow-up, the present study suggests that steroid dependency and mild disease represent good predictors of favorable clinical response to GMA treatment. The improvement in QoL related to the steroid-sparing effects was also relevant in the present series, suggesting an indication for GMA in pediatric UC patients, in whom steroid dependence is expected to interfere with a normal growth and development.

In conclusion, GMA may represent a useful therapeutic tool in steroid-dependent UC patients with mild to moderate disease, although further well designed sham-controlled studies are needed.

ACKNOWLEDGMENTS

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COMMENTS

Background

In a proportion of ulcerative colitis patients, the current pharmacological therapy is responsible for side-effects. A non-pharmacological approach, such as granulocyte-monocyte apheresis (GMA), proves effective in these patients and displays an excellent safety profile. Data are also available from studies involving pediatric patients showing good results, without side-effects. The efficacy of GMA has been documented by several studies, but few data are available on long-term follow-up and on factors predicting clinical response.

Research frontiers

This study showed that steroid dependency [Odds ratio (OR) = 4.097, $P = 0.01$] and low clinical activity index levels at baseline (OR = 0.745, $P = 0.02$) proved to be effective as factors predicting clinical response. GMA may be a valid therapeutic option for steroid-dependent ulcerative colitis patients with mild-moderate disease and its clinical efficacy appears to persist for 12 mo.

Innovations and breakthroughs

These findings support other published evidence on the association of clinical efficacy with an extremely favorable safety profile. The study also indicates that steroid dependency and low activity of the disease help identify those ulcerative colitis (UC) patients who are most likely to respond to treatment. Thus, GMA

represents a promising non-pharmacological approach to UC, to be used when stable remission cannot be obtained by conventional therapy. Its use in pediatric UC patients, in whom steroid dependence is expected to interfere with a normal growth and development, could be an important challenge.

Peer review

The paper is generally well written and results analysed with appropriate statistical methods. The size of the cohort is fair and is enough to make some meaningful recommendations. The results are clearly tabulated and noted in the results section. The discussion is clearly written and the conclusions are appropriate to the findings.

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Gastrointestinal stromal tumors: Thirty years experience of an Institution

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Abstract

AIM: To report our experience of gastrointestinal stromal tumors (GISTs) during the last 29 years.

METHODS: Thirty two cases of GIST referred to our Institution from the 1st January 1981 to the 10th June 2010 were reviewed. Metastases, recurrence and survival data were collected in relation to age, history, clinical presentation, location, size, resection margins and cellular features.

RESULTS: Mean age was 63.7 years (range, 40-90) and incidence was slightly higher in males (56%). R0 resection was performed in 90.7% of cases, R1 in 6.2% (2 cases) and R2 in 3.1% (one case). Using Fletcher's classification 8/32 (25%) had high risk, 9/32 (28%) intermediate and 15/32 (47%) low risk tumors. Follow-up varied from 1 mo to 29 years, with a median of 8 years; overall survival was 75% (24/32), disease-free survival was 72% and tumor-related mortality was 9.3%. Three patients with high risk GIST were treated with imatinib mesylate: one developed a recurrence after 36 mo, and 2 are free from disease at 41 mo.

CONCLUSION: Surgical treatment remains the gold standard therapy for resectable GISTs. Pathological and

biological features of the neoplasm represent the most important factors predicting the prognosis.

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Keywords: Gastrointestinal stromal tumors; Fletcher's classification; Resection margins; Recurrence

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that arise from the gastrointestinal tract and account for < 1% of all gastrointestinal neoplasms^[1,2]. The term "stromal tumor" was first used by Mazur and Clark^[3] in 1983; later the acronym GIST was introduced^[4,5] to define a well established pathological entity, whose constitutive elements derive from the interstitial cell of Cajal, an intestinal pacemaker cell, and express a highly specific marker called KIT (CD117).

The estimated incidence of GISTs is approximately 10-20 per million people annually worldwide^[6]. This tumor affects men slightly more often than women and the mean age at the time of diagnosis is 60 years^[7,8]. The majority of GISTs arise in the stomach (60%) and small bowel (30%); the remaining 10% in the esophagus and rectum^[9].

Clinical presentation is heterogeneous, even if GISTs are usually asymptomatic and are diagnosed incidentally during endoscopy, radiological imaging or abdominal exploration^[10,11]. Preoperative biopsy is not recommended for resectable masses^[12], because of the fragility and predisposi-

tion to hemorrhage of these masses, and the possibility that the biopsy needle touches a necrotic portion of the tumor. Biopsy is justified only for masses preoperatively judged unresectable, in that a definitive pathological diagnosis would allow medical treatment using imatinib to commence.

Surgery represents the gold standard treatment for resectable GISTs. Principles of a correct procedure include negative margins on the specimen and integrity of the pseudocapsule^[13]. GISTs do not metastasize through lymphatic spread, so systematic lymphadenectomy is not indicated.

Survival after 5 years is extremely variable in the reported series, ranging from 48% to 80%. This variability could be explained by the amount of knowledge of the disease and, most of all, by the introduction of the inhibitors of tyrosine kinases. Imatinib mesylate was first used as medical therapy for GIST in 2000^[14]. Several clinical trials later confirmed that imatinib was safe and effective in the treatment of advanced and metastatic GISTs^[15-17]. In 2009 the American College of Surgeons Oncology Group presented the results of a randomised Phase III Multicentre Trial that showed the effectiveness of imatinib as adjuvant therapy for primary GISTs in term of recurrence free and overall survival^[18].

The purpose of this study is to analyze pathological features and treatment modality of 32 cases of GIST operated on at San Luigi Teaching Hospital (Turin) during the last 29 years.

MATERIALS AND METHODS

The study is a retrospective analysis of 32 cases of GIST referred to our Institution between 1st January 1981 and 10th June 2010. In every case, the diagnosis of GIST was confirmed by a positive immunohistochemical assay for CD117. Before GISTs were recognized as well defined pathological entities and the CD 117 assay was available, GISTs were diagnosed as leiomyoma, fibroleiomyoma or leiomyosarcoma. All specimens labeled with these diagnoses were tested for CD 117 and the positive ones classified as GISTs and included in the study. In this group, metastases, recurrence and survival data were collected in relation to age, history, clinical presentation, location, size, resection margins and cellular features such as mitotic index and immunohistochemistry (Table 1).

RESULTS

During the last 29 years, 32 patients underwent surgical intervention for GIST at San Luigi Teaching Hospital. Mean age was 63.7 years (range, 40-90) and prevalence was slightly higher in males (56%). Among males the mean age was 62, while among females it was 66. Gastrointestinal bleeding with acute or chronic anemia represented the most common clinical presentation (16 cases, 50%), followed by non-specific abdominal pain (12 cases, 37.5%), dysphagia (one case, 3.1%) and lumbar pain (one case, 3.1%); in 2 cases (6.3%) GISTs were found incidentally on the resected specimen. The medical histories did not show any association between GISTs and associated pathologies, which were extremely heterogeneous.

The majority of tumors were located in the stomach (18 cases, 56.3%); 2 in the antrum, 3 in the fundus, 13 in the corpus (5 in the anterior wall, 3 in the posterior wall, 2 along the great curve and 3 along the small curve). The others were found in the small bowel (10 cases, 31.3%; 7 in the ileum, 2 in the jejunum and one in the duodenum), mesentery (2 cases, 6.2%), esophagus (one case, 3.1%) and rectum (one case, 3.1%). Tumor size varied between 1 and 30 cm, with a mean diameter of 7 cm. Even though wedge or segmental resection was the most frequent surgical procedure, one anterior rectal resection, 4 total gastrectomies, one subtotal and one extended to the pancreatic tail, were performed. Only 4 operations (12.5%) were executed laparoscopically for tumors with a mean diameter of 4.5 cm: one segmental resection of the ileum and 3 gastric resections, one of which was converted. R0 resection was performed in 90.7% of the cases, R1 in 6.2% (2 cases) and R2 in 3.1% (one case). Using Fletcher's classification^[19], 8 out of 32 cases (25%) were high risk, 9 (28%) intermediate and 15 (47%) low risk tumors.

Follow-up varies from 1 mo to 29 years, with a median of 8 years. To date, 24 patients (75%) are alive; 8 patients (25%) died: 3 patients (9.3%) died due to recurrence at 13, 14, and 36 mo after the first operation, one died from lung cancer, 2 from gastric cancer, one from gallbladder cancer and one died of AIDS. Among the 24 surviving patients, only one, who had a high risk GIST, developed a recurrence and underwent a second surgical intervention, so that to date 23 patients (72%) are actually free from disease. If we consider a 3-year follow-up, we can include 25 patients from our series. In this group overall survival is 68% (17 out of 25) and disease-free survival is 64% (16 out of 25).

The rate of metastatic disease did not exceed 9.3% (3 cases); in 2 cases the primary tumor was a high risk GIST (a rectal GIST with liver metastasis and a mesenteric GIST with ileal metastasis), but, interestingly, in one case the patient was affected by a low risk jejunal tumor with lung metastasis.

Two patients underwent an R1 resection and one an R2 resection; they were treated with imatinib mesylate: one patient (R2) developed a recurrence after 36 mo that required a second surgical intervention followed by continued therapy with imatinib (no other tyrosine kinase inhibitors were available); 2 patients (R1) are free from disease at 41 mo. Tumors were located in the rectum, ileum and stomach, respectively.

DISCUSSION

During the last 10 years, since the GIST has been recognized as a well-defined pathological entity with its own characteristics, the surgical management of GISTs has changed. The lack of lymphatic spread of this kind of tumor makes lymphadenectomy unnecessary, so the only oncological criteria is to maintain the integrity of the capsule and to perform an R0 resection. Wedge resection is a correct procedure from an oncological point of view, but if technically unfeasible, as for esophageal or rectal GISTs, a segmental resection becomes necessary. Our experience was over a period of 29 years. Patients operated before 2000 underwent more extensive resections than patients operated after 2000, without any difference in overall survival or disease-

Table 1 Distribution of gastrointestinal stromal tumors by site and their features

Organs	Number of cases	Low risk	Moderate risk	High risk	Mitotic index > 5 × 50 HPF	Mean size (cm)	CD117+	CD34+	Ki67 > 10%
Esophagus	1	1	/	/	/	2.5	1	/	/
Stomach	18	11	6	1	5	5.4	18	8	7
Duodenum	1	/	1	/	/	5.0	1	/	/
Jejunum	2	1	/	1	1	8.4	2	1	1
Ileum	7	2	2	3	3	9.7	7	4	4
Rectum	1	/	/	1	1	15.0	1	1	1
Mesentery	2	/	/	2	1	8.0	2	1	1
Total	32	15	9	8	11	7.0	32	15	14

HPF: High power field.

free survival. The types of intervention were extremely heterogeneous, demonstrating that there is no standardized procedure to approach this kind of neoplasm. Epidemiological features found in our series are comparable with the literature: DeMatteo *et al*^[20] reported a median age of 58, with predominant localization of the tumors in the stomach (39%) followed by small bowel (32%); Ahmed *et al*^[21] reported a mean age of 64.4, with tumors mainly localized in the stomach (52%) and colon (13%). Clinical presentation is extremely heterogeneous in the literature as in our series: a recent Swedish study demonstrated that 70% of GISTs had associated symptoms, 20% had none and 10% were detected at autopsy^[22]. Symptoms were generally non specific: nausea, vomiting, abdominal pain or discomfort; sometimes GISTs caused gastrointestinal bleeding, because of the erosion of gastric or small bowel mucosa; dysphagia occurred rarely, and was associated with a tumor located in the esophagus; biliary obstruction could occur if the tumor was located in the duodenum; and intussusception, could occur if the tumor was located in the small bowel.

The R0 resection rate was higher in our series (90.7%) than in others: DeMatteo *et al*^[20] reported 47%; Ahmed *et al*^[21] 51%. The difference can be explained by the larger number of patients in those series and the larger number of locally advanced or metastatic GISTs treated. In our series, 9.3% of the patients died of disease. Ahmed reported 11% with a mean follow-up of 6.8 years and De Matteo 50% with a mean follow-up of 24 mo. The difference is due to the large number of patients with GISTs at low and moderate risk of relapse in our series compared with that of De Matteo.

Four laparoscopic operations (12.5%) were performed in our series for tumors with a mean diameter of 4.5 cm located in the anterior wall of the stomach and in the jejunum; none of these patients developed local or distant recurrence. DeMatteo and Ahmed did not deal with a laparoscopic approach to GISTs. Even if our laparoscopic series is limited to 4 operations we report results of other series, avoiding every comparison with our own. Novitsky *et al*^[23] reported a series of 50 laparoscopic gastric resections for GISTs. Mean diameter of the neoplasm was 4.4 cm; the conversion rate was 0% and disease-free survival 92% at 36 mo. Huguet *et al*^[24] reported a series of 33 patients affected by gastric GISTs of mean diameter of 3.9 cm and treated with a laparoscopic approach. The conversion rate was 6% and disease-free survival 100% with a median

follow-up of 13 mo. Similar results were obtained by Basu *et al*^[25], Nishimura *et al*^[26] and Pitsinis *et al*^[27]. Tabrizian *et al*^[28] reported a series of 76 laparoscopic resections for GISTs. Of these, 72% were located in the stomach and 28% in the small bowel, with mean diameter of 4.2 and 3.9 cm, respectively. The conversion rate was 14% and disease-free survival 78% at 41 mo (77% gastric *vs* 82% small bowel). Laparoscopic treatment of GIST is a safe and effective procedure, but should only be performed at centers with excellent laparoscopic experience, taking strict oncological precautions to avoid rupture of the pseudocapsule and spreading of neoplastic cells. If these precautions cannot be assured, the surgeon must not hesitate to convert, because this kind of mistake can change a curable disease into a poor prognostic one and this must be avoided. International guidelines recommend the employment of laparoscopic surgery only for tumors smaller than 5 cm^[13].

In conclusion, surgical treatment remains the gold standard therapy for resectable GISTs. Surgical strategies are different and heterogeneous, as the only oncologic criteria imposes the preservation of the integrity of the capsule and the avoidance of infiltration of the resection margins. The laparoscopic approach is considered safe and effective for masses not exceeding 5 cm, in centers experienced in advanced laparoscopic surgery. In the presence of resectable and non metastatic masses, correctly removed by the surgeon, pathological and biological features of the tumor, expressed by Fletcher's classification, remain the most important factors for predicting the prognosis. For high risk or metastatic tumors as for non resectable masses, molecular therapy with the tyrosine kinase inhibitor imatinib mesylate has improved survival. The use of the laparoscopic technique in combination with molecular therapy will permit the development of a minimally invasive treatment for this type of neoplasm, improving patients' survival and quality of life.

COMMENTS

Background

Gastrointestinal stromal tumors (GISTs) are tumors arising from the wall of the gastrointestinal tract, from the esophagus to the rectum. As in the heart, where pacemaker cells regulate the beat, so in the intestinal tract there are similar pacemaker cells regulating intestinal motility (peristalsis) called interstitial cells of Cajal. GISTs are a neoplastic proliferation of this kind of cell and account for < 1% of all gastroin-

testinal tumors.

Research frontiers

A correct definition of GIST is quiet recent. During the last 15 years knowledge about the biological behavior and natural history of these tumors has improved, but treatment is not yet perfectly standardized.

Innovations and breakthroughs

The purpose of this article is to report the cases of GIST treated during the last 29 years at San Luigi University Hospital. Many changes occurred in diagnosis and treatment, but it is possible to extract from these data relevant information about epidemiology, natural history and therapy of GISTs. Data are comparable with other important series from the USA, UK, and Japan. The authors can conclude that surgical treatment remains the gold standard therapy for resectable tumors. For advanced or metastatic GISTs medical therapy with imatinib mesylate, an inhibitor of tyrosine kinase, is safe and effective.

Applications

The study is a further confirmation of the epidemiological features and correct treatment modality for GISTs.

Terminology

Stromal refers to the connective tissue, that is the structural tissue of the organs. Tyrosine kinases are molecules implicated in cell proliferation and tyrosine kinase inhibitors block this cellular pattern, controlling tumor proliferation.

Peer review

The article presented by the authors is interesting.

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Feasibility of a finger prick-based self-testing kit in first- and second-degree relatives of children with coeliac disease

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CONCLUSION: Our study indicates that Biocard™ test is a reliable, easy to use and well-accepted tool for home testing of first- and second-degree relatives of CD patients.

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Key words: Coeliac disease; Self-testing kit; Second-degree relatives

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Abstract

AIM: To assess feasibility of a finger prick-based kit as method for self-testing of first and second-degree relatives of coeliac disease (CD) patients.

METHODS: A total number of 379 subjects were invited to participate in this study, consisting of 197 first-degree and 182 second-degree relatives of CD patients. The self-testing kit (Biocard™) was sent out with included instructions for use. Completed tests were sent back to the study coordinator for assessment.

RESULTS: One hundred and ninety-six invited relatives carried out the Biocard™ test at home. Amongst these, 70% were children. In 97% of the cases the test was performed correctly. Three tests revealed a positive result, all of which were later confirmed by serology and histology as coeliac disease.

INTRODUCTION

Coeliac disease (CD) is an autoimmune disorder induced by gliadin in genetically predisposed individuals. The "classical" gastrointestinal malabsorption syndrome is characterised by the acute onset of diarrhoea, steatorrhoea, weight loss and anaemia, which typically occurs in children under the age of one year following their first gluten exposure during the weaning process. However, the vast majority of CD patients frequently present later with mild and less specific symptoms such as abdominal discomfort, bloating, altered stool habit and reduced energy levels. An increasing number of children are completely asymptomatic but are detected as part of screening programs^[1,2]. Disease prevalence has been rapidly increasing and several serological screening studies from Europe, South America, Australasia and the USA

have shown a prevalence of up to 0.5%-1% with disease affecting both children and adults equally^[3]. Given the fact that CD is a lifelong condition that can be treated by strict gluten exclusion, early diagnosis is desirable as it helps avoid unnecessary symptoms and reduces long-term complications^[4,5].

While a screening of all healthy individuals is difficult to perform, routine testing of asymptomatic individuals in high-risk groups is being increasingly recommended. Amongst the groups at greater risk are first- and second-degree relatives of patients with CD^[6]. Currently, the most frequently used screening tools are based on the detection of specific IgA against endomysial antibody (EMA) and tissue transglutaminase (tTG) and are routinely performed on patients in primary and secondary care^[7]. Whilst testing symptomatic individuals can often be done by venepuncture at their primary visit, screening asymptomatic patients would be made much easier if this were possible outside a healthcare environment and without the need for a trained phlebotomist. Testing first- and second-degree relatives with such a test should improve acceptability and hence uptake of the test. The Biocard™ Coeliac Test (ANI Biotech) measures serum levels of anti-tTG IgA antibodies and total IgA levels in 15 min using a finger prick blood sample. This test has been specifically designed for use by non-professionals and has already demonstrated high efficacy in clinical settings^[8]. The result is stable over time and hence can be returned by mail for confirmation.

The aim of this study was to assess the feasibility of using the Biocard™ Coeliac Test as a method for home self-testing of first- and second-degree relatives of CD patients.

MATERIALS AND METHODS

Patient recruitment and testing procedure

All first- and second-degree relatives of children with known CD who attended an outpatient clinic for follow-up at the Centre for Paediatric Gastroenterology, Royal Free Hospital (London, UK) were invited to participate in the study. CD of all paediatric index cases had been diagnosed according to the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria^[9]. One or both parents of eligible families were contacted by telephone or asked during their child's clinic appointment to participate in the study. All invited participants had not been tested for CD previously. Detailed information on the study was provided and written consent obtained from parents and relatives, as well as from older children as appropriate. Following recruitment, all families received Biocard kits with included written instructions and contact details of the study coordinator by post. Adult participants were asked to perform the test at home using manufacturer's instructions only. Parents were asked to perform the test on their children. Completed tests were returned in a pre-paid envelope. All patients with a positive test result for the Biocard™ Coeliac Test were followed up in hospital and underwent serum testing

Table 1 Participant characteristics

Invited family members	379
Male	174 (46%)
Female	205 (54%)
Age (yr), mean (range)	37.5 (1.3-85.1)
0-18	70 (18.5%)
> 18	309 (81.5%)
First-degree relatives	197
Second-degree relatives	182

for anti-tTG IgA, EMA IgA and total IgA. In the case of a confirmed positive test result, an upper endoscopy was offered to affected individuals.

In the period from March 2008 to March 2009, 67 families were contacted and a total of 423 family members were invited to take part in the study. Amongst these, 379 subjects agreed to try out the test (197 first-degree and 182 second-degree relatives of CD patients) (Table 1). The mean age of subjects was 37.5 years (range, 1.3-85.1 years); including 70 children (0-18 years) and 309 adults (> 18 years).

Description of Biocard test

The test requires 1 drop (10 µL) of blood, obtained by performing a finger prick with a sterile lancet. The drop of blood is collected in a capillary tube which is inserted into the testing tube with the reagent solution. Following the insertion of the sample strip into the test reagents, results are available after 15 min. There are two separate indicator fields on the test strip. A test is considered positive if a line appears both in the control field as well as in the test field. The line in the test field indicates that there are anti-tTG IgA antibodies present in the blood sample, while the control line indicates normal levels of total IgA. Hence, a test is considered positive if both lines are visible, and negative if a line is present only in the control field but not in the test field. If there is no line or only a faint line in the control field, the result is indeterminate, indicating insufficient IgA antibody levels (or IgA deficiency) requiring further serological tests (tTG IgG and/or EMA IgG and total IgA).

Ethical approval

Ethical approval was obtained prior to the start of the study (Royal Free Hospital and Medical School Research Ethic Committee, 08/H0720/29).

RESULTS

Patient compliance

Of 379 subjects who agreed to participate in the study, 196 (51%) carried out the Biocard test at home. Amongst these, 70% were children (Table 2). In 100% of the cases the test was performed correctly, confirmed by clear lines in the appropriate test/control field on return of the test strip. One hundred and eighty-eight (49%) of the enrolled participants did not perform the test (adult 82%, children 18%). Families who failed to return the test strip received

Table 2 Feasibility and results of Biocard test

Tests performed	196
Correctly performed at home	196 (100%)
Test not performed	188 (49%)
Change of mind	185
Afraid	2
Felt too unwell to perform test	1

a phone call to record reasons for not completing the test; 98.5% of enrolled subjects admitted to changing their mind after initially agreeing to take part. Only 2 subjects (1%) stated they were afraid of performing the test, while 1 subject had felt too unwell.

Test results and follow-up

Of the 196 subjects who performed the Biocard test correctly, 3 tested positive with clear lines in control and test field. CD was then confirmed by serological testing as well as on histology following duodenal biopsy. One of these 3 individuals had a positive line in the test field and no line in the control field, indicating an IgA deficiency, which was also confirmed on more formal testing.

DISCUSSION

First- and second-degree relatives of patients with CD have been identified as one of the main groups at higher risk of silent CD and hence are recommended to be screened. Additionally, doctors involved in the care of CD patients are often confronted with great anxiety that other family members could be affected. Hence, in such situations, reliable, minimally invasive and easy to perform tests should be offered. However, current screening tests are cost- and labour-intensive as well as requiring venepuncture performed by trained health professionals. In this study, we aimed to assess the feasibility of using a finger prick-based kit as a method for home self-testing of first- and second-degree relatives of CD patients. A recent study validating this test has demonstrated sensitivity and specificity comparable to those of conventional serological coeliac screening^[8]. Moreover, the test can easily be performed by non-professionals following only written instructions. Results are available within 15 min and include a reference test line to confirm adequate levels of total IgA antibodies. Of all relatives enrolled in our study, 51% ($n = 196$) performed the test at home. Despite the fact that 49% of potential family members chose not to perform the test, the majority (98.5%) did so for reasons unrelated to the test itself, a phenomenon frequently encountered amongst populations undergoing voluntary screening programs. Importantly, all returned tests revealed valid results as assessed by the study coordinator, indicating that it was performed correctly. Also, the fact that our study included a significant proportion of children further highlights the potential of this test to be used in this population. However, it is important to state that a positive test result currently still requires further investigation and follow-up by appropriate paediatric medi-

cal and dietetic health professionals.

In summary, results of our study indicate that the Biocard™ Coeliac Test is suitable for use in home self-testing of first- and second-degree relatives of children with CD. Moreover, given that the test can reliably be performed by non-professionals combined with its high validity, the application of this test could be further extended and offered to high risk groups in a non-specialist outpatient setting such as general practitioners surgeries or health practitioner clinics.

COMMENTS

Background

Frequently, coeliac disease (CD) presents with mild, non-specific abdominal symptoms or is even diagnosed in asymptomatic individuals. Being at an increased risk, first- and second-degree relatives of CD patients are often screened routinely or request to be screened. Highly reliable, cost effective and easy to use tests are required to meet this growing demand. The aim of this study was to assess the feasibility of using a finger prick-based kit as a method for home self-testing of first- and second-degree relatives of CD patients.

Research frontiers

The Biocard™ Coeliac Test (ANI Biotech) measures serum levels of anti-tissue transglutaminase IgA antibodies and total IgA levels in 15 min using a finger-prick blood sample. This test has been specifically designed for use by non-professionals and has already demonstrated high efficacy in clinical settings.

Innovations and breakthroughs

First- and second-degree relatives of patients with CD have been identified as one of the main groups at higher risk of silent CD and hence are recommended to be screened for the disease. Doctors involved in the care of CD patients are often confronted with great anxiety that other family members could be affected. Hence, in such situations, reliable, minimally invasive and easy to perform tests should be offered. However, current screening tests are cost and labour intensive, as well as requiring venepuncture performed by trained health professionals.

Applications

The authors' study indicates that the Biocard™ Coeliac Test is suitable for use in home self-testing of first- and second-degree relatives of children with CD. Moreover, given the test can reliably be performed by non-professionals combined with its high validity, the application of this test could be further extended and offered to high risk groups in a non-specialist outpatient setting such as general practitioners surgeries or health practitioner clinics.

Peer review

The manuscript presents original data on feasibility of self-testing for CD among patients' relatives. This strategy could improve the compliance to a screening test among asymptomatic subject, while maintaining good sensitivity and reliability.

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Role of ERCP in the era of laparoscopic cholecystectomy for the evaluation of choledocholithiasis in sickle cell anemia

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cholangiography. Sequential endoscopic sphincterotomy and stone extraction followed by LC is beneficial in these patients. Endoscopic sphincterotomy may also prove to be useful in these patients as it may prevent the future development of biliary sludge and bile duct stones.

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Key words: Sickle cell anemia; Cholelithiasis; Choledocholithiasis; Laparoscopic cholecystectomy; Cholangiography; Endoscopic retrograde cholangiopancreatography

Peer reviewer: Ashok Kumar, MD, Department of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, 226014, India

Abstract

AIM: To evaluate the role of endoscopic retrograde cholangiopancreatography (ERCP) for choledocholithiasis in patients with sickle cell anemia (SCA) in the era of laparoscopic cholecystectomy (LC).

METHODS: Two hundred and twenty four patients (144 male, 80 female; mean age, 22.4 years; range, 5-70 years) with SCA underwent ERCP as part of their evaluation for cholestatic jaundice (CJ). The indications for ERCP were: CJ only in 97, CJ and dilated bile ducts on ultrasound in 103, and CJ and common bile duct (CBD) stones on ultrasound in 42.

RESULTS: In total, CBD stones were found in 88 (39.3%) patients and there was evidence of recent stone passage in 16. Fifteen were post-LC patients. These had endoscopic sphincterotomy and stone extraction. The remaining 73 had endoscopic sphincterotomy and stone extraction followed by LC without an intraoperative cholangiogram.

CONCLUSION: In patients with SCA and cholelithiasis, ERCP is valuable whether preoperative or postoperative, and in none was there a need to perform intraoperative

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INTRODUCTION

Sickle cell anemia (SCA) is one of the commonest hemoglobinopathies in the Eastern Province of Saudi Arabia with a sickle cell trait frequency of about 25% and a sickle cell disease frequency of about 2% in some areas^[1,2]. One of the common complications of SCA is cholelithiasis and choledocholithiasis. The prevalence of cholelithiasis in patients with SCA is variable, ranging from 17% to 55%, but the frequency increases with age^[3-5]. In the Eastern Province of Saudi Arabia, an overall frequency of cholelithiasis of 19.7% was reported in children with SCA. This frequency however, increased to 36% in those 15-18 years of age^[6]. The incidence of choledocholithiasis in patients with SCA undergoing cholecystectomy was reported to be around 30%^[7,8]. In the past, and based on this high incidence of common bile duct (CBD) stones, routine intraoperative cholangiography (IC) was recommended during cholecystectomy as this may necessitate

CBD exploration^[7]. This however is not the case in the era of LC. Laparoscopic IC and CBD exploration, although feasible, is not easy to perform, requires expertise, and may be time-consuming. Add to this the possibility of converting a LC into an open one once CBD stones are diagnosed and found to be difficult to retrieve laparoscopically. The question now is whether routine laparoscopic IC is necessary for patients with SCA undergoing LC.

MATERIALS AND METHODS

Two hundred and twenty four patients with SCA underwent endoscopic retrograde cholangiopancreatography (ERCP) as part of their evaluation for cholestatic jaundice (CJ). Their medical records were reviewed for: age at diagnosis, sex, indication for ERCP, hemoglobin (Hb) electrophoresis, liver function tests, including total and direct bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase, and abdominal ultrasound. The indications for ERCP were divided into 3 categories based on ultrasound findings: (1) patients with CJ and normal ultrasound; (2) patients with CJ and dilated bile ducts on ultrasound; and (3) patients with CJ and CBD stones on ultrasound. All ERCPs were performed in the radiology department using Olympus TJF 240 or JF 260 side-viewing duodenoscopes under general anesthesia, with nasotracheal intubation for children less than 10 years old, and under deep sedation using meperidine (1 mg/kg) and diazepam (midazolam) (0.1-0.2 mg/kg) for those above 10 years of age. The ampulla of Vater was cannulated with tapered or regular catheters and the biliary ducts were visualized under fluoroscopy using Hexabrix (320 mg diluted to 50%). Appropriate radiographs were obtained and, where indicated, sphincterotomy was performed using a 5F sphincterotomy (Olympus); bile duct stones, if found, were extracted with a basket, balloon extractor or mechanical lithotripter.

RESULTS

There were 144 male and 80 female patients, mean age 22.4 years (range, 5-70 years). Mean HbS was 76.8% (range, 64.7%-92.3%); their mean HbF was 20.4% (range, 5.1%-34.0%); mean total bilirubin was 224 mg/L (range, 55-395 mg/L); mean direct bilirubin was 134 mg/L (range, 40-263 mg/L); mean alkaline phosphatase was 486 IU/mL (range, 81-1189 IU/mL; normal: 50-136 IU/mL); mean ALT was 234.3 IU/mL (range, 50-761 IU/mL; normal: 30-50 IU/mL) and mean AST was 206.3 IU/mL (range, 63-317 IU/mL; normal: 15-37 IU/mL). The indications for ERCP were: CJ only in 97 patients, CJ and dilated bile ducts on ultrasound in 103, and CJ and CBD stones on ultrasound in 42. In those with CJ only, ERCP revealed CBD stones in 18 (18.6%), and the ampulla of Vater was edematous and inflamed in 4, suggestive of recent stone passage. In those with CJ and dilated bile ducts, ERCP revealed CBD stones in 48 (46.6%), and inflamed edematous ampulla in 8. In those with CJ and CBD stones, ERCP revealed stones in 22 (52.4%), and the ampulla was inflamed

and edematous in 4. In total, CBD stones were found in 88 (39.3%) patients, and there was evidence of recent stone passage in 16. Fifteen of these were post-cholecystectomy patients, who had endoscopic sphincterotomy and stone extraction. The remaining 73 had endoscopic sphincterotomy and stone extraction followed by LC without an IC. Two (2.3%) patients developed minor bleeding from the sphincterotomy site. This was controlled with local diluted adrenaline injection. Two (2.3%) developed transient mild pancreatitis.

DISCUSSION

In the general population with cholelithiasis the incidence of CBD stones has been reported as 5%-15% whereas in those with SCA it ranges from 18% to 30%^[7-10]. Because of the high incidence of choledocholithiasis in patients with SCA, routine IC was advocated^[7]. With the recent advances in LC, exclusion of CBD stones is of great importance. This is specially so in patients with SCA who frequently present with CJ and are known to have a high incidence of cholelithiasis and choledocholithiasis. There are those who advocate routine laparoscopic IC to delineate the anatomy and to detect CBD stones^[11-16]. Lillemoe *et al*^[17] on the other hand advocate the selective use of laparoscopic cholangiography. Although laparoscopic IC is feasible, it has several disadvantages. It requires operators who are experienced in laparoscopic surgery, it is not technically easy, and it is known to increase the operative time. It also makes it difficult to decide, when CBD stones are diagnosed and difficult to retrieve, whether to convert the operation to open or wait and do a post-LC ERCP. Add to this a 20%-25% false positive rate of IC that may lead to unnecessary CBD exploration or conversion to open cholecystectomy^[16]. In a prospective study of CBD calculi in patients undergoing cholecystectomy, Nathanson *et al*^[18] found choledocholithiasis in 3.4%, and concluded that laparoscopic IC would result in unnecessary interventions in 50% of patients who had either false positive results or subsequently passed the stones. Another study in 343 patients who underwent LC concluded that routine IC should be discouraged in view of the low yield and significant rate of false positive results^[19]. However, Targarona *et al*^[20] stated that the choice of diagnostic and therapeutic strategies for CBD stones should depend on local circumstances and available expertise. We found ERCP to be valuable both for the diagnosis and management of CBD stones in patients with SCA who were scheduled to have LC, or in those who presented with retained CBD stones following LC. Our policy is that all SCA patients with cholelithiasis who have a dilated CBD on ultrasound, biochemical evidence of obstructive jaundice (elevated alkaline phosphatase, elevated total bilirubin of more than 50 mg/L), or a history of pancreatitis either alone or in combination, and those who have choledocholithiasis detected on ultrasound, should undergo ERCP to confirm and extract the stones, followed by LC. We, like others, support a policy of preoperative ERCP for those with risk factors for CBD stones followed by LC^[21,22]. However, magnetic resonance

cholangiopancreatography and or endoscopic ultrasound, if available, should replace ERCP as a diagnostic method to detect CBD stones as this will reduce the number of negative ERCPs and avoid the risks and complications of ERCP particularly for those with dilated CBD without stones.

In conclusion, considering the high incidence of CBD stones in patients with SCA, it is important to exclude them as a cause of CJ whether pre- or post-cholecystectomy. This is specially so in the era of LC. Laparoscopic IC, although feasible, is not easy to perform, is time-consuming, requires expertise, and may necessitate conversion to open surgery if CBD stones are identified. We found ERCP valuable in this regard whether pre- or post-LC, and in none of our patients was there a need to perform laparoscopic IC. Sequential endoscopic sphincterotomy and stone extraction followed by LC is beneficial in these patients^[23,24]. Since we started using ERCP, none of our patients required CBD exploration and all CBD stones, whether diagnosed preoperatively or postoperatively, were managed by endoscopic sphincterotomy and stone extraction. Endoscopic sphincterotomy may also prove to be useful in these patients as it may prevent the future development of biliary sludge and bile duct stones.

COMMENTS

Background

Cholelithiasis and choledocholithiasis are common in patients with sickle cell anemia, and it is important to exclude choledocholithiasis in those undergoing cholecystectomy. In the past and at the time of open cholecystectomy, routine intraoperative cholangiography (IC) was part of the operative procedure as they have a high incidence of choledocholithiasis. The question now is whether routine laparoscopic IC is necessary for patients with Sickle cell anemia (SCA) undergoing laparoscopic cholecystectomy.

Research frontiers

Common bile duct (CBD) stones were found in 88 (39.3%) of 224 patients and there was evidence of recent stone passage in 16. Fifteen of these were post-laparoscopic cholecystectomy patients and had endoscopic sphincterotomy and stone extraction. The remaining 73 had endoscopic sphincterotomy and stone extraction followed by laparoscopic cholecystectomy without IC.

Innovations and breakthroughs

Laparoscopic IC, although feasible, is not easy to perform, is time-consuming, requires expertise, and may necessitate conversion to open surgery if bile duct stones are identified. We found endoscopic retrograde cholangiopancreatography (ERCP) valuable whether pre- or post-laparoscopic cholecystectomy and in none of our patients was there a need to perform laparoscopic IC. Sequential endoscopic sphincterotomy and stone extraction followed by laparoscopic cholecystectomy are beneficial in these patients. Since we started using ERCP, none of our patients required CBD exploration and all CBD stones whether diagnosed preoperatively or postoperatively were managed by endoscopic sphincterotomy and stone extraction. Endoscopic sphincterotomy may also prove to be useful in these patients as it may prevent the future development of biliary sludge and bile duct stones.

Applications

All patients with SCA and cholelithiasis who have a dilated CBD on ultrasound, elevated alkaline phosphatase, elevated total bilirubin of more than 50 mg/L, or a history of pancreatitis either alone or in combination, and those who have choledocholithiasis detected on ultrasound should undergo ERCP to confirm and extract stones followed by laparoscopic cholecystectomy. However, magnetic resonance cholangiopancreatography and or endoscopic ultrasound should replace ERCP as a diagnostic method to detect CBD stones as this will reduce the number of negative ERCPs and avoid the risks and complications of ERCP particularly for those with dilated CBD without stones.

Terminology

ERCP: Endoscopic retrograde cholangio pancreatography. SCA: Sickle cell anemia, a hereditary hemoglobinopathy resulting from a single change of one amino acid, valine instead of glutamic acid of the hemoglobin B-chain. This will lead to a change in the shape of red blood cells causing their adherence together, blocking the small blood vessels and their hemolysis.

Peer review

The authors discussion of routine cholangiography in patients with SCA with high suspicion of choledocholithiasis is highly appreciated.

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Thrombotic microangiopathy-like disorder after living-donor liver transplantation: A single-center experience in Japan

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in liver transplantation, because TMA is an infrequent but life-threatening complication in the transplantation field.

METHODS: A total of 206 patients who underwent living-donor liver transplantation (LDLT) were evaluated, and the TMA-like disorder (TMALD) occurred in seven recipients.

RESULTS: These TMALD recipients showed poor outcomes in comparison with other 199 recipients. Although two TMALD recipients successfully recovered, the other five recipients finally died despite intensive treatments including repeated plasma exchange (PE) and re-transplantation. Histopathological analysis of liver biopsies after LDLT revealed obvious differences according to the outcomes. Qualitative analysis of antibodies against a disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs (ADAMTS-13) were negative in all patients. The fragmentation of red cells, the microhemorrhagic macules and the platelet counts were early markers for the suspicion of TMALD after LDLT. Although the absolute values of von Willebrand factor (vWF) and ADAMTS-13 did not necessarily reflect TMALD, the vWF/ADAMTS-13 ratio had a clear diagnostic value in all cases. The establishment of adequate treatments for TMALD, such as PE for ADAMTS-13 replenishment or treatments against inhibitory antibodies, must be decided according to each case.

CONCLUSION: The optimal induction of adequate therapies based on early recognition of TMALD by the reliable markers may confer a large advantage for TMALD after LDLT.

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Key words: Thrombotic microangiopathy; Liver transplan-

Abstract

AIM: To investigate thrombotic microangiopathy (TMA)

tation; von Willebrand factor; A disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs; Complication

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INTRODUCTION

Thrombotic microangiopathy (TMA) is a microvascular occlusive disorder induced by facilitation of endothelial damage and primary platelet (PLT) aggregation^[1]. Currently, the concept of TMA encompasses two previous entries, i.e., thrombotic thrombocytopenic purpura and hemolytic uremic syndrome^[2]. Although the specific pathophysiological mechanism is still not understood, many investigators have focused on a disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs (ADAMTS)-13 as a metalloproteinase that specially cleaves the multimeric von Willebrand factor (vWF)^[3-5]. The vWF is an essential factor for the adhesion/aggregation of circulating PLTs associated with high shear stresses^[6,7]. Following the identification of ADAMTS-13 in 2001^[3-5], some investigators suggested that a deficiency of ADAMTS-13 activity^[3,8] and/or inhibitory autoantibodies against ADAMTS-13^[2,9] may cause TMA *via* a pathway in which unusually large vWF multimers elevate the shear stress and lead to excessive PLT clumping^[6,7,10]. As a consequence, PLTs would be consumed in TMA patients, and finally result in organ failure because of microvascular occlusion.

Clinically, TMA patients develop thrombocytopenia accompanied by hemolytic anemia and show a hemorrhagic tendency^[1,2,11]. The TMA is clinically defined as thrombocytopenia and microangiopathic hemolytic anemia with no apparent alternative explanations^[1,12,13]. The diagnostic criteria for TMA are defined as follows^[1,2,14,15]: (1) the presence of thrombocytopenia (PLT count < $5.0 \times 10^4/\text{mm}^3$) or the progressive decline in PLT counts (decrease of $> 3.0 \times 10^4/\text{mm}^3$ within 24 h); (2) microangiopathic hemolytic anemia [hemoglobin (HB) < 8.0 g/dL]; (3) sharply elevated levels of serum lactate dehydrogenase (LDH) (typically > 500 IU/L); (4) the presence of fractionated erythrocytes in a blood smear; and (5) severe deficiency in ADAMTS-13 activity (< 5% in normal plasma)

or prevalence of ADAMTS-13-specific antibodies (Abs) categorized as immunoglobulin G (IgG) isotypes.

TMA is well known as a fatal complication after transplantation, and its frequencies are documented to be 6% after bone marrow transplantation^[16] and 3%-5% after solid-organ transplantation^[17,18]. The first case of TMA in the liver transplantation (LT) field was reported in 1984^[19], and thereafter some researchers have reported that the frequency of TMA after LT is 3.8%-5.0%^[2,14]. Repeated plasma exchange (PE)/exchange transfusion (ET) is considered to be the standard therapy for TMA from the viewpoint of the replenishment of ADAMTS-13 and the depletion of inhibitory Abs^[13,15,20-22]. To date, a total of 739 pediatric and 669 adult LTs have been performed in our institution. Although we have no experience of cases that completely fulfilled the TMA criteria described above, seven cases were retrospectively considered to be TMA-like disorder (TMALD) after living-donor liver transplantation (LDLT). Here, we focused on post-LDLT TMA cases and present our results for TMALD patients. The pathophysiological basis, the early marker for the recognition of TMALD, the reliable factors for diagnosis and further clinical strategies are discussed in the LDLT field.

MATERIALS AND METHODS

Patients

A total of 155 adult and 51 pediatric recipients who underwent LDLT at Kyoto University Hospital from April 2006 to March 2009 were evaluated in this study. Seven patients (five adults and two pediatric recipients) showed thrombocytopenia and microangiopathic hemolytic anemia with no apparent alternative causes. They were given the intensive TMALD treatments.

These seven patients comprised one male and six females, and their age range was 0.8-65.2 years. The primary diseases for LDLT included two cases each of primary biliary cirrhosis and liver cirrhosis caused by hepatitis C virus (HCV), and one case each of liver cirrhosis caused by autoimmune hepatitis, biliary atresia (post-Kasai's portoenterostomy) and fulminant hepatic failure (etiology unknown). The United Network for Organ Sharing statuses were estimated to be four cases of II A, two of II B and one of I. The mean Child-Pugh score was 12.0 ± 1.7 points (range, 10-14 points). The mean score of the model for end-stage liver disease (MELD) or pediatric end-stage liver disease (PELD) was 23.7 ± 8.8 points (range, 17-41 points). The ABO blood groups were characterized as three cases each of identical and incompatible and one case of compatible. The donor relationships were four spouses, one grandmother, one father and one son.

The protocol of the study was approved by the Ethics Review Committee for Clinical Studies of Kyoto University Graduate School of Medicine.

Operation

There were three left-lobe grafts, two extended lateral-segment grafts and one right-lobe graft without the middle

Table 1 Recipient profiles

Recipients	ABO ¹	TMALD ²	Clinical courses after TMALD	PE/ET ³	Additional surgery ⁴	Outcome
Pediatric	Incompatible	21	Herpes simplex sepsis	0	None	Alive
Pediatric	Incompatible	6	Pneumonitis	4	None	Alive
Adult	Identical	14	Hepatic encephalopathy, intraperitoneal bleeding	9	Surgical hemostasis (16)	Dead
Adult	Incompatible	9	Hepatic encephalopathy, brain edema gastroesophageal varices rupture, intrathoracic intraperitoneal, bleeding sepsis, aspergillus infection	8	Surgical hemostasis (21) Re-transplantation (22)	Dead
Adult	Compatible	4	Acute respiratory distress, syndrome intraperitoneal bleeding	5	None	Dead
Adult	Compatible	10	Intraperitoneal bleeding	3	Surgical hemostasis	Dead
Adult	Compatible	14	Hepatic arterial thrombosis	10	None	Dead

¹ABO blood group; ²The definite diagnosis of thrombotic microangiopathy like disorder (TMALD) [postoperative day (POD)]; ³The frequency of repeated plasma exchange/exchange transfusion (PE/ET) (Number of times); ⁴ Additional surgery for the complications (POD).

hepatic vein. The range of the graft/recipient weight ratios was 0.74–4.58. Normal findings for histopathological analyses of biopsy specimens during the donor operation were confirmed in all cases. The mean operative time was 690.7 ± 150.0 min (range, 480–873 min) and the mean blood loss was 4180.0 ± 2843.3 mL (range, 370–7700 mL). The mean cold ischemic time, warm ischemic time and anhepatic phase were 49.4 ± 18.9 min (range, 27–77 min), 63.0 ± 32.9 min (range, 40–133 min) and 150 ± 76.6 min (range, 51–267 min), respectively. All the recipients received blood transfusions of a red cell concentrate and fresh-frozen plasma (FFP) during LDLT, and two recipients received a PLT transfusion. The patient profiles are shown in Table 1.

Immunosuppression

Immunosuppression after LDLT was started with tacrolimus and methylprednisolone. The trough level of tacrolimus was maintained at 8–15 ng/mL during the early postoperative period, based on the clinical findings in each case. Calcineurin inhibitors (CNIs) were converted to cyclosporin A from tacrolimus at postoperative day (POD) 15 in one case. Methylprednisolone was given intravenously (1 mg/kg) once daily from POD 1 to POD 3 followed by 0.5 mg/kg once daily for the next three days. On POD 7, 0.3 mg/kg of methylprednisolone was given intravenously. Steroid administration was switched to oral prednisolone 0.3 mg/kg once daily on POD 8. This dose was reduced to 0.1 mg/kg at one month after LDLT. We had already overcome ABO-incompatibility in LDLT, and our regimens for these recipients, including heparin usage, were described previously^[23,24].

The exclusion of other diseases

To exclude other diseases, such as humoral rejection (HR), hematological diseases, heparin-induced thrombocytopenia and autoimmune diseases, detailed examinations, such as bone-marrow puncture, liver needle biopsy, immunological assays and measurements of anti-platelet factor 4/heparin Abs and anti-PLT autoantibodies were performed.

Measurements of individual variables

In our institution, laboratory examinations were routinely performed at least every 8–12 h in all recipients during the early postoperative period after LDLT and in critical LDLT recipients. The appearances of fragmentation of

red cells (FRC) and microhemorrhagic macules (MHMs) were checked, and temporal changes in the counts or levels of PLTs, HB, LDH, prothrombin time-international normalized ratio (PT-INR) and total bilirubin (T-BIL) were estimated.

The values for vWF and ADAMTS-13 were measured by enzyme-linked immunosorbent assays, and subsequently used to calculate the vWF/ADAMTS-13 ratio. Quantitative determination of inhibitory IgG (anti-ADAMTS-13 Abs) was performed by the Bethesda method (the detection limit was 0.5 Bethesda units/mL).

Normal ranges of vWF, ADAMTS-13 and the vWF/ADAMTS-13 ratio were calculated in a control group, with 10% rejection region. A control group was composed of 54 healthy volunteers (26 males and 29 females). Normal values of vWF, ADAMTS-13 and the vWF/ADAMTS-13 ratio were 60%–170%, 70%–120%, and 0.51–2.43, respectively. The anti-ADAMTS-13 Abs were non-existent in healthy individuals (under the detection limit).

The definition of TMALD

We still do not have any experience of patients who strictly fulfilled all of the TMA criteria. In this study, TMALD was defined as the LDLT recipients who fulfilled the diagnostic criteria of TMA without the absolute value of ADAMTS-13 activity or the presence of ADAMTS-13-specific Abs.

The risk factors for LT outcomes

Previous investigators have documented the important factors for LT outcomes, such as recipient age, disease, donor age, MELD/PELD score, ABO incompatibility, lymphocyte cross-match, cold ischemic time, operative time, blood loss, graft-recipient weight ratio, the type/number of anastomosis and conventional liver function test at early postoperative period^[23,25–33]. There were no statistical differences between the 2 groups in each risk factor for LDLT outcomes, respectively.

Histopathological analysis

A protocol liver needle biopsy (LNB) is not employed in our institution. However, all cases underwent an LNB before and after the treatments for TMALD, except for one case in which an LNB could not be performed because of a severe hemorrhagic tendency. The graft damage

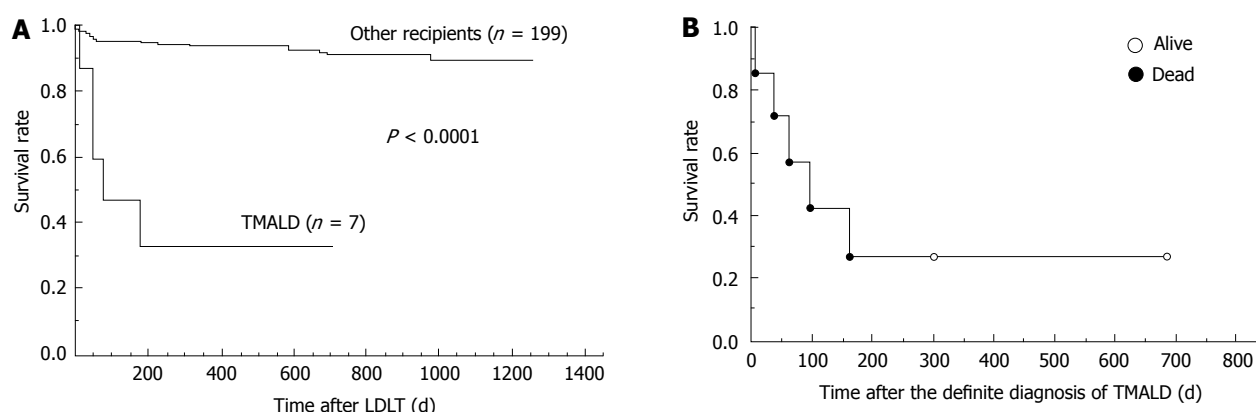


Figure 1 Outcomes of thrombotic microangiopathy like disorder after living-donor liver transplantation. A: The seven thrombotic microangiopathy like disorder (TMALD) patients clearly showed a poor prognosis compared with the other 199 patients ($P < 0.0001$). TMALD after living-donor liver transplantation (LDLT) therefore had a negative impact upon the LDLT outcomes. A total of 206 LDLT recipients at Kyoto University Hospital (from April 2006 to March 2009) were evaluated; B: The thrombotic microangiopathy patients showed a poor prognosis despite intensive treatments after the diagnosis of TMALD. The open circles represent two recipients who were successfully treated, and the closed circles represent five recipients who finally died.

score was estimated according to established guidelines^[34], and was calculated as the total of points of the following parenchymal features: hepatocyte ballooning (0 point = no, 1 = yes), hepatocyte necrosis (0 = none, 1 = small foci, 2 = confluent areas, 3 = bridging necrosis), congestion (0 = no, 1 = yes), microvesicular fat (0 = none, 1 = 1/3 hepatocytes, 2 = between 1/3 and 2/3 hepatocytes, 3 = 2/3 hepatocytes), neutrophil aggregate (0 = none, 1 = minimal, 2 = moderate, 3 = extensive), and cholestasis (0 = none, 1 = mild, 2 = moderate, 3 = severe).

Statistical analysis

The results are expressed as the mean \pm SD. The differences between unpaired continuous or discontinuous data between two groups were analyzed by Student's *t*-test. Survival rates were calculated by the Kaplan-Meier method, and the log-rank test was used for between-group comparisons. All calculations were performed using SPSS Software Version 16.0 (SPSS Inc., Chicago, IL 60606, USA). Differences with *P* values of < 0.05 were considered to be statistically significant.

Ethical approval

The protocol of this study was approved by the Ethics Review Committee for Clinical Studies of Kyoto University Graduate School of Medicine.

RESULTS

TMALD onset after LDLT

TMALD was confirmed in seven cases, and the frequency in our institution was 3.4%. The definite diagnoses were reached at a mean time point of POD 11.1 ± 5.7 (range, 4-21 d).

Clinical courses after TMALD and outcomes in TMALD patients

Five adult recipients clearly showed prolonged jaundice after TMALD [T-BIL peak, 38.9 ± 11.2 mg/dL (range,

21.0-51.0 mg/dL)]. Four of these five cases suffered massive bleeding because of a hemorrhagic tendency in TMALD, and surgical hemostasis was emergently required in three cases. Thrombosis in the hepatic blood flow occurred after TMALD in one of the five cases. Consequently, these five cases fell into graft loss after TMALD, and two of the five recipients suffered hepatic encephalopathy. The details of the clinical courses are shown in Table 1.

On the other hand, two pediatric recipients (ABO-incompatible combinations) showed good responses to the treatments for TMALD, and recovered from their severe graft damage (Table 1).

In comparisons of the survival rates, the TMALD patients clearly showed poor outcomes compared with the other patients who underwent LDLT during the same period in our institution (Figure 1A).

Critical events occurred after TMALD in five recipients and their clinical conditions tended to become worse despite the intensive treatments (Table 1). All of these five recipients with TMALD finally died despite intensive treatments (Figure 1B), and their mean survival time after the TMALD diagnosis was only 70 ± 63 d (range, 5-161 d). Although one of the five patients underwent a re-transplantation because of graft loss, she also finally died (Table 1).

The values of diagnostic early markers and important signs

The fragmentation of red cells was obviously detected in all cases. The haptoglobin level was also decreased to < 5.0 mg/dL in all cases. MHMs were observed in five patients. The mean lowest value of the PLT counts was $1.2 \pm 5.5 \times 10^4/\text{mm}^3$ (range, $0.6\text{--}2.1 \times 10^4/\text{mm}^3$) while that of HB was 6.61 ± 0.68 g/dL (range, 5.9-7.6 g/dL). The mean peak value of the LDH level was 1202.3 ± 603.9 IU/L (range, 518-2089 IU/L). The PT-INR value was prolonged to > 1.5 in only one case at the time of a definite diagnosis, although the mean value for the most prolonged PT-INR after TMALD was 2.66 ± 1.85 (range, 1.22-5.64).

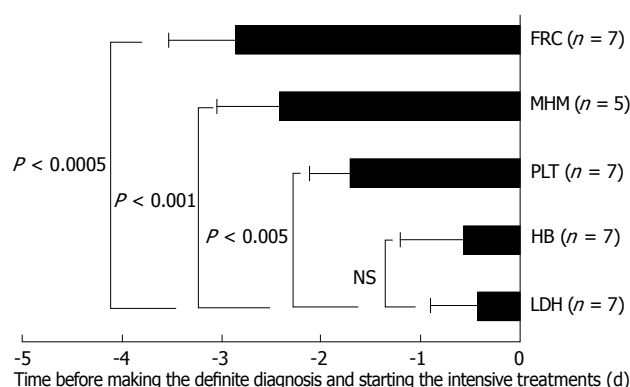


Figure 2 Temporal changes in important markers and signs for thrombotic microangiopathy. The statistical significances of the differences in each factor in comparison with the time points for lactate dehydrogenase (LDH) are shown. Note that although microhemorrhagic macules (MHMs) were only observed in five of the seven cases (71.4%), the MHMs unexpectedly appeared at the early phase of thrombotic microangiopathy like disorder (TMALD) onset after living-donor liver transplantation, as well as fragmentation of red cells (FRC). In each case, the LDH levels proved decisive for making a diagnosis of TMALD, while the appearance of sufficient elevations of the LDH level were mostly late, similar to the case for hemoglobin (HB). PLT: Primary platelet.

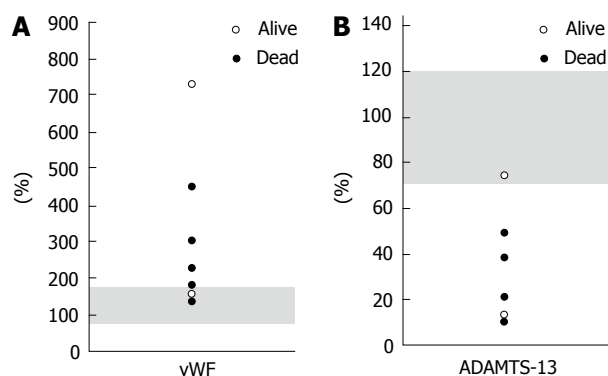


Figure 3 Absolute values of von willebrand factor and a disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs-13. A The mean absolute value of von Willebrand factor (vWF) was $306.6\% \pm 212.0\%$ (range, 135%-723%), and the vWF levels were within the normal range in two cases. The shaded area represents the normal range of vWF. The open circles represent two recipients who were successfully treated, and the closed circles represent five recipients who finally died; B The mean absolute value of a disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs (ADAMTS)-13 was $31.1\% \pm 24.3\%$ (range, 10%-75%), and the level of ADAMTS-13 was within the normal range in one case. The shaded area represents the normal range of ADAMTS-13. The open circles represent two recipients who were successfully treated, and the closed circles represent five recipients who finally died. Consequently, the degrees of abnormalities in the absolute values of both vWF and ADAMTS-13 did not seem to precisely reflect the thrombotic microangiopathy like disorder outcomes after living-donor liver transplantation.

Changes over time in important indicators for TMA

In two cases, the PLT counts before TMA onset were continuously $< 5.0 \times 10^4/\text{mm}^3$. Progressive decreases of $> 3.0 \times 10^4/\text{mm}^3$ within 24 h were considered to be the time points of fulfilling the TMALD criteria in these cases. Although MHMs were not necessarily observed in all cases, MHMs surprisingly appeared at the early phase of TMALD onset after LDLT, as well as FRC (Figure 2). In each case, elevated LDH levels proved decisive for making

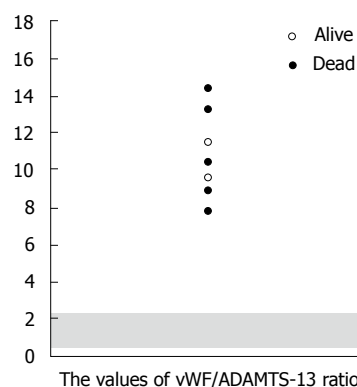


Figure 4 Balance between von willebrand factor and a disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs-13. From the viewpoint of an imbalance between von Willebrand factor (vWF) and a disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs (ADAMTS)-13, the ratio of vWF/ADAMTS-13 strictly revealed the abnormalities in all thrombotic microangiopathy like disorder (TMALD) recipients after living-donor liver transplantation (LDLT). The shaded area represents the normal range. The mean value of the vWF/ADAMTS-13 ratio was 11.0 ± 2.4 (range, 7.8-14.6). The open circles represent two recipients who were successfully treated, and the closed circles represent five recipients who finally died.

a diagnosis of TMALD, although their appearances were mostly late, similar to the case for HB (Figure 2).

Absolute values of vWF and ADAMTS-13 and the vWF/ADAMTS-13 ratio

In two cases, the vWF levels were within the normal range. The mean absolute value of vWF was $306.6\% \pm 212.0\%$ (range, 135%-723%) (Figure 3A). The ADAMTS-13 level also revealed no abnormalities in one case. The mean absolute value of ADAMTS-13 was $31.1\% \pm 24.3\%$ (range, 10%-75%) (Figure 3B). Therefore, the degrees of alterations in these absolute values did not seem to precisely reflect the TMALD outcomes after LDLT (Figure 3).

On the contrary, from the viewpoint of an imbalance between vWF and ADAMTS-13, the ratio of vWF/ADAMTS-13 strictly revealed the abnormalities in all of the TMALD recipients after LDLT (Figure 4). The mean value for the vWF/ADAMTS-13 ratio was 11.0 ± 2.4 (range, 7.8-14.6).

Inhibitors of ADAMTS-13

Qualitative analysis of anti-ADAMTS-13 Abs produced negative results in all cases. Even in the quantitative determinations, only two cases showed subtle elevations at approximately the cut-off level, although some TMA patients in the hematological field in our hospital showed obvious elevation of anti-ADAMTS-13 Abs.

Repeated PE/ET

Intensive care procedures for secondary complications, such as continuous hemodiafiltration for renal failure, respiratory control for pulmonary dysfunction and intravenous administration of antibiotics for sepsis, were performed in each case based on real-time estimations of their physical conditions, if necessary.

PE/ET (FFP 80-100 mL/kg per day) were repeated

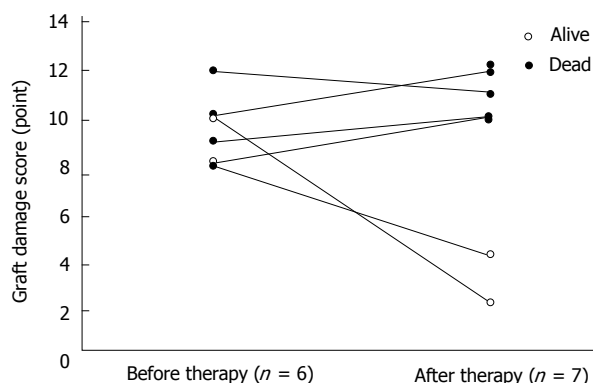


Figure 5 Graft parenchymal damage before and after thrombotic microangiopathy like disorder treatments. The mean graft damage scores before and after thrombotic microangiopathy like disorder (TMALD) treatments in all patients were 8.9 ± 2.2 points (range, 5-12 points) and 8.7 ± 4.0 points (range, 2-12 points), respectively. In comparisons of the histopathological findings before and after TMALD treatments, the graft damage scores clearly demonstrated that five recipients who had poor outcomes (closed circles) showed no improvements despite repeated plasma exchange (PE). The mean graft damage score after TMALD treatments in these five recipients was 8.8 ± 2.6 points (range, 5-12 points). On the other hand, the two surviving recipients (open circles) recovered from their graft parenchymal damage. The graft damage scores after TMALD treatments in these two recipients were two and four points.

as the standard therapy for TMALD in six cases immediately after making a definite diagnosis of TMALD. The mean number of times of repeated PE/ET was 6.5 ± 2.9 (range, 3-10). Repeated PE therapy was not introduced in only one patient who successfully recovered from TMALD. It has been suggested since that repeated PE for the liver failure before LDLT can cause a recurrence of herpes simplex infection after LDLT because of the depletion of antigen-specific Abs by PE and the non-specific immunosuppression after LDLT.

Graft parenchymal damage

Histopathological analysis of LNB specimens clearly revealed that the five recipients who had poor outcomes suffered graft loss after TMALD.

The mean graft damage scores before and after TMALD treatments in all patients were 8.9 ± 2.2 points (range, 5-12 points) and 8.7 ± 4.0 points (range, 2-12 points), respectively (Figure 5).

In comparisons of the histopathological findings before and after TMALD treatments, the results clearly demonstrated that the five recipients who had poor outcomes still showed severe graft parenchymal damage despite of repeated PE [mean graft damage score after TMALD treatments, 8.8 ± 2.6 points (range, 5-12 points)], although the two surviving recipients recovered from their graft parenchymal damage after repeated ET (four times) or no PE/ET treatment. The graft damage scores after TMALD treatments in the successfully treated recipients decreased to two or four points.

DISCUSSION

Although the specific mechanism remains unknown, previ-

ous reports have documented that CNIs and infections, including cytomegalovirus (CMV) and HCV infections, may be possible causes of TMA after LT^[2,17,35-39]. Despite the fact that our institution is a major transplant center in Japan, the frequency of TMA in our institution may be slightly lower than those in previous reports^[16,18]. A possible explanation for this is the well-controlled conditions aimed at preventing CMV and HCV infections after LDLT in our institution^[40,41]. In particular, our clinical strategy brings about excellent long-term outcomes after LDLT for recipients with HCV^[40], and the frequency of TMALD in such recipients is 3.2% (2 TMALD cases among 62 HCV patients after LDLT) in our institution. Paradoxically, uncontrolled HCV infection may be a trigger of TMA onset as previously described^[2,42]. TMA occurs as a long-term complication after LT by association with viral infections^[2], whereas our patients presented with TMALD during the early postoperative period. One of the possible explanations is the good control of viral infections as described above, although we are sometimes troubled by Epstein-Barr virus infections, including long-term complications of post-transplantation lymphoproliferative disorders^[43]. Regarding CNIs, our results that CNIs were used in all cases (tacrolimus was switched to cyclosporin A in 1 case) reveal an undeniable possibility of CNI effects, as previously documented^[17,35,36,39]. We also cannot negate the possibility that CNI introduction caused TMALD during the early postoperative period in our cases. However, we consider that complete CNI-free LDLT for preventing TMA is a distant ideal, since CNIs are key immunosuppressants for LDLT during this period^[15,44].

The mortality of TMA patients without effective treatments can be as high as 90%^[1,16], and 10%-20% of TMA patients still die even after PE/ET^[13,45]. The outcomes of TMALD patients are truly poor in our institution. Serious problems, such as massive bleeding, vascular thrombosis and organ failures, actually appeared in these recipients because of PLT consumption and microvascular occlusion mediated by TMALD. Five TMALD patients died without long survival times despite receiving continuous intensive treatments including re-transplantation for graft loss. Our own results indicate that further improvements in clinical strategies, including diagnostic methods and effective treatments based on the TMA mechanism after LDLT, are still needed.

Currently, the criteria for TMA are established as described above. We still do not have any experience of patients who strictly fulfilled all of the TMA criteria. Therefore, we consider that our patients were TMALD rather than TMA. However, we think that our recipients are homogeneously the same as other TMA patients in their clinical events. Based on our results in assays for TMA diagnosis, TMA recipients after LT may not be considered in the same manner as congenital or autoimmune TMA patients purely in the hematological field, when making a definite diagnosis. Inherently, TMA involves microvascular occlusion and unusually large vWF multimers are present at its inception^[1,7]. Although we

initially focused on vWF and ADAMTS-13, similar to previous studies^[2-5,8,9,14], we found that these absolute values were not necessarily reliable for the confirmation of TMA in LDLT recipients. Our results revealed only tendencies toward high vWF and low ADAMTS-13, while a few cases showed normal absolute values. However, the balance between vWF and ADAMTS-13 was clearly broken down. We suggest that this imbalance is a true entity in TMA after LDLT, and that evaluation of the vWF/ADAMTS-13 ratio is more suitable after LDLT to establish the patient's condition and achieve a precise diagnosis of TMA. Since a precise diagnosis is always required in order to administer appropriate treatments^[2,14,15], we suggest that this variable is one of the keys for improving the TMALD outcomes after LDLT.

Immunologically and pharmaceutically, numerous unpreventable influences exist in LDLT recipients. The phenomena of microvascular occlusive disorders accompanied by endothelial damage, PLT aggregation and hemolysis will be observed for other reasons, such as HR. On making a diagnosis of TMA after LDLT, many examinations including hematological, immunological and histopathological assays are therefore required to rule out other possible reasons, such as HR, sepsis and autoantibodies as well as HIT and drug-induced disorders^[2,14]. Therefore, reaching a definite diagnosis of TMA usually requires many considerations and much time. Actually, in our results, we retrospectively considered that TMALD onsets were earlier rather than at the point of diagnosis and/or treatment induction. We suggest that although the LDH level has a specific sensitivity for TMA/TMALD this variable will not work as an early marker for TMA/TMALD. Decreases in PLT counts can cause serious problems which sometimes require additional surgery. FRC and PLT decreases were observed at earlier points, and unexpectedly, MHMs were also reliable as an early sign of TMALD after LDLT if they appeared. A possible explanation is the time-lag between an initial high shear stress and the resultant microvascular occlusion^[6,7]. Ironically, transplant surgeons cannot decide actual treatments based on FRC, MHMs and PLT decreases even with earlier appearances, while TMA basically contraindicates a PLT transfusion. Although the changes over time in vWF and ADAMTS-13 could not be estimated in these patients because of cost-saving reasons, we hypothesize that the ratio of vWF/ADAMTS-13 is the earliest marker after LDLT for ruling out TMA and deciding appropriate treatments from the viewpoint of an essential cause of TMA.

Basically, the treatments must be chosen based on each cause of TMA, i.e. the lack of ADAMTS-13 and/or the inhibition of ADAMTS-13 via specific Abs^[2,3,8,9]. We suggest that adequate therapies, i.e. PE/ET for ADAMTS-13 replenishment or additional treatments against inhibitory Abs, must be decided according to each cause of TMA/TMALD. The PE/ET has effects on both replenishment of ADAMTS-13 and depletion of inhibitory Abs against ADAMTS-13. Therefore, PE/ET (80-100 mL/kg per day) is widely considered

as the standard therapy for TMA^[13,15,20-22]. As described above, the essence of TMA after LDLT is an imbalance between vWF and ADAMTS-13, and we expect that PE/ET will have a sufficient effect from the viewpoint of the replenishment of ADAMTS-13. However, the five TMALD patients who finally died revealed that the graft loss caused by TMALD resulted in poor outcomes, even in a re-transplanted case, and that graft parenchymal damage became worse one-sidedly despite the intensive treatments. On the contrary, the other two TMALD patients were successfully treated, even though the treatment in one case excluded PE/ET. The graft damage caused by TMALD exhibited sufficient responses to the administered treatments in these successfully treated patients. Our results indicate that further improvements for TMALD after LDLT are truly required. We suggest that PE/ET itself has an anticipated efficacy in TMALD patients who lack ADAMTS-13^[3,8]. Furthermore, optimal timing of the PE/ET induction according to the TMALD onset can improve the outcomes in these patients, even though the same treatment is performed^[2,13-15,45]. The morbidity and mortality may increase if we hesitate to introduce the therapy until the criteria for TMA are completely fulfilled. We therefore suggest that earlier induction will improve the outcomes in ADAMTS-13-deficient patients when TMALD is suspected after LDLT based on early markers or an imbalance of the vWF/ADAMTS-13 ratio. Since our results showed that successful treatments are difficult once the TMA criteria are completely fulfilled, we consider that some data and/or signs accompanied by a PLT decrease are sufficient for PE/ET induction after LDLT, e.g. FCR, MHMs and a discrepancy of LDH elevation (even under the criteria) compared with other transaminases. On the other hand, PE/ET also has a benefit in TMALD patients accompanied by inhibitors from the viewpoint of Ab depletion^[2,9,15]. However, our results for the TMALD patients who died might sustain the previous opinion that PE/ET has a limited usage for the depletion of specific Abs^[15,46], and the actual results of poor outcomes oblige us to challenge some other therapies for TMALD patients after LDLT. Although we have no experience of Ab-positive TMA after LDLT, we now take anti-CD20 monoclonal Ab or intravenous high-dose IgG administration under consideration, if repeated PE/ET has insufficient effects on TMA progress.

Previous investigators suggested the diagnostic and therapeutic advices for TMA, and previous reports which focused TMA after LT were summarized in Table 2^[2,14,15,37-39,47,48]. In our institution, the outcomes of TMALD recipients were poor despite the intensive treatments, especially in adult LDLT recipients. Previous researchers investigated the risk factors for TMA after LT^[15,39], and Nishi *et al.*^[15] suggested that the onset POD, the value of urea nitrogen and the level of albumin were important for TMA outcomes after LDLT. In our institution, the levels of urea nitrogen were not measured routinely. A possible explanation for the poor outcomes of TMALD after LDLT in our institution was the early

Table 2 Previous series and case reports with TMA after liver transplantation

Year	The first author	Patient number	PE therapy	Focus	Diagnostic advices	Therapeutic advices
2008	Oya	1	+	The risk factor TMA, the relation of TMA and ABO compatibility	-	Reduction of CNI PE Intravenous infusion of high-dose γ -globulin
2007	Miyata	4	+	Plasminogen activator inhibitor type 1, the relation of TMA and ABO compatibility, early diagnosis of TMA for appropriate therapy	Plasminogen activator inhibitor type 1 the vWF value	Early recognition of TMA
2007	Akamatsu	1	+	The reation of TMA and CNI	-	CNI conversion
2006	Nishi	18	+	Poor outcome of TMA recipients PE therapy for TMA, the risk factors of TMA after LDLT	-	PE was not sufficient
2005	Banno	5	+	The analyzer of red blood cell fragmentation	Red blood cell fragmentation	-
2005	Taura	10	+	The relation of TMA and HCV infection	-	PE CNI conversion
2003	Nakazawa	1	+	The vWF-cleaving protease, the inhibitors against this protease	The vWF-cleaving protease, the inhibitors against this protease	PE
2003	Ramasubbu	1	+	The relation of TMA and CMV	-	-

PE: Plasma exchange; TMA:Thrombotic microangiopathy; CNI: Calcineurin inhibitor; LDLT: Living-donor liver transplantation; HCV: Hepatitis C virus; vWF: von Willebrand factor; CMV: Cytomegalovirus.

postoperative onset of TMALD within 30 d after LDLT (Table 1), though the albumin levels showed no statistical differences between groups. Current therapeutic strategies were still not enough, and we therefore speculated that an earlier induction of adequate treatments based on the early markers may improve the prognosis of TMALD after LDLT. We considered that the earlier recognition of TMALD and the earlier induction of treatments even under a suspicion of TMALD are crucial for LDLT recipients with TMALD.

TMA/TMALD stands as an infrequent but life-threatening complication in the LT field. Although retransplantation may seem to be a therapeutic option, this is strictly limited from the viewpoints of donor shortage and donor safety. We suggest that adequate therapies, i.e. PE/ET for ADAMTS-13 replenishment or additional treatments against inhibitory Abs, must be decided according to each cause of TMA/TMALD. We conclude that the optimal induction of these therapies based on earlier or reliable markers and the establishment of more advanced therapeutic strategies confers a large advantage for TMA/TMALD patients after LDLT and consequently improves LT outcomes.

However, the balance between vWF and ADAMTS-13 was clearly broken down. This balance reflected the condition of TMA after LDLT. Moreover, this variable is reliable along with earlier markers for TMALD recognition.

Applications

On making a diagnosis of TMA after LDLT, many examinations are required to rule out other possible reasons. Therefore, reaching a definite diagnosis of TMA usually requires many considerations and much time. The authors suggest that although the LDH level has a specific sensitivity for TMA/TMALD, this variable will not work as an early marker for TMA/TMALD. Fragmentation of red cell (FRC) and primary platelet (PLT) decreases were observed at earlier points and unexpectedly, microhemorrhagic macules were also reliable as an early sign of TMALD after LDLT if they appeared. Ironically, transplant surgeons cannot decide actual treatments based on FRC, MHMs and PLT decreases even with earlier appearances, while TMA basically contraindicates a PLT transfusion. They suggested that the ratio of vWF/ADAMTS-13 is the earliest marker after LDLT for ruling out TMA and deciding appropriate treatments from the viewpoint of an essential cause of TMA.

Terminology

The establishment of adequate treatments for TMALD, such as plasma exchange for ADAMTS-13 replenishment or treatments against inhibitory antibodies, must be decided according to each case. The optimal induction of these therapies based on early recognition of TMALD by the early and/or reliable markers may confer a large advantage for TMALD recipients after LDLT. Consequently, their outcomes will be improved.

Peer review

A very interesting manuscript for a rare disorder. It should be accepted with minor revisions.

COMMENTS

Background

The thrombotic microangiopathy (TMA) is an infrequent but life-threatening complication in the transplantation field. Here, the authors focused on post-living-donor liver transplantation (LDLT) TMA cases.

Research frontiers

Some researchers have reported that the frequency of TMA after LT is 3.8%-5.0%. To date, a total of 1408 LTs have been performed in the authors' institution. However, although the authors have no experience of cases that completely fulfilled the TMA criteria, seven cases were retrospectively considered to be thrombotic microangiopathy like disorder (TMALD) after LDLT. The pathophysiological basis, the early marker for the recognition of TMALD, the reliable factors for diagnosis and further clinical strategies are discussed in the LDLT field.

Innovations and breakthroughs

Only tendencies toward high von Willebrand factor (vWF) and low a disintegrin-like domain and metalloproteinase with thrombospondin type 1 motifs (ADAMTS)-13 were seen, while a few cases showed normal absolute values.

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Characteristics of non-erosive gastroesophageal reflux disease refractory to proton pump inhibitor therapy

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Abstract

AIM: To investigate whether potent acid inhibition is effective in non-erosive reflux disease (NERD) refractory to standard rabeprazole (RPZ) treatment.

METHODS: We treated 10 Japanese patients with NERD resistant to standard dosages of RPZ: 10 mg or 20 mg od, 20 mg bid, or 10 mg qid for 14 d. All patients completed a frequency scale for symptoms of gastroesophageal reflux disease questionnaire frequency scale for the symptoms of GERD (FSSG); and underwent 24 h pH monitoring on day 14.

RESULTS: With increased dosages and frequency of

administration of RPZ, median intragastric pH significantly increased, and FSSG scores significantly decreased. With RPZ 10 mg qid, potent acid inhibition was attained throughout 24 h. However, five subjects were refractory to RPZ 10 mg qid, although the median intragastric pH in these subjects (6.6, range: 6.2-7.1) was similar to that in the remaining five responsive subjects (6.5, range: 5.3-7.3). With baseline RPZ 10 mg od, FSSG scores in responsive patients improved by > 30%, whereas there was no significant decrease in the resistant group.

CONCLUSION: NERD patients whose FSSG score fails to decrease by > 30% after treatment with RPZ 10 mg od for 14 d are refractory to higher dosage.

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Key words: Non-erosive reflux disease; Rabeprazole; CYP2C19

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined as the presence of acid-reflux-related symptoms, or esophageal mucosal damage, caused by the abnormal reflux of gastric contents into the esophagus^[1]. The diagnosis of GERD is therefore relatively easy when patients complain of typical acid-reflux-related symptoms (i.e. heartburn

Table 1 Frequency scale for the symptoms of gastroesophageal reflux disease

Question	Never	Occasionally	Sometimes	Often	Always
1 Do you get heartburn?	0	1	2	3	4
2 Does your stomach get bloated?	0	1	2	3	4
3 Does your stomach ever feel heavy after meals?	0	1	2	3	4
4 Do you sometimes subconsciously rub your chest with your hand?	0	1	2	3	4
5 Do you ever feel sick after meals?	0	1	2	3	4
6 Do you get heartburn after meals?	0	1	2	3	4
7 Do you have an unusual (e.g. burning) sensation in your throat?	0	1	2	3	4
8 Do you feel full while eating meals?	0	1	2	3	4
9 Do some things get stuck when you swallow?	0	1	2	3	4
10 Do you get bitter liquid (acid) coming up into your throat?	0	1	2	3	4
11 Do you burp a lot?	0	1	2	3	4
12 Do you get heartburn if you bend over?	0	1	2	3	4

Patients were asked to score each question as never = 0; occasionally = 1; sometimes = 2; often = 3; or always = 4. In the frequency scale for the symptoms of gastroesophageal reflux disease (FSSG), there are seven acid-reflux-related symptoms (questions 1, 4, 6, 7, 9, 10 and 12), and five dysmotility-like symptoms (questions 2, 3, 5, 8 and 11). The acid reflux, dysmotility and total scores (acid reflux and dysmotility scores) were calculated, and a total score of ≥ 8 was considered to indicate probable GERD/NERD.

and regurgitation), and/or esophageal mucosal breaks are seen by gastroduodenal endoscopy. Recently, non-erosive gastroesophageal reflux disease (NERD) has been defined as the presence of acid-reflux-related symptoms without esophageal mucosal breaks^[2]. NERD is classified into two types: grade M and grade N. Grade M is characterized by minimal mucosal changes, such as erythema, without sharp demarcation, whitish turbidity, and/or translucency in the lower esophageal mucosa; and grade N reveals no endoscopic abnormality^[3]. The clinical characteristics of patients with NERD—that they are less likely to smoke or have an esophageal hiatal hernia, and more likely to be female, underweight, and have *Helicobacter pylori* (*H. pylori*) infection—differ from those of erosive GERD patients^[2,4,5]. Furthermore, the fact that esophageal mucosal sensitivity in NERD patients tends to be higher than those with erosive GERD is another important clinical characteristic of patients with NERD^[6]. These findings suggest that NERD is not simply a milder type of erosive GERD, and that the pathophysiology of erosive GERD differ from that of NERD^[7].

Proton pump inhibitors (PPIs), which potently inhibit gastric acid secretion, improve acid-reflux heartburn symptoms and esophageal mucosal breaks^[8–12]. Meta-analyses of treatment for erosive GERD patients have shown that PPIs are much more effective in curing esophageal erosions and acid-reflux-related symptoms than are H₂ receptor antagonists (H₂RAs) or prokinetics^[13,14]. However, improvement of heartburn associated with NERD using standard PPI dosages are lower (around 30%–60%) than for erosive GERD^[2,15,16]. This raises the question whether PPI-resistant NERD is an acid-related disease. Although PPIs potently inhibit acid secretion, standard PPI dosages do not sufficiently control intragastric pH throughout 24 h^[17–19]. For patients with NERD refractory to a standard PPI dosage, therefore, treatment with potent acid inhibition will be required to determine whether PPI-resistant NERD is caused by insufficient acid inhibition.

To the best of our knowledge, no earlier studies have investigated whether acid-related symptoms in patients with NERD refractory to standard PPI dosages improve

when sufficient acid inhibition is attained using PPI qid therapy. In this study, we investigated the effects of frequent PPI dosing on subjects with PPI-resistant NERD, with the aim of determining the clinical characteristics of subjects with PPI-refractory NERD that was resistant to potent acid inhibition.

MATERIALS AND METHODS

Subjects

After obtaining written informed consent, we invited 15 Japanese NERD patients with acid-reflux symptoms more than once a week to participate in our study. They underwent testing for CYP2C19 genotyping and gastroduodenal endoscopy. Endoscopy was performed in all subjects after fasting overnight, and the presence of esophageal mucosal breaks was assessed according to the Los Angeles classification (grade A–D)^[20]. In addition, grade M NERD was defined as mucosal findings of redness, edema or white granules in the esophagocardial junction (EC) junction, and grade N as normal mucosa, in subjects with acid-reflux-related symptoms. Subjects were administered a standard PPI dosage (rabeprazole 10 mg od) for 4 wk. Of the 15 enrolled subjects, 10 *H. pylori*-negative subjects with a score higher than 8 on the Frequency Scale for the Symptoms of GERD (FSSG) questionnaire (Table 1) were diagnosed with PPI-resistant NERD, and were enrolled in the study proper^[21,22]. However, because a score on the FSSG questionnaire in the remaining five NERD patients decreased to < 7 after PPI treatment (PPI-responded NERD), we did not enroll them in the study.

Study protocol

All subjects were administered the four different regimens in the following order: Rabeprazole (Pariet®; Eisai Co. Ltd., Tokyo, Japan) 10 mg od [RPZ(10)] at 08:00 h, rabeprazole 20 mg od [RPZ(20)], rabeprazole 20 mg bid [RPZ(20*2)] at 08:00 and 19:00 h, and rabeprazole 10 mg qid [RPZ(10*4)] at 07:00, 13:00, 19:00 and 0:00 h for 14 d each. On day 14 of each regimen, subjects filled in the FSSG question-

Table 2 Demographic characteristics of subjects with non-erosive gastroesophageal reflux disease

		Grade N	Grade M	Total	P value
Number		3	7	10	
Age		22.3 ± 5.8	22.1 ± 0.7	22.2 ± 0.6	0.73
Height		159.7 ± 3.2	156.7 ± 8.3	157.6 ± 7.0	0.31
Weight		52.2 ± 2.5	52.7 ± 6.0	52.6 ± 5.0	0.91
CYP2C19	RM/IM/PM	1/2/0	3/2/2002	5/2/2003	0.41
24 h pH	Gastric pH	2.6 (2.6-2.6)	2.4 (1.5-2.8)	2.5 (1.5-2.8)	0.31
	Esophageal pH	6.6 (6.4-7.6)	6.6 (6.4-7.5)	6.6 (6.4-7.6)	0.75
FSSG		19 (15-24)	22 (19-31)	21 (15-31)	0.3

Age, height, body weight and height are given as mean ± SD. Twenty-four hour pH and frequency scale for the symptoms of gastroesophageal (FSSG) score are given as median (range). RM: Rapid metabolizer; IM: Intermediate metabolizer; PM: Poor metabolizer.

naire and underwent 24 h intraesophageal and intragastric pH monitoring. All subjects were provided three meals a day (breakfast at 07:00 h, lunch at 13:00 h, and dinner at 19:00 h). Mineral water was allowed *ad libitum*, but no other beverages (e.g. grapefruit juice) were permitted. There was a washout period of at least 2 wk between the study periods. There were no rescue drugs during and between the study periods. No subjects drank alcohol or smoked. No subjects had taken any medications for at least 1 mo prior to the study, nor were they allowed during the study. All protocols for each subject were completed within 6-10 mo.

The protocol was approved in advance by the Human Institutional Review Board of the Hamamatsu University School of Medicine. Written informed consent was again obtained from each subject before participation in each of the four trial phases.

Twenty-four hour intraesophageal and intragastric pH monitoring

Intraesophageal and intragastric pH readings were recorded using a DigiTrapper pH 400 (Medtronic Functional Diagnostic A/S, Skovlunde, Denmark). On day 14 of each trial phase, after fasting overnight, an antimony pH catheter (Medtronic Inc., Minneapolis, MN, USA) was inserted transnasally under local anesthesia and placed 5 cm distal to the gastric cardia, and pH readings were recorded for 24 h.

Evaluation of reflux symptoms

GERD-related symptoms were evaluated using the FSSG, which includes seven acid-reflux-related questions and five dysmotility related questions^[21,22]. Subjects answered questions about the frequency of their symptoms, scoring them as follows: never, 0; occasionally, 1; sometimes, 2; often, 3; and always, 4. We calculated the acid reflux, dysmotility and total scores, with a total score of ≥ 8 considered to indicate probable GERD/NERD.

CYP2C19 genotyping PPIs are mainly metabolized by hepatic CYP2C19, and there are genetic differences in the activity of this enzyme^[17-19]. In poor metabolizers (PMs) of CYP2C19, the plasma PPI concentrations are markedly increased and the pharmacodynamic effects of PPIs are enhanced in comparison with those in rapid metabolizers (RMs) or intermediate metabolizers (IMs). Therefore, we tested CYP2C19.

DNA was extracted from each subject's leukocytes

using a commercially available kit (IsoQuick; ORCA Research Inc., Bothell, WA, USA). Genotyping procedures for identifying the CYP2C19 wild-type (*1) gene and the two mutated alleles, CYP2C19*2 (*2) and CYP2C19*3 (*3), were performed using an allele-specific primers-polymerase chain reaction method with allele-specific primers^[23]. CYP2C19 genotypes were classified into three groups, RMs (*1/*1), IM (*1/*2 or *1/*3), and PM (*2/*2, *3/*3 or *2/*3).

Data analysis

Differences between different regimens and groups were determined using Wilcoxon's signed rank test, when significant differences were obtained using Friedman's test. All *P* values were two-sided, and *P* < 0.05 was taken to indicate statistical significance.

RESULTS

Japanese subjects with NERD resistant to standard PPI dosages enrolled in this study exhibited no demographic differences in age, body weight, CYP2C19 genotype status, median intragastric and intraesophageal pH, or baseline FSSG score between subjects with NERD grade N and grade M (Table 2). No severe adverse events occurred with any of the study regimens, and all regimens were well tolerated by all subjects.

Twenty-four hour pH profiles according to dosage regimen

The median 24 h intragastric pH at baseline and on day 14 for the RPZ(10), RPZ(20), RPZ(20*2) and RPZ(10*4) regimens was 2.5 (range: 1.5-2.8), 5.0 (3.3-7.5), 6.1 (3.8-6.8), 6.2 (4.9-7.4) and 6.5 (5.3-7.3), respectively (Figure 1A). Median intragastric pH significantly increased in a dosage and dosing frequency-dependent manner, and was significantly higher with RPZ(10*4) than with RPZ(20*2), although the total daily dosage (40 mg) was the same (Figure 1A). The median percentage of intragastric pH < 4.0 in a day with the RPZ(20), RPZ(20*2) and RPZ(10*4) regimens was 17.2% (range: 0.8%-66.6%), 13.0% (0.0%-43.6%) and 5.2% (0.0%-36.8%), respectively, which was significantly lower than those at baseline [85.6% (78.3%-97.0%), *P* < 0.01] and with the RPZ(10) regimen [34.8% (3.5%-73.7%), *P* < 0.01] (Figure 1B).

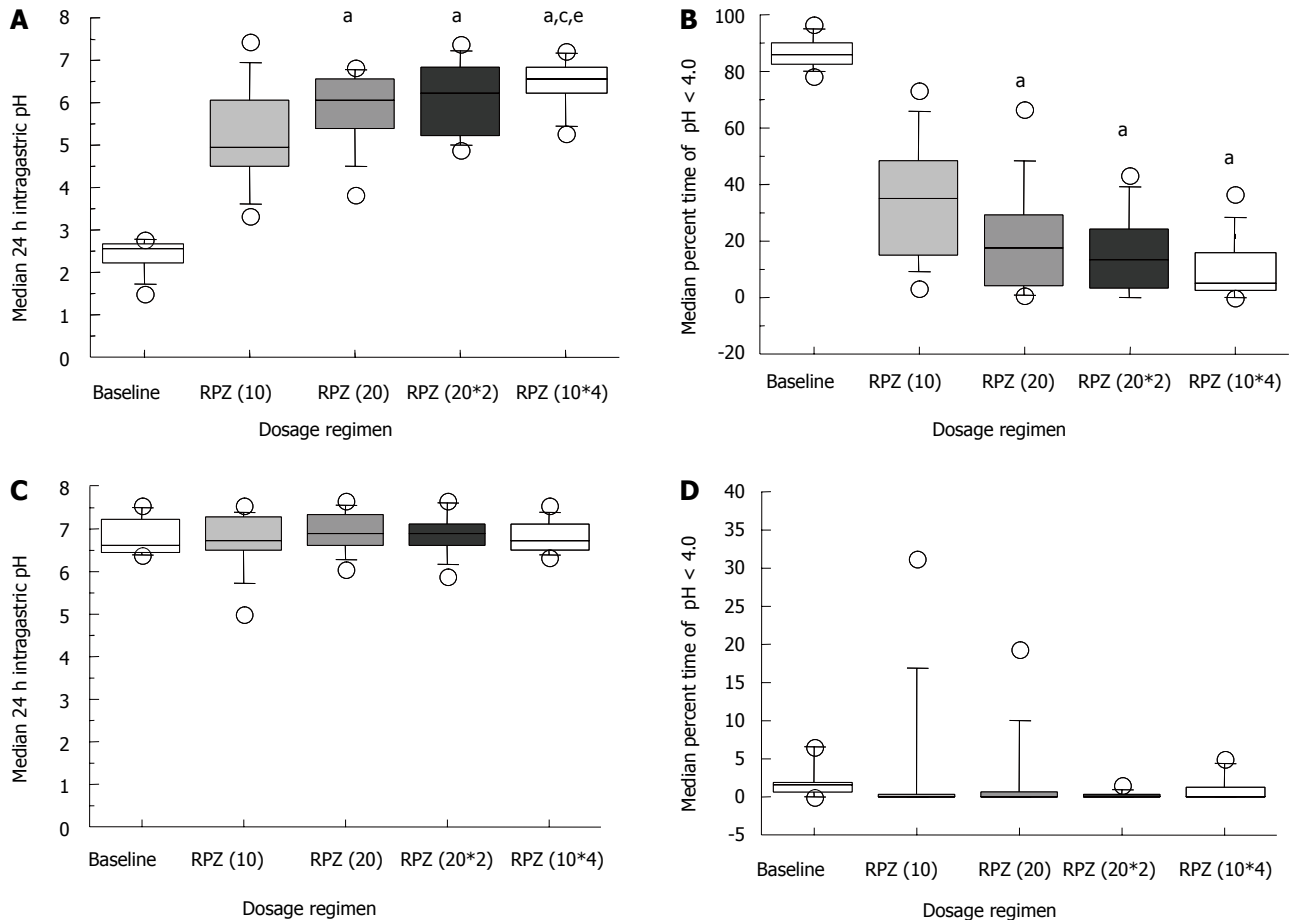


Figure 1 Median 24 h intragastric and intraesophageal pH (A and C) and median percentage time with intragastric and intraesophageal pH < 4.0 (B and D) with five different treatment regimens on day 14. ^a $P < 0.05$ [vs RPZ (10)]; ^c $P < 0.05$ [vs RPZ (20)]; ^e $P < 0.05$ [vs RPZ (20*2)]. RPZ: Rabeprazole.

Median 24 h intraesophageal pH and the percentage of intraesophageal pH < 4.0 in a day were similar for the four different RPZ dosage regimens (Figure 1C and 1D). Most baseline measurements showed no abnormal acid reflux; defined as > 5% of patients with intraesophageal pH < 4.0 (Figure 1D). The median reflux frequency of gastric acid to the esophagus at baseline, RPZ(10), RPZ(20), RPZ(20*2) and RPZ(10*4) was 58.5 (range: 7-137), 5 (3-244), 4.5 (0-180), 13.5 (0-251) and 4.5 (0-506), respectively (Figure 2). Differences were not statistically significant in comparison with baseline.

Median 24 h intraesophageal and intragastric pH, the percentage of intraesophageal and intragastric pH < 4.0 in a day, and the frequency of reflux of gastric acid to the esophagus were similar between patients with different CYP2C19 genotype status (data not shown).

FSSG score and acid inhibition

FSSG scores improved significantly in a dose-dependent manner (Figure 3A). The median FSSG score at baseline was 20.5 (range: 15-31), which significantly decreased with each RPZ dosage regimen [RPZ(10), 13.5 (10-31); RPZ(20), 10.5 (1-28); RPZ(20*2), 10.0 (0-24); and RPZ(10*4), 6.5 (0-20), $P < 0.05$] (Figure 3A). When patients with NERD were administered RPZ 10 mg od, their FSSG scores were all > 8. FSSG scores with the RPZ(20),

RPZ(20*2) and RPZ(10*4) regimens were significantly lower than that with the RPZ(10) regimen (Figure 3A).

When FSSG scores were subdivided into acid reflux and dysmotility scores (Table 1), these were seen to decrease significantly with changes in RPZ dosage schemes in acid reflux scores (Figure 3B and C). The FSSG acid reflux scores with the RPZ(20*2) [3.5 (0-17)], RPZ(20*2) [3 (0-12)] and RPZ(10*4) [2.5 (0-11)] regimens were significantly lower than that with RPZ(10) [6.5 (3-18)] ($P < 0.05$).

Also in dysmotility scores, these were seen to decrease significantly with changes in RPZ dosage schemes (Figure 3C). The FSSG acid reflux scores with the RPZ(20*2) [6.5 (1-13)], RPZ(20*2) [6 (0-12)] and RPZ(10*4) [4 (0-9)] regimens were significantly lower than that with RPZ(10) [7.5 (6-13)] ($P < 0.05$) (Figure 3C). The FSSG dysmotility score with the RPZ(10*4) regimen was significantly lower than that with RPZ(20*2), although the total daily dosage was the same (40 mg) (Figure 3C).

When patients were classified into a responsive (FSSG score: < 8, $n = 5$) and resistant (FSSG score: > 8, $n = 5$) group for the RPZ(10*4) regimen, the median intragastric pH was similar for the two groups [responsive group: 6.5 (5.3-7.3) and resistant group: 6.6 (6.2-7.1)] (Figure 4), which indicated that sufficient acid inhibition was attained in both subjects with PPI-resistant as well responsive NERD. Median total, acid reflux and dysmotility FSSG scores signifi-

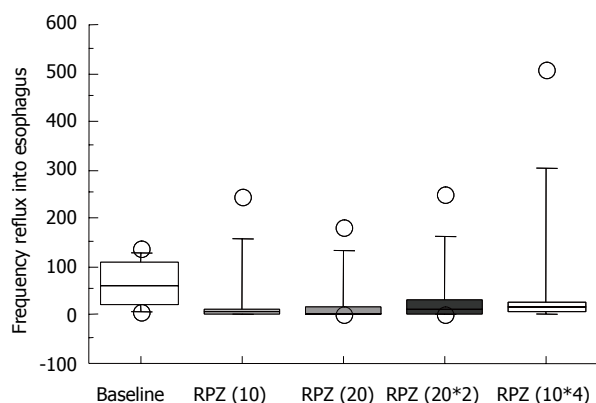


Figure 2 Number of reflux episodes of gastric contents to the esophagus with five different treatment regimens on day 14. RPZ: Rabeprazole.

cantly decreased in a dose-dependent manner in subjects who responded to, or were refractory to, the RPZ(10*4) regimen (Figure 5A-D). Acid reflux and dysmotility FSSG scores both differed significantly between the two groups (Figure 5A-D). FSSG scores in subjects with NERD responsive to the RPZ(10*4) regimen improved by > 30% in comparison with baseline scores with RPZ 10 mg od. On the other hand, in subjects with NERD refractory to the RPZ(10*4) regimen, FSSG scores decreased by < 30% compared with the baseline with RPZ 10 mg od.

DISCUSSION

Recently, the rising prevalence of NERD worldwide^[24], and its adverse impact on quality of life^[25], have led to the acceptance of NERD as a gastrointestinal disease that requires treatment. Intraesophageal and intragastric pH directly correlate with the degree of esophageal mucosal damage and the degree of sensitivity^[26], therefore, treatment strategies for NERD recommend that pH levels in both the esophagus and stomach should be maintained above 4.0 by optimal use of acid inhibitory agents^[27]. We previously have reported that it is difficult to maintain the intragastric pH above 4 throughout 24 h with standard PPI dosages (omeprazole 20 mg, lansoprazole 30 mg and rabeprazole 10 mg)^[17-19], but that PPI qid therapy^[18] and H2RA + PPI combination therapy^[28] provide profound inhibition of gastric acid secretion. These therapies may be the main strategies for treating PPI-resistant, acid-related GERD with/without nocturnal acid breakthrough, as well as peptic ulcers and *H. pylori* infection. Complete remission of endoscopic esophageal mucosal injury and reflux-related symptoms are achieved in around 30%-60% of patients with NERD using standard PPI dosages^[2,15,16], which suggests that PPI-resistant NERD may be due to insufficient acid inhibition by standard PPI dosages. In this study, we demonstrated that the qid RPZ regimen maintained intraesophageal and intragastric pH above 4.0 throughout 24 h, and that our subjects with PPI-resistant NERD responded to varying degrees. Accordingly, we assume that cases of PPI-resistant NERD are caused by insufficient acid inhibition, and that the pathogenesis of NERD may be related

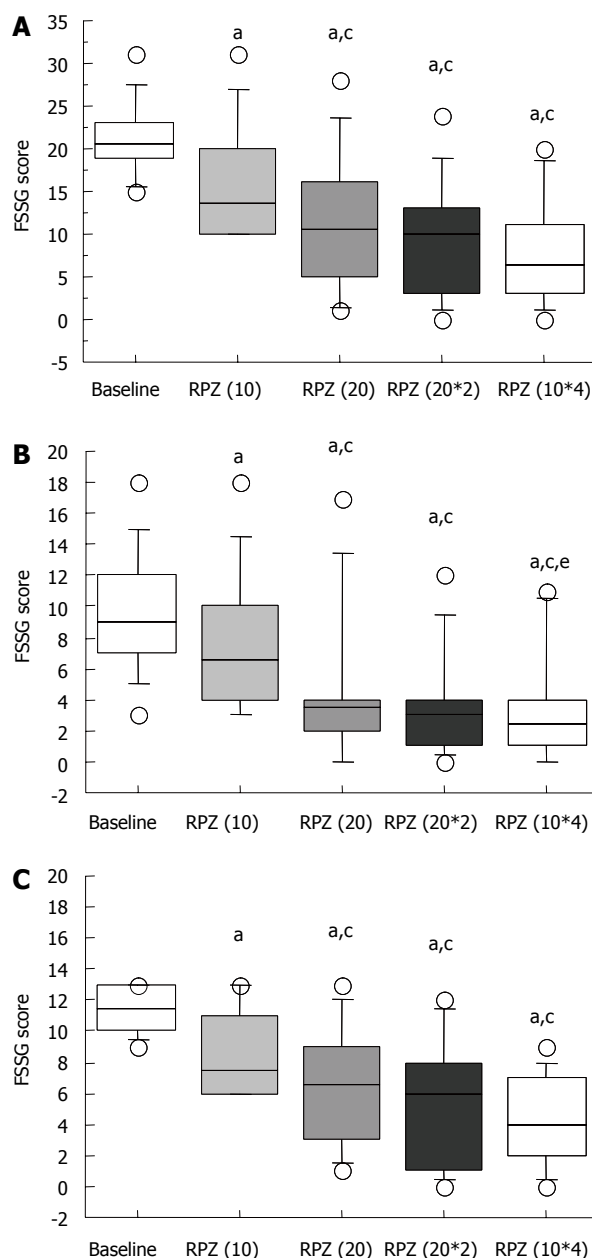


Figure 3 Median total (A), acid reflux (B) and dysmotility (C) Frequency scale for the symptoms of gastroesophageal reflux disease scores. Frequency scale for the symptoms of gastroesophageal reflux disease scores in RPZ(20), RPZ(20*2), and RPZ(10*4) regimens significantly improved compared with that at baseline and RPZ(10). ^a*P* < 0.05 [vs baseline (10)]; ^c*P* < 0.05 [vs RP (10)]; ^e*P* < 0.05 [vs RP (20)]. RPZ: Rabeprazole; FSSG: Frequency scale for the symptoms of gastroesophageal reflux disease.

to weak acid reflux into the esophagus, and not observed as abnormal reflux and not inhibited by standard PPI dosages. In subjects with NERD responsive to potent acid inhibition by RPZ 10 mg qid, FSSG scores decreased by > 30% compared with baseline after RPZ 10 mg od for 14 d. Therefore, NERD patients whose FSSG reduces by > 30% by a standard dosage of PPI will respond to a higher dose of PPI, although the clinical effects of standard PPI dosages on their symptoms are limited.

Cases of NERD that are resistant to high PPI dosages appear to be acid-independent, and are considered to have

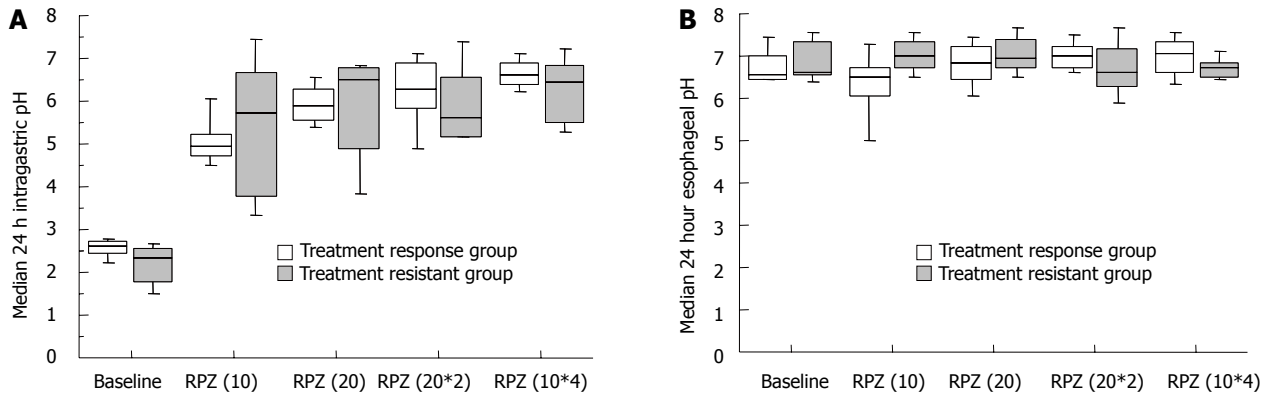


Figure 4 Median intragastric and intraesophageal pH in subjects responsive ($n = 5$) and refractory ($n = 5$) to rabeprazole 10 mg qid. Median intragastric pH was the same in patients responsive and refractory to RPZ 10 mg qid. RPZ: Rabeprazole.

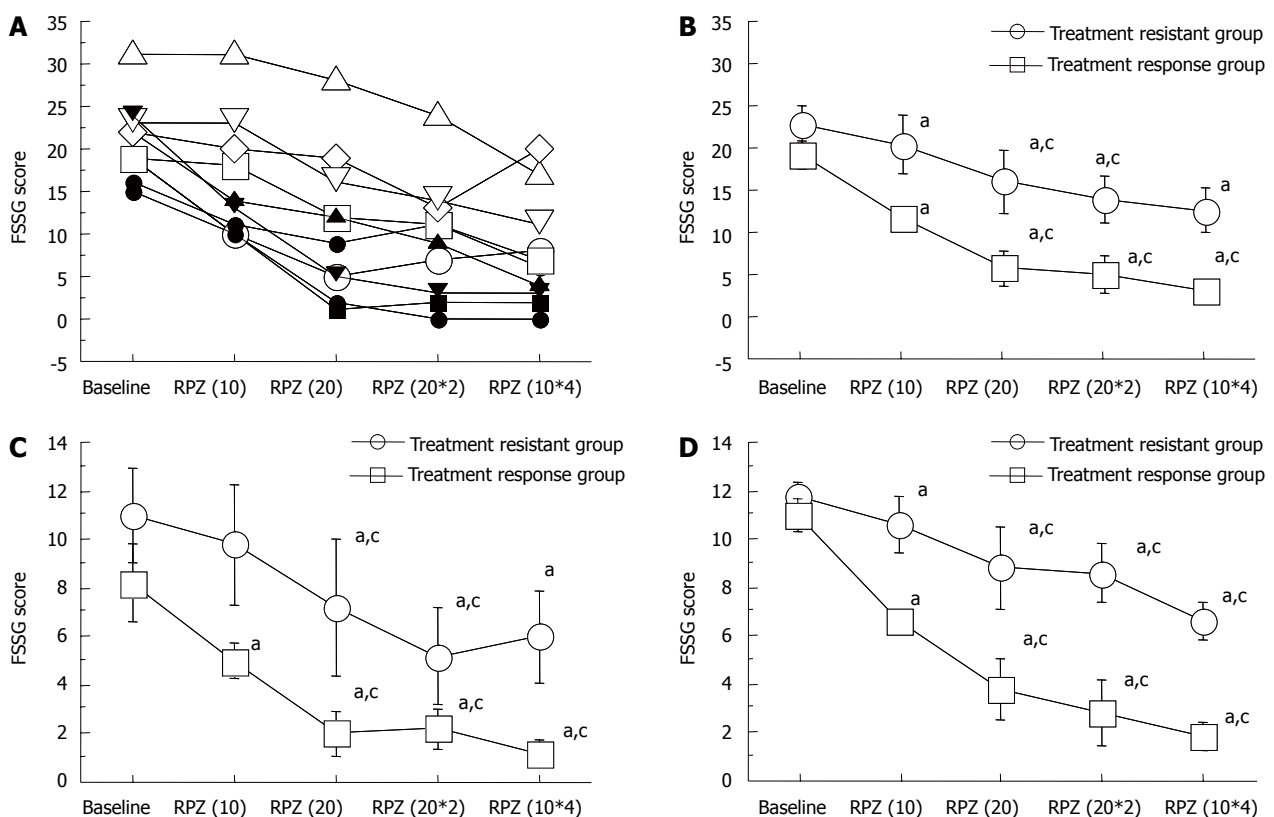


Figure 5 Each patient's (A), total (B), acid reflux (C) and dysmotility (D) FSSG scores in subjects with non-erosive gastroesophageal reflux disease responsive and refractory to the rabeprazole(10*4) regimen. Frequency scale for the symptoms of gastroesophageal reflux disease (FSSG) scores in subjects with non-erosive gastroesophageal reflux disease (NERD) who responded to the RPZ(10*4) regimen improved > 30% compared with baseline with rabeprazole 10 mg od. RPZ: Rabeprazole. ^a $P < 0.05$ [vs baseline]; ^c $P < 0.05$ [vs RP (10)].

been caused by other factors, such as esophageal hypersensitivity and/or functional heartburn. In fact, the prevalence of NERD grade N patients with abnormal acid reflux into the esophagus (> 5% with intraesophageal pH < 4.0) is reported to be only 11.8% in Japanese^[29] and 33%-50% in Caucasians^[30,31]. In this study, also, no abnormal acid reflux was detected in most of the baseline trials in subjects with NERD. Moreover, in some patients, NERD resembles postprandial distress syndrome type functional dyspepsia, which is characterized by dysmotility symptoms, and there is considerable overlap between NERD and functional

dyspepsia^[32,33]. It is not easy to distinguish NERD patients from functional heartburn using either the Montreal definition or the Rome III criteria^[34].

Although double-dosage PPI therapy is recommended for patients with PPI-resistant NERD, this study shows that additional PPI therapy is not indicated for all NERD patients refractory to standard-dosage PPI. In patients with NERD resistant to potent acid inhibition by PPIs, FSSG scores improve < 30% with a standard PPI dosage compared with baseline. We can therefore easily select PPI-resistant patients using the FSSG questionnaire; the simplest

and time-saving method of diagnosing GERD/NERD refractory to higher PPI dosages^[21,22]. Recently, Futagami *et al.*^[35] have reported that combination therapy with a PPI and mosapride citrate significantly improves acid reflux symptoms in patients with PPI-resistant NERD. It may therefore be better to treat PPI-resistant patients with other drugs, such as prokinetics, rather than with increased PPI dosages.

Patients with grade M NERD are more likely to have pathological acid reflux than those with grade N disease, and acid reflux symptoms in patients with grade M disease are more likely to be attributable to acid reflux^[29]. However, there have been a number of reports of similar rates of complete resolution of heartburn, clinical features and quality of life scores with PPI therapy in patients with grade M and N NERD^[16,29,36,37]. Also in this study, there were no significant differences in FSSG scores and intraesophageal pH values between patients with grade M and grade N NERD (data not shown). The pathophysiological differences between the two types of NERD are unclear, and further studies are required to clarify the pathogenesis of NERD.

In this study, we demonstrated the effectiveness of RPZ on both dyspeptic and acid-reflux-related symptoms, which improved in a dose-dependent manner in subjects with PPI-resistant NERD. Miner *et al.*^[8] and Kusano *et al.*^[22] previously have reported that RPZ significantly improves dyspeptic symptoms in patients with NERD. It is reasonable to conclude that dyspeptic symptoms in patients with NERD can be expected respond to PPI treatment.

The limitations of this study included low sample power and a lack of placebo effect. However, in this study the most important point was to prove that NERD patients who were refractory to a standard PPI dosage, caused by insufficient acid inhibition, were improved by a greater dosage of PPI. Moreover, we analyzed intragastric and intraesophageal pH with each regimen in each subject, and were able to demonstrate the characteristics of subjects with PPI-resistant NERD using the FSSG. We believe that the FSSG score after RPZ 10 mg od for 14 d can be used to predict whether a patient with NERD refractory to a standard dosage of a PPI will respond to a higher dosage of that PPI (e.g. RPZ 10 mg qid).

In conclusion, we demonstrated that patients with NERD with a > 30% decrease in their FSSG score with RPZ 10 mg od responded to a higher PPI dosage, although they appeared to be refractory to the standard PPI dosage. On the other hand, symptoms in patients completely resistant to a standard dosage of a PPI (FSSG reduction < 30% after RPZ 10 mg od) do not resolve even if the PPI dosage is increased (e.g. RPZ 10 mg qid). We recommend that such patients should be treated with other agents such as prokinetics rather than increasing the PPI dosage^[35]. The FSSG score after rabeprazole 10 mg od for 14 d shows promise in determining the optimal treatment for patients with NERD refractory to a standard PPI dosage. The clinical usefulness of the FSSG in the PPI treatment of NERD requires verification in further studies with larger subject numbers.

COMMENTS

Background

Half of patients with non-erosive reflux disease (NERD) are resistant to treatment with standard proton pump inhibitor (PPI) dosages, due to insufficient control of acid secretion throughout 24 h. However, no earlier studies have investigated whether acid-related symptoms in patients with NERD refractory to standard PPI dosages improve when sufficient acid inhibition is attained using PPI qid therapy.

Research frontiers

PPIs are rapidly eliminated from the systemic circulation (*t*_{1/2}: 2-3 h). H⁺,K⁺-ATPase newly generated or activated in gastric parietal cells after the rapid elimination of PPI can secrete gastric acid. Frequent PPI dosing sustains plasma PPI levels for a longer time to achieve sufficient acid inhibition over 24 h. Sugimoto *et al.* have investigated the effects of frequent PPI dosing on subjects with PPI-resistant NERD, with the aim of determining the clinical characteristics of subjects with PPI-refractory NERD resistant to potent acid inhibition.

Innovations and breakthroughs

Sugimoto *et al.* have highlighted the clinical characteristics of PPI-refractory NERD patients. In subjects with NERD that is responsive to potent acid inhibition, the Frequency Scale for the Symptoms of GERD (FSSG) scores decreased by > 30% compared with baseline after a standard dose of PPI. Therefore, NERD patients whose FSSG reduces by > 30% after a standard dosage of PPI will respond to a higher dose of PPI, although the clinical effects of standard PPI dosages on their symptoms are limited.

Applications

The FSSG score after PPI treatment may show promise in determining the optimal treatment for patients with NERD that is refractory to a standard PPI dosage; increasing the PPI dosage or other agents such as prokinetics. The clinical usefulness of the FSSG in the PPI treatment of NERD requires verification in further studies with larger subject numbers.

Peer review

The study analyzed the problem of patients with symptoms of GERD but refractory to PPIs. This is a very important problem in the treatment of these patients. The study was well designed and the results are clearly described.

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Clinicopathologic significance of GLUT1 expression and its correlation with Apaf-1 in colorectal adenocarcinomas

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gender ($P = 0.009$), non-mucinous tumor type ($P = 0.045$), poorer differentiation ($P = 0.001$), lymph node metastasis ($P < 0.001$), higher AJCC and Dukes stage ($P < 0.001$ and $P < 0.001$, respectively). There was a significant inverse correlation between GLUT1 expression and Apaf-1 expression ($P = 0.001$). In univariate survival analysis, patients with GLUT1 expression demonstrated poor overall survival and disease-free survival ($P = 0.047$ and $P = 0.021$, respectively, log-rank test).

CONCLUSION: GLUT1 expression was frequently increased in adenocarcinomas and metastatic lesions. GLUT1 expression was significantly correlated with poorer clinicopathologic phenotypes and survival of patients with colorectal adenocarcinomas.

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Key words: Adenocarcinoma; Colorectum; Glucose transporter 1; Apoptosis-activating factor-1; Prognosis; Survival

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Abstract

AIM: To investigate the role of glucose transporter 1 (GLUT1) expression in colorectal carcinogenesis and evaluate the correlation with clinicopathological parameters and apoptosis-activating factor-1 (Apaf-1) expression in colorectal adenocarcinomas.

METHODS: We used tissue microarrays consisting of 26 normal mucosa, 50 adenomas, 515 adenocarcinomas, and 127 metastatic lesions. Medical records were reviewed and clinicopathological analysis was performed.

RESULTS: GLUT1 expression was absent in normal mucosa and low or moderately apparent in 19 cases (38.0%) of 50 adenomas. However, GLUT1 expression was detected in 423 (82.1%) of 515 adenocarcinomas and in 96 (75.6%) of 127 metastatic lesions. GLUT1 expression was significantly correlated with female

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related death in men and women in the industrialized nations^[1,2]. There have been marked advances in the understanding of the carcinogenesis in colorectal cancer and cancer biology; however, the specific therapeutic problem continues to persist^[3]. Most patients with colorectal ma-

lignancy, except for the advanced stage, undergo curative resection. Stage II patients with obstruction, perforation or certain tumor markers, and stage III patients, receive adjuvant chemotherapy after surgical resection^[4]. However, there is no appropriate targeted therapy to further improve the clinical outcome. The molecular prognostic factors associated with a distinct prognostic outcome would be of great help for patients who are likely to benefit from adjuvant therapies, leading to an improvement in prognosis^[5].

The transition from normality to malignancy through the adenoma-carcinoma carcinogenesis sequence is accompanied by various alterations in the expression of a number of genes associated with the maintenance of cellular homeostasis^[6]. Previous studies have revealed an enhancement of glycolytic metabolism in malignant tumors. Increased glucose uptake and use is one of the major characteristics found in many malignant tumors. This process is mediated by the glucose transporters (GLUTs) which are membrane proteins responsible for the transport of glucose across cellular membranes^[7].

Among seven cloned glucose transporters, GLUT1 is an isoform that is expressed in erythrocytes, the blood-tissue barriers such as the blood-brain and blood-nerve barriers, and the placenta^[8]. GLUT1 protein expression can be altered by a number of different conditions, including cellular differentiation and transformation, and also can be altered under the influence of growth factors, insulin, glucose, and even stress^[9]. The increased expression of GLUT1 mRNA and protein has been demonstrated in various cancer tissues which indicates that GLUT1 may play an important role in glucose uptake by various cancers and that GLUT1 expression could be useful as a marker for malignant transformation^[10-15].

In this study, we immunohistochemically determined the GLUT1 expression in a large series of 26 normal mucosa, 50 tubular adenomas, 515 adenocarcinomas, and 127 metastatic lesions. This was to investigate the membranous GLUT1 expression in colorectal carcinogenesis and to evaluate the correlation between GLUT1 expression and the clinicopathological parameters and between GLUT1 expression and cytoplasmic apoptosis-activating factor-1 (Apaf-1) expression, as well as its effect on survival of patients with colorectal adenocarcinomas.

MATERIALS AND METHODS

Patients and tissue samples

Our study enrolled a consecutive series of 515 patients with colorectal adenocarcinoma. All patients were diagnosed and treated at the Hanyang University Hospital (Seoul, Korea) between January 1991 and August 2001. There were 293 male and 222 female patients. The mean age of patients was 58 years. The tumor growth pattern was fungating in 239 cases and infiltrative in 276 cases. The tumors consisted of 489 non-mucinous adenocarcinomas and 26 mucinous adenocarcinomas. The tumors were located in the cecum ($n = 18$), ascending colon ($n = 75$), hepatic flexure ($n = 12$), transverse colon ($n = 25$), splenic

flexure ($n = 4$), descending colon ($n = 25$), sigmoid colon ($n = 107$), and rectum ($n = 249$). The mean tumor size was 5.7 cm. The mean follow-up interval was 6.0 years. One hundred and eighty (35%) patients died and 335 (65%) patients survived. Twenty-six cases of normal mucosa, 50 cases of tubular adenomas, 127 metastatic lesions (lymph nodes and distant organs) were randomly selected to evaluate the role of GLUT1 in the multistep carcinogenesis.

Tissue microarray construction

Tissue microarrays were constructed from archival formalin-fixed, paraffin-embedded tissue blocks using a manual tissue arrayer (Quick-Ray Manual Tissue Microarrayer, Unitma Co, Ltd, Seoul, Korea). As described previously^[16], for each sample, areas rich in tumor cells were identified by light microscopic examination of hematoxylin-eosin-stained sections and then selected for use in tissue microarrays. Tissue cylinders with a diameter of 2 mm were punched from the previously marked tumor area of each block (donor block) and then transferred to a recipient paraffin block. This resulted in a 6×10 array for 60 cases.

Immunohistochemical staining

For immunohistochemical staining, multiple 4 μ m sections were cut with a Leica microtome. Sections were transferred to adhesive-coated slides. Tissue microarray (TMA) slides were dewaxed by heating at 55°C for 30 min and by three washes, 5 min each, with xylene. Tissues were rehydrated by a series of 5 min washes in 100%, 90% and 70% ethanol and phosphate buffered saline (PBS). Antigen retrieval was performed by microwaving the samples for 4 min 20 s at full power in 250 mL of 10 mmol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase for 20 min. The primary mouse monoclonal GLUT1 antibody (ab40084, Abcam, Cambridge, UK) was diluted 1:100 using goat serum and the primary polyclonal rabbit Apaf-1 antibody (Novocastra Laboratories, Newcastle upon Tyne, UK) was diluted 1:200 using goat serum and incubated at room temperature for 1 h. After three washes, 2 min each with PBS, the sections were incubated with a biotinylated goat anti-mouse secondary antibody for 30 min (DAKO, Carpinteria, CA, USA). After three washes, 2 min each with PBS, horseradish peroxidase-streptavidin (DAKO, Carpinteria, CA, USA) was added to the sections for 30 min, followed by another three washes, 2 min each with PBS. The samples were developed with 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) for 1 min and counterstained with Mayer's hematoxylin. Then, the slides were dehydrated following a standard procedure and sealed with coverslips. Negative controls were performed by omitting the GLUT1 and Apaf-1 antibodies during the primary antibody incubation.

Interpretation of GLUT1 and Apaf-1 immunostaining

The GLUT1 and Apaf-1 expression was evaluated semi-quantitatively by two independent pathologists (Jang SM and Paik SS) without prior knowledge of the clinical follow-up data for each case. The GLUT1 immunostaining

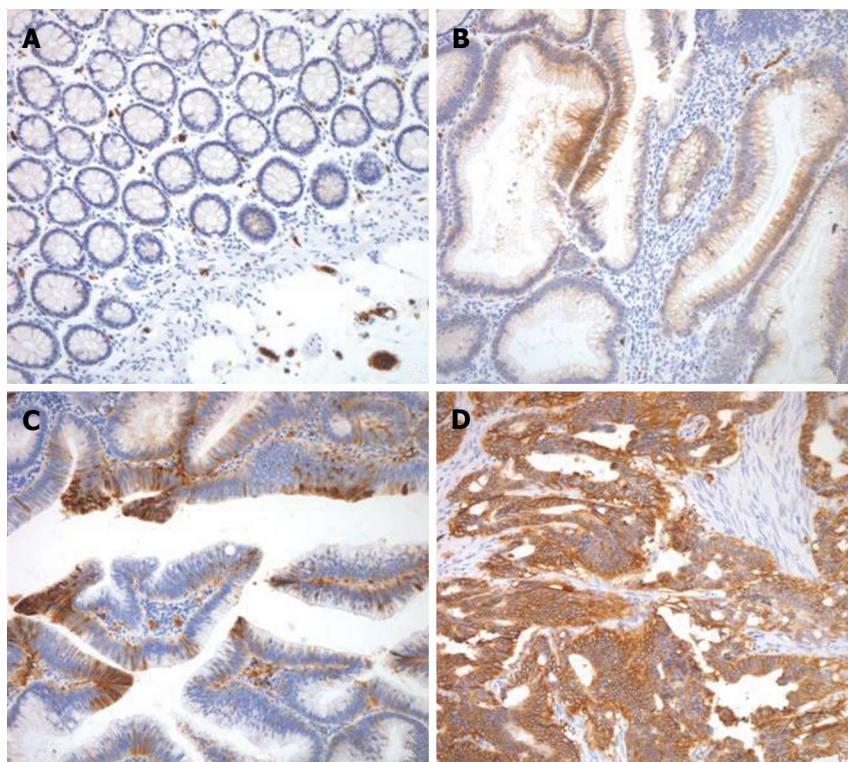


Figure 1 Representative photomicrograph of glucose transporter 1 immunostaining in normal mucosa (A), tubular adenoma with low grade dysplasia (B), tubular adenoma with high grade dysplasia (C), and adenocarcinoma (D). The glucose transporter 1 was stained in the cytoplasmic membrane of the cells.

was semi-quantitated by grading the proportion of cells that were GLUT1-positive, as described previously^[7,11]; grade 0: negative (positive cells are 0%), grade 1: low positive (positive cells are less than 10%), grade 2: moderate positive (positive cells are 10%-50%) and grade 3: high positive (positive cells are more than 50%). For purposes of statistical analysis, a cut-off value of 50% was adopted. Each tissue section was classified as either < 50% or > 50% GLUT1-positive. Apaf-1 expression was evaluated based on the staining intensity and staining extent, as described previously^[17]. Staining intensity for Apaf-1 was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Staining extent was scored as 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (76%-100%) according to the percentage of positive-stained cells. The sum of intensity and extent scores was used as the final staining score. All cases were divided into four expression groups according to their sum scores which were as follows: 0 = negative; 1-3 = weak; 4-5 = moderate; and 6-7 = strong. If the sum scores were moderate or strong, cases were classified as Apaf-1-positive. If the sum scores were negative or weak, cases were classified as Apaf-1-negative. In cases of discrepant assessments, slides were reinvestigated by both pathologists under a multi-head microscope and an agreement was obtained.

Statistical analysis

Statistical analysis was performed using SPSS software (version 12.0, SPSS, Chicago, IL, USA). The chi-square test for linear trend, Fisher exact test, and one-way

ANOVA test were used to examine the association between the GLUT1 expression and clinicopathological parameters including age, gender, tumor location, tumor size, tumor gross, tumor type, differentiation, TNM category, AJCC stage, Dukes stage, and Apaf-1 expression. The Kaplan-Meier method was used to calculate overall survival and disease-free survival curves. Univariate survival analysis with the log-rank test was used to compare the difference between the survival rates of the patient subgroups. Multivariate survival analysis with the Cox proportional hazards regression model was used to evaluate the independent prognostic factors. A difference of $P < 0.05$ between groups was considered significant.

RESULTS

Pattern of GLUT1 expression

The GLUT1 expression was evaluated in 26 normal mucosa, 50 tubular adenomas, 515 adenocarcinomas, and 127 metastatic lesions. As expected, erythrocyte membranes were strongly GLUT1-positive. Various grades of membranous GLUT1 expression were observed in the included tissue samples. The representative photomicrographs of GLUT1 immunostaining are shown in Figure 1. All 26 normal mucosa specimens (100%) were negative for GLUT1 expression with no exception. Twenty-one (80.8%) of 26 tubular adenomas with low grade dysplasia were negative for GLUT1 expression and only 5 cases (19.2%) revealed a low grade of GLUT1 expression. Ten (41.7%) of 24 tubular adenomas with high grade dyspla-

Table 1 Glucose transporter 1 expression in normal mucosa, tubular adenomas, adenocarcinomas, and metastatic lesions (*n* = 718)

Tissue samples	<i>n</i>	Expression of GLUT1				<i>P</i> value (χ^2 -test)
		Negative (%)	Low (%)	Moderate (%)	High (%)	
Normal mucosa	26	26 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	< 0.001 ¹
Tubular adenomas with LGD	26	21 (80.8)	5 (19.2)	0 (0.0)	0 (0.0)	
Tubular adenomas with HGD	24	10 (41.7)	9 (37.5)	5 (20.8)	0 (0.0)	
Adenocarcinomas	515	92 (17.9)	75 (14.6)	161 (31.2)	187 (36.3)	
Metastatic lesions	127	31 (24.4)	15 (11.8)	22 (17.3)	59 (46.5)	

¹Chi-square test for linear trend. LGD: Low grade dysplasia; HGD: High grade dysplasia; GLUT1: Glucose transporter 1; Metastatic lesions; lymph nodes and distant organs.

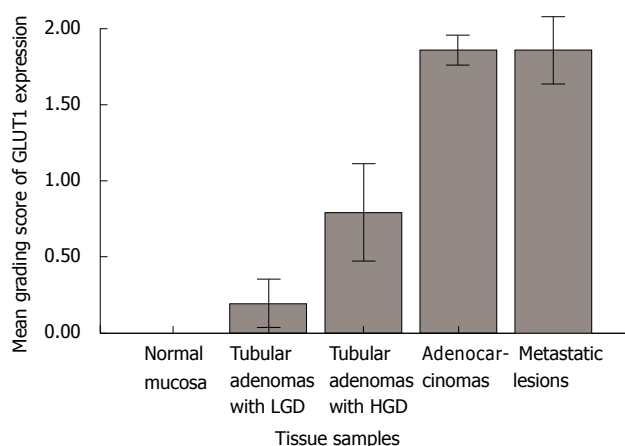


Figure 2 Representation showing the difference of mean grading score of glucose transporter 1 expression in normal mucosa, tubular adenomas with low grade dysplasia, tubular adenomas with high grade dysplasia, adenocarcinomas, and metastatic lesions (lymph nodes and distant organs). One-way ANOVA test was used. GLUT1: Glucose transporter 1; LGD: Low grade dysplasia; HGD: High grade dysplasia.

sia were negative for GLUT1 expression, 9 cases (37.5%) revealed a low grade of GLUT1 expression, and 5 cases (20.8%) showed a moderate grade of GLUT1 expression. However, in colorectal adenocarcinomas, 187 (36.3%) of 515 cases revealed a high grade of GLUT1 expression and 161 cases (31.2%) and 75 cases (14.6%) showed moderate grade and low grade of GLUT1 expression, respectively. Only 92 (17.9%) of 515 cases were negative for GLUT1 expression. In metastatic lesions, 59 (46.5%) of 127 cases showed high grade of GLUT1 expression and 22 cases (17.3%) and 15 cases (11.8%) showed moderate grade and low grade of GLUT1 expression, respectively. Thirty-one cases (24.4%) were negative for GLUT1 expression. A significant difference in the GLUT1 expression among normal mucosa, tubular adenomas, adenocarcinomas, and metastatic lesions was observed (Table 1 and Figure 2).

Correlation between GLUT1 expression and clinicopathologic parameters and Apaf-1 in colorectal adenocarcinomas

To assess the clinicopathologic significance of GLUT1 expression, we evaluated the correlation between GLUT1 expression and the clinicopathologic parameters in 515 colorectal adenocarcinomas. We found that a higher ex-

pression of GLUT1 correlated with more aggressive phenotypes of colorectal adenocarcinomas. GLUT1 expression was significantly correlated with female gender ($P = 0.009$), non-mucinous tumor type ($P = 0.045$), poorer differentiation ($P = 0.001$), frequent lymph node metastasis ($P < 0.001$), higher AJCC and Dukes stage ($P < 0.001$ and $P < 0.001$, respectively). There was a significant inverse correlation between GLUT1 expression and Apaf-1 expression ($P = 0.001$) (Table 2).

Correlation between GLUT1 expression and overall survival and disease-free survival

We examined the impact of GLUT1 expression on patient survival. As we expected, a significant prognostic influence of patient age, tumor differentiation, AJCC stage, and vascular invasion on overall and disease-free survival was found in univariate and multivariate analyses (Table 3). Notably, GLUT1 expression was significantly correlated with poor overall survival ($P = 0.047$, log-rank test) and disease-free survival ($P = 0.021$, log-rank test) in univariate analysis. However, in multivariate survival analysis with the Cox proportional hazards model, GLUT1 expression was not an independent prognostic factor for overall survival and disease-free survival ($P = 0.534$ and $P = 0.416$, respectively). Kaplan-Meier survival curves showed a significant difference in patient survival according to GLUT1 expression (Figure 3).

DISCUSSION

In the present study, we investigated the expression of GLUT1 in normal mucosa, tubular adenomas, adenocarcinomas and metastatic lesions and evaluated the correlation with the clinicopathologic parameters and patient survival in patients with adenocarcinomas. GLUT1 expression was absent in 26 normal mucosa specimens with no exception. Only 5 of 26 tubular adenomas with low grade dysplasia showed low GLUT1 expression and 14 of 24 tubular adenomas with high grade dysplasia revealed low or moderate GLUT1 expression. On the other hand, 423 of 515 adenocarcinomas and 96 of 127 metastatic lesions showed GLUT1 expression with variable grades. Furthermore, the GLUT1 expression was closely correlated with poor prognostic parameters.

The activation of GLUT1 gene expression is a molecular feature of malignant phenotype in a variety of

Table 2 Correlation between glucose transporter 1 expression and clinicopathologic factors in colorectal adenocarcinomas (n = 515) n (%)

Factors	n	Expression of GLUT1		P value
		< 50% (%) (n = 328)	≥ 50% (%) (n = 187)	
Age (yr)				0.069 ¹
< 58	244	164 (67.2)	80 (32.8)	
≥ 58	271	164 (60.5)	107 (39.5)	
Gender				0.009 ¹
Male	293	200 (68.3)	93 (31.7)	
Female	222	128 (57.7)	94 (42.3)	
Tumor location				0.506 ¹
Colon	266	169 (63.5)	97 (36.5)	
Rectum	249	159 (63.9)	90 (36.1)	
Tumor size (cm)				0.507 ¹
< 5.7	282	180 (63.8)	102 (36.2)	
≥ 5.7	233	148 (63.5)	85 (36.5)	
Tumor gross				0.216 ¹
Fungating	239	157 (65.7)	82 (34.3)	
Infiltrative	276	171 (62.0)	105 (38.0)	
Tumor type				0.045 ¹
Non-mucinous	489	307 (62.8)	182 (37.2)	
Mucinous	26	21 (80.8)	5 (19.2)	
Differentiation				0.001 ²
Well	21	19 (90.5)	2 (9.5)	
Moderate	386	252 (65.3)	134 (34.7)	
Poor	108	57 (52.8)	51 (47.2)	
T category				0.226 ²
Tis, T1	16	13 (81.3)	3 (18.8)	
T2	35	22 (62.9)	13 (37.1)	
T3	452	286 (63.3)	166 (36.7)	
T4	12	7 (58.3)	5 (41.7)	
N category				< 0.001 ²
N0	226	170 (75.2)	56 (24.8)	
N1	130	73 (56.2)	57 (43.8)	
N2	159	85 (53.5)	74 (46.5)	
M category				0.145 ¹
M0	495	318 (64.2)	177 (35.8)	
M1	20	10 (50.0)	10 (50.0)	
AJCC stage				< 0.001 ²
0, I	41	30 (73.2)	11 (26.8)	
II A, II B	183	139 (76.0)	44 (24.0)	
III A, III B, III C	271	149 (55.0)	122 (45.0)	
IV	20	10 (50.0)	10 (50.0)	
Dukes stage				< 0.001 ²
A	12	10 (83.3)	2 (16.7)	
B1, B2	208	156 (75.0)	52 (25.0)	
C1, C2	275	152 (55.3)	123 (44.7)	
D	20	10 (50.0)	10 (50.0)	
Apaf-1				0.001
Negative	401	241 (60.1)	160 (39.9)	
Positive	114	87 (76.3)	27 (23.7)	

¹Fisher's exact test, ²Chi-square test for linear trend. GLUT1: Glucose transporter 1; Apaf-1: Apoptosis-activating factor-1.

cancers and has been shown to be associated with malignant transformation. Various malignant tumors, including colorectal cancers, show increased glucose metabolism and utilization^[12-14]. Increased GLUT1 expression in neoplastic tissue reflects an increased glycolytic metabolism and is observed under conditions that induce greater dependence on glycolysis as an energy source, such as ischemia or hypoxia^[7,18-20]. Previous studies suggest that GLUT1 expression may play an important role in the

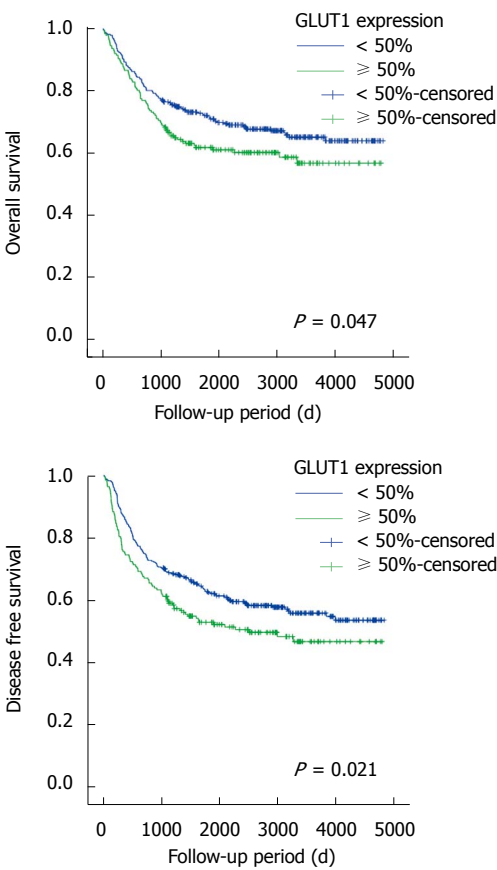


Figure 3 Cumulative overall survival curves (A) and disease-free survival curves (B) according to glucose transporter 1 expression in 515 colorectal cancer patients (Kaplan-Meier method with log-rank test). GLUT1: Glucose transporter 1.

survival of tumor cells by promoting an adequate energy supply^[21,22]. Two possible mechanisms were suggested to explain the activation of GLUT1 gene expression in cancers. Firstly, increased glycolysis and concomitant GLUT1 expression may be a constitutive feature of the malignant phenotype in many cancers. Secondly, local hypoxia in the tumor microenvironment may result in an adaptive increase in glycolytic metabolism and GLUT1 expression^[11].

Sakashita *et al*^[7] demonstrated that GLUT1 expression was positive in 18% of low-grade adenomas and in 63% of high-grade adenomas. In our results, GLUT1 expression was demonstrated in 19.2% (5/26) of tubular adenomas with low grade dysplasia and in 58.3% (14/24) of tubular adenomas with high grade dysplasia. Haber *et al*^[11] demonstrated GLUT1 expression in 101 (90%) of 112 colorectal adenocarcinomas. GLUT1 expression was undetected in 11 cases (9.8%) and detected in < 10% of the tumor cells in 39 cases (34.8%), in 10%-50% of the tumor cells in 42 cases (37.5%), and in > 50% of the tumor cells in 20 cases (17.9%). In our study, GLUT1 expression was demonstrated in 423 (82.1%) of 515 colorectal adenocarcinomas and undetectable in 92 cases (17.9%). Furthermore, we evaluated GLUT1 expression in 127 metastatic lesions, including lymph nodes and distant organs. The GLUT1 expression was significantly different between normal mucosa, tubular adenomas with low grade dyspla-

Table 3 Survival analyses of variables predicting the risk of death with colorectal adenocarcinomas

Variables	Significance univariate ¹	Significance multivariate ²	Hazard ratio	95% CI
Overall survival				
GLUT1 expression (< 50% <i>vs</i> ≥ 50%)	0.047	0.534	1.101	0.813-1.491
Patient age (< 58 yr <i>vs</i> ≥ 58 yr)	< 0.001	< 0.001	1.884	1.389-2.555
Differentiation (low <i>vs</i> high)	< 0.001	< 0.001	1.846	1.344-2.536
AJCC stage (0, I, II <i>vs</i> III, IV)	< 0.001	< 0.001	2.715	1.909-3.863
Vascular invasion (absent <i>vs</i> present)	0.001	0.005	2.993	1.393-6.430
Disease free survival				
GLUT1 expression (< 50% <i>vs</i> ≥ 50%)	0.021	0.416	1.118	0.855-1.461
Patient age (< 58 yr <i>vs</i> ≥ 58 yr)	0.002	0.003	1.497	1.150-1.947
Differentiation (low <i>vs</i> high)	< 0.001	0.002	1.593	1.193-2.126
AJCC stage (0, I, II <i>vs</i> III, IV)	< 0.001	< 0.001	2.940	2.161-3.999
Vascular invasion (absent <i>vs</i> present)	0.014	0.035	2.264	1.057-4.848

¹Log-rank test, ²Cox proportional hazards model. GLUT1: Glucose transporter 1; CI: Confidence interval.

sia, tubular adenomas with high grade dysplasia, adenocarcinomas and metastatic lesions (Table 1, $P < 0.001$). Our findings indicate that GLUT1 expression may play an important role at the late stage in the adenoma-carcinoma carcinogenesis sequence.

Some studies have reported the correlation between GLUT1 expression and the clinicopathologic parameters in colorectal adenocarcinomas. Sakashita *et al.*^[7] reported that GLUT1 expression was significantly different between well differentiated and less differentiated groups (positivity of 67% *vs* 93%, $P < 0.05$). The rate of GLUT1 expression, both moderate and strong, was also significantly different between these two groups (49% *vs* 74%, $P < 0.05$). Ito *et al.*^[15] demonstrated that GLUT1 immunostaining was stronger in tumors with less differentiation in lung adenocarcinomas. However, Younes *et al.*^[10] and Haber *et al.*^[11] reported that there was no correlation between GLUT1 expression and histologic differentiation. In our results, GLUT1 expression was < 50% of tumor cells in 19 cases (90.5%) and > 50% of the tumor cells in 2 cases (9.5%) of well differentiated adenocarcinomas. In moderately differentiated adenocarcinomas, GLUT1 expression was < 50% of tumor cells in 252 cases (65.3%) and > 50% of the tumor cells in 134 cases (34.7%). In poorly differentiated adenocarcinomas, GLUT1 expression was < 50% of tumor cells in 57 cases (52.8%) and > 50% of the tumor cells in 51 cases (47.2%). There was a significant correlation between GLUT1 expression and the histologic differentiation ($P = 0.001$).

The relationship between GLUT1 expression and the depth of invasion has been reported in colorectal adenocarcinomas. Sakashita *et al.*^[7] reported that GLUT1 expression was significantly different between T1 and T2 groups (positivity of 61% *vs* 97%, $P < 0.01$). The rate of moderate and strong GLUT1 expression was also significantly different between these two groups (45% *vs* 74%, $P < 0.01$). However, Younes *et al.*^[10] demonstrated that there was no significant difference between GLUT1 expression and the depth of invasion. Our results revealed that there was no significant correlation between GLUT1 expression and the depth of invasion. Younes *et al.*^[10] documented that there was a close correlation

between strong GLUT1 expression and the frequency of lymph node metastasis in colorectal adenocarcinomas. Sakashita *et al.*^[7] reported that the rate of GLUT1 expression in colorectal carcinomas with nodal metastasis was higher than that in those without, but the difference was not significant due to the small size of lymph node metastases-positive carcinomas. In our study, there was a close correlation between GLUT1 expression and the presence of lymph node metastasis ($P < 0.001$). This result indicates that GLUT1 may be important for maintaining the high-energy requirements of aggressive cancers. The immunohistochemical detection of GLUT1 in biopsies of colorectal cancers may be useful as a marker of aggressive biologic behavior, especially in lymph node metastasis^[10].

There has been no documented report as to the relationship between GLUT1 expression and tumor stages in colorectal adenocarcinomas. Haber *et al.*^[11] reported the association of GLUT1 staining status with Dukes stage; however, no statistical significance was revealed. Our results documented that there was a close correlation between GLUT1 expression and tumor stages, AJCC and Dukes stages ($P < 0.001$ and $P < 0.001$, respectively). The correlation between GLUT1 expression and survival in colorectal adenocarcinomas has been reported^[11]. There was a significant increase in mortality in those patients whose tumors had more than 50% of GLUT1-positive cells (relative risk, 2.4; $P = 0.02$ by the log rank test). Our study showed that GLUT1 expression was significantly correlated with poor overall survival ($P = 0.047$) and disease-free survival ($P = 0.021$) in univariate analysis. However, in multivariate analysis with the Cox proportional hazards model, GLUT1 expression was not an independent prognostic factor of overall survival and disease-free survival ($P = 0.534$ and $P = 0.416$, respectively).

There are multiple interactions between the cellular machinery involved in glucose uptake and metabolism, and the cellular mechanism of programmed cell death or apoptosis. Glucose deprivation can promote apoptosis in a variety of cells. The induction of glucose uptake and metabolism can prevent or reduce apoptosis^[23,24]. Enhanced GLUT1 expression has been shown to inhibit cytochrome c release and downstream caspase activa-

tion during hypoxia^[25,26]. Vesely *et al.*^[27] documented that GLUT1 prevents hypoxia-induced apoptosis in vascular smooth muscle cells and cardiac myocytes largely *via* a mitochondrial, caspase 9-dependent pathway. In this study, we evaluated the correlation between GLUT1 expression and the expression of Apaf-1, one of the key regulators in the mitochondrial apoptotic pathway^[4,28]. Our results revealed that there is a significant inverse correlation between GLUT1 expression and Apaf-1 expression ($P = 0.001$). The GLUT1 expression may prevent apoptosis through the suppression of Apaf-1 expression *via* a mitochondrial apoptotic pathway.

In conclusion, we tried to clarify the clinicopathologic significance of GLUT1 expression in a large cohort consisting of 26 normal mucosa, 50 tubular adenomas, 515 adenocarcinomas, and 127 metastatic lesions. The GLUT1 expression pattern suggested an important role in colorectal cancer development, especially at the late stage of the adenoma-carcinoma sequence, and GLUT1 expression was closely correlated with poor clinicopathologic factors in colorectal adenocarcinomas.

COMMENTS

Background

Colorectal cancer is the second leading cause of cancer-related death in men and women in the industrialized nations. There have been marked advances in the understanding of the carcinogenesis in colorectal cancer and cancer biology; however, the relevant therapeutic problem continues to persist. Previous studies revealed an enhancement of glycolytic metabolism in malignant tumors. The increased expression of glucose transporter 1 (GLUT1) mRNA and protein has been demonstrated in various cancer tissues which indicates that GLUT1 may play an important role in glucose uptake by various cancers and that GLUT1 expression could be useful as a marker for malignant transformation.

Research frontiers

This study was to investigate the membranous GLUT1 expression in colorectal carcinogenesis and to evaluate the correlation between GLUT1 expression and the clinicopathological parameters, and between GLUT1 expression and cytoplasmic Apaf-1 expression, as well as its effect on survival of patients with colorectal adenocarcinomas.

Innovations and breakthroughs

GLUT1 expression was significantly correlated with female gender, non-mucinous tumor type, poorer differentiation, lymph node metastasis, higher AJCC and Dukes stage. There was a significant inverse correlation between GLUT1 expression and Apaf-1 expression. Patients with GLUT1 expression demonstrated poor overall survival and disease-free survival in univariate survival analysis.

Applications

The authors evaluated the correlation between GLUT1 expression and expression of Apaf-1, one of the key regulators in the mitochondrial apoptotic pathway. The results revealed that there is a significant inverse correlation between GLUT1 expression and Apaf-1 expression. These results warrant further careful and well-designed studies of GLUT1 and Apaf-1 expression in colorectal cancers for clinical therapeutic application.

Terminology

GLUTs are membrane proteins responsible for the transport of glucose across cellular membranes. GLUT1 is an isoform that is restricted to erythrocytes and blood-tissue barriers such as the blood-brain and blood-nerve barriers. Apaf-1 is one of the key regulators in the mitochondrial apoptotic pathway. Apaf-1 binds to a protein called cytochrome-c, which is released from mitochondria under the control of p53, and this complex activates caspase-9, which then triggers executioner caspases, leading to apoptosis.

Peer review

The paper investigated the role of GLUT1 expression in colorectal carcinogenesis and evaluated the correlation with the clinicopathological parameters and Apaf-1 expression in colorectal adenocarcinomas. It is very interesting.

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Acoustic radiation force impulse elastography for hepatocellular carcinoma-associated radiofrequency ablation

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were brighter than surrounding cirrhotic parenchyma. Another 13 (34.2%) were darker. The others (5 cases, 13.2%) were seen as the same color as the adjacent liver parenchyma. Post-RFA lesions were darker than previous tumor and surrounding parenchyma in all 38 cases. However, recurred HCCs were brighter than the treated site in all 8 cases.

CONCLUSION: Using ARFI technique is helpful for differential diagnosis in order to detect recurred HCCs more easily in patients with confusing status.

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Key words: Hepatocellular carcinoma; Elastography; Radiofrequency ablation

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Abstract

AIM: To evaluate the potential usefulness of acoustic radiation force impulse (ARFI) images for evaluation of hepatocellular carcinomas (HCC)-associated radiofrequency ablation.

METHODS: From January 2010 to June 2010, a total of 38 patients with HCC including recurred HCCs after RFA underwent ARFI elastography. The brightness of tumor was checked and the shear wave velocity was measured for the quantification of stiffness. According to the brightness, the tumors were classified as brighter, same color and darker compared with adjacent parenchyma. Using the same methods, 8 patients with recurred HCCs after RFA state were evaluated about the brightness compared with adjacent RFA ablation area.

RESULTS: In the 38 patients with HCCs, 20 (52.6%)

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INTRODUCTION

In recent decades, there has been an increasing interest in assessing the viscoelastic properties of tissues with ultrasound. Ultrasonography (US) tissue-strain analysis can be performed under compression using Hitachi Real-time Tissue elastography (HI-RTE, Hitachi Medical Systems Europe, Zurich, Switzerland), eSie Touch (Siemens, Erlangen, Germany) or Elasticity Imaging (Simems)^[1-8].

Acoustic radiation force impulse (ARFI) imaging is a new ultrasound imaging modality to evaluation of tissue

stiffness by radiation forced-based imaging method that is provided with conventional B-mode US.

In ARFI imaging, an initial ultrasonic pulse is transmitted at diagnostic intensity levels to obtain a baseline signal for later comparison. A short-duration (approximately 0.3 s), high intensity acoustic “pushing pulse” is then transmitted by the same transducer, and followed by a series of diagnostic intensity pulses, which are used to track the displacement of the tissue caused by the pushing pulse^[9-11]. The response of the tissue to the radiation force is observed using conventional B-mode imaging pulses and it is possible to display the quantitative shear wave velocity (SWV, m/s) of ARFI displacement^[12,13]. Because the velocity of the shear wave depends on the tissue stiffness, it is possible to apply ARFI technology to elastography^[14-19].

Until now, there are no studies that have evaluated ARFI elastography’s usefulness in differentiating HCC, post RFA HCC and recurrent HCC after RFA and no studies have been reported on the quantification of tumor stiffness using shear wave velocity, which may help in the diagnosis.

The present study was performed to investigate the potential usefulness of ARFI elastography for evaluation of HCC associated RFA, assuming that different features can be shown on ARFI elastography images according to the HCC, post RFA HCC and recurrent HCC after RFA.

MATERIALS AND METHODS

Patients

Between January 2010 and June 2010, a total of 38 patients with viable HCC were evaluated by ARFI elastography. Eight patients had recurrent HCC after RFA. Because every patient each had one HCC, a total of 38 HCCs were included in this study.

In cases of technical failure such as patient motion, the presence of a deep-seated lesion or the patient’s inability to hold their breath properly, recurrent HCC after TACE or operation, and multiple HCCs were excluded from this study.

Mean tumor diameter was 2.4 cm (0.8-3.5 cm).

The diagnosis of all HCCs was based on the typical findings determined using either computed tomography (CT) or magnetic resonance (MR) imaging and/or biopsy. The following imaging findings were considered for the diagnosis of HCC: (1) tumors showing typical enhancement pattern (early enhancement on the arterial phase and wash-out on the portal and delayed phases); and/or (2) mass showing high signal intensity on the T2 weighted image.

A detailed medical history was obtained from all 38 patients. All patients had underlying liver disease which was established according to serum markers and liver biopsy as follows: chronic viral hepatitis was diagnosed as serum positive HBV-DNA and/or HCV-RNA with an elevated serum alanine amino transferase (ALT). Chronic alcoholic hepatitis was diagnosed as a history of long term alcohol consumption with an elevated serum ALT. Liver cirrhosis was diagnosed based on cross-sectional imaging findings and/or biopsy.

Table 1 Stiffness of hepatocellular carcinomas on acoustic radiation force impulse imaging

Acoustic radiation force impulse imaging	Darker (stiffer)	Same color (equally stiff)	Bright (softer)
Pre-RFA HCCs (38)	13	5	20
Recurrent HCCs (8)	0	0	8
Post-RFA HCCs (38)	38	0	0

HCCs: Hepatocellular carcinomas; RFA: Radiofrequency ablation.

Image protocol and analysis

B-mode standard US scanning and ARFI elastography were performed using a SIEMENS Acuson S2000 using a 4-1 MHz curved array probe. Two experienced radiologists participated in the US scanning. Prior to RFA, the radiologists scanned the liver to locate the HCC detected on the other cross-sectional imaging technique, such as CT, MRI, and/or prior US. After fitting the ARFI image box to cover the lesion, an ARFI image was obtained with a corresponding B-mode image. The SWV was obtained from the HCC and hepatic parenchyma, three times. After RFA, ARFI images and SWV were obtained, as before RFA.

Two radiologists, who performed US, reviewed all of the B-mode and ARFI images. The stiffness of the pre- and post-RFA HCCs were analyzed on the ARFI images. The HCCs were categorized according to the brightness, darker (stiffer), same color (equally stiff), or brighter (softer), based on the HCCs brightness relative to the hepatic parenchyma on the ARFI images. The SWV of HCCs and hepatic parenchyma were averaged. These SWVs were used for quantification of stiffness.

Discrepancies between the two reviewers were resolved through consensus.

RESULTS

Table 1 summarized the stiffness of HCCs on the ARFI image. Before RFA, 20 HCCs (52.6%) appeared as a bright color, which meant the HCC were softer than hepatic background (Figure 1). When compared with the hepatic parenchyma, 13 HCCs (34.2%) had a darker appearance, which indicated HCCs were stiffer than surrounding hepatic parenchyma (Figure 2). The remaining HCCs (5 cases, 13.2%) were seen as the same color as the adjacent hepatic parenchyma, which indicated the HCC had the same stiffness as the hepatic background. After RFA, all HCCs (38 cases, 100%) revealed a darker color (Figure 3). All recurrent HCC after RFA (8 cases), showed a bright appearance compared to the RFA site (Figure 4). This means that RFA ablation site was harder than before RFA, so the recurrent HCC were softer than the surrounding area of the prior RFA site. 5 cases of recurrent HCC after RFA were treated by re-RFA. The others were treated by TACE (2 cases), or operation (1 case).

The tumor diagnosis of recurrence after RFA was based on the typical findings determined using either CT or MRI and serum tumor marker elevation (AFP and/or PIVKA).

Two cases showed typical imaging findings of tumor re-

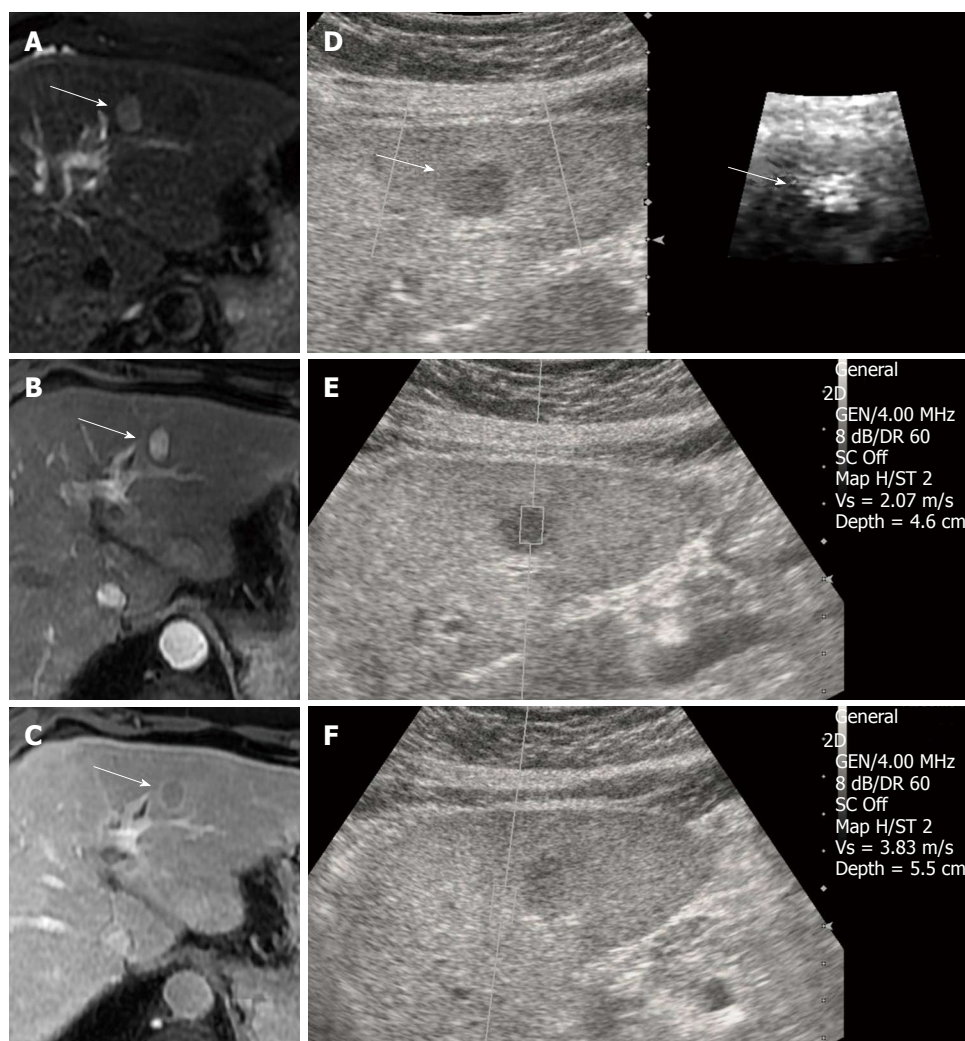


Figure 1 Hepatocellular carcinoma in a 59-year-old man with underlying liver cirrhosis. A: T2-weighted magnetic resonance (MR) image shows a high signal intensity mass in segment 2 of the liver (arrow); B: On dynamic study, the mass revealed an arterial enhancement; C: Delayed wash-out, which is a typical finding of hepatocellular carcinoma (HCC) (arrow); D: On B-mode image, the HCC appears as a well defined, hypoechoic mass and the HCC have a bright (softer) color on the acoustic radiation force impulse (ARFI) image (arrows); E: The shear wave velocity (SWV) of HCC is 2.07 m/s; F: SWV of surrounding cirrhotic hepatic parenchyma measures 3.83 m/s. GEN: General; SC: Spatial compounding; Vs: Velocity.

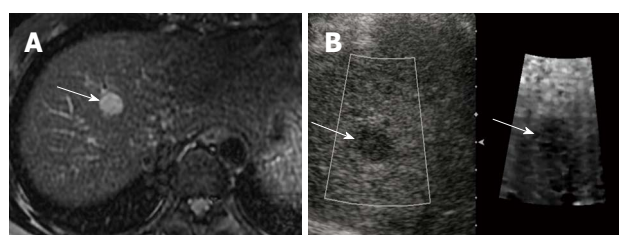


Figure 2 Hepatocellular carcinoma in a 45-year-old woman with underlying liver cirrhosis. A: T2-weighted magnetic resonance image shows a high signal intensity hepatocellular carcinoma (HCC) in segment 4 of the liver (arrow); B: On a B-mode image, the HCC is seen as an ovoid, hypoechoic mass (arrow), that appears darker (stiffer) than hepatic background on the acoustic radiation force impulse image (arrow). The shear wave velocity of HCC and surrounding hepatic parenchyma were measured as 2.20 m/s and 1.57 m/s, respectively (not shown).

currence at CT, but serum tumor marker level was normal. But one showed typical imaging findings of tumor recurrence on MRI, another imaging study. The other recurrent HCC was confirmed after surgery.

Table 2 summarized the SWV of HCCs and hepatic

parenchyma. Cirrhotic liver parenchyma and non-cirrhotic liver parenchyma had a mean SWV of 2.62 m/s and 1.04 m/s, respectively. The mean SWVs of pre-RFA HCCs and recurrent HCCs after RFA were 2.04 m/s and 2.02 m/s. All post-RFA HCCs were uncheckable, and their numerical value was shown 'X.XX'. This nonnumerical value is due to abrupt tissue degeneration in the RFA site preventing a reliable reading.

DISCUSSION

Recently, ARFI sonoelastography has been used to noninvasively generate internal mechanical excitation and has attracted great attention for its use in the measurement of tumor stiffness^[20-21]. To our knowledge, no other investigators have evaluated the use of ARFI sonoelastography for the evaluation of post-RFA recurrent HCC. In our study, pre-RFA HCCs showed variable hardness as compared with the liver parenchyma on ARFI images. These results seem to be inconsistent

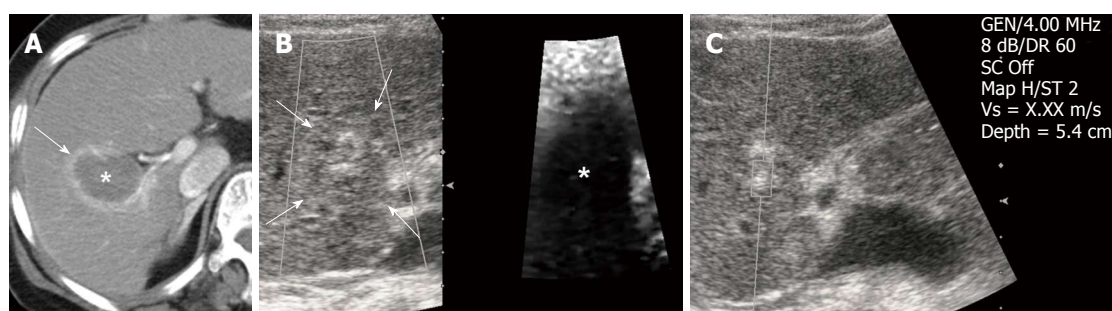


Figure 3 Post-radiofrequency ablation hepatocellular carcinoma in a 71-year-old woman with underlying liver cirrhosis. A: Contrast-enhanced arterial phase computed tomography scan obtained immediately after radiofrequency ablation (RFA) shows the ablation zone of low attenuation (asterisk) with a surrounding ring of benign enhancement (arrows) in segment 5 of the liver; B: Next day, follow up B-mode image of post-RFA hepatocellular carcinoma (HCC) is seen as an ill-defined heterogeneous echogenic lesion (arrow) and the HCC appears darker on the acoustic radiation force impulse image (asterisk); C: The shear wave velocity of post-RFA HCC is uncheckable, shown as X.XX m/s. GEN: General; SC: Spatial compounding; Vs: Velocity.

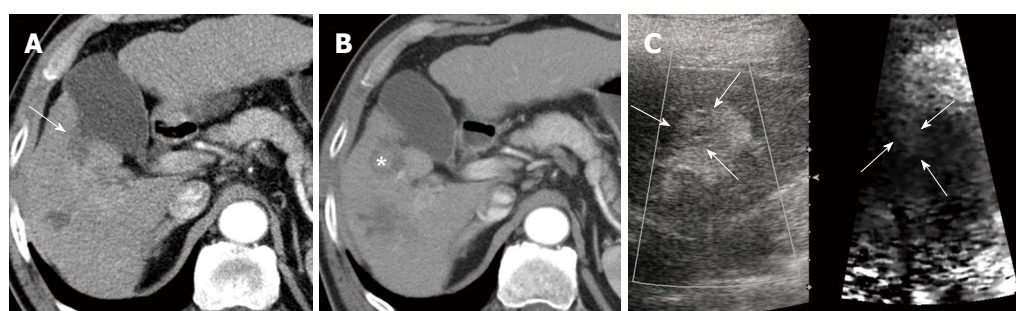


Figure 4 Recurred hepatocellular carcinoma after radiofrequency ablation in a 65-year-old man with underlying liver cirrhosis. A: Follow-up post-radiofrequency ablation (RFA) computed tomography (CT) scan shows the ablation zone of low attenuation without residual or recurrent tumor in segment 5 of the liver (arrow); B: Contrast-enhanced CT scan obtained after 6 mo shows enhancing nodule in the ablated lesion (asterisk). The serum alpha-fetoprotein increased from 2.6 ng/mL to 16.8 ng/mL; C: On a B-mode image, prior RFA site and recurrent hepatocellular carcinoma (HCC) are seen as homogeneous hyperechoic lesions and subtle heterogeneous echogenic nodules with a thin hypoechoic rim (arrows). The recurrent HCC appears as a brighter color (softer) with a distinct border on the acoustic radiation force impulse image.

Table 2 Stiffness of hepatic parenchyma and hepatocellular carcinomas on shear wave velocity

Mean shear wave velocity (m/s)				
Cirrhosis	Non-cirrhosis	Pre-RFA HCCs	Recurred HCCs	Post-RFA HCCs
2.62	1.04	2.04	2.02	uncheckable

HCCs: Hepatocellular carcinomas; RFA: Radiofrequency ablation.

with the results of Fahey's study^[22,23], in which all HCCs were softer than the surrounding liver. This discordancy was thought to be the difference in parenchymal hardness between chronic hepatitis and liver cirrhosis. Additionally, differences in severity of liver cirrhosis also might be contributing to the discrepancy.

Actually according to the other report^[24], there was no statistically significant difference at the HCCs in terms of tumor stiffness suggested by ARFI elastography ($P > 0.05$).

In all post-RFA HCCs, thermal ablated necrotic lesions were seen as a darker color on the ARFI images and had uncheckable SWV. In the RFA site, the system constantly provided a nonnumerical value. It was considered an unknown mechanical error that was associated with immediate post-RFA state^[25].

Although remarkable advances in surgical and imaging

techniques have improved the prognosis of HCC patients, the high incidence of intrahepatic recurrence remains a major challenge in HCC therapy^[26,27]. However, sometimes B-mode imaging for assessment of HCC recurrence is not satisfactory.

On ARFI imaging, most of the recurrent HCCs appeared brighter than the pre-RFA ablation sites. Moreover, most of the cases revealed distinct borders, unlike the B-mode images. The RFA ablation site comprised hard lesions that showed coagulative necrosis and fibrotic scarring. As a result, recurrent HCCs were found to be softer with improved contrast compared to the surrounding pre-RFA ablation area. Thus, we thought that the ARFI images were superior to the B-mode images for evaluating HCC recurrence after RFA and also useful to guide the second round of RFA for recurrent HCC.

COMMENTS

Background

Recently, acoustic radiation force impulse (ARFI) sonoelastography has been used to noninvasively generate internal mechanical excitation and has attracted great attention for its use in the measurement of tumor stiffness.

Research frontiers

To our knowledge, no other investigators have evaluated the use of ARFI sonoelastography for the evaluation of post-radiofrequency ablation (RFA) recurrent hepatocellular carcinoma (HCC).

Innovations and breakthroughs

The RFA ablation site was comprised of hard lesions that showed coagulative necrosis and fibrotic scarring. As a result, recurrent HCCs were found to be softer with improved contrast compared to the surrounding pre-RFA ablation area.

Applications

ARFI images are superior to B-mode images for evaluating HCC recurrence after RFA and also useful to guide the second round of RFA for recurrent HCC.

Terminology

ARFI imaging is a new ultrasonography (US) imaging modality used to evaluate tissue stiffness by a radiation force-based imaging method that is provided with conventional B-mode US.

Peer review

This is an interesting report of the effect of RFA on HCC by use of ARFI techniques.

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Cetuximab plus irinotecan in pretreated metastatic colorectal cancer patients: The ELSIE study

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Abstract

AIM: To evaluate the efficacy and safety of cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer (mCRC) patients from South-East Asia and Australia.

METHODS: In this open-label, phase II study, the main eligibility criteria were epidermal growth factor receptor-positive mCRC with progressive disease within 3 mo of an irinotecan-based regimen as the most recent chemotherapy. Patients received cetuximab 400 mg/m² initially, then 250 mg/m² every week, with the same regimen of irinotecan on which the patients had progressed (4 pre-defined regimens allowed). The primary objective was evaluation of progression-free survival (PFS) at 12 wk. Secondary objectives included a further investigation of PFS, and an assessment of the overall response rate (ORR), duration of response, time to treatment failure (TTF), overall survival and the safety profile.

RESULTS: One hundred and twenty nine patients were enrolled from 25 centers in the Asia-Pacific region and of these 123 received cetuximab plus irinotecan. The most common recent irinotecan regimen used was 180 mg/m² every 2 wk which had been used in 93 patients (75.6%). The PFS rate at 12 wk was 50% (95% confidence interval (CI, 41-59) and median PFS time was 12.1 wk (95% CI: 9.7-17.7). The ORR was 13.8% (95% CI: 8.3-21.2) and disease control rate was 49.6% (95% CI: 40.5-58.8). Median duration of response was 31.1 wk (95% CI: 18.0-42.6) and median overall survival was 9.5 mo (95% CI, 7.5-11.7). The median TTF was 11.7 wk (95% CI: 9.1-17.4). Treatment was generally well tolerated. The most common grade 3/4 adverse events were diarrhea (13.8%), neutropenia (8.9%), rash (5.7%) and vomiting (5.7%).

CONCLUSION: In patients from Asia and Australia, this study confirms the activity and safety of cetuximab plus irinotecan observed in previous studies in Europe and South America.

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Key words: Epidermal growth factor receptor; Cetuximab; Irinotecan; Metastatic colorectal cancer; Asia

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INTRODUCTION

Based on 2002 estimates, there are approximately 1 million new cases of colorectal cancer (CRC) annually worldwide, with 529 000 deaths from the disease^[1]. Approximately 25% of patients with CRC present with metastatic CRC (mCRC), and 25%-30% of newly diagnosed patients with CRC will eventually develop metastatic disease^[2]. Five-year survival rates for patients with newly diagnosed mCRC have improved from 9.1% during 1990-1997 to 19.2% for 2001-2003 to a predicted value of 32% for 2004-2006^[3]. The observed increase in survival is associated with the adoption of hepatic resection and improved chemotherapy regimens^[3]. In 2002, the annual incidence and mortality associated with CRC in Eastern and South-East Asia were approximately 307 600 and 160 000 cases, respectively, and in Australia, they were around 12 300 and 4 900 cases, respectively^[1]. While the incidence and mortality rates for CRC are generally stabilizing in Western and developed countries, those in economically developing countries and in Eastern and South East Asia are increasing^[1,4,5].

The epidermal growth factor receptor (EGFR) is frequently expressed in CRC^[6-9], where high levels of expression have been reported to be associated with reduced survival time in patients with mCRC^[10]. Cetuximab (Erbix[®]) developed by Merck KGaA (Darmstadt, Germany) under license from ImClone Systems, a wholly-owned subsidiary of Eli Lilly, Branchburg, NJ, USA) is a monoclonal immunoglobulin G1 antibody that specifically targets the EGFR with high affinity, and exerts an inhibitory effect on tumor cell proliferation, survival, motility, invasion and angiogenesis^[11]. Cetuximab has been shown to be efficacious and well tolerated in patients failing irinotecan therapy either in combination with irinotecan or as monotherapy^[7], and in combination with irinotecan in patients failing first-line oxaliplatin-based therapy^[12]. In the pivotal registration study (BOND) for cetuximab conducted in European mCRC patients failing irinotecan-based therapy, cetuximab

plus irinotecan was well tolerated and was associated with a disease control rate of 56%, a median time to progression of 4.1 mo and median overall survival of 8.6 mo^[7]. The NCIC-CO.17 trial demonstrated that cetuximab monotherapy significantly improved response rate, progression-free survival (PFS) and overall survival time in heavily pretreated patients with mCRC with *KRAS* wild-type tumors compared with patients receiving best supportive care alone^[13]. Findings from the BOND study have been confirmed in a community practice setting in the large Monoclonal Antibody Erbitux in a European Pre-License Study (MABEL)^[14]. This study investigated the combination of different irinotecan regimens with cetuximab in 1147 patients with mCRC progressing on irinotecan. To date the MABEL study is the largest published cetuximab study in this setting, reporting a PFS rate at 12 wk of 61% and an estimated median survival time of 9.2 mo^[14]. Data from the Latin American Erbitux Pre-License Study (LABEL) in Latin American patients with EGFR-expressing mCRC progressing on irinotecan suggest cetuximab to be active and tolerable, with a safety profile similar to that described in European patients^[15].

At the time of the design of this study there were no standard treatment options available for patients in this setting. Approval following the BOND trial, for cetuximab in combination with irinotecan for mCRC patients in this setting therefore provided the rationale for this study. Thus the phase II Erbitux[®] pre-License Study In the East (ELSIE) was designed to investigate the combination of irinotecan-containing regimens with cetuximab in irinotecan-refractory patients with mCRC from Asia and Australia.

MATERIALS AND METHODS

Patients

All patients provided written informed consent. Main inclusion criteria were age ≥ 18 years, Karnofsky performance status (KPS) ≥ 80 , histologically confirmed diagnosis of adenocarcinoma of the colon or rectum, metastatic disease not suitable for curative treatment, immunohistochemical evidence of EGFR expression in the primary tumor or metastasis, previous treatment with 1 of 4 pre-defined irinotecan regimens for ≥ 6 wk as the most recent chemotherapy, disease progression on or within 3 mo of the most recent irinotecan regimen, able to tolerate irinotecan continuation, no more than 3 previous lines of chemotherapy, and adequate hepatic, renal and bone marrow function. Main exclusion criteria were known or suspected brain metastases, radiotherapy or major surgery within the 4 wk prior to study entry, previous EGFR-targeted therapy, coronary heart disease or history of myocardial infarction or uncontrolled arrhythmia, other malignancy within 5 years (except basal cell carcinoma of the skin or pre-invasive carcinoma of the cervix), and pregnancy.

Study design

This was an open-label, uncontrolled, multicenter, phase II study carried out in 25 centers in 8 countries in the Asia-Pacific region. Patients initially received an infusion of ce-

tuximab 400 mg/m² over 2 h followed by weekly infusions of 250 mg/m² over 1 h in addition to irinotecan treatment at the same dosage regimen (including previous dose reductions) on which the subject had become refractory to irinotecan. Irinotecan was administered intravenously over 30–90 min with at least 1 h between the end of cetuximab and the start of infusion of irinotecan. The irinotecan regimens allowed as pre-study treatment and continued during the study were 100 or 125 mg/m² weekly for 4 wk then 2 wk rest (1 cycle); 100 or 125 mg/m² weekly for 2 consecutive wk out of 3 (1 cycle); 180 or 210 mg/m² every 2 wk (1 cycle); and 300 or 350 mg/m² every 3 wk (1 cycle). The first 3 pre-study irinotecan regimes could have been administered either as single-agent irinotecan or in combination; however, only single-agent irinotecan was accepted for the fourth regimen. Study treatment was continued until disease progression or unacceptable toxicity. In case of an irinotecan-related toxicity, irinotecan could be stopped and treatment could be continued with cetuximab monotherapy. Two irinotecan dose reductions were allowed for toxicity prior to study entry and during the study for all regimens except for patients receiving 100 mg/m² weekly where only 1 dose reduction was allowed.

The primary objective was the assessment of PFS rate after 12 wk of treatment. Secondary objectives included further evaluation of PFS, and the assessment of best overall confirmed response rate, duration of response, time to treatment failure (TTF), overall survival time, safety and toxicity.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki as well as the International Conference on Harmonization-Good Clinical Practice (ICH-GCP, 1996).

Assessments

Pre-screening of patients was performed within 3 mo after confirmation of disease progression on irinotecan-based chemotherapy and included informed consent, collection of demographic data, tumor diagnosis and the determination of tumor EGFR expression which was performed by designated pathology laboratories in Hong Kong, India and China by immunohistochemical staining of tumor tissue slides using a standardized protocol with the DAKO EGFR pharmDX kit (DAKO Corporation, Carpinteria, CA, USA). Baseline evaluations were performed within 21 d prior to the start of study treatment; pre-study staging by computed tomography (CT) or magnetic resonance imaging (MRI) was performed within 28 d. After the start of treatment, imaging (CT or MRI) was performed every 6 wk. Based on these images, the tumor response was assessed according to modified World Health Organisation (WHO) criteria every 6 wk during study treatment. Follow-up for survival occurred every 12 wk from the end of the study visit and included recording of subsequent treatments.

Adverse events (AEs) were reported weekly at each infusion visit, at the time of disease progression or study discontinuation, and up to 6 wk after the last infusion of study treatment. AEs were reported by severity according to National Cancer Institute Common Toxicity Criteria (NCI-

CTC) version 2 using Medical Dictionary for Regulatory Activities (MedDRA) version 10 preferred terms as event category and MedDRA body system/primary system organ class (SOC) as SOC category.

Statistical analysis

Safety and efficacy analyses were carried out considering the intention-to-treat (ITT)/safety population, which comprised all patients who had received any dose of study treatment. As patients were not randomized to treatment and the number of patients per individual treatment regimen was expected to be small, all data were analyzed independently of the on-study irinotecan regimen. The primary objective of this study was to determine the PFS rate at 12 wk after initiation of cetuximab treatment. Time-to-event analyses were based on Kaplan-Meier estimates. Confidence intervals (CIs) for the median were calculated according to Brookmeyer and Crowley^[16]. CIs for the event-free rate estimates, including the primary variable, were derived from the Kaplan-Meier curve at defined time points. The estimate of the standard error was computed using Greenwood's formula^[17]. PFS was defined as the time from the first study medication to the first observation of radiologically confirmed disease progression, symptomatic deterioration leading to discontinuation of study treatment (unless imaging confirmed absence of progressive disease), or death due to any cause within 60 d of the last on-study tumor assessment. The best overall confirmed response was defined as the best confirmed response from start of treatment until disease progression. Duration of response in patients with a complete response (CR) or partial response (PR) was defined as the time from first assessment of CR/PR to the first time disease progression was documented or death within the trial or until death within 60 d after last tumor assessment, whichever occurred first. The time to TTF was defined as the time from the start of the first cetuximab treatment until the date of the first occurrence of an event defining treatment failure, i.e. withdrawal of any study treatment due to AEs or progressive disease or withdrawal of consent or death, whichever occurred first. The overall survival time was defined as the time from the day of first infusion of study medication to death. For subjects who were still alive at the last survival follow-up, or who were lost to follow-up, survival was censored at the last recorded date that the subject was known to be alive.

A subgroup analysis that used a landmark method for the analysis of time-dependent covariates was carried out to examine whether the development and severity of acne-like rash that occurred during the first 21 d of treatment was associated with prolonged survival time. To avoid selection bias as a consequence of the early exclusion of patients, this analysis was limited to all patients in the ITT/safety population who were still under on-study treatment at day 21. Consequently, overall survival time was analyzed for this subgroup by using the landmark of study day 22 (and not day 1) as the start day. Survival was further analyzed in other subgroups based on known prognostic variables.

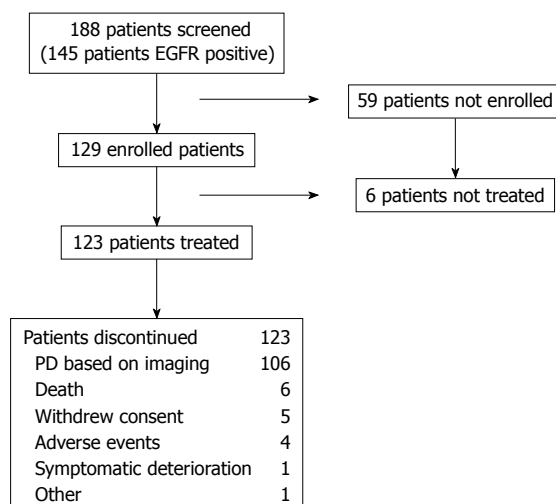


Figure 1 Disposition of patients at each stage of the study. EGFR: Epidermal growth factor receptor; PD: Progressive disease.

To ensure adequate precision of estimates, the sample size determination was based on a confidence limit approach. The expected rate of progression-free patients 12 wk after start of study treatment was 50%. It was calculated that with the enrollment of 150 patients, the observed 95% CI for this point estimate would have a range of approximately $\pm 8\%$.

RESULTS

Patient demographics

Patient characteristics are summarized in Figure 1. Between November 2005 and September 2006, 188 patients were screened at 25 centers in Australia (3 centers), China (6), Hong Kong (2), India (3), Korea (5), Singapore (1), Taiwan (3), and Thailand (2). Of these patients, 145 (77%) had EGFR detected in their tumor sample (data were missing for 4 patients), 129 patients were enrolled and of these, 123 patients were treated and formed the ITT/safety population. The main reason for study discontinuation was disease progression (86.2%). There were 6 patients who were treated until death (5 of these deaths due to disease progression, 1 due to chemotherapy-related AEs, 4 patients stopped treatment due to AEs and 5 patients withdrew consent).

Patient and disease characteristics at baseline are summarized in Table 1. The majority of patients were male (59.3%), the median age was 56 years (range, 24-78 years) and 74.8% of patients were aged < 65 years. The most common primary tumor site was the colon (57.7%), and 74.8% of patients had liver metastases, 56.1% had lung metastases and more than 30% of patients had other organs involved. The median duration of mCRC at baseline was 8.8 mo (range, 1-56).

The most common recent irinotecan regimen used was 180 mg/m² every 2 wk, which had been used in 93 patients. Approximately half of the patients (49.6%) had a time interval of 1-3 mo between the last pre-study dose of irinotecan and the first dose of cetuximab and the interval was < 1 mo in 35% of patients. All other patients had an interval

Table 1 Patients and disease characteristics at baseline (intention-to-treat/safety population)

Characteristic	Patients (n = 123)
Patient characteristics	
Median age, yr (range)	56 (24-78)
Age categories, n (%)	
< 65 yr	92 (74.8)
≥ 65-75 yr	28 (22.8)
≥ 75 yr	3 (2.4)
Gender, n (%)	
Male	73 (59.3)
Female	50 (40.7)
Ethnic origin, n (%)	
Caucasian	9 (7.3)
Asian (Chinese)	66 (53.7)
Asian (non Chinese)	48 (39.0)
Karnofsky performance status, n (%)	
80	39 (31.7)
90	52 (42.3)
100	32 (26.0)
Disease characteristics	
Median duration of CRC, mo (range)	15.7 (2-78)
Median duration of mCRC, mo (range)	8.8 (1-56)
Localization of tumor, n (%)	
Colon	71 (57.7)
Rectum	49 (39.8)
Colon and rectum	3 (2.4)
No. of organs with metastasis, n (%)	
1	51 (41.5)
2	53 (43.1)
3	12 (9.8)
> 3	7 (5.7)
Previous treatment, n (%)	
No. of previous treatment lines (non-adjuvant)	
1	66 (53.7)
2	37 (30.1)
3	20 (16.3)
Previous adjuvant chemotherapy	37 (30.1)
Previous non-adjuvant oxaliplatin therapy	50 (40.7)
Most recent irinotecan therapy, n (%)	
100 or 125 mg/m ² weekly for 4 consecutive wk followed by 2 wk of rest	1 (0.8)
100 or 125 mg/m ² weekly for 2 consecutive wk out of 3	15 (12.2)
180 or 210 mg/m ² every 2 wk ¹	94 (76.4)
300 or 350 mg/m ² every 3 wk	9 (7.3)
Other	4 (3.3)
Type of therapy, n (%)	
Irinotecan monotherapy	10 (8.1)
Irinotecan + 5-FU or analog	104 (84.6)
Irinotecan + 5-FU or analog + other	8 (6.5)
Irinotecan + other	4 (3.3)
Median duration, wk (Q1-Q3)	14.3 (7.3-22.4)
Best overall response to most recent irinotecan treatment, n (%)	
Complete response	3 (2.4)
Partial response	17 (13.8)
Stable disease	43 (35.0)
Progressive disease	59 (48.0)
Missing	1 (0.8)

¹One patient received 210 mg/m² and 93 received 180 mg/m² every 2 wk. CRC: Colorectal cancer; 5-FU: 5-fluorouracil; mCRC: Metastatic colorectal cancer; PD: Progressive disease; Q1-Q3: Interquartile range.

of less than 6 mo between the most recent irinotecan dose and the start of study treatment.

Table 2 Extent of exposure to cetuximab and irinotecan (intention-to-treat/safety population)

Characteristic	Patients (n = 123)	
	Cetuximab ¹	Irinotecan ²
Duration, wk		
Median	12.0	12.0
Q1-Q3	6.0-25.0	6.0-25.6
Total number of infusions, n (%)		
0	0	2 (1.6)
1-5	13 (10.6)	57 (46.3)
6-10	42 (34.1)	35 (28.5)
11-20	30 (24.4)	19 (15.4) ²
21-30	20 (16.3)	6 (4.9)
31-40	7 (5.7)	2 (1.6)
41-50	5 (4.1)	1 (0.8)
> 50	6 (4.9)	1 (0.8)
Cumulative dose, mg/m ²		
Median	3143.7	894.8
Q1-Q3	1650.8-6110.6	532.0-1980.4
Cetuximab dose intensity, mg/m ² per week		
Median	246.7	
Q1-Q3	236.0-250.3	
Irinotecan dose intensity, mg/m ² per 6 wk		
Median		507.8
Q1-Q3		407.6-539.5
Relative dose intensity, n (%)		
< 60%	2 (1.7)	5 (4.3)
60% to 80%	5 (4.2)	21 (17.9)
80% to 90%	6 (5.0)	15 (12.8)
≥ 90%	107 (89.2)	76 (65.0)
Patients who stopped irinotecan and received cetuximab, n (%)	14 (11.4)	
Median duration of cetuximab monotherapy after stopping irinotecan, wk (range)	2.5 (0.9-23.0)	
Patients who stopped combination treatment and received irinotecan only, n (%)	0	

¹Relative dose intensity calculated for patients receiving at least 1 maintenance dose only, the initial dose was excluded for the calculation of relative dose intensity; ²Duration, cumulative dose by cycle and relative dose intensity overall and by cycle were not calculated for 4 patients who received "other" irinotecan regimens (n = 119 for these calculations).

Treatment exposure

Treatment exposure is summarized in Table 2. At least 50% of patients received cetuximab treatment for ≥ 12 wk and were administered ≥ 12 infusions. The longest treatment duration was 98 wk and almost 5% of patients received more than 50 infusions of cetuximab. Relative dose intensity (RDI) of cetuximab of ≥ 90% was achieved in 89.2% of patients. Furthermore, 118 patients (95.9%) were treated at the planned standard dose of cetuximab. Four patients (3.3%) had at least 1 dose reduction of cetuximab and information was missing for 1 patient. Dose reductions were required at the occurrence of second or third incidences of grade 3 skin toxicity according to the protocol.

The median duration of irinotecan exposure was 12 wk, with patients receiving a median of 6 cycles. RDI of ≥ 90% of irinotecan was achieved in 65% of patients. Approximately three-quarters of patients (74%, n = 91) received ≥ 80% of the planned dose of irinotecan. In total, 74 patients (60.2%) required at least 1 dose reduction of irinotecan, which included pre-study dose reductions.

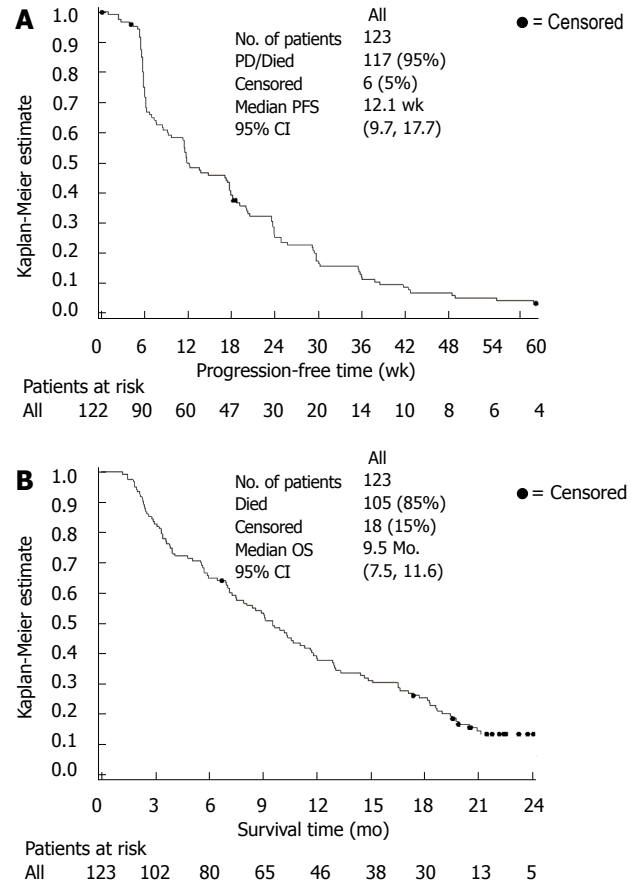


Figure 2 Kaplan-Meier curve. A: Progression-free survival (PFS); B: Overall survival (OS) in the intention-to-treat/safety population. CI: Confidence interval.

Treatment outcome

Study results are summarized in Table 3. The 12-wk PFS rate was found to be 50% (95% CI: 41-59). The median PFS time was 12.1 wk (95% CI: 9.7-17.7) (Figure 2A). In general, there were no marked or unexpected differences in PFS time for any of the prognostic variables or characteristics of previous treatment which were considered in the pre-specified subgroup analyses (data not shown).

The median overall survival time was 9.5 mo (95% CI: 7.5-11.6) (Figure 2B), with a 1-year survival rate of 38% (95% CI: 29-46) (Table 3). The median follow-up time for survival was 22.4 mo (95% CI: 21.4-24.4). There were generally no marked or unexpected differences for any of the prognostic variables or characteristics of previous treatment which were considered in pre-specified subgroup analyses of overall survival time (Table 4). A landmark analysis investigated the impact of early occurrence of acne-like rash (during the first 21 d of treatment) on overall survival time. Median overall survival was longer in patients with acne-like rash compared with those patients who experienced no acne-like rash (9.5 mo *vs* 6.2 mo). Median overall survival time was shorter in patients with grade 3/4 early acne-like rash (4 patients only) compared with grade 1/2 acne-like rash. As the sample size in the subgroup with grade 3/4 acne-like rash was very small, this observation is likely to be an artifact due to differences between patients with and

Table 3 Treatment activity (intention-to-treat/safety population)

Parameter	Patients (n = 123)
Response, n (%)	
Complete response	1 (0.8)
Partial response	16 (13.0)
Stable disease	44 (35.8)
Progressive disease	52 (42.3)
Not evaluable	10 (8.1)
Objective response (95% CI)	17 (13.8) (8.3-21.2)
Disease control rate (95% CI)	61 (49.6) (40.5-58.8)
PFS	
No. of events, n (%)	117 (95.1)
Median PFS time, wk (95% CI)	12.1 (9.7-17.7)
PFS rate, % (95% CI)	
6 wk	72 (64-80)
12 wk	50 (41-59)
18 wk	39 (30-48)
24 wk	25 (17-33)
30 wk	17 (10-23)
36 wk	11 (6-17)
Overall survival	
No. of events, n (%)	105 (85.4)
Median survival time, mo (95% CI)	9.5 (7.5-11.6)
Survival rate, % (95% CI)	
6 mo	65 (53-73)
12 mo	38 (29-46)
18 mo	25 (18-33)
24 mo	14 (7-20)

PFS: Progression-free survival; CI: confidence interval.

without early acne-like rash with regard to other prognostic factors, such as gender, age and KPS.

The objective response rate was 13.8% (95% CI: 8.3-21.2) (Table 3). The median duration of response was 31.1 wk (95% CI: 18.0-42.6). In addition, 44 patients (35.8%) had stable disease and the disease control rate was 49.6% (95% CI: 40.5-58.8). A higher response rate of 25.0% (5/20 patients) was observed for patients with CR and PR as best response to the most recent irinotecan schedule as compared with subjects with progressive disease and stable disease who had a response rate of 11.8% (12/102 patients). In addition, patients with KPS \leq 80 at baseline had lower response rates [7.7% (95% CI: 1.6-20.9)] than the KPS $>$ 80 subgroup [16.7% (95% CI: 9.4-26.4)]. There were generally no marked or unexpected differences in response rates between other pre-specified subgroups (data not shown). Disease control rates in the subgroups were consistent with those observed for response rate. The median TTF was 11.7 wk (95% CI, 9.1-17.4).

Ninety patients (73.2%) received post-study anti-cancer treatments, consisting of best supportive care (66 patients; 53.7%), chemotherapy (59 patients, 48.0%), surgery (6 patients, 4.9%), radiotherapy (15 patients, 12.2%) and other treatments (15 patients, 12.2%).

Adverse events

Treatment was generally well tolerated. The most common AEs of any grade according to MedDRA SOC were skin and subcutaneous tissue disorders (85.4%), gastrointestinal disorders (85.4%) and general disorders and administration

Table 4 Overall survival in patient subgroups (intention-to-treat/safety population)

Prognostic factors	n	No. of deaths n (%)	Median survival	
			Months	95% CI
Age (yr)				
< 65	92	77 (83.7)	9.6	(7.9-11.8)
\geq 65	31	28 (90.3)	7.5	(5.7-12.9)
Gender				
Male	73	64 (87.7)	9.5	(7.3-11.7)
Female	50	41 (82.0)	8.9	(6.4-16.5)
Stage based on TMN classification at first diagnosis				
I / II	11	10 (90.9)	12.8	(5.6-14.6)
III	24	22 (91.7)	9.3	(5.1-11.3)
IV	80	67 (83.8)	9.5	(7.5-13.0)
unknown	8	6 (75.0)	4.9	(2.9-NA)
Karnofsky performance status				
\leq 80	39	36 (92.3)	5.7	(2.6-11.3)
$>$ 80	84	69 (82.1)	10.3	(8.9-13.3)
No. of previous non-adjuvant lines				
1	66	57 (86.4)	11.2	(9.1-13.3)
2	37	31 (83.8)	5.9	(3.8-9.1)
\geq 3	20	17 (85.0)	9.3	(3.6-17.7)
Time from end of last course of most recent irinotecan treatment to progression				
\leq 30 d	86	76 (88.4)	8.8	(7.1-11.3)
$>$ 30 d	37	29 (78.4)	10.6	(5.7-14.6)
Best response of most recent irinotecan schedule ¹				
CR/PR	20	14 (70.0)	15.6	(8.6-9.4)
PD/SD	102	90 (88.2)	8.9	(7.0-11.0)
No. of metastatic sites				
1	51	44 (86.3)	10.6	(7.9-13.3)
\geq 2	72	61 (84.7)	8.4	(6.9-11.0)
BSA				
\leq 1.6 m ²	42	34 (81.0)	8.6	(6.4-16.5)
$>$ 1.6 m ² to \leq 1.8 m ²	48	41 (85.4)	10	(6.9-13.0)
$>$ 1.8 m ²	33	30 (90.9)	9.5	(5.7-11.8)
Early acne-like rash during the first 21 d ²				
No	38	34 (89.5)	6.2	(3.3-11.3)
Yes	85	71 (83.5)	9.5	(7.2-12.3)
Grade 1 or 2	81	67 (82.5)	9.6	(7.4-13.7)
Grade 3 or 4	4	4 (100)	3	(2.1-10.9)

¹Best response was missing for 1 patient who died at 2.5 mo; ²Landmark analysis. BSA: Body surface area; NA: Not available; TMN: Tumor, node, metastasis; CR: Complete response; PR: Partial response; PD: Progressive disease; SD: Stable disease.

site conditions (44.7%). The most common AEs according to MedDRA preferred terms occurring in at least 20% of the patients were diarrhea (71 patients, 57.7%), rash (70 patients, 56.9%), nausea (55 patients, 44.7%), vomiting (48 patients, 39.0%), anorexia (35 patients, 28.5%), neutropenia (30 patients, 24.4%), constipation (27 patients, 22.0%) and stomatitis (26 patients, 21.1%). Grade 3/4 AEs occurred in 68 patients (45.5%) (Table 5). The most common grade 3/4 AEs were diarrhea in 17 patients (13.8%), neutropenia in 11 patients (8.9%) and rash and vomiting both in seven patients (5.7%) (Table 5). Composite AE categories were considered for AEs known to be related to cetuximab, na-

Table 5 Adverse events

Preferred term	Grade 3 or 4	Any grade
Any, <i>n</i> (%)	68 (55.3)	123 (100)
Diarrhea	17 (13.8)	71 (57.7)
Neutropenia	11 (8.9)	30 (24.4)
Rash	7 (5.7)	70 (56.9)
Vomiting	7 (5.7)	48 (39.0)
Abdominal pain	4 (3.3)	22 (17.9)
Anemia	4 (3.3)	12 (9.8)
Fatigue	4 (3.3)	21 (17.1)
Febrile neutropenia	4 (3.3)	4 (3.3)
Nausea	4 (3.3)	55 (44.7)
Blood alkaline phosphatase increased	3 (2.4)	9 (7.3)
Dehydration	3 (2.4)	4 (0.3)
Hypersensitivity	3 (2.4)	9 (7.3)
Infection	3 (2.4)	4 (3.3)
Neutrophil count	3 (2.4)	3 (2.4)
Paronychia	3 (2.4)	16 (13.0)
White blood cell count decreased	3 (2.4)	7 (5.7)
Anorexia	2 (1.6)	35 (28.5)
Constipation	2 (1.6)	27 (22.0)
Stomatitis	1 (0.8)	26 (21.1)
Composite AE categories ¹		
Infusion-related reactions ²	3 (2.4)	19 (15.4)
Acne-like rash ³	10 (8.1)	99 (80.5)

Grade 3/4 AEs (Adverse events) in > 2% and any grade AEs in > 20% of patients in the intention-to-treat/safety population and composite AE categories of special interest. ¹Composite categories where several Medical Dictionary for Regulatory Activities preferred terms have been pooled; ²Terms included acute myocardial infarction, acute respiratory failure, anaphylactic reaction, anaphylactic shock, anaphylactoid reaction, anaphylactoid shock, angina pectoris, apnea, asthma, blood pressure decreased, bronchial obstruction, bronchospasm, cardiac failure, cardiopulmonary failure, chills, clonus, convulsion, cyanosis, drug hypersensitivity, dyspnea, dyspnea at rest, dyspnea exacerbated, dyspnea exertional, epilepsy, hyperpyrexia, hypersensitivity, hypotension, hypoxia, infusion-related reaction, loss of consciousness, myocardial infarction, myocardial ischemia, orthopnea, pyrexia, respiratory distress, respiratory failure, shock, sudden death, and syncope; ³Terms included acne, acne pustular, dermatitis acneiform, dry skin, erythema, folliculitis, pruritus, rash, rash erythematous, rash follicular, rash generalized, rash macular, rash maculo-papular, rash papular, rash pruritic, rash pustular, skin exfoliation, skin hyperpigmentation, telangiectasia, and xerosis; Preferred terms included in the infusion-related reactions category regardless of when they occurred; all other preferred terms in this category were included only if the onset date of the AE was on the day of the first cetuximab administration.

mely infusion-related reactions and acne-like rash (Table 5). Grade 3/4 infusion-related reactions occurred in 3 patients (2.4%; 2 patients with Grade 3, 1 patient with Grade 4). Ten patients (8.1%) had grade 3 acne-like rash (Table 4), with no occurrences of grade 4 rash reported. Overall, 7 patients (5.7%) had AEs that led to the discontinuation of cetuximab and 13 patients (10.6%) had AEs leading to the discontinuation of chemotherapy. Three patients (2.4%) had AEs leading to the discontinuation of both.

At the time of database closure (February 27th, 2009), 105 patients in the ITT/safety population had died, of which 8 died within 30 d after the last dose of study medication. Seven deaths (5.7%) occurred due to disease progression and 1 patient (0.8%) died due to events related to irinotecan-based therapy. There were no deaths related to cetuximab treatment.

DISCUSSION

The ELSIE study was designed to further investigate, in patients from the Asia-Pacific region, the observations initially made in the BOND study^[7] relating to efficacy and safety of the combination of cetuximab with irinotecan in mCRC patients progressing on irinotecan-based chemotherapy. The PFS rate at 12 wk of 50% (95% CI: 41-59) met the expectation for the primary endpoint of the study made at the time of study planning. A sample size of 150 patients was considered sufficient to meet the primary study objective in the current study; however, only 129 patients were actually enrolled. The low recruitment rate in some of the participating countries is thought to be due to the fact that irinotecan was not a preferred treatment option in many of the study centers. Consequently, few patients fulfilled the protocol requirement of having received irinotecan-based treatment as the most recent therapy.

Cross comparisons of the results of this study and similar studies in relation to the interpretations of the efficacy data should be undertaken with some degree of caution, particularly where differences exist in study design^[18]. The ELSIE study was comparable in design to the combination arm of the BOND study and the LABEL study^[7,15], although all 3 differed slightly in study design from the MABEL study, particularly in the interval for imaging assessment of the tumor^[14]. Except for the notable differences in geographical location, there were only minor variations in the patient populations of these studies. Furthermore, the disposition of these populations with respect to the disease under investigation, namely, the number of previous treatment lines, the number of metastatic sites, and best response to most recent irinotecan treatment were considered comparable^[7,14,15,19].

A comparison of the treatment outcomes of patients receiving cetuximab in combination with irinotecan-based chemotherapy in the ELSIE, BOND, MABEL and LABEL studies is shown (Table 6). The PFS rate at 12 wk in the present study was slightly lower than that reported in the MABEL and LABEL studies and in the combination therapy arm of the BOND study (PFS rate at 3 mo). The variations across the studies may also reflect differences in the CT scanning schedules for tumor assessment. Tumors were assessed according to modified WHO criteria in these studies every 6 wk by the investigators in the ELSIE and LABEL studies^[15], every 12 wk in the MABEL study^[14], and every 6 wk by an independent review committee in the BOND study^[7]. In addition, differences may reflect other factors relating to patient characteristics, such as different KPS.

Consistent with the primary endpoint, tumor response rate and median PFS (12.1 wk) were also slightly lower in the current study than reported in the MABEL and LABEL studies and the combination arm of the BOND study. However, the treatment with cetuximab in combination with irinotecan results translated into very similar overall survival across all four studies in this irinotecan refractory setting (Table 6). Importantly, the median survival

Table 6 Outcome in metastatic colorectal cancer patients receiving cetuximab with irinotecan-based chemotherapy from different geographical regions

	ELSIE	LABEL	MABEL	BOND
Reference	Current study	Buzard <i>et al</i> ^[15]	Wilke <i>et al</i> ^[14]	Cunningham <i>et al</i> ^[7]
No. of patients	123	79	1147	218
Geographical region	Asia-Pacific	Latin America	Europe	Europe
Response rate, % (95% CI)	13.8 (8.3-21.2) ¹	26.6 (17.3-37.7) ¹	20.1 (17.9-22.6) ²	22.9 (17.5-29.1) ³
Disease control rate, % (95% CI)	49.6 (40.5-58.8) ¹	55.7 (44.1-66.9) ¹	45.2 (42.3-48.2) ²	55.5 (48.6-62.2) ³
Median PFS, wk (95% CI)	12.1 (9.7-17.7) ¹	17.4 (11.7-18.9) ¹	14.1 (13.0-17.1) ¹	18.0 (12.0-18.0) ³
PFS rate at 12 wk, % (95% CI)	50 (41-59) ¹	57 (46-68) ¹	61 (58-64) ¹	56 ⁴ (48.7-62.6) ³
Median survival, mo (95% CI)	9.5 (7.5-11.7)	9.2 (7.9-10.8)	9.2 (8.6-9.8)	8.6 (7.9-9.6)
OS rate at 1 yr, % (95% CI)	38 (29-37)	38 (27-48)	38 (35-41)	29 (22-37)

¹World Health Organisation (WHO) criteria by investigator; ²Subjective assessment by investigator; ³WHO criteria by independent review committee; ⁴In the BOND study the PFS rate was assessed at 3 mo. ELSIE: Erbitux® pre-License Study In the East; LABEL: Latin American Erbitux Pre-License Study; MABEL: Monoclonal Antibody Erbitux in a European Pre-License Study.

time obtained with cetuximab in combination with irinotecan in this and the other studies is in the upper range of results reported for irinotecan or oxaliplatin/5-fluorouracil (5-FU)/folinic acid in less heavily pre-treated patients^[20-24].

The current study demonstrates that cetuximab plus irinotecan is generally well tolerated in patients from the Asia-Pacific region. The most frequently reported grades 3 to 4 AEs were typical for irinotecan (e.g. diarrhea and neutropenia), or cetuximab (e.g. composite AE of acne-like rash). Cetuximab did not appear to increase the incidence or severity of irinotecan-associated AEs and *vice versa*. Diarrhea was the most common grade 3/4 AE reported in the current study (13.8%), in BOND (21%), MABEL (19%) and LABEL (20%) with neutropenia and rash also frequently observed in each of the studies^[7,14,15]. Severe infusion-related reactions occurred in 2.4% of patients in the present study, which is the same proportion as observed in the MABEL study^[14]. Interestingly, results from the MABEL study suggest that the incidence of severe infusion-related reactions may be minimized by prophylactic premedication with an antihistamine/corticosteroid combination^[25].

In the ELSIE study all-grade and grade 3/4 acne-like rash (composite AE category) were reported in 80.5% and 8.1% of patients respectively, which is comparable with that reported in other studies^[14,26]. An association between any early acne-like rash with prolonged overall survival was found. Saltz and colleagues described a correlation between the presence and severity of acne-like rash and survival; median survival in heavily pretreated mCRC patients experiencing no rash was 1.9 mo compared with 9.5 mo in patients experiencing any rash ($P = 0.02$)^[26]. A trend was also observed between the increasing severity of rash and prolonged survival^[26]. Comparable results between the presence and severity of acne-like rash with clinical outcome (PFS) have also been reported in the MABEL study^[14].

Randomized studies in the first-line treatment of mCRC patients^[27,28] and in heavily pretreated mCRC patients^[13], have recently demonstrated that the benefit conferred from cetuximab in combination with chemotherapy occurs mainly in patients whose tumors are wild-type for the *KRAS* gene. In a study of cetuximab plus irinotecan, 5-FU, and leucovorin as first-line treatment for mCRC, pa-

tients with *KRAS* wild-type tumors had a response rate of 59.3% compared with 36.2% in those with mutated *KRAS* tumors^[27]. These data led to revised guidance from regulatory and advisory authorities concerning the administration of cetuximab only to patients with *KRAS* wild-type mCRC. Thus *KRAS* mutation testing of tumors should be routine clinical practice when cetuximab is to be administered in this setting. At the time of the ELSIE study design this knowledge was not available and therefore material was not collected for tumor *KRAS* testing. This might suggest that the benefit observed from combining cetuximab with irinotecan in unselected patients with mCRC in the current study would be smaller than the effect in patients with *KRAS* wild-type tumors. The future tailoring of this cetuximab-containing regimen to selected patients is therefore likely to enhance the efficacy and cost-effectiveness of treatment in this patient population.

To conclude, the ELSIE study has demonstrated that the combination of cetuximab with irinotecan is well tolerated and active in mCRC patients from Asia and Australia. The efficacy and safety profiles of the combination are similar to those described in previous studies in European and Latin American patient populations. The use of cetuximab and irinotecan may be particularly beneficial in patients with wild-type *KRAS*, and further studies in this patient population are warranted.

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COMMENTS

Background

The incidence and mortality rates for colorectal cancer (CRC) in Eastern and South-East Asia are increasing. Patients with metastatic CRC (mCRC) who progress following first-line therapy have few treatment options. This open-label, phase II study evaluated the efficacy and safety of cetuximab plus irinote-

can in irinotecan-refractory mCRC patients from South-East Asia and Australia.

Research frontiers

Research in the field of mCRC has recently focused on the development of targeted therapies in disease treatment. Epidermal growth factor receptor (EGFR) is frequently expressed in mCRC and is associated with tumor growth and metastasis. Cetuximab is an EGFR-targeted monoclonal antibody which binds EGFR inhibiting its activity. Registration of cetuximab was based on the pivotal BOND trial in which patients with mCRC progressing on an irinotecan-containing regimen achieved a higher overall survival rate when cetuximab was added to irinotecan compared with irinotecan alone. The subsequent Monoclonal Antibody Erbitux in a European Pre-License Study (MABEL) and Latin American Erbitux Pre-License Study (LABEL) studies confirmed the efficacy of this treatment combination in a community-based setting.

Innovations and breakthroughs

The safety and efficacy of cetuximab in combination with irinotecan in mCRC patients failing on irinotecan-containing regimens has been demonstrated in phase II studies. The ELSIE study supports these findings in patients from the Asia-Pacific region. Median overall survival was comparable with that reported in the LABEL and MABEL studies and the combination arm of the BOND study. There were slightly lower tumor response rates, median PFS and PFS rate at 12 wk in the ELSIE study. Patients were unselected in the ELSIE and the observed treatment benefit may be greater in patients selected for KRAS wild-type tumors.

Applications

The results of this study confirm the activity of cetuximab in combination with irinotecan in patients with mCRC from Asia and Australia. In the future cetuximab-containing regimens are likely to be tailored to selected patients in this setting with KRAS wild-type tumors.

Peer review

The paper on the whole is well written and the abstract reflects the basic design and findings.

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Risk factors for residual tumor after resection of hepatocellular carcinoma

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Abstract

AIM: To identify the clinicopathological risk factors correlated with residual tumor in hepatocellular carcinoma (HCC) patients after resection.

METHODS: From January 2001 to April 2007, 766 HCC patients who had undergone resection were included in this research. Lipiodol angiography was performed within 2 mo after surgery and followed by post-Lipiodol computed tomography (CT) 4 wk later for all 766 patients to monitor tumor in the remnant liver. Tumor detected within the first 3-mo postoperative period was defined as residual tumor. Patients were divided into 2 groups: disease or disease-free within the first 3 mo after surgery. Risk factors for residual tumor were investigated among various clinicopathological variables.

RESULTS: A total of 63 (8.22%) patients were found to

have residual tumor after surgery. Three independent factors associated with residual tumor were identified by multivariate analysis: preoperative serum α -fetoprotein (AFP) level [odds ratio (OR) = 1.68 (95% confidence interval (CI): 1.20-2.36)], tumor size [OR = 1.73 (95% CI: 1.29-2.31)] and microvascular invasion [OR = 1.91 (95% CI: 1.12-3.24)].

CONCLUSION: Residual tumor is related to AFP level, tumor size and microvascular invasion. Patients at high risk should undergo closer follow-up and could be candidates for multimodality therapy.

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Key words: Risk factors; Residual tumor; Hepatocellular carcinoma; Radical resection; Lipiodol angiography

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Chen XH, Zhang BH, Xin Y, Ren ZG, Fan J, Qiu SJ, Zhou J. Risk factors for residual tumor after resection of hepatocellular carcinoma. *World J Gastroenterol* 2011; 17(14): 1889-1894 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i14/1889.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i14.1889>

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 80%-90% of primary liver cancer and is a global health problem^[1,2]. About 55% of all HCC incidences are identified in China^[1]. Surgical resection has a major role in the treatment for HCC and offers a chance of cure for patients^[3,4]. Although resection is complete, there is still a possibility of early recurrence which occurs mainly because of pre-existing microscopic tumor foci that are undetected by

imaging modalities before or during operation^[5]. Tumor detected soon after resection is thought to be related to the presence of residual tumor, and it is associated with a poor prognosis.

Determination of factors predicting residual tumor may allow for identification of patients who are more likely to have this problem after so-called radical resection. In view of Lipiodol angiography with its post-Lipiodol computed tomography (CT) being regarded as the most sensitive means to confirm the presence of tumor, we identify in this study the risk factors correlated with residual tumor in HCC patients who received Lipiodol angiography within 2 mo after resection followed up by CT scan 4 wk later. Knowledge of these risk factors could be useful for clinicians to assess patient prognosis and determine treatment strategies, for the purpose of improving the long-term outcome.

MATERIALS AND METHODS

Patients

From January 2001 to April 2007, pathologically confirmed HCC patients who underwent their first radical hepatic resection at the Liver Cancer Institute & Zhongshan Hospital of Fudan University were recruited for this study if they met the following entry criteria: (1) received no preoperative anticancer treatment; (2) age between 18-80 years; (3) liver function classified as Child Pugh Grade A or B; (4) recovered within 6 wk of the operation; and (5) general health satisfactory for toleration of the Lipiodol angiography within 2 mo after resection.

We defined radical resection in this study as: (1) complete removal of all tumor nodules and the cut surface being free of cancer by histological examination; (2) no cancerous thrombus found in the portal vein (main trunk or two major branches), hepatic veins, or bile duct by imaging and histological examination; (3) no demonstrable evidence of residual disease in the remnant on intraoperative ultrasonographic examination; (4) the number of tumor nodules not exceeding three^[6]; and (5) no extrahepatic metastasis found.

There were 766 patients who satisfied the selection criteria. This study was approved by the Research Ethics Committee of Zhongshan Hospital.

Lipiodol angiography

Lipiodol angiography was performed within 2 mo after resection in all these 766 patients. The hepatic artery supplying the liver remnant was selectively catheterized *via* the femoral artery under fluoroscopic guidance. An approximately 3 mL suspension of iodized oil was injected into the hepatic artery. A contrast CT scan of the liver was performed an average of 4 wk later. If there was early local lesion, it would be shown as dense foci of Lipiodol uptake, or as enhancing nodule not present in the preoperative CT.

Residual tumor and patient grouping

The injection of Lipiodol into the hepatic artery is effective for aiding in the diagnosis of tumor by post-Lipiodol

CT and it can detect the lesion in its earliest period. In this study, patients received Lipiodol angiography within 2 mo after resection, followed up by CT scan 4 wk later to monitor tumor in the remnant liver; so, if there was no demonstrable evidence of lesion within the first 3 mo after resection, the surgery could be regarded as a truly curative resection. Therefore, residual tumor was defined as tumor detected within the first 3-mo postoperative period. We divided the 766 patients into two groups: Group 1 = disease detected within 3 mo after surgery; Group 2 = no disease detected within 3 mo after surgery.

Follow-up and statistics

Follow-up cutoff date was April 2008. All surviving patients had a minimum follow-up of 12 mo, 16 patients were lost during follow-up. The median follow-up period of all 766 patients was 26 mo (range: 3-87 mo). Statistically significant differences between categorical variables were examined using the Chi-square or Fisher's exact test where appropriate. The logistic regression model was applied to evaluate the risk factors related to residual tumor. Diagnostic accuracy of predictive risk factors was evaluated using receiver operating characteristic (ROC) analysis. Survival analysis was studied by the Kaplan-Meier method with a log-rank test to detect the statistical difference. The statistical analysis was performed using Stata 7.0. *P* values < 0.05 were considered statistically significant.

RESULTS

Survival of patients with or without residual tumor

Tumor was detected in 314 (314/766, 40.99%) patients during the follow-up period. Of the 766 patients, 63 patients were found to have residual tumor (Group 1), whereas the other 703 patients showed no lesion detected within 3 mo after resection (Group 2). Respective cumulative survival rates at 1-, 2-, 3-, 4-, 5-years in Group 1 were 44.44%, 35.90%, 23.08%, 17.31% and 0.00% compared with 92.18%, 80.99%, 71.08%, 62.48% and 56.95% in Group 2 (*P* < 0.0001) (Figure 1).

Clinicopathologic characteristics of patients with or without residual tumor

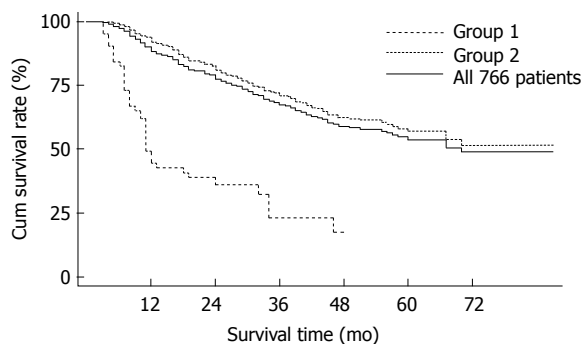
The distribution of selected clinical and pathological characteristics between Group 1 and Group 2 is shown in Table 1. No significant differences were observed regarding sex, age, HBsAg-positive rate, cirrhotic nodules, liver function status, serum alanine aminotransferase (ALT) level, tumor number, cell differentiation grade and the percentage of incomplete/absent capsule. However, serum gamma-glutamyl transferase (GGT) level ($\chi^2 = 4.6062$, *P* = 0.032) and α -fetoprotein (AFP) level ($\chi^2 = 16.0745$, *P* < 0.0001) were statistically different between the two groups. The ratio of large tumor size in Group 1 was significantly higher than that in Group 2 ($\chi^2 = 23.3257$, *P* < 0.0001), and there was a significantly higher incidence of microvascular invasion ($\chi^2 = 9.7556$, *P* = 0.002) noted in Group 1 compared with that in Group 2. As for tumor staging, there was a significantly poorer degree of pTNM staging ($\chi^2 = 15.1735$, *P* = 0.001) in Group 1; BCLC clas-

Table 1 Main characteristics of 766 hepatocellular carcinoma patients with (Group 1) or without (Group 2) residual tumor *n* (%)

Characteristics		Group 1	Group 2	χ^2	P-value
Sex	Female	11 (17.46)	96 (13.66)	0.6964	> 0.05
	Male	52 (82.54)	607 (86.34)		
Age (yrs)	≤ 30	3 (4.76)	17 (2.42)	1.2490	> 0.05
	30-60	48 (76.19)	549 (78.09)		
	> 60	12 (19.05)	137 (19.49)		
Child-Pugh score	Class A	62 (98.41)	692 (98.44)	0.0002	> 0.05
	Class B	1 (1.59)	11 (1.56)		
Cirrhotic nodule	No	7 (11.11)	121 (17.21)	1.5462	> 0.05
	Yes	56 (88.89)	582 (82.79)		
HBsAg	Negative	8 (12.70)	90 (12.80)	0.0006	> 0.05
	Positive	55 (87.30)	613 (87.20)		
ALT level ¹ , U/L	≤ 80	57 (90.48)	621 (88.34)	0.2605	> 0.05
	> 80	6 (9.52)	82 (11.66)		
GGT level ¹ , U/L	≤ 60	24 (38.10)	367 (52.20)	4.6062	< 0.05
	> 60	39 (61.90)	336 (47.80)		
AFP level, ng/mL	≤ 20	8 (12.70)	259 (36.84)	16.0745	< 0.01
	20-400	20 (31.75)	192 (27.31)		
	≥ 400	35 (55.56)	252 (35.85)		
Tumor size ² , cm	≤ 2	3 (4.76)	82 (11.66)	23.3257	< 0.01
	2-5	19 (30.16)	333 (47.37)		
	5-8	15 (23.81)	166 (23.61)		
	> 8	26 (41.27)	122 (17.35)		
Capsule	complete/present	31 (49.21)	390 (55.48)	0.9183	> 0.05
	incomplete/absent	32 (50.79)	313 (44.52)		
Microvascular invasion	No	30 (47.62)	472 (67.14)	9.7556	< 0.01
	Yes	33 (52.38)	231 (32.86)		
Tumor number	solitary nodule	49 (77.78)	597 (84.92)	3.8413	> 0.05
	2 nodules	13 (20.63)	86 (12.23)		
	3 nodules	1 (1.59)	20 (2.84)		
Cell differentiation grade	Grade I - II	41 (65.08)	506 (71.98)	1.3475	> 0.05
	Grade III-IV	22 (34.92)	197 (28.02)		
pTNM staging	Stage I	21 (33.33)	409 (58.18)	15.1735	< 0.01
	Stage II	34 (53.97)	250 (35.56)		
	Stage III A	8 (12.70)	44 (6.26)		
BCLC classification	Stage A	49 (77.78)	607 (86.34)	3.4501	> 0.05
	Stage B	14 (22.22)	96 (13.66)		

¹ALT value (normal value, ≤ 75 IU/L), using 80 IU/L as cutoff, and GGT level > 60 IU/L (normal range, 11-50 IU/L) was used as the elevated level [7].

²Tumor size: Diameter of the largest nodule was used as tumor size when multiple.

**Figure 1** Survival curves for all 766 patients and for patients with (Group 1) or without (Group 2) residual tumor.

sification also showed a trend towards significance ($\chi^2 = 3.4501$, $P = 0.063$) between the two groups.

Predictive factors for residual tumor

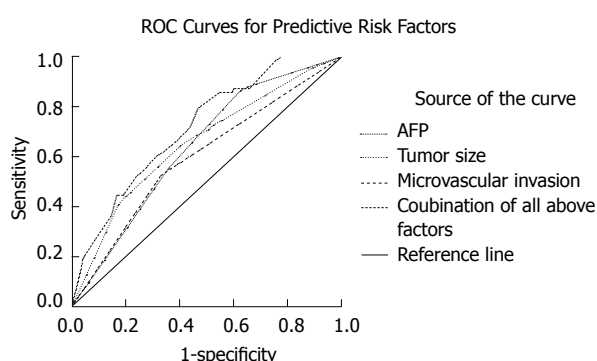
A multivariate stepwise logistic regression model was constructed to predict risk factors for residual tumor including

sex, age, HBsAg, cirrhosis nodules, Child-Pugh class, ALT, GGT and AFP levels, tumor size (diameter of the largest nodule was used as tumor size when multiple), tumor number, capsule, microvascular invasion, and cell differentiation grade. Only serum AFP level [odds ratio (OR) = 1.68 (95% confidence interval (CI): 1.20-2.36)], tumor size [OR = 1.73 (95% CI: 1.29-2.31)] and microvascular invasion [OR = 1.91 (95% CI: 1.12-3.24)] were revealed as independently predictive factors for residual tumor (Table 2).

Analysis of the prevalence of these three parameters among the entire population of 766 patients showed that the incidence of residual tumor was very low (nil, 0/24) when none of these three factors was present, and the simultaneous presence of the risk factors increased the probability of residual tumor: this rate increased to 28.15% (9/32) when AFP > 400 ng/mL, tumor size > 8 cm and the presence of microvascular invasion were present at the same time. ROC curves for the combination of these three risk factors showed an area under the curve (AUC) of 0.717 (95% CI: 0.657-0.778) to predict residual tumor. ROC curves for serum AFP level had an AUC of 0.640

Table 2 Multivariate stepwise logistic regression analysis of independent risk factors related to residual tumor

Risk factor		Coefficient	OR (95% CI)	P-value
AFP level (ng/mL)	≤ 20 = 0	0.52	1.68 (1.20-2.36)	< 0.01
	20-400 = 1			
	> 400 = 2			
Tumor size (cm)	≤ 2 = 0	0.55	1.73 (1.29-2.31)	< 0.01
	2-5 = 1			
	5-8 = 2			
	> 8 = 3			
Microvascular invasion	No = 0	0.65	1.91 (1.12-3.24)	0.02
	Yes = 1			

**Figure 2** Receiver operating characteristic curves for predictive factors (α -fetoprotein, Tumor size, Microvascular invasion and the combination of all above three factors) related to residual tumor. ROC: Receiver operating characteristic; AFP: α -fetoprotein.

(95% CI: 0.575-0.704), for tumor size had an AUC of 0.655 (95% CI: 0.582-0.728), and for microvascular invasion had an AUC of 0.598 (95% CI: 0.523-0.672). Significant difference was observed among these four curves ($P < 0.0001$) (Figure 2).

DISCUSSION

The distinction between recurrence after a radical resection and residual tumor after a palliative resection is crucial^[8]. Tumor detected soon after resection is thought to be related to the presence of residual tumor. Residual tumor cells in the remnant liver can acquire more malignant characteristics, and may accelerate tumor progression and induce intrahepatic metastasis due to the enhanced growth and increased neovascularization after surgery^[9]. The shorter is the disease-free interval time; the poorer is the prognosis^[10]. In this study, we showed that postoperative patients with residual tumor carried a significantly poorer prognosis than those who did not (Figure 1).

Complete surgical excision is an important factor for long-term outcome. However, it is difficult to remove all tumor cells in most patients^[11]. Even with so-called radical resection, there is still a possibility of residual tumor with a rate of up to 37.6%^[12]. The curative operation for HCC is difficult to define and the presence of residual tumor influences the evaluation of radical resection for

HCC. Determination of predictive factors for residual tumor can help the selection of patients suitable for more aggressive therapy to supplement surgery.

With regard to the definition of residual tumor, there is no consensus on this definition in the literature. Some authors have suggested the demonstration of intrahepatic disease found by imaging studies (ultrasonography, angiography, and post-Lipiodol CT) within one or two months after resection as residual tumor^[10]. In this study, positive finding by angiography followed by Lipiodol-CT within the first 3-mo postoperative period was defined as residual tumor. We then investigated the risk factors for residual tumor among various pathologic and clinical factors.

Results suggested that only high preoperative level of serum AFP, large tumor size and the presence of microvascular invasion were significantly associated with residual tumor at the univariate and multivariate analysis. When none of these three factors was present, the incidence of residual tumor was nil and increased up to 28.15% when AFP > 400 ng/mL, tumor size > 8 cm and the presence of microvascular invasion were present in the same patient. ROC analysis suggested that combination of these three factors was more sensitive to predict residual tumor than any single factor.

HCC is characterized by its propensity for vascular invasion. Numerous previous studies have demonstrated that the presence of microvascular invasion is the risk factor for early tumor occurrence after resection of HCC^[13-16]. In addition, tumor size, especially > 5 cm, also predicts a high risk of tumor recurrence after resection^[15,17,18]. Large tumor size is always correlated with increased invasiveness, as demonstrated by a higher incidence of intrahepatic metastasis and portal venous invasion^[13,15,17,19,20]. The larger the tumor is, the earlier the lesion occurs^[21].

As for preoperative serum AFP level, Hanazaki *et al*^[22] reported that AFP ≥ 1000 ng/mL was an independently significant factor for poor disease-free survival; Imamura *et al*^[23] identified serum AFP level > 32 ng/mL as a factor for early (< 2 years) recurrence; and Furihata *et al*^[24] also suggested that patients with AFP/volume > 20.0 were likely to experience recurrence within 6 mo after radical hepatectomy. Serum AFP level may represent a marker for either tumor bulk or aggressive tumor biology, such as tumor cell proliferation and spread^[25]. An HCC patient with a high serum AFP concentration (≥ 400 ng/mL) tends to have greater tumor size, bilobar involvement, massive or diffuse type of recurrence, portal vein thrombosis^[26,27]. All of these may be due to the ability of AFP to elicit the escape of carcinoma cells from the host's lymphocyte immune surveillance^[28,29]. However, there are several studies which have shown no relation between serum AFP level and recurrence^[30,31]. In this present study, we support the concept of elevated serum AFP level as a candidate for early tumor occurrence^[7].

Here, our study showed tumor number was not a predictive factor for residual tumor. This might be because, in this study, we defined radical resection as the number of tumor nodules not exceeding three (since multiple tumors

may be the sign of intrahepatic metastasis^[16]). Accordingly, radical resection could be achieved as long as tumor number was no more than three.

High preoperative level of serum AFP, large tumor size or the presence of microvascular invasion may indicate an increased biological aggressiveness of tumor and a greater possibility of systemic diffusion. The simultaneous presence of these factors increases the risk of residual tumor, and patients presenting these three risk factors in association are prone to have residual tumor. This information is beneficial for better clinical decision-making and future trial design. Some aggressive therapies can be tested in selected patients, such as utilizing neoadjuvant or adjuvant therapy (including hepatic artery chemotherapy or chemoembolization, immunotherapy, targeted therapy and differentiation therapy, *etc.*) to supplement surgery for a better chance of a cure or at least a longer survival. Patients with high risk factors for residual tumor should be monitored very carefully for early detection, and the surveillance interval also needs to be shortened to have a chance to eradicate the residual tumor at its earlier period, because long-term survival after recurrence is still possible if appropriated therapy is adopted^[32].

One limitation in the present study is the low discriminative power of the identified predictive factors (with the specificity < 75%). In other words, even if patients have all these three risk factors, many are unlikely to have residual tumor after surgery. To resolve these issues, a more discriminatory method is required, such as molecular analysis. In addition, in our hospital, Lipiodol angiography is usually recommended to each postoperative patient for the purpose of early detection of residual tumor. A patient can receive this examination as long as his general condition allows. It may be that there is a bias in patient selection, but considering the large sample size in this study and the eligible patients presenting various clinicopathologic characteristics, this bias should be reduced. Certainly, further studies to confirm the present results are still needed.

In conclusion, this study showed that factors reflecting tumor behavior were correlated with residual tumor. High preoperative serum AFP level, large tumor size and the presence of microvascular invasion were independent predictors associated with an increased risk of residual tumor detected by angiography followed by Lipiodol-CT. This finding may have clinical implications in determining rational strategies in surveillance, prevention and management of postoperative residual tumor.

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COMMENTS

Background

Even with so-called radical resection for hepatocellular carcinoma (HCC), there

is still a possibility of residual tumor. Determination of factors predicting residual tumor can help the selection of patients suitable for more aggressive therapy to supplement surgery. However, the definition of residual tumor and its prognostic factors have not been fully determined.

Research frontiers

Complete surgical excision offers a chance of cure to HCC patients, while recurrence is common and is the main cause of death. A large number of studies have investigated the prognostic risk factors for recurrence appearing at different times.

Innovations and breakthroughs

Lipiodol angiography followed by post-lipiodol computed tomography (CT) is the most sensitive means to confirm the presence of tumor. In this study, high preoperative serum α -fetoprotein (AFP) level, large tumor size and the presence of microvascular invasion were independent predictors associated with an increased risk of residual tumor detected by angiography followed by Lipiodol-CT. The simultaneous presence of these factors increases the risk of residual tumor.

Applications

Patients with high risk factors for residual tumor should be monitored very carefully for early detection, and the surveillance interval also needs to be shortened to have a chance to eradicate the residual tumor at its earlier period.

Terminology

Lipiodol angiography is an imaging technique for HCC using the injection of Lipiodol into the hepatic artery. Lipiodol-CT, which involves computed tomography after intrahepatic arterial injection of Lipiodol, is regarded as the most sensitive imaging modality for HCC.

Peer review

This is a large experience with HCC treated with resection, with careful follow-up using sensitive imaging studies. Recurrence within the first 3 mo was appropriately considered as residual tumor. The three independent factors associated with residual tumor were AFP level, tumor size, and microscopic invasion.

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Down-regulation of miR-622 in gastric cancer promotes cellular invasion and tumor metastasis by targeting *ING1* gene

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Abstract

AIM: To evaluate the biological and clinical characteristics of miR-622 in gastric cancer.

METHODS: We analyzed the expression of miR-622 in 57 pair matched gastric neoplastic and adjacent non-neoplastic tissues by quantitative real-time polymerase chain reaction. Functional analysis of miR-622 expression was assessed *in vitro* in gastric cancer cell lines with miR-622 precursor and inhibitor. The roles of miR-622 in tumorigenesis and tumor metastasis were analyzed using a stable miR-622 expression plasmid in nude mice. A luciferase reporter assay was used to assess the effect of miR-622 on inhibitor of growth family, member 1 (ING1) expression.

RESULTS: Expression of miR-622 was down-regulated in gastric cancer. MiR-622 was found involved in differentia-

tion and lymphatic metastasis in human gastric cancer. Ectopic expression of miR-622 promoted invasion, tumorigenesis and metastasis of gastric cancer cells both *in vitro* and *in vivo*. ING1 is a direct target of miR-622.

CONCLUSION: These findings help clarify the molecular mechanisms involved in gastric cancer metastasis and indicate that miR-622 modulation may be a bona fide treatment of gastric cancer.

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Key words: MicroRNA; MiR-622; Gastric cancer; Metastasis; Inhibitor of growth family member 1

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INTRODUCTION

Gastric cancer is the fourth most common cancer and is the second most common cause of death from cancer in the world^[1-3]. For most solid malignancies, metastasis is the predominant cause of cancer death^[4-6]. Elucidation of the molecular mechanisms that regulate the sequential steps of metastasis formation is critical for the reduction of cancer mortality. The regulatory mechanism involved in the development of gastric cancer is not well understood; the discovery of critical carcinogenic pathways and the identification of new therapeutic targets for gastric cancer are crucial for local and global public health.

Recently, researchers discovered a novel class of short,

endogenous non-coding RNAs, called microRNAs (miRNAs) described in not only animals and plants, but also in humans as well^[7-9]. It is clear that miRNAs play pivotal roles in a wide array of biological processes, including cell proliferation, differentiation and apoptosis^[10,11]. MiRNAs regulate the expression of protein-coding genes by degrading target mRNAs or by inhibiting gene translation^[12]. Emerging evidence strongly suggests that abnormal miRNA expression is a common and important feature of human malignancies^[13,14], and some studies show that some miRNAs are associated with metastasis from gastric cancer^[15-18].

In this study, we investigated the biological effects and potential mechanisms of miR-622 in gastric carcinoma. We examined the expression of miR-622 in gastric cancer and found that miR-622 was associated with differentiation and lymphatic metastasis of human gastric cancer. Exogenous expression of miR-622 promotes invasion, tumorigenesis and metastasis formation both *in vitro* and *in vivo*. Using bioinformatics analysis, we identified inhibitor of growth family, member 1 (*ING1*) as a putative miR-622 target. Subsequent experiments confirmed that up-regulation of miR-622 repressed the expression of *ING1* at translational level.

MATERIALS AND METHODS

Cell lines and tissue samples

MKN-45, AGS, MKN-28, SGC-7901 and human embryonic kidney cell lines 293T (HEK 293T) were maintained in our central laboratory. Gastric cancer cell lines SNU-1 and NCI-N87 were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. Primary gastric tumor tissues and adjacent non-tumor gastric tissues were collected from either routine therapeutic surgery or gastrointestinal endoscopy at our department. All samples were obtained with informed consent from the patients and approval by the hospital institutional review board.

Analysis of miRNA expression using TaqMan real-time polymerase chain reaction

Total RNA from tissue samples and cell lines was isolated using the mirVana miRNA Isolation Kit (Ambion, USA). Expression of mature miRNAs was detected using the Taqman MicroRNA Assay (Applied Biosystems, Carlsbad, CA, USA) specific for hsa-miR-622. Briefly, 5 ng small RNA or total RNA was reversely transcribed using specific stem-loop RT primers. The reverse transcription products were then amplified and detected using PCR with specific primers and TaqMan probes. The PCR was run in a 7900 HT Fast Real-Time polymerase chain reaction (RT-PCR) System (Applied Biosystems) and SDS2.2.2 software (Applied Biosystems) was used for comparative $\Delta\Delta C_t$ analysis. U6 snRNA (RNU6B, Applied Biosystems) was used as an endogenous control.

MiRNA target prediction

The miR-622 sequence was obtained from miRBase (<http://www.microrna.sanger.ac.uk>). Predicted miRNA targets were determined using the TargetScan^[19], PicTar^[20], and Miranda^[7] algorithms.

Construction of miR-622 expression plasmids

For miR-622 expression, human miR-622 precursor (622 bp) was cloned into pSilencer 4.1 (Ambion, Austin, TX, USA) using the following primers: Forward: 5'-ATCCCAGGGAGACAGAGATCGAGG-3', Reverse: 5'-AAGCTTGGTGGTGGACTTTTGGTTGT-3'. The plasmid was named pS-miR-622, and the control plasmid, consisting of a scrambled sequence (Ambion), was named pS-control. Transfected cells were selected with puromycin 48 h after transfection and then diluted for clonal selection. Mature miRNA expression in selected clones was assessed by RT-PCR as described above. All constructs were verified by sequencing. Stability-enhanced miR-622 precursor and negative control 1 ribo-oligonucleotides were obtained from Ambion. To inhibit miR-622 function, an Ambion miRNA inhibitor for miR-622 (AS-miR-622) was used, along with the negative control (AS-control).

Transfection

For transfection, a complex of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 200 nmol/L RNAs described above was prepared according to the manufacturer's instructions and directly mixed with cells in 24-well cell culture plates at a density of 4×10^4 cells per well. The level of miR-622 expression in transfected cell lines was assayed by real-time RT-PCR 72 h after transfection.

Invasion and scratch healing assays

Cell invasion assays were performed using insert membranes coated with diluted Matrigel (BD Biosciences, San Jose, CA, USA). Cells (1×10^5) were added to the upper chamber and cultured for 48 h. Finally, the insert membranes were cut and stained with Crystal violet (0.04% in water, 100 mL) and permeable cells were counted under an inverted microscope and photographed. For the scratch assay, cells were treated with 10 mg/mL mitomycin C (Sigma) for 3 h and then wounded with a pipette tip. Fresh, full medium was added, and wound healing was observed for 48 h. Photographs were taken every 6 h.

Tumorigenesis and metastasis assay in nude mice

Cells (1×10^6), transfected with pS-miR-622 or pS-control, were collected and inoculated subcutaneously into right flank regions of 4-wk-old male BALB/c nude mice (Institute of Zoology, Chinese Academy of Sciences, Shanghai). In another group, male nude mice were injected through the tail vein. Tumor nodules were measured every 4 d with a caliper. Mice were sacrificed at the end of one month and the number of metastatic tumors in the liver; tumor growth rate and rate of inhibition were calculated. Two independent experiments were performed for each experimental group.

Immunohistochemistry

Mouse tissues were fixed in 10% neutralized formalin and embedded in paraffin blocks. Sections were then prepared for immunohistochemical examination. After deparaffinization and rehydration, antigen retrieval was performed by boiling samples in 10 mmol/L citrate buffer (pH 6.0) for 10 min. After inhibition of endogenous peroxidase activity for 30 min with methanol containing 0.3% H₂O₂, sections were blocked with 2% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min and then incubated with human anti-rabbit ING1 monoclonal antibody (Abcam, dilution 1:500). The immune complex was visualized using the Dako REATM EnvisionTM Detection System and Peroxidase/DAB (Dako) according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin.

Western blotting

ING1 protein levels were quantified by standard Western blotting procedures, using the anti-rabbit ING1 monoclonal antibody (Abcam, dilution 1:500). Protein levels were normalized to the total glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a rabbit monoclonal anti-GAPDH antibody (Sigma).

Luciferase miRNA target reporter assay

Total cDNA from SGC-7901 cells was used to amplify the 3'-UTR of *ING1* by PCR, using the forward primer: 5'-TAGTCGAGTGGTTCCTCTCTCGTGC-3' and the reverse primer: 5'-TTACAGCTCACATACAGCAG-GAAG-3'. After digestion of the PCR product by *Spe* I and *Hind* III, the *ING1* 3'UTR was cloned into the *Spe* I and *Hind* III sites of pMir-Report (Ambion), yielding pMir-Report-ING1. Mutations were introduced at potential miR-622 binding sites using the QuikChange site-directed mutagenesis kit (Stratagene). HEK 293T cells were transfected with the pMir-Report vectors containing the 3'UTR variants, and 5 h after transfection, the cells were transfected again with 100 nmol/L of miR-622 precursor, miR-622-inhibitor or control plasmids. Five ng of the pRL-TK vector (Promega) harboring the Renilla luciferase gene was co-transfected as an internal control to determine the transfection efficiency. Cells were harvested 48 h after transfection and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and the GloMaxTM 20/20 detection system (E5331, Promega).

Statistical analysis

Statistical analysis was performed using SPSS 15.0 software (SPSS, USA). Data were expressed as the mean \pm SD from at least three separate experiments. Differences between groups were analyzed using Student's *t* test and the χ^2 test. *P* < 0.05 was considered statistically significant.

RESULTS

Relationship between miR-622 expression levels and clinicopathological factors in patients with gastric cancer

To determine whether miR-622 expression is associated

Table 1 Clinicopathological features and miR-622 expression in gastric cancer patients

Factors	No. of patients	Mean expression of miR-622	P value
Age (yr)			
< 65	42	4.84 \pm 3.78	0.973 ¹
\geq 65	15	4.89 \pm 4.65	
Gender			
Male	30	5.51 \pm 3.89	0.191 ¹
Female	27	4.12 \pm 4.03	
Cell differentiation			
Poor	19	5.71 \pm 4.14	0.02 ^{1a}
Moderate	38	3.14 \pm 3.07	
Tumor size (cm)			
< 5	48	4.60 \pm 3.79	0.259 ¹
\geq 5	9	6.24 \pm 4.91	
Gross appearance			
Borrmann I + II type	10	3.72 \pm 3.69	0.325 ¹
Borrmann III + IV type	47	5.10 \pm 4.04	
Site of tumor			
Cardia	9	3.60 \pm 4.00	0.37 ¹
Body	8	3.84 \pm 5.57	
Antrum	40	5.34 \pm 3.682	
Lymphatic metastasis			
Positive	31	6.03 \pm 4.16	0.04 ^{1a}
Negative	26	3.87 \pm 3.60	
Depth of cancer invasion			
T2	11	4.71 \pm 5.03	0.586 ¹
T3	44	5.02 \pm 3.78	
T4	2	2.02 \pm 1.37	
TNM Stage			
I	2	0.82 \pm 0.72	0.385 ¹
II	12	4.02 \pm 3.31	
III	35	5.34 \pm 4.12	
IV	8	5.00 \pm 4.49	
Distal metastasis			
Positive	6	3.67 \pm 3.22	0.4441
Negative	51	5.00 \pm 4.07	

¹Chi-square test. ^a*P* < 0.05; TNM: Tumor-node-metastasis.

with gastric cancer, we examined and compared miR-622 expression in primary gastric cancer tissues and in pair-matched adjacent non-tumor tissues using quantitative RT-PCR. A decrease of miR-622 expression was found in 70.18% of the 57 patients with gastric cancer with a median change by about 1.83-fold (*P* < 0.05, Figure 1). We then investigated the relationship between miR-622 expression levels and clinicopathological factors in the 57 patients. We found that the expression level of miR-622 was associated with the differentiation and lymphatic metastasis (*P* < 0.05, Table 1). As shown in Table 1, miR-622 expression levels were not associated with tumor size and distal metastasis, TNM stage or invasion.

Expression of miR-622 and ING1 in gastric cancer cell lines

We detected miR-622 and ING1 expression using qRT-PCR in six different gastric cancer cell lines (AGS, SNU-1, NCI-N87, SGC-7901, MKN-45 and MKN-28). Based on the miR-622 expression levels in these cell lines, we chose to further analyze SGC-7901 and NCI-N87 cells for miR-622 gain-of-function and loss-of-function studies, respectively

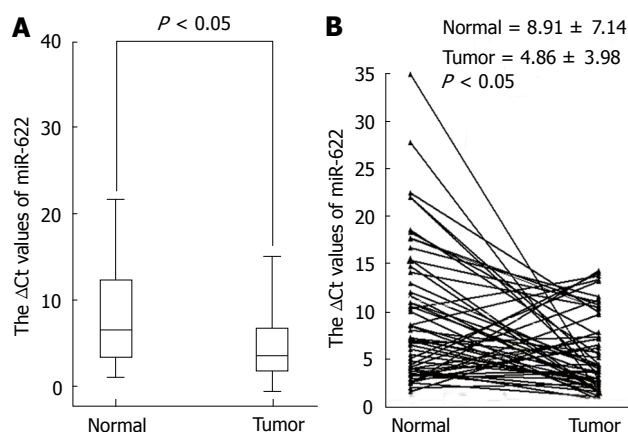


Figure 1 Expression of miR-622 was down-regulated in gastric cancer. A: Level of miR-622 expression in gastric cancer tissues ($n = 57$) was lower than that in non-tumor tissues ($n = 57$). B: The values (ΔC_t) are relative to those of U6 small RNA.

(Figure 2A). After transfection, we examined the expression of miR-622 in cancer cells by real-time PCR. The expression level of miR-622 was significantly up-regulated in SGC-7901 cells transfected with miR-622 precursor ($P < 0.01$, Figure 2B) and the expression level of miR-622 was significantly down-regulated in NCI-N87 cells transfected with miR-622 inhibitor, compared with the negative controls ($P < 0.01$, Figure 2B). We also analyzed the expression of *ING1* in the same gastric cancer cells. The expression of miR-622 was inversely correlated with the expression of *ING1* (Figure 2A).

Ectopic expression of miR-622 promotes invasion and migration of gastric cancer cells *in vitro*

We examined cell invasion and migration ability using the transwell invasion (Figure 2C) and scratch healing assays (Figure 2D). The number of invasive cells in the miR-622 precursor-transfected sample was significantly increased compared with the control (731.51 ± 3.16 vs 362.24 ± 5.18 , $P < 0.01$). MiR-622 precursor-transfected cells healed the wound 48 h after scratching, whereas control-transfected cells were unable to heal the wound. The mean wound distances of the experimental sample and the control 48 h after scratching were significantly different (11.23 ± 7.19 μm vs 176.31 ± 7.24 μm , $P < 0.01$). Conversely, miR-622 inhibitor-transfected cells showed inhibited invasion (127.31 ± 11.62 vs 329.18 ± 5.18 , $P < 0.05$) and migration ability (384.06 ± 9.35 μm vs 176.31 ± 7.24 μm , $P < 0.01$) compared with the control group (Figure 2C and D).

Ectopic expression of miR-622 promotes tumorigenesis and metastasis *in vivo*

We then tested whether ectopic expression of miR-622 could promote tumor growth *in vivo*. We constructed a miR-622 expression vector (pS-miR-622) and selected SGC-7901 cells stably transfected with pS-miR-622. Subsequently, pS-miR-622-transfected SGC-7901 cells and pS-control-transfected SGC-7901 cells were injected subcutaneously into nude mice, and tumor formation was monitored. After 30 d, the animals were euthanized,

and the tumor volume was measured. Tumor growth was significantly promoted in the mice injected with pS-miR-622-transfected SGC-7901 cells compared with pS-control-transfected SGC-7901 cells (Figure 3A and B). The average tumor volume of mice inoculated with pS-miR-622-transfected SGC-7901 cells at day 30 was 29.91 ± 2.13 cm^3 , which was significantly larger than that of mice inoculated with pS-control-transfected SGC-7901 cells (23.81 ± 1.95 cm^3 , $P < 0.05$, Figure 3A and B). Thus, miR-622 can promote tumorigenesis *in vivo*. We found that ectopic expression of miR-622 promoted migration and invasion of gastric cancer cells *in vitro*; therefore, we further tested whether ectopic expression of miR-622 could affect tumor metastasis *in vivo*. SGC-7901 cells stably transfected with pS-miR-622 or pS-control vector were injected into 4-wk-old male nude mice through the tail vein. One month after the injection, the mice were euthanized. The average number of hepatic metastatic nodes per mouse was much larger in mice injected with SGC-7901 cells stably transfected with pS-miR-622 vector than that in the control group (3 ± 0.68 vs 12.32 ± 0.21 , $P < 0.01$) (Figure 3C). In addition, immunohistochemical analysis confirmed that the *ING1* protein was down-regulated in pS-miR-622-transfected SGC-7901 cells compared with pS-control-transfected SGC-7901 cells (Figure 3D). Thus, these results indicate that miR-622 has the ability to promote metastasis of gastric cancer cells *in vivo*, which is consistent with the data obtained from the *in vitro* migration and invasion assays.

MiR-622 directly inhibits the expression of *ING1* through *ING1* 3'UTR

Bioinformatics analysis identified several candidate miR-622 target genes. As shown in Figure 4A, a target sequence for miR-622 was found in the 3'UTR of *ING1* at position 253-260 nt. To test whether the 3'UTR of *ING1* is a functional target of miR-622, we engineered a reporter plasmid containing the wild-type 3'UTR of *ING1* in the 3' position of the firefly luciferase reporter gene. In parallel, we engineered another reporter plasmid in which the conserved target sequence within nt253-260 was specifically mutated, a modification that was predicted to inhibit miR-622-nt253-260 interactions (Figure 4A). To investigate the influence of miR-622 on *ING1* expression, we searched for changes in *ING1* protein and mRNA levels in HEK 293T cells transfected with the miR-622 precursor or the miR-622 inhibitor or in control-transfected cells. As shown in Figure 4B, miR-622 precursor transfection led to a significant decrease in *ING1* protein levels ($P < 0.05$, Figure 4B). In contrast, miR-622 inhibitor transfection did not significantly alter *ING1* protein levels compared with control-transfected cells.

These findings suggest an interaction between miR-622 and *ING1* that we further investigated by luciferase assay analysis. To show a direct interaction between miR-622 and the 3'UTR of *ING1*, we cloned the 3'UTR region that is predicted to interact with miR-622 into a luciferase reporter vector. We then assessed luciferase activity by co-transfection the luciferase reporter vector bearing

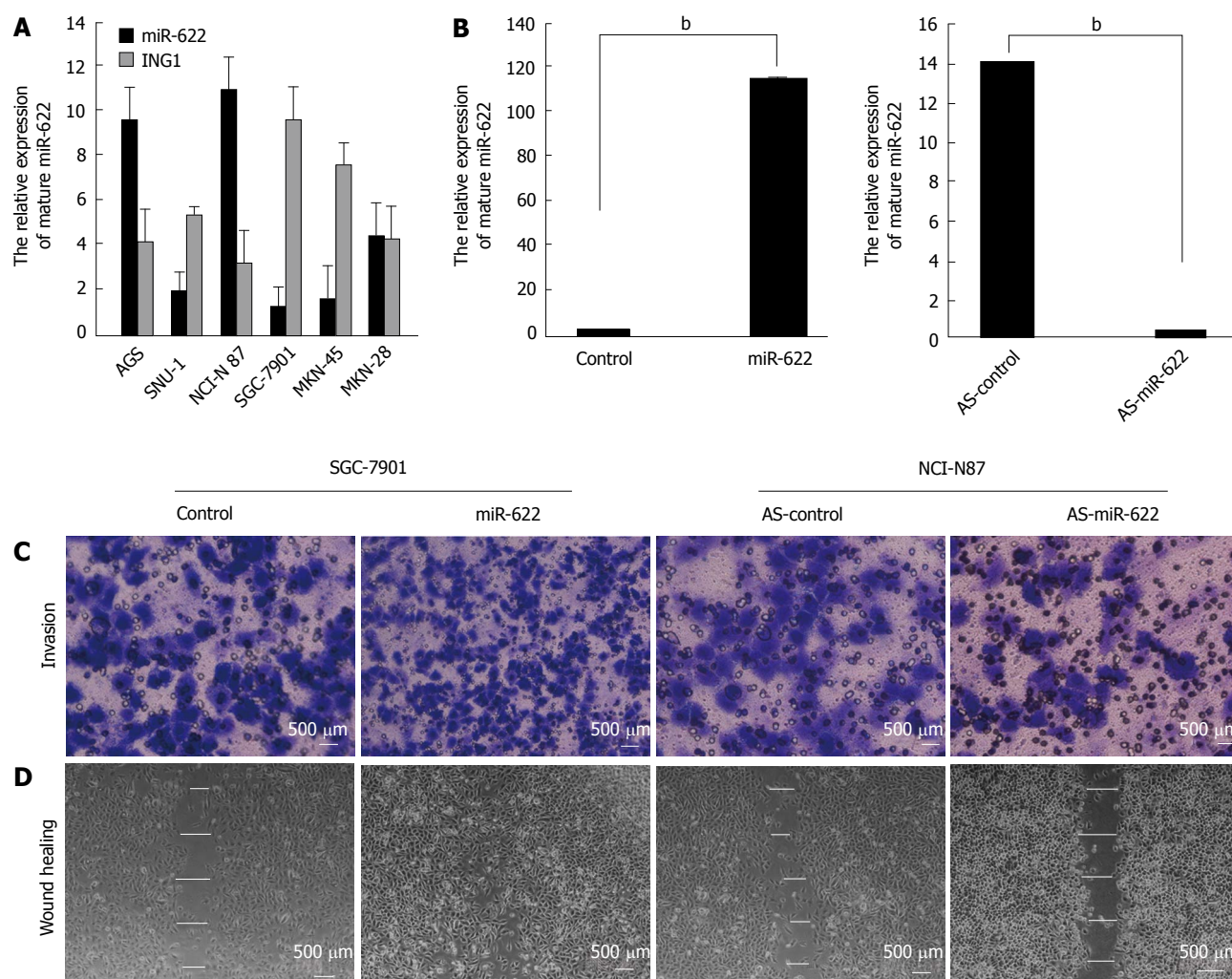


Figure 2 Restoration of miR-622 promotes gastric cancer cell invasion and migration. **A:** q-real time polymerase chain reaction for the expression of miR-622 and ING1 was performed using six gastric cancer cell lines. The lowest and highest levels of miR-622 expression among the six gastric cancer cell lines were found in SGC-7901 and NCI-N87 cells, respectively, and the expression of miR-622 was inversely correlated with the expression of ING1; **B:** Expression of miR-622 was restored or suppressed in gastric cancer cells after miR-622 precursor or miR-622 inhibitor transfection, compared with the controls ($P < 0.01$); **C** and **D:** Over-expression of miR-622 promotes increased cell invasion and migration compared with the miR-control transfectants. However, down-regulation of miR-622 inhibited cell invasion and migration compared with AS-control transfectants. ^b $P < 0.01$, compared with miR-control transfectants. Scale bars: 500 μ m. AS-miR-622: An ambion miRNA inhibitor for miR-622.

the 3'UTR of *ING1* with the miR-622 precursor or the miR-622 inhibitor or with control plasmids. Luciferase activity was markedly diminished in the cells transfected with the miR-622 precursor and wild-type 3'UTR reporter plasmid, compared with the cells transfected with the miR-622 precursor and mutant 3'UTR reporter plasmids (Figure 4C, $P < 0.05$). Conversely, a significant increase in luciferase activity was observed after transfection with miR-622 inhibitor (Figure 4C). Taken together, these data imply that miR-622 may attenuate the expression of *ING1* by directly targeting the *ING1* 3'UTR.

DISCUSSION

Invasion and metastasis, two of the most important hallmarks of cancer, are the leading factors of malignant cancer that lead to lethality, especially for gastric cancer. The long-term survival of patients with gastric cancer after curative resection is confounded by a high recur-

rence rate, which is mainly due to the spread of lymphatic metastasis^[4,21]. Therefore, the identification of metastatic factors and an understanding of the underlying molecular pathways involved in the progression of metastasis are critical issues. Recent studies have shown that miRNAs play a fundamental role in the invasion and metastasis of gastric cancer^[15,16,18], thereby opening a novel avenue to investigate the molecular mechanism of gastric cancer progression and to develop potential therapeutics against gastric cancer.

Prior to this study, very little was known about miR-622 expression in gastric carcinoma and its correlation with the clinicopathologic features of these patients. To address these issues, miR-622 expression levels and the clinicopathologic characteristics of 57 patients with gastric cancer were examined. There was a significant association between miR-622 expression and differentiation as well as lymphatic metastasis. However, no relationship was found between miR-622 expression and gender, tumor size, differentiation grade, distant metastasis or TNM stage. Given

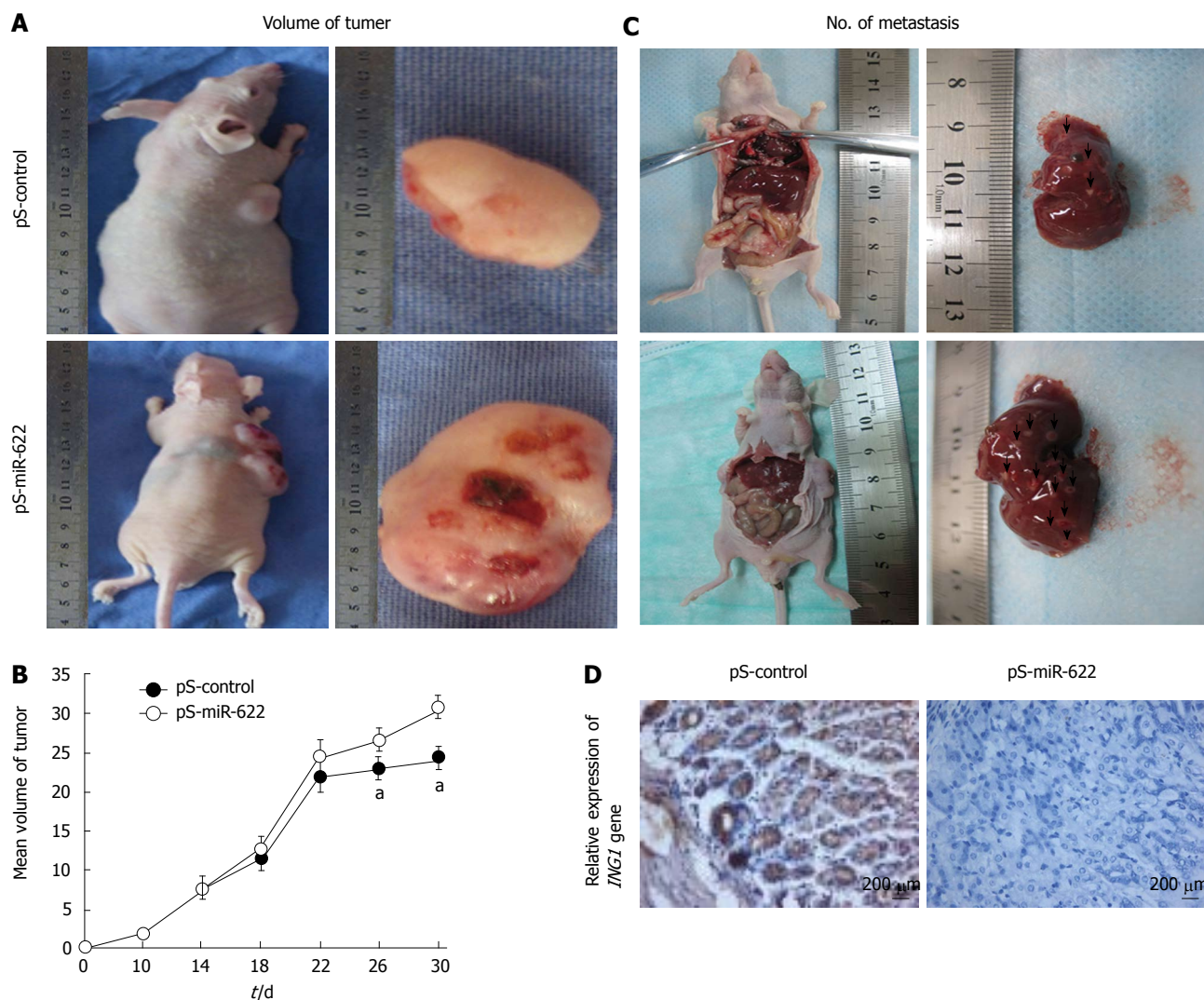


Figure 3 MiR-622 promotes tumorigenesis and metastasis *in vivo*. A: Photographs of tumors in nude mice resulting from injection of miR-622 expression vector (pS-miR-622) or control plasmid (pS-control) stably transfected SGC-7901 cells; B: Tumorigenesis curves. Rapid tumor growth was observed in the pS-miR-622 group (data expressed as mean \pm SD; $P < 0.05$); C: Photographs of the number of liver metastatic nodes in nude mice injected with pS-miR-622 or pS-control vector stably transfected SGC-7901 cells. The arrows indicate liver metastatic nodes; D: Histological examination found that the expression of *ING1* was markedly reduced in the pS-miR-622-transfection group compared with the control groups. ^a $P < 0.05$, compared with miR-control transfectants. Scale bars: 200 μ m. pS-control: Control plasmid; pS-miR-622: miR-622 expression vector.

that the expression levels of miR-622 were significantly associated with lymphatic metastasis and differentiation, we speculated that up-regulation of miR-622 might promote the malignant phenotypes of gastric cancer cells. Therefore, we used miR-622 precursor and inhibitor constructs to perform miR-622 gain-of-function or loss of-function studies in human gastric cells. Invasion and migration ability assays revealed that miR-622 over-expression induced invasion and migration of gastric cancer cells *in vitro*. Conversely, miR-622 inhibitor can inhibit invasion and migration. These findings suggest that miR-622 functions as a key mediator of cell invasion and migration in gastric cancers. Cell migration plays an important role in many diverse biological processes^[22]. Aberrant activation of cell migration in neoplastic cells results in tumor metastasis, which is the principal event leading to death in the majority of cancer patients^[5].

We observed that miR-622 promotes cell invasion and migration ability in gastric cancers; however it was unclear

whether up-regulation of miR-622 also affects gastric cancer cell tumorigenesis and metastasis. Therefore, we constructed specific miR-622 plasmids and established permanent transfected cell lines to investigate the potential role of miR-622 in tumorigenesis and metastasis of gastric cancer. We showed, for the first time, that miR-622 over-expression significantly induced tumorigenesis and metastasis of gastric cancer cell lines in nude mice. Cellular tumorigenesis assays and metastasis assays both *in vitro* and *in vivo*, revealed that miR-622 over-expression resulted in the promotion of tumorigenesis and metastasis of SGC7901 cells. These findings lend evidence that miR-622 may indeed function as a key mediator of cell tumorigenesis and metastasis and that it will be a promising target for gastric cancer treatment.

The fundamental function of miRNAs is to regulate target genes by direct cleavage of the mRNA and/or by inhibition of protein synthesis, according to the degree of

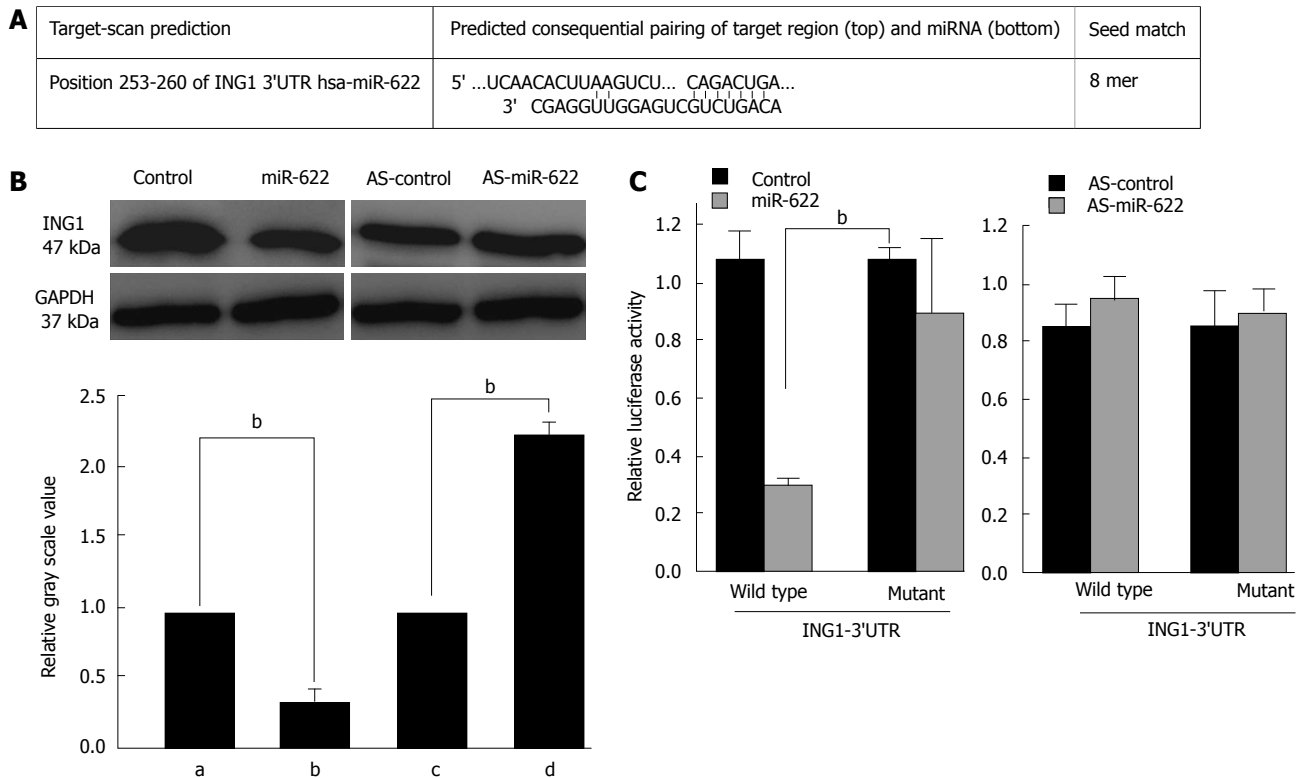


Figure 4 MiR-622 binds to the 3'UTR of Inhibitor of growth family, member 1 to post-transcriptionally repress the protein levels. A: Representative nucleotide sequence matches between possible target genes and miRNAs. Putative binding site of miR-622 in the *ING1* 3'UTR region (detected by TargetScan). Only matched nucleotides with miRNA seed sequences are indicated with vertical lines; B: Effects of miR-622 on *ING1* protein levels. HEK 293T cells were transfected with 100 nmol/L of miR-622 precursor or miR-622 inhibitor. Whole-cell lysates were prepared 72 h after transfection and subjected to immunoblot analysis using antibodies against *ING1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative gray scale values were compared with GAPDH; C: Luciferase reporter assay. A total of 1×10^5 HEK 293T cells were transfected with 100 ng wild-type-UTR-reporter or mutant-UTR-reporter constructs together with 100 nmol/L of miR-622 precursor or miR-622 inhibitor as indicated. $^bP < 0.01$. Each bar represents mean values \pm SE from three independent experiments. *ING1*: Inhibitor of growth family, member 1; AS-miR-622: An ambion miRNA inhibitor for miR-622.

complementarity with the target mRNA 3'UTR^[23]. Computational algorithms have been the major driving force in predicting miRNA targets, which are based mainly on base pairing of miRNAs and target gene 3'UTRs^[24]. To explore the molecular mechanism underlying miR-622 function, we searched for its direct target genes using bioinformatics analysis of miRNA-mRNA 3'UTR matching. Among the putative targets for miR-622, *ING1* was detected by the TargetScan program. This gene encodes a tumor suppressor protein that can induce cell growth arrest and apoptosis; the encoded protein is a nuclear protein that physically interacts with the tumor suppressor protein TP53 and is a component of the p53 signaling pathway^[25-28]. The bioinformatics analysis revealed that the conserved binding sites on *ING1* that can be recognized by miR-622 are located in the 3'UTR. To test this assumption, we investigated whether miR-622 affects *ING1* protein levels. We found that miR-622 leads to a significant decrease in *ING1* protein levels, suggesting that *ING1* is a functional target of miR-622. Lastly, results from our dual-luciferase reporter assays suggest that *ING1* is a functional downstream target of miR-622.

In summary, we have shown that expression levels of miR-622 in gastric cancer tissues correlates with the differentiation and lymphatic metastasis. Using gain-of-function

and loss-of-function studies, we demonstrated that up-regulation of miR-622 expression promotes gastric cancer cell invasion, tumorigenesis and metastasis both *in vitro* and *in vivo*. Therefore, miR-622 is likely to play an important role in tumorigenesis and metastasis of human gastric cancer and is a promising molecular target for gastric cancer therapy.

ACKNOWLEDGMENTS

We thank the colleagues from our central laboratory of Shandong Provincial Hospital affiliated to Shandong University for their excellent technical support.

COMMENTS

Background

Gastric cancer is a worldwide cancer with poor prognosis. Identification of diagnostic biomarkers and effective therapeutic targets is important in the treatment and diagnosis of gastric cancer. Recently, researchers discovered a novel class of short, endogenous non-coding RNAs, called microRNAs (miRNAs), in plants, animals, and humans. It is clear that miRNAs play pivotal roles in a wide array of biological processes, including cell proliferation, differentiation and apoptosis.

Research frontiers

MiRNAs are 18-22 nucleotides, non-coding RNAs that regulate gene expression in a post-transcriptional manner. The fact that miRNAs are widely expressed in various species and tissues indicates that miRNAs may play an essential role in cell growth, differentiation, apoptosis as well as carcinogenesis. MiRNAs regu-

late the expression of protein-coding genes by degrading target mRNAs or by inhibiting gene translation. Emerging evidence strongly suggests that abnormal miRNA expression is a common and important feature of human gastric cancer.

Innovations and breakthroughs

The present study demonstrated that miR-622 is associated with differentiation and lymphatic metastasis in human gastric cancer. Exogenous expression of miR-622 promotes invasion, tumorigenesis and metastasis, both *in vitro* and *in vivo*. Using bioinformatics analysis, the authors identified the inhibitor of growth family, member 1 (ING1) as a putative miR-622 target. Subsequent experiments confirmed that up-regulation of miR-622 represses the expression of ING1 at the translational level.

Applications

In this study, the expression of miR-622 was found down-regulated in gastric cancer. MiR-622 was involved in differentiation and lymphatic metastasis in human gastric cancer. The ectopic expression of miR-622 promoted invasion, tumorigenesis and metastasis of gastric cancer cells both *in vitro* and *in vivo*. *ING1* was a direct target of miR-622. These findings help clarify the molecular mechanisms involved in gastric cancer metastasis and indicate that miR-622 modulation may be a *bona fide* treatment of gastric cancer.

Terminology

MiRNAs are 18-22 nucleotides, non-coding RNAs that regulate gene expression in a post-transcriptional manner. ING1 is an inhibitor of growth family, member 1. This gene encodes a tumor suppressor protein that can induce cell growth arrest and apoptosis. The encoded protein is a nuclear protein that physically interacts with the tumor suppressor protein TP53 and is a component of the p53 signaling pathway.

Peer review

The authors evaluated the possible pathogenetic role of miR-622 in metastasis formation and invasiveness of gastric cancer using not only human samples and miRNA expression arrays, but also functional assays on cell lines and mouse animal model. The topic is of significant clinical importance and their results may be used in clinical research of gastric cancer, and it may give some evidences for new therapeutic strategies.

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Salvianolate inhibits cytokine gene expression in small intestine of cirrhotic rats

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group, medium-dose salvianolate (24 mg/kg) treatment group, and high-dose salvianolate (48 mg/kg) treatment group, and treated for 2 wk. Another 10 healthy rats served as a normal control group. Mortality of cirrhotic rats in each group was evaluated after treatment with salvianolate. Serum samples were taken from portal vein for the detection of endotoxin. Morphological changes in tissue samples from the ileocecum were observed under a light microscope. Expression of TNF- α and IL-6 mRNA in the small intestine of rats was analyzed by real-time reverse-transcriptase polymerase chain reaction.

RESULTS: The mortality of cirrhotic rats in the non-treatment group was 37.5%. No cirrhotic rat died in the high-dose salvianolate treatment group. The serum endotoxin level was significantly higher in the non-treatment group than in the salvianolate treatment and normal control groups. The intestinal mucosal and villous atrophy, necrosis and shedding of the intestinal mucosal epithelium, observed in the non-treatment group, were reversed in different salvianolate treatment groups. The TNF- α and IL-6 mRNA expression levels in small intestine were significantly lower in different salvianolate treatment groups than in the non-treatment group.

CONCLUSION: Salvianolate can reduce the endotoxin level, ameliorate the injury of intestinal mucosa, and inhibit the expression of TNF- α and IL-6 mRNA in small intestine of cirrhotic rats.

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Abstract

AIM: To study the effect of salvianolate on expression of tumor necrosis factor (TNF)- α and interleukin (IL)-6 mRNA in small intestine of cirrhotic rats.

METHODS: Cirrhosis in rats was induced using CCl₄ (0.3 mL/kg). Rats were randomly divided into non-treatment group, low-dose salvianolate (12 mg/kg) treatment

Key words: Salvianolate; Cirrhosis; Endotoxin; Intestinal mucosa; Tumor necrosis factor- α ; Interleukin-6

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INTRODUCTION

In liver cirrhosis patients, disruption of intestinal barrier function (IBF) and increased intestinal permeability lead to bacterial translocation (BT) and endotoxemia^[1-6], which increase susceptibility to infection, with spontaneous bacterial peritonitis (SBP) being the most frequent and severe^[1,4]. Endotoxemia, resulting from BT^[4], may provoke sustained activation of the immune system with release of proinflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukins (IL)-1, 6 and 8, and nitric oxide (NO), which in turn decrease IBF and increase severe complications^[7,8]. Intestinal cytokines play an important role in the pathogenesis of intestinal injury and inflammation^[9,10], especially TNF- α and IL-6, which may contribute to the systemic hemodynamic derangement of liver cirrhosis^[11] and lead to liver failure^[12]. Therefore, restoration of the intestinal barrier integrity and inhibition of the cytokine expression are the important goals in preventing intestinal endotoxemia. However, no effective remedy is available at present for the prevention and treatment of intestinal endotoxemia.

Radix Salviae Miltiorrhizae, a traditional Chinese medical herb known as “danshen”, has been widely used in treatment of various cardiovascular diseases^[13,14]. Its extracts contain lipid-soluble diterpene quinones (tanshinones) and water-soluble phenolic acid derivatives, such as salvianolic acids A and B as well as lithospermic acid B^[15]. Recent pharmacological studies showed that *Salviae Miltiorrhizae* (*S. miltiorrhiza*) can eliminate oxygen free radicals, enhance antioxidant activity, decrease serum levels of cytokines, and inhibit endotoxemia^[16]. It has been demonstrated that *S. miltiorrhiza* can block the lethal toxicity of lipopolysaccharide (LPS) in mice by suppressing TNF- α release^[17]. Salvianolate is a new water-soluble phenolic compound that is one of the most bioactive compounds in *S. miltiorrhiza* Bge. As far as we are know, no reports are available at present on the pharmacological activities of salvianolate in liver cirrhosis patients. TNF- α and IL-6 are the most frequent cytokines associated with liver dysfunction in cirrhosis patients^[18], and show an increased local production in mesenteric lymph nodes in response to BT induced by intestinal injury^[19].

The present study was designed to investigate the effect of salvianolate on endotoxin level in the portal vein and expression of TNF- α and IL-6 mRNA in small intestine of rats with CCl₄-induced liver cirrhosis. Whether different doses of salvianolate can enhance the intestinal mucosal barrier function and prevent intestinal endotoxemia is also

studied. The results of the present study provide a new strategy for the treatment of liver cirrhosis.

MATERIALS AND METHODS

Animals

Ninety male Sprague-Dawley rats weighing 180-220 g were provided by Department of Animal Care, Zhejiang Traditional University (Hangzhou, China). Experimental animals were housed in individual cages at 22-25°C in a 12-h light/dark cycle with free access to standard laboratory diet and tap water.

Experimental protocol

The rats were randomly divided into normal control group ($n = 10$) and model group. Rats in the model group received subcutaneous injection of 40% CCl₄ in a 2:3 mixture with olive oil (0.3 mL/kg), once a week for 12 wk. Liver cirrhosis was induced in 55 rats at the end of 12 wk, as shown by liver histological evaluation (Figure 1). The 55 rats in model group were further divided into non-treatment group (group B, $n = 14$), low-dose salvianolate (12 mg/kg) treatment group (group C, $n = 14$), medium-dose salvianolate (24 mg/kg) treatment group (group D, $n = 14$), and high-dose salvianolate (48 mg/kg) treatment group (group E, $n = 13$). Rats in group A were intraperitoneally (ip) injected with 5% glucose solution, once a week for 2 wk. Rats in groups C-E were ip injected with different doses of salvianolate dissolved in a 5% glucose solution, once a week for 2 wk. At the same time, 40% CCl₄ was continued for an experimental period of 14 wk. At the end of the 14-wk experimental period, all rats were anesthetized with 3% chloral hydrate and dissected. Blood samples were taken from the portal vein and intestinal tissue for further analysis.

Measurement of serum endotoxin level

Five milliliters of blood was taken from the portal vein and immediately put into a tube containing heparin. Plasma was taken after the blood was centrifuged at 3000 r/min for 1 min at 0°C. Endotoxin level in blood was measured by photometry, using a MB-80 microbiology kinetic rapid reader (Beijing, Gold Mountainriver Tech Development Co., Ltd, China).

Assessment of morphological changes in intestinal mucous membrane

At the end of the 14-wk experimental period, a horizontal incision was made along the mid-section to expose the abdominal cavities of all rats with their intestines excised. Ileal tissue samples were taken immediately and washed 3 times with cold physiological saline, fixed in a 10% formalin solution, dehydrated and embedded in paraffin. Each sample was cut into 4 μ m-thick sections which were stained with hematoxylin and eosin (H and E), and examined under a light microscope (Olympus BX50; Tokyo, Japan).

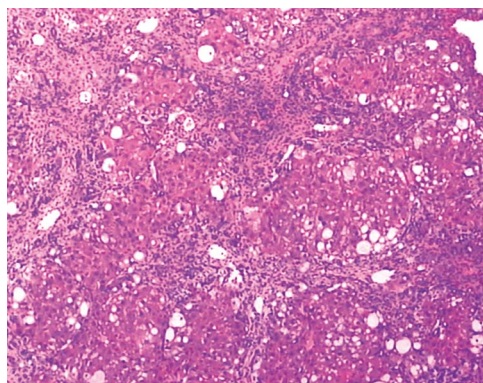


Figure 1 Liver histology in a cirrhotic rat model (HE staining, × 200).

Table 1 Forward and reverse primers used in quantitative real-time polymerase chain reaction

Gene	Primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
β-actin	5'-ACTGCCG-CATCTCTTCCTC-3' 5'-ACTCCTGCTTGCTGATC-CACAT-3'	55	598
Rat TNF-α	5'-GGCAGGTCTACTTTG-GAGTCATTGC-3' 5'-ACATTGGGGATCCAGT-GAGCTCCG-3'	58	318
Rat IL-6	5'-GGATACCACCACAA-CAG-3' 5'-GGTCCTTAGC-CATCCTT-3'	58	451

TNF: Tumor necrosis factor; IL: Interleukin.

Isolation and analysis of mRNA expression by real-time reverse transcriptase polymerase chain reaction

Total RNA was isolated from snap-frozen ileal tissue samples using the Trizol method (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free water. Single-stranded cDNA was synthesized from the total RNA as follows. In brief, 1 μg RNA was pre-incubated with 1 μL oligo (dT)₁₅ primer, and diethylpyrocarbonate (DEPC)-treated water was added to a total volume of 9.5 μL at 70°C for 10 min, and then rapidly chilled on ice. To the annealed primer/template, 4 μL 5 × RT (reverse transcriptase) buffer, 0.5 μL dNTP (10 mmol/L each), 25 U ribonuclease inhibitor (Takara, Dalian, China), 200 U Moloney murine leukaemia virus reverse transcriptase (Takara) and DEPC-treated water were added to a final volume of 20 μL. The reaction was incubated at 42°C for 1 h and terminated by placing it on ice after deactivation at 70°C for 10 min. The resultant cDNA was used as a template for subsequent polymerase chain reaction (PCR).

The PCR mixture contained 5 μL 10 × Taq buffer (Takara), 4 μL dNTP (10 mmol/L each), 2 μL gene-specific primers, 2.5 U Taq DNA polymerase (Takara) and 2 μL cDNA in a total volume of 50 μL. Thirty cycles of PCR amplification were performed with an initial incubation

Table 2 Mortality of rats in different groups

Group	n	Mortality values
A	10	0 (0/10) ^a
B	14	37.5% (6/14)
C	14	21.42% (3/14)
D	14	21.42% (3/14)
E	13	0 (0/13) ^a

^a*P* < 0.05 vs non treatment group. A: Normal control group; B: Non treatment group; C: Low-dose salvianolate treatment group; D: Medium-dose salvianolate treatment group; E: High-dose salvianolate treatment group.

at 94°C for 3 min and a final extension at 72°C for 7 min. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. The primer sequences used for PCR are shown in Table 1.

The quantities of cDNA that produced an equal amount of β-actin PCR product were used in PCR with the primers for IL-6 and TNF-α. Following reverse transcription polymerase chain reaction (RT-PCR), 5 μL samples of the amplified products was resolved by electrophoresis on 1% agarose gel and stained with ethidium bromide. The level of each PCR product was semi-quantitatively evaluated using a digital camera and an image analysis system (Vilber Lourmat, Marne La Vallée, France), and normalized to GAPDH.

DNA was amplified and detected by BioRad iCycler iQ PCR (BioRad Laboratories, California, USA) in a final volume of 20 μL, using SYBR green master mix reagent at a final concentration of 1 × (Applied Biosystems, Foster City, CA, USA). The PCR amplification conditions for DNA were 95°C for 3 min and 40 cycles at 95°C for 30 s at 55°C for 30 s, and at 72°C for 30 s. A melting curve analysis was carried out after amplification. The threshold cycle (Ct) values and baseline settings were determined by automatic analysis settings. Data were analyzed using the Opticon Monitor 3 software, which was supplied by The BioRad iCycler iQ PCR. Data about relative mRNA copies were expressed as relative quantification (RQ), which was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct$ (cirrhosis group) - ΔCt (normal group), $\Delta Ct = (Ct_{\text{sample}} - Ct_{\beta\text{-actin}})$.

Statistical analysis

Statistical analysis was performed with the SPSS version 13.0 (Chicago, IL, USA). Mortality of rats was compared using Fisher's exact test. Endotoxin level was analyzed using Kruskal-Wallis *H* test. Results of quantitative RT-PCR were assessed by ANOVA. Data were expressed as mean ± SD. *P* < 0.05 was considered statistically significant.

RESULTS

Mortality

At the end of 14-wk experimental period, 6 rats in group B, 3 rats in groups B and C, and no rats in groups A and E died. The mortality rate of rats was significantly higher in group B than in groups A and E (*P* < 0.05, Table 2).

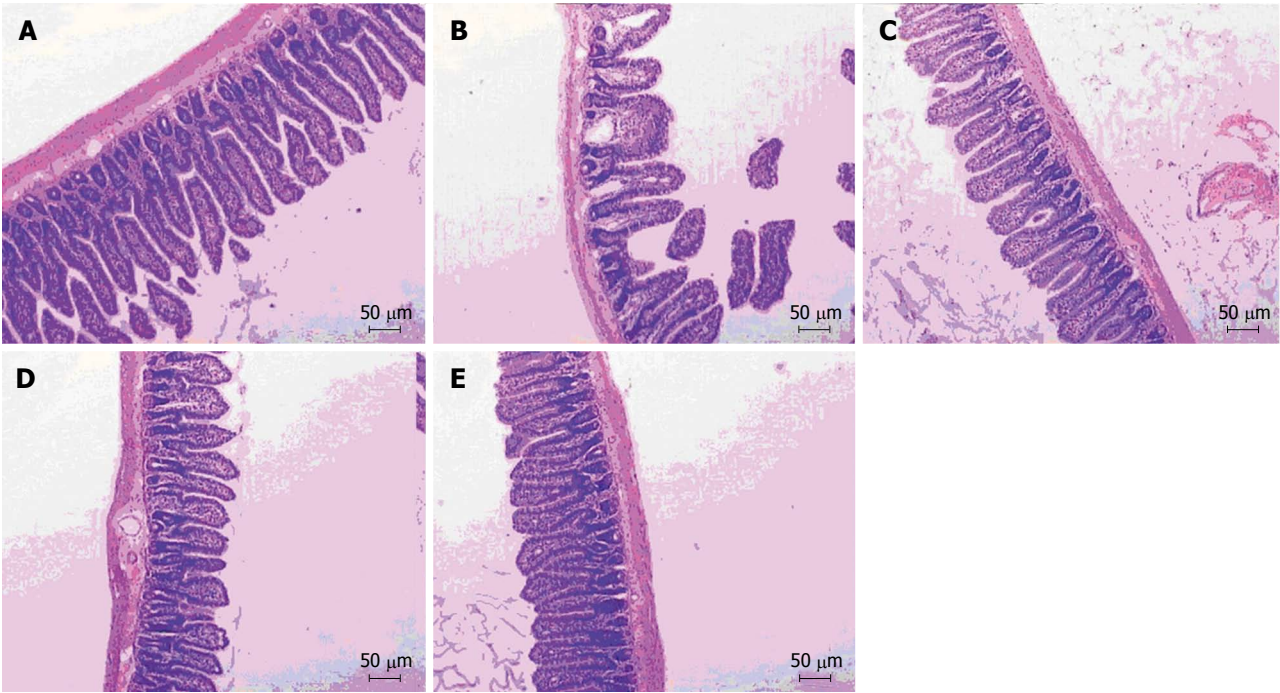


Figure 2 Mucosal morphology of ileal tissue in normal control group (A), non-treatment group (B), low-dose salvianolate treatment group (C), medium-dose salvianolate treatment group (D), and high-dose salvianolate treatment group (E) (HE staining, × 100).

Table 3 Distribution of endotoxin levels in serum of different groups							
Group	n	Dose (mg/kg)	Levels of endotoxin (pg/L)				Mean rank
			0-1	1-10	10-20	> 20	
A	10	-	10	0	0	0	15.35 ^b
B	14	-	2	2	3	7	55.71
C	14	12	8	4	2	0	47.54 ^a
D	14	24	12	1	1	0	29.14 ^b
E	13	48	12	1	0	0	14.57 ^b

^a*P* < 0.05, ^b*P* < 0.01 *vs* non treatment group. A: normal control group; B: non treatment group; C: low-dose salvianolate treatment group; D: medium-dose salvianolate treatment group; E: high-dose salvianolate treatment group.

Plasma endotoxin level

The plasma endotoxin level was < 20 pg/L in groups A and E, > 20 pg/L in 7 rats of group B, and significantly higher in the non-treatment group than in different salvianolate treatment groups and normal control group (*P* < 0.01). No marked difference was found in the plasma endotoxin level between the normal control and high-dose salvianolate treatment groups (Table 3).

Histological changes in ileal tissue

As shown in Figure 2, the intestinal mucosa in normal control group was intact and the villi were presented in an orderly fashion. No inflammatory cell infiltration occurred in the chorioepithelioma. In contrast, the intestinal mucosal villi in rats of the non-treatment group were atrophic, shorter and fractured. Some epithelial cells were necrotic. The mucous membrane showed signs of thinning. The intestinal

mucosa was infiltrated with inflammatory cells (Figure 2B) and repaired gradually in different salvianolate treatment groups. The intestinal mucosal villi in rats of different salvianolate treatment groups were in good order and the mucous membrane became thicker. Inflammatory cell infiltration was decreased, especially in the high-dose salvianolate treatment group (Figure 2A-E).

Expression of TNF-α and IL-6 mRNA in small intestine

The bands (300-500 bp) of RT-PCR amplification products were visualized by 1% agarose gel electrophoresis (Figure 3). Real-time RT-PCR showed that the IL-6 and TNF-α mRNA expression levels were significantly higher in the non-treatment group than in the highest salvianolate dose (3.82 ± 1.30 *vs* 1.71 ± 0.27, 6.13 ± 4.13 *vs* 1.57 ± 0.31) treatment group (*P* < 0.01, *P* < 0.05, Figure 4).

DISCUSSION

To the best of our knowledge, this is the first study to show that salvianolate can decrease the plasma level of endotoxin in the portal vein and restores intestinal mucosal injury in rats with CCl₄-induced liver cirrhosis. During the development of cirrhosis, impaired intestinal mucosal barrier^[10,20] and decreased function of hepatocytes and Kupffer cells can lead to invasion of enteric organisms/endotoxin in blood and formation of bacteremia and intestinal endotoxemia^[21,22]. Endotoxin itself can destroy mitochondria and lysosomes in enteric epithelial cells, leading to cell autolysis. Bacteria and their products (e.g. LPS) can activate innate immune responses by triggering a complex gene program in intestinal epithelium^[23], which can increase secretion

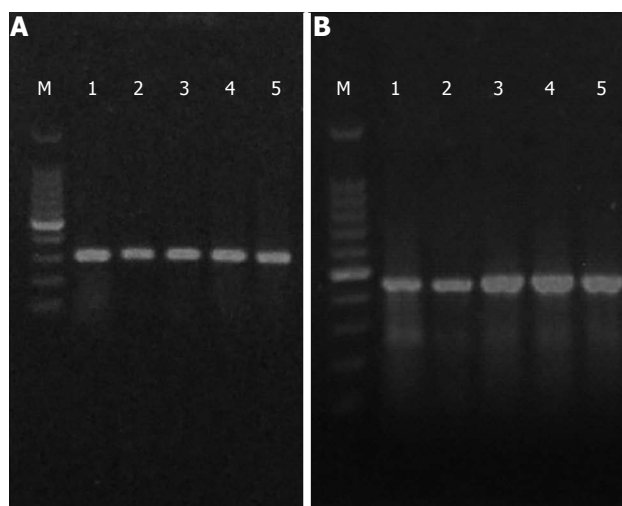


Figure 3 Electrophoretograms of tumor necrosis factor- α (A) and IL-6 (B). M: Marker; lane 1: Normal control group; lane 2: Untreated group; lane 3: Low-dose salvianolate-treated group; lane 4: Medium-dose salvianolate-treated group; lane 5: High-dose salvianolate-treated group.

of cytokines in the intestine and intestinal inflammatory disorders^[24,25]. Ultimately, a vicious cycle can arise between intestinal endotoxemia and increased permeability of enteric mucosa, and lead to liver injury and sepsis, resulting in a high mortality^[12,26]. In the present study, the plasma endotoxin level and mortality of cirrhotic rats were significantly higher in non-treatment group than in different salvianolate treatment groups.

In this study, the intestinal histopathological changes in cirrhotic rats were improved after treatment with salvianolate, indicating that salvianolate can protect intestine against villous atrophy, epithelial cell necrosis, and inflammatory cell infiltration. In parallel with these findings, endotoxemia was significantly reduced in rats after treatment with salvianolate, suggesting that salvianolate exerts its effect on the intestine by protecting the mucosal barrier integrity.

In this study, the altered IBF in rats with liver cirrhosis was found to be associated with the up-regulation of TNF- α and IL-6 mRNA expression levels in intestinal wall. It has shown that serum TNF- α and IL-6 expression levels are significantly increased in cirrhosis patients^[21]. TNF- α is a cytokine that is mainly released by mononuclear cells in response to inflammatory stimuli. The gut and its associated lymphoid tissue, including mesenteric lymph nodes (MLN), have been shown to produce TNF- α in response to BT induced by intestinal injury^[25,27]. More recently, increased TNF- α production by MLN with BT has been detected in cirrhotic rats^[19]. It was reported that local production of TNF- α in MLN is increased in patients with advanced liver cirrhosis, especially ascites^[19], which in common with experimental cirrhosis may also be induced by BT. It seems that enhanced TNF- α expression in gut of cirrhotic rats can result from increased intestinal leakiness, which allows penetration of bacteria and endotoxin into the gut wall. In return, TNF- α can affect the structure of intestinal mucosa, decrease the expression of

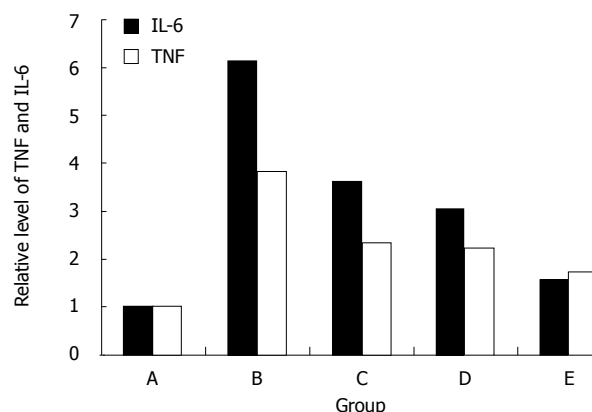


Figure 4 Real-time reverse transcription polymerase chain reaction for the expression of relative mRNA level of TNF and IL-6 in normal control group (A), non-treatment group (B), low-dose salvianolate treatment group (C), medium-dose salvianolate treatment group (D), and high-dose salvianolate treatment group (E). TNF: Tumor necrosis factor; IL: Interleukins.

tight junction (zona occludens 1), and change the morphology of colon in a mouse model of acute liver failure (ALF)^[28]. It may also participate in the pathophysiology of SBP complicating ALF. In this context, the reduced intestinal TNF- α levels observed in rats with cirrhosis after treatment with salvianolate might reflect the improvement of IBF. The increased production of TNF- α in intestine is a crucial event that leads to loss of IBF and BT, and anti-TNF- α therapy may prevent BT and SBP.

IL-6 is a pleiotropic cytokine which can regulate the biological responses of several target cells, including hepatocytes. Similarly, a link between IL-6 and liver fibrosis/cirrhosis has also been reported^[29,30]. Toda *et al*^[21] reported that IL-6 has a direct mitogenic effect on hepatic stellate cells. The activation status of intestinal immune system cells is much higher than that of analogous peripheral cells. Porowski *et al*^[31] reported that the intestine is an important source of IL-6 in patients with liver cirrhosis. Increased production of IL-6 is induced by LPS in intestinal injury^[32,33]. In the present study, the intestinal IL-6 levels were higher in the non-treatment group and lower in different salvianolate treatment groups, suggesting that salvianolate has a direct anti-inflammatory effect on the intestine, and can thus prevent IBF by inhibiting the expression of TNF- α and IL-6 mRNA.

In conclusion, salvianolate can reduce endotoxin level, restore intestinal mucosal injury, and inhibit expression of TNF- α and IL-6 in small intestine of cirrhotic rats. Clinical trials are needed to determine whether the aqueous extract from *Radix Salviae Miltiorrhizae* can favorably influence the natural history of liver cirrhosis, and reduce the risk of SBP and other septic complications in cirrhotic patients.

COMMENTS

Background

In liver cirrhosis, disruption of intestinal barrier function (IBF) leads to bacterial translocation and endotoxemia, which increase susceptibility to spontaneous

bacterial peritonitis. Intestinal cytokines play an important role in the pathogenesis of IBF disruption and intestinal endotoxemia. Inhibition of cytokine gene expression in small intestine is an important goal in enhancing IBF in cirrhotic patients.

Research frontiers

Currently, no effective remedy is available for the prevention and treatment of IBF disruption in liver cirrhosis patients. Recent studies have shown that soluble phenolic acid derivatives can eliminate oxygen free radicals, enhance antioxidant activity, decrease serum levels of cytokines, and inhibit endotoxemia. In the present study, the authors demonstrated that salvianolate, a new water-soluble phenolic compound, could enhance IBF in cirrhotic rats.

Innovations and breakthroughs

Recent studies have highlighted the anti-inflammatory effects of soluble phenolic acid derivatives in *Salvia miltiorrhiza* Bge. The present study is the first to investigate the pharmacological activities of salvianolate in liver cirrhosis, showing that salvianolate decreases the plasma endotoxin level in the portal vein and restores intestinal mucosal injury in cirrhotic rats. The authors demonstrated that salvianolate could protect small intestine of cirrhotic rats by inhibiting tumor necrosis factor (TNF)- α and interleukin (IL)-6 gene expression and enhancing the intestinal mucosal barrier function, thus preventing intestinal endotoxemia.

Applications

By demonstrating the effects of salvianolate on expression of TNF- α and IL-6 mRNA in small intestine of cirrhotic rats, this study provides a new strategy for the treatment of liver cirrhosis. Salvianolate can be applied in clinical practice due to its potential pharmacological activities.

Terminology

Radix Salviae Miltiorrhizae is a traditional Chinese medical herb known as "dan-shen". Salvianolate is a new water-soluble phenolic compound that is isolated from *Radix Salviae Miltiorrhizae* and one of the most bioactive compounds in *S. miltiorrhiza* Bge.

Peer review

The authors have illustrated the pharmacological activity of salvianolate using molecular biology techniques in an animal model of cirrhosis. The results of the study provide a new strategy for the treatment of liver cirrhosis. Further studies are needed to establish the mechanism of action of antifibrotic activity of salvianolate in cirrhotic rats.

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Computational prediction and experimental validation of novel markers for detection of STEC O157:H7

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Abstract

AIM: To identify and assess the novel makers for detection of Shiga toxin producing *Escherichia coli* (STEC) O157:H7 with an integrated computational and experimental approach.

METHODS: High-throughput NCBI blast (E-value cutoff e-5) was used to search homologous genes among all sequenced prokaryotic genomes of each gene encoded

in each of the three strains of STEC O157:H7 with complete genomes, aiming to find unique genes in O157:H7 as its potential markers. To ensure that the identified markers from the three strains of STEC O157:H7 can serve as general markers for all the STEC O157:H7 strains, a genomic barcode approach was used to select the markers to minimize the possibility of choosing a marker gene as part of a transposable element. Effectiveness of the markers predicted was then validated by running polymerase chain reaction (PCR) on 18 strains of O157:H7 with 5 additional genomes used as negative controls.

RESULTS: The blast search identified 20, 16 and 20 genes, respectively, in the three sequenced strains of STEC O157:H7, which had no homologs in any of the other prokaryotic genomes. Three genes, *wzy*, Z0372 and Z0344, common to the three gene lists, were selected based on the genomic barcode approach. PCR showed an identification accuracy of 100% on the 18 tested strains and the 5 controls.

CONCLUSION: The three identified novel markers, *wzy*, Z0372 and Z0344, are highly promising for the detection of STEC O157:H7, in complementary to the known markers.

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Key words: Shiga toxin producing *Escherichia coli* O157:H7; Diagnosis; Marker genes; Infectious diseases

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INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) O157:H7 is a food-borne pathogen that can cause both epidemic outbreaks and sporadic cases of diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura^[1]. In recent years, epidemic outbreaks of STEC O157:H7 occurred in the United States, Japan and other industrial countries as well as in developing nations, thus posing a serious threat to human health and economic developments^[2]. The effectiveness of current treatment remains frustratingly limited with major side effects, antibiotics-based treatment of patients with STEC O157:H7 infection increases the risk of hemolytic uremic syndrome, especially in children and seniors^[3]. The potential for large-scale outbreaks of STEC O157:H7 infection and the lack of effective treatment have inspired intensive researches on the early detection of O157:H7.

A number of methods have been developed for the detection of STEC O157:H7. Morphological analysis and serotype identification are time-consuming, laborious and not always reliable^[4]. A fast, highly sensitive and reliable technique, polymerase chain reaction (PCR) assay, has been employed to detect the specific target genes associated with STEC O157:H7^[5]. A number of virulence genes can be used in detecting STEC O157:H7, such as representative virulence gene (*eaeA*) and *stx*^[6]. However, these marker genes have unacceptably high false positive and negative rates^[7]. It is, therefore, urgently necessary to identify novel and more effective diagnostic markers for the detection of STEC O157:H7 with a high sensitivity and reliability.

One of the key reasons for the sub par performance of existing markers is that studies that identified the current markers have not fully taken the advantages of available genomic sequence data of STEC O157:H7 and hundreds of other prokaryotes with complete genomes. In this paper, we present an integrated study that combined large-scale genome sequence comparisons, sequence feature analysis and PCR-based experimental validation of marker identification, and report three marker genes based on our sequence feature analysis and experimental validation. These genes represent the promising complements to the known marker genes.

MATERIALS AND METHODS

Genome sequence data

Seven hundred and fifty completely sequenced prokaryote genomes, including 3 strains of STEC O157:H7, were downloaded from the NCBI Prokaryotic Genome Database (<ftp://ftp.ncbi.nih.gov/genomes/Bacteria/>) in January 2009.

Computational identification of candidate marker genes

NCBI blast was used to identify the candidate marker genes across any of the 750 prokaryotic genomes analyzed for each gene encoded by each of the 3 strains of STEC O157:H7 with complete genomes as previously

Table 1 Characteristics and genotypes of Shiga toxin producing *Escherichia coli* O157:H7 isolates

Isolate No.	Source	Sorbitol fermentation	<i>stx1</i>	<i>stx2</i>
1	Raw milk	Negative	+	+
2	Raw milk	Negative	+	+
3	Meat	Negative	+	+
4	Meat	Negative	+	+
5	Meat	Negative	+	+
6	Cattle	Negative	+	+
7	Human	Negative	+	+
8	Human	Negative	+	+
9	Human	Negative	+	+
10	Human	Negative	+	+
11	Cattle	Negative	+	+
12	Human	Negative	+	+
13	Human	Negative	+	+
14	Human	Negative	+	+
15	Human	Negative	+	+
16	Human	Negative	+	+
17	Human	Negative	+	+
18	Human	Negative	+	+

described^[8]. A gene of STEC O157:H7 was considered a potential marker gene if it did not have a blast hit with *E-value* < 10⁻⁵ and identity > 95%, which was identical in the 3 strains of STEC O157:H7.

Stability analysis of candidate marker genes

The following strategy was employed to predict the instability of a gene. A gene was considered stable in STEC O157:H7 if the flanking region (1500 bps on each side of the gene) had a higher sequence identity than 50% with the corresponding flanking region of its orthologous genes in the other two strains of STEC O157:H7. A stable gene should also have no transposons^[9,10] or phages^[11,12] in the flanking region. *rRNA* was closely associated with the pathogenicity islands^[13], thus genes within 3000 bps of *rRNA* genes on the safe side were excluded.

A genomic barcode scheme was developed for visualizing a genome, which demonstrated that genomic barcodes can effectively identify “abnormal” genes^[14]. A key step of the approach was to calculate the 4-*mer* frequencies of each 4-*mer* together with its reverse complement, representing these 4-*mer* frequencies as a vector of the number of combined 4-*mer* frequencies and their corresponding reverse complements, with 136 arranged in the alphabetical order. A key interesting observation was that the majority of fragments in a genome had highly similar 4-*mer* frequencies calculated throughout a genome. Sequence fragments with distinct 4-*mer* frequencies often indicate horizontal gene transfers. The distance between two vectors of 4-*mer* frequencies was expressed as the Euclidean distance between the two vectors.

Validation of predicted marker genes by PCR

Ten STEC O157:H7 isolates were obtained from the University of Maryland and 8 STEC O157:H7 isolates were obtained from the Center for Disease Control of China (Table 1). The isolates were cultured on

Table 2 Oligonucleotide primer sequences and parameters used for detection Shiga toxin producing *Escherichia coli* O157:H7

Gene	sequence (5' to 3')	Expected length (bp)	PCR condition Ta/ Te (°C)
<i>eaeA</i>	F-AAGCGACTGAGGTCACCT R-ACGCTGCTCACTAGATGT	450	55/60
<i>wzy</i>	F-GAACGATTCTTTCCGACACC R-GCGCAATTTATCGAGCTATG	276	50/60
Z0372	F-AGAATCTCATCTCGCATTT R-TCTCGCAGTTTCGCATCTTAT	342	52/60
Z0344	F-ATTGTCAGGGAATTAGCGTG R-TGCTGTAAATGTTGAACCGA	121	51/60

F: Forward; R: Reverse; Ta: Annealing temperature; Te: Elongation time; PCR: Polymerase chain reaction.

sorbitol-substituted MacConkey agar and serologically typed for O and H antigens. Shiga toxin genes (*stx1* and *stx2*) of all the isolates were detected by PCR as previously described^[15]. Five non-STEC O157 isolates were used as negative controls in PCR, namely *Escherichia coli* (*E. coli*) w3110, *E. coli* ATCC25922, *S. aureus* ATCC25923, *P. aeruginosa* ATCC27853, and *K. pneumonia* ATCC700603, which were obtained from Clinical Test Center of Ministry of Public Health, China.

PCR was carried out in a 50 µL reaction mixture containing 5 × Flexi buffer, 25 mmol/L MgCl₂, 10 mmol/L dNTP, Taq DNA polymerase (Promega, USA), 10 pmol of each primer (IDT, USA) and 3 µL bacterial lysates. PCR amplification conditions were optimized to obtain the optimal reaction parameters. The PCR amplification products were visualized by separation on a 2% agarose gel stained with ethidium bromide and by UV transillumination. The primer, annealing temperature and expected product size for each gene are listed in Table 2.

RESULTS

Candidate marker genes for Shiga toxin producing *E. coli* O157:H7

The three sequenced genomes of STEC O157:H7 were scanned with 20, 16 and 20 genes identified in STEC O157:H7 Sakai, STEC O157:H7 EDL933 and STEC O157:H7 EC4115, respectively (Table 3), and no whole-genome homology was observed in any of the 750 prokaryotic genomes as detected by blast with E-value < 5 and sequence identity > 95% presented in the 3 STEC O157:H7 genomes. Functional analyses, based on Pfam_Scan^[16] and Blast2GO^[17], indicated that most of these genes encoded the hypothetical proteins except for *wzy* (O antigen polymerase). These genes could potentially serve as markers for STEC O157:H7. Virulence marker genes, such as *eaeA*, *stx* and *nidA*, were not included in these genes, because most of them are part of (horizontally transferred) phages, plasmids and pathogenicity islands, with homologs in other prokaryotic genomes.

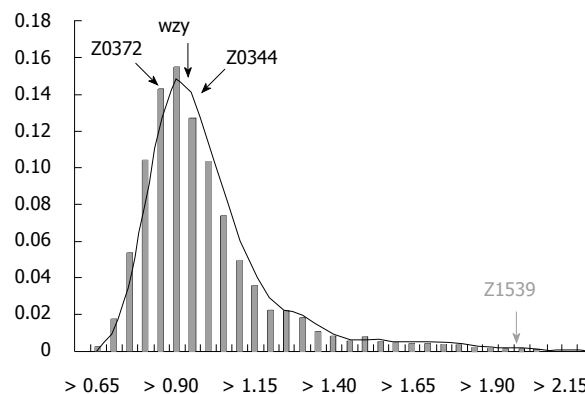


Figure 1 Distance distribution histogram for the barcode k-mer compositions of each gene compared with the average k-mer compositions of the whole genome. The x-axis is the k-mer genomic barcode distance, and the y-axis is the frequency. The bar indicates the frequency of different ranges of k-mer distance throughout the whole genome. The curve is the fitting curve to the histogram.

Assessment of instability of candidate marker genes

The instability of candidate marker genes was assessed if each predicted marker overlapped a known mobile genetic element. In addition, whether the flanking region (1500 bps on each side) of each gene is well conserved across the flanking regions of its orthologous genes in the other two strains of STEC O157:H7 was evaluated using sequence identity 50% as the cutoff, which showed that four genes, *wzy*, Z0372, Z0344 and Z1539 (Table 3), are probably not part of any mobile genetic elements.

Genomic barcode analysis

A conserved approach was taken by removing candidate marker genes with substantially different nucleotide compositions from the rest of the genome, measured with the genomic barcode. The distance distribution between the k-mer compositions of each gene in the genome and the average k-mer compositions of the whole genome is shown in Figure 1, which reveals that among the four candidate genes, Z1539 had a large k-mer composition-based distance to the average k-mer compositions of the genome, while the other three candidate genes had highly similar k-mer compositions to those of the whole genome, thus Z1539 was removed from our list of the candidate markers.

In preparation of our manuscript, the genome of a new strain of STEC (TW14359) was available with the 3 marker genes as we predicted here.

PCR validation

PCR was performed using the three predicted markers, *wzy*, Z0372, Z0344, and one STEC O157:H7 representative virulence gene (*eaeA*), on 18 STEC O157:H7 strains and 5 control organisms, including two non-O157 *E. coli* strains. The 18 STEC O157:H7 strains were detected using the three genes, with an accuracy of 100% compared to 77.8% using the existing marker *eaeA* (Figure 2). The virulence genes *stx1* and *stx2* were present in the 18 STEC

Table 3 Candidate marker genes for Shiga toxin producing *Escherichia coli* O157:H7

EDL933	Sakai	EC4115	3000 bp flanking regions				Function
			Ident %	tRNA	Tpase	phage	
<i>wzy</i>	ECs2844	ECH74115_2973	78	-	-	-	O antigen polymerase
Z0372	ECs0334	ECH74115_0348	56	-	-	-	Hypothetical protein
Z0344	ECs0307	ECH74115_0324	50	-	-	-	Hypothetical protein
Z1539	ECs1281	ECH74115_1278	50	-	-	-	Hypothetical protein
Z3621	ECs3239	ECH74115_3589	13	-	-	-	Hypothetical protein
Z3271	ECs2909	ECH74115_3086	0	-	-	-	Hypothetical protein
Z0948	ECs0804	ECH74115_0880	9	-	-	+	Hypothetical protein
Z1328	ECs1061	ECH74115_1143	32	-	-	+	Hypothetical protein
Z1430	ECs1165	ECH74115_3572	0	-	-	+	Hypothetical protein
Z3348	ECs2979	ECH74115_3543	0	+	-	+	Hypothetical protein
Z3118	ECs2755	ECH74115_2802	0	-	-	+	Hypothetical protein
Z0244	ECs0212	ECH74115_0230	38	-	+	+	Hypothetical protein
Z1153/Z1592	ECs5413	ECH74115_1331	50	-	-	-	Hypothetical protein
Z2107/Z2378/ Z6055/Z3108	ECs1960/ ECs2748	ECH74115_2190/ ECH74115_2260/ ECH74115_2792	0	+	+	+	Hypothetical protein
Z1782/Z6064	ECs2271	ECH74115_3158/ ECH74115_1841/ ECH74115_2270/ ECH74115_1520	2	-	-	+	Hypothetical protein

"Ident %" is the identity percentage of 3000-bp flanking regions among the three genomes. "+" and "-" in column "tRNA", "Tpase" and "phage" represent if the 3000-bp flanking regions contain any tRNA, transposase or phage. The gene names and their functions were retrieved from the NCBI Prokaryotic Genome Database, as described in materials and methods.

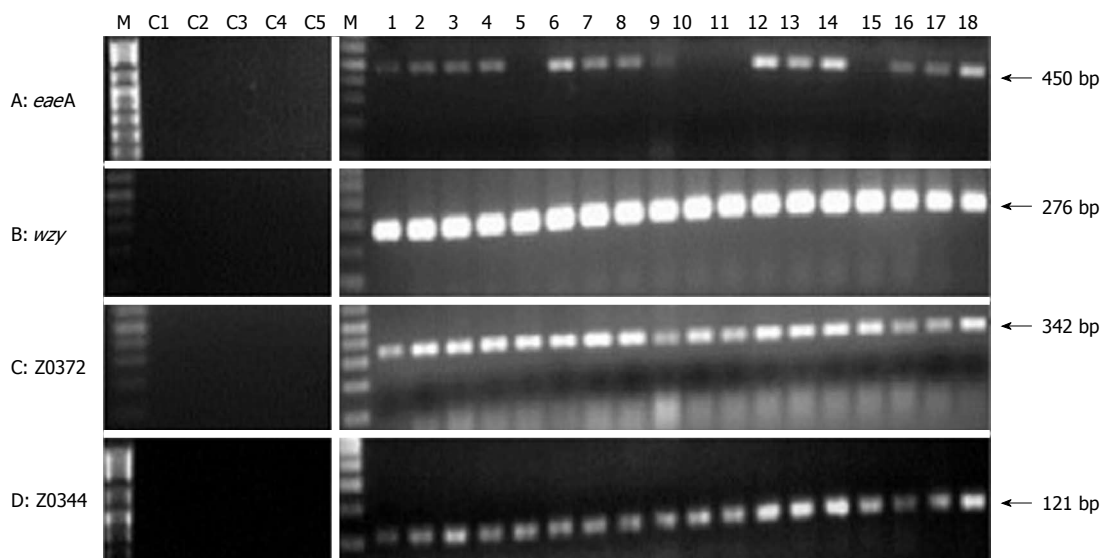


Figure 2 Diagnostic specificity of O157:H7 by one representative gene (*eaeA*) and three of our new markers. Lane M: E-Gel® 1 Kb Plus DNA Ladder (Invitrogen); Lane C1: *E. coli* W3110; Lane C2: *E. coli* ATCC25922; Lane C3: *S. aureus* ATCC25923; Lane C4: *P. aeruginosa* ATCC27853; Lane C5: *K. pneumonia* ATCC700603; Lanes 1-18: O157:H7 bacterial isolates.

O157:H7 strains. Both sets of markers did equally well without any false predictions on the control samples.

DISCUSSION

The accelerated production of microbial genome sequences provides a unique opportunity for the early diagnosis of pathogenic microbes based on comparative genomic studies. Due to the extremely complex and varied STEC genotype, the current diagnostic targets cannot meet the demand for rapid, accurate diagnosis of this pathogen. Furthermore, a number of multi-drug resistant bacteria

strains survive in the natural environment and pose a great threat to the human health, and the multi-drug resistance results from the horizontally transferable genes. So it is essential to identify these genes specific to a group of closely related pathogenic microbes, and the microbes should be early diagnosed by detecting these marker genes.

In the present study, such gene islands in STEC O157:H7 were identified with three completely sequenced strains of STEC O157:H7. Through a large-scale existence scanning of the genes in the three genomes, 20, 16 and 20 genes in the three strains were identified, respectively. Our previous study^[14] suggested that genes with barcodes significantly different to the host genomes are usually acquired,

and may easily excise the host genomes. These genes were removed from the further analysis in our study. Our computational pipeline reached 3 genes for each of the three strains. The wet laboratory PCR experiments confirmed their existence in 18 clinically retrieved STEC O157:H7 strains but not in 5 control pathogenic microbial samples. We believe that these three marker genes can complement the current detection technique of STEC O157:H7.

We are also working on the identification of marker genes for other human pathogenic microbes, especially for multi-drug resistant strains.

COMMENTS

Background

Shiga toxin producing *Escherichia coli* (STEC) O157:H7 is an important food-borne pathogen of human gastrointestinal diseases. The potential for large-scale outbreaks of STEC O157:H7 and the lack of effective treatments have inspired intensive researches on the early detection of this pathogen.

Research frontiers

Traditional morphological analysis and serotype identification for detecting STEC O157:H7 are time-consuming, laborious and not always reliable. Polymerase chain reaction (PCR) is a highly desirable method to detect specific target genes associated with O157:H7. However, existing marker genes have unacceptably high false-positive and negative rates. The advantages of available genomic sequence data of O157:H7 and hundreds of other prokaryotes with complete genomes will provide us a great opportunity to select diagnostic markers for rapid and reliable detection of STEC O157:H7.

Innovations and breakthroughs

To the best of the authors' knowledge, this is the first study to use bioinformatics approach for high-throughput screen of diagnostic markers for detection of pathogens. Furthermore, the authors combined computational biology and molecular biology to solve biological problems, which will provide us a new vision for the prevention of infectious diseases.

Applications

The authors identified and validated three novel and highly promising markers, *wzy*, Z0372 and Z0344, which may outperform the existing markers for rapid and reliable detection of STEC O157:H7 in food and patients.

Terminology

Genomic barcode is a computational technique, representing the *k-mer* nucleotide sequence frequency distributions across a whole genome as a 2-D image. In this paper, the authors used this technique to visualize the genome, showing that parts of the genome may have foreign origins.

Peer review

This manuscript is a well-planned, executed study. Although the work was not carried out in gastrointestinal tissue or cells, it is a good example of using bioinformatics approach for the prevention and management of intestinal diseases.

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Oxidative stress and hypoxia-induced factor 1 α expression in gastric ischemia

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Abstract

AIM: To investigate the relation of reactive oxygen species (ROS) to hypoxia induced factor 1 α (HIF-1 α) in gastric ischemia.

METHODS: The animal model of gastric ischemia reperfusion was established by placing an elastic rubber band on the proximal part of the bilateral lower limb for ligation for 3 h and reperfusion for 0, 1, 3, 6, 12 or 24 h. Ischemic post-conditioning, three cycles of 30-s reperfusion and 30-s femoral aortic reocclusion were conducted before reperfusion. Histological and immunohistochemical methods were used to assess the gastric oxidative damage and the expression of HIF1- α in gastric ischemia. The malondialdehyde (MDA) content and superoxide dismutase (SOD), xanthine oxidase (XOD) and myeloperoxidase (MPO) activities were determined by colorimetric assays.

RESULTS: Ischemic post-conditioning can reduce

post-ischemic oxidative stress and the expression of HIF-1 α of gastric tissue resulting from limb ischemia reperfusion injury. MDA, SOD, XOD and MPO were regarded as indexes for mucosal injuries from ROS, and ROS was found to affect the expression of HIF-1 α under gastric ischemic conditions.

CONCLUSION: ROS affects HIF-1 α expression under gastric ischemic conditions induced by limb ischemia reperfusion injury. Therefore, ROS can regulate HIF-1 α expression in gastric ischemia.

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Key words: Oxidative stress; Reactive oxygen species; HIF-1 α expression; Gastric ischemia; Limb ischemia reperfusion

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INTRODUCTION

Recent studies have suggested that limb ischemia can induce ischemia reperfusion (IR) of remote organs (lung, liver, kidney and gastrointestinal tract), and limb ischemia reperfusion-induced gastric mucosal injury can cause the occurrence of stress ulcer. Although the incidence of acute postoperative gastric ulcers decreased after the introduction of H₂ receptor-blockers and HK-pump inhibitors, there are still some problems to be solved. Oxidative stress plays a very important role through the free radicals and or reactive oxygen species (ROS)^[1] in gastric ischemia reperfusion injury, and ROS production during reperfusion may

play a role in the pathogenesis of gastric mucosal injury induced by IR. Many studies demonstrated that besides the lesions induced by ischemia, the reperfusion causes additional cellular damage not only in the primary sites but also in remote structures^[2,3], such as gastric tissues.

The main factor involved in tissue lesions in the course of ischemia is hypoxia. It initiates intracellular signaling pathways, hence leading to the activation of the hypoxia-induced factor 1 (HIF-1)^[4]. HIF-1 is a critical regulator of the transcriptional response to low-oxygen (O₂) conditions (hypoxia/anoxia) in mammalian cells under both physiological and pathophysiological circumstances^[5]. Heterodimeric protein is composed of a constitutively expressed HIF-1 β subunit, and an O₂-regulated HIF-1 α subunit. Since the clinical data first indicated that HIF-1 α may play an important role in human cancer progression in the 1999^[6], significant knowledge has been accumulated and it has played a major role in gastric tumor and ischemia through activation of various genes that are linked to regulation of angiogenesis, cell survival, energy metabolism, and apoptotic and proliferative responses^[7-9].

The mechanism of ROS in HIF-1 α expression in ischemia could be solely related to H₂O₂ initial concentrations and production. Some studies have shown increased ROS expression in hypoxia, and increased HIF-1 α expression has been found to contribute to mitochondrial activity, and especially ROS formation during hypoxia^[10,11]. However, little is known about oxidative stress and the role of HIF-1 α in rat gastric injury after limb ischemia reperfusion injury (LI-RI). We aimed to investigate the oxidative stress and HIF-1 α protein expression in gastric ischemia induced by limb ischemia reperfusion, and to test if HIF-1 α is regulated by reactive oxygen species. We used rat hind limbs ischemia as the primary lesion and the gastric mucosa as the remote site in order to find out a potential etiologic factor of acute gastric ulcers.

MATERIALS AND METHODS

Animal and reagent

Male Wistar rats weighing 220-250 g were purchased from the Animal Experimental Center, Gansu College of Traditional Chinese Medicine (Lanzhou, China). All procedures were performed in accordance with the Declaration of Helsinki of the World Medical Association. The kits used to determine superoxide dismutase (SOD), malondialdehyde (MDA), xanthine oxidase (XOD) and myeloperoxidase (MPO) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and rat HIF-1 α assay kits were purchased from Wuhan Boster Bioengineering Institute (Wuhan, China).

Model and grouping

Animals were divided into three groups randomly, and each group contained 36 rats. Group 1, (sham-operated, control), a rubber band was used without any constriction; group 2, (ischemia/reperfusion, I/R), gastric ischemia/reperfusion injury (GI-RI) was induced with bilateral lower

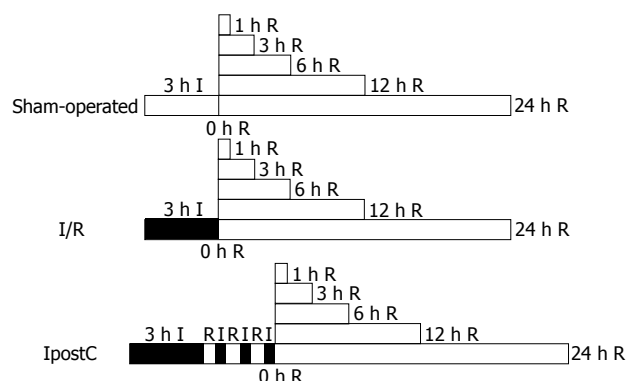


Figure 1 Experimental protocol. In the sham-operated group ($n = 36$) there was no intervention; ischemic/reperfusion (I/R, $n = 36$) was elicited by 3 h I followed by 0, 1, 3, 6, 12 or 24 h R; ischemic post-conditioning (IpostC) ($n = 36$) was performed by 3 circles of 30 s of R followed by 30 s of I before 0, 1, 3, 6, 12 or 24 h of R, respectively. R: reperfusion; I: ischemia.

limb ligated for 3 h by placing an elastic rubber band under a pressure of 290-310 mmHg on the proximal part of the lower limb^[12,13] and then released to allow reperfusion; and group 3, (ischemic post-conditioning, IpostC), at the start of reperfusion, three cycles of 30-s reperfusion and 30-s femoral aortic reocclusion^[14] were conducted before reperfusion as shown in Figure 1. Global ischemia in hind limb was verified by the absence of blood flow in femoral aorta and vein. Each group was housed in wire mesh cages at room temperature and in a 12/12 h day/night cycle. Prior to the experiment, all rats were fasted for 24 h and allowed access to tap water ad libitum. The animals were anesthetized by inhalation 2%-3% isoflurane^[15] and the anesthesia was only maintained in the course of the experimental operation. The three groups underwent 0, 1, 3, 6, 12 or 24 h reperfusion. Following reperfusion, blood samples from the inferior vena cava were collected, and six rats were humanely killed by venous bloodletting and the stomachs were immediately removed to collect tissue samples at each time point, respectively.

Measurement of malondialdehyde content and activity of superoxide dismutase, xanthine oxidase and myeloperoxidase

The stomach was homogenized in 0.9% saline solution using a homogenizer. The homogenate was then centrifuged at 2000-3000 rpm for 10 min at 4°C. The supernatant obtained was used to determine the MDA content and SOD, XOD and MPO activities according to the manufacturer's instructions. MDA content was determined spectrophotometrically at 532 nm by the thiobarbituric acid method, and was expressed in nmol/mg of protein. The protein concentrations were determined by Coomassie brilliant blue protein assay. SOD activity was evaluated spectrophotometrically at 550 nm by the xanthine oxidase method, and SOD activity was expressed in U/mg of protein. XOD was determined spectrophotometrically at 530 nm using a commercial XOD kit, and XOD activity was expressed in U/g of protein.

MPO activity was determined spectrophotometrically at 460 nm by the O-dianisidine method, and MPO activity was expressed as U/g of wet tissue. Each measurement was performed in triplicate.

Measurement of gastric mucosal injury

The murine stomach was incised along the lesser gastric curvature and fixed in 10% phosphate-buffered formalin, paraffin-embedded and sectioned at 4 μ m in thickness. After deparaffinization and gradual hydration, they were examined using hematoxylineosin staining. Based on a cumulative-length scale where an individual lesion was limited to the mucosal epithelium (including pinpoint erosions, ulcers, and hemorrhagic spots), the index was scored according to its length: 1, \leq 1 mm; 2, $>$ 1 mm and \leq 2 mm; and 3, $>$ 2 mm and \leq 3 mm. For lesions $>$ 1 mm in width, the score was doubled. The sum total of the scores of all lesions represented the gastric mucosal injury index as outlined by Zhang *et al.*^[16]. To avoid bias, the index was determined by a researcher who was blind to the treatment.

Histological examination

The stomach fixed in 10% phosphate-buffered formalin was paraffin-embedded and sectioned 4 μ m thick. After deparaffinization and gradual hydration, it was examined using hematoxylin-eosin staining. Morphologic assessment was performed by an experienced pathologist who was unaware of the treatment under a light microscope.

Immunohistochemical staining of HIF-1 α

The best tissue section for immunohistochemistry was selected and the corresponding formalin-fixed, paraffin-embedded resection specimens were obtained. Immunohistochemical detection of HIF-1 α was performed using the image pro-plus 6.0 analysis system (Media Cybernetics Co., America) based on a StreptAvidin-Biotin Complex formation. Sections 4 mm in thickness were deparaffinized and the antigen was retrieved by microwaving in 10 mmol/L citrate buffer (pH 6.0) for 20 min followed by blocking steps according to the manufacturer's protocol. Mouse monoclonal antibody (Wuhan Boster Co., China), diluted at 150-200, was applied and the slides were incubated overnight at 41 °C. The biotinylated goat anti-rat secondary antibody (Wuhan Boster Co., China), was applied using additional blocking precautions to minimize the amplification of nonspecific background. The antibody was visualized using diaminobenzidine and the sections were counterstained with haematoxylin, dehydrated and mounted. Substitution of the primary immunoadsorption with immunizing peptide served as negative control. Batch-to-batch variation was assessed by choosing two sections showing high and low HIF-1 α expressions and running additional sections from these biopsies in each batch.

Assessment of HIF-1 α staining in tissue sections

The extent of hypoxia gastric tissue staining was quantified on 24-h specimens. Digital images of the gastric tissue

overlying 3 regions of the lesser gastric curvature (mucosae, muscularis mucosae and glands) were obtained using a microscope at \times 20 magnification. The total thickness of the gastric tissue and positive staining cells were measured using Image Pro Software. In each field, 5 measurements were obtained and averaged. The HIF-1 α protein level was expressed as the sum of the integrated optical density (SUMIOD) value in different groups. HIF-1 α was also assessed by an experienced pathologist who was unaware of the treatment.

Statistical analysis

Data were entered into a database and analyzed using SPSS software (SPSS, Chicago, IL, USA) and were expressed as mean \pm SD. Data were analyzed by Repeated Measures Analysis, and the means of all groups were compared using the least significant difference (LSD) test for multiple comparisons. $P < 0.05$ was considered as significant difference.

RESULTS

Gastric oxidative stress and lipid peroxidation after gastric I/R injury

Significant elevation in MDA content and decrease in SOD activity were observed in the gastric tissue of I/R group when compared with the sham-operated group. Treatment with IpostC prevented marked elevation in MDA content and decrease in SOD activities (Figure 2A and B). XOD and MPO activities were also much higher than in the sham-operated group, whereas administration of IpostC reversed this change (Figure 2C and D).

Effects of ischemic postconditioning on pathological changes of gastric mucosa

Gastric IR resulted in significant injury as evidenced by gastric mucosal edema, gastric epithelial hemorrhage, hyperemia and erosion, and was infiltrated with inflammatory cells between the muscularis mucosa and the glands. In contrast, IpostC treatment ameliorated severe gastric damages (Figure 3A-C). According to the Yong-Mei Zhang scores, 3 h gastric ischemia followed by 6 h reperfusion resulted in severe acute gastric lesions. Quantitative analysis showed dramatically increased scores in the I/R group compared with the sham-operated group and decreased scores in the IpostC group compared with the I/R group (Figure 3D).

Expression of HIF-1 α in gastric tissue

Photomicrographs of HIF-1 α staining in the gastric tissues of all groups are shown in Figure 4A-C. The thickness of gastric specimens containing HIF-1 α -positive gastric cells was determined at a 6-h time point. HIF-1 α was seen in the glandular epithelial cytoplasm and the vascular endothelial cytoplasm or nucleus of normal gastric tissues, but expression increased in density and intensity with progression to GI-RI (Figure 4D). Compared with group I/R, HIF-1 α expression level was decreased signifi-

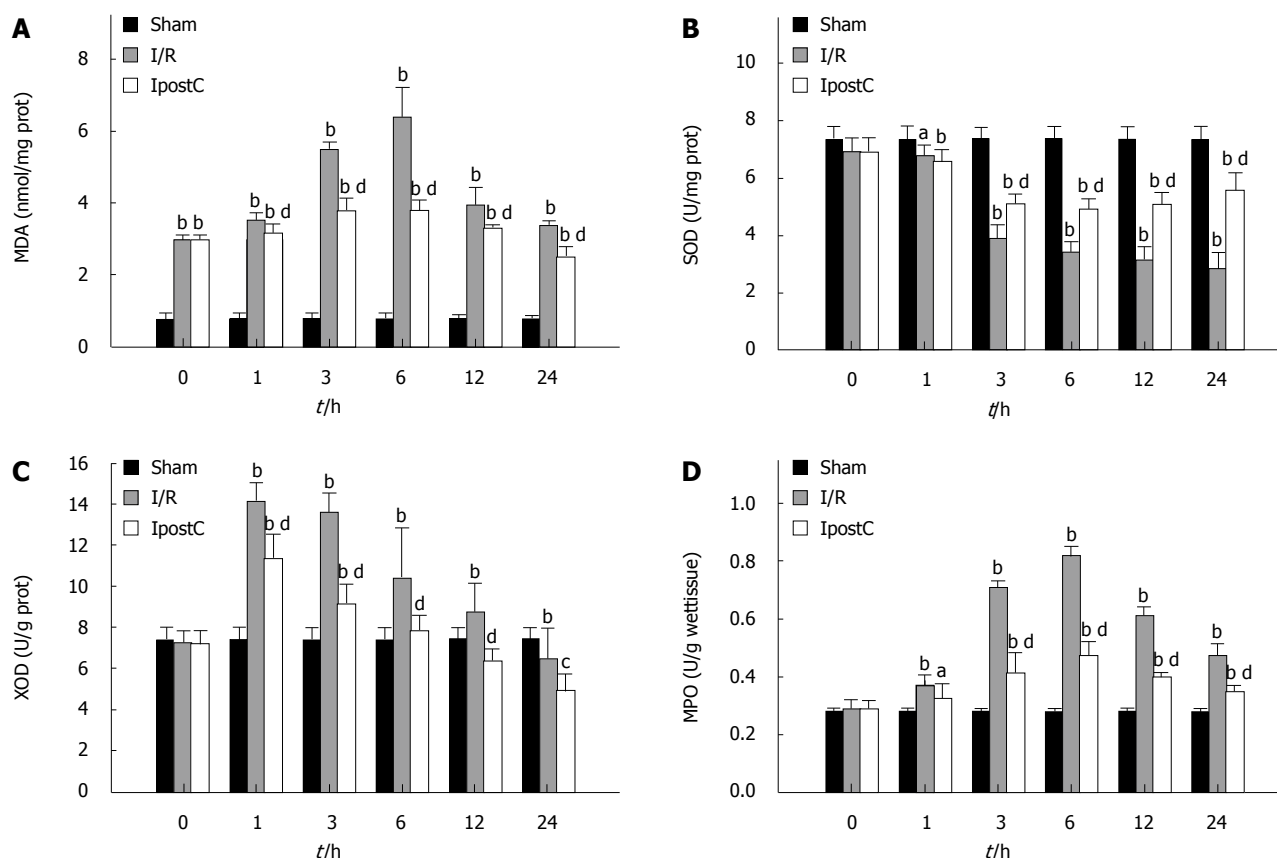


Figure 2 Gastric oxidative stress and lipid peroxidation after gastric ischemia/reperfusion injury of 3 h of ischemia and 24 h of reperfusion (mean \pm SE, $n = 36$). A: The level of malondialdehyde (MDA); B: The activity of superoxide dismutase (SOD); C: The activity of xanthine oxidase (XOD); D: The activity of myeloperoxidase (MPO). The activity of SOD decreased with the activities of XOD, MPO and the level of MDA increased in the ischemia/reperfusion (I/R) group compared with those of the sham-operated group at 1, 3, 6, 12, and 24 h. However, ischemic post-conditioning (IpostC) treatment significantly increased the activity of SOD, and decreased the activities of XOD, MPO and the level of MDA at each time. ^a $P < 0.05$, ^b $P < 0.01$ vs sham group; ^c $P < 0.05$, ^d $P < 0.01$ vs I/R group.

cantly in the glandular epithelial cytoplasm, and the vascular endothelial cytoplasm and nucleus of gastric tissues of the IpostC group (Figure 4D).

Changes of malondialdehyde content, superoxide dismutase, xanthine oxidase and myeloperoxidase activities and expression of HIF-1 α in gastric tissue

Changes of MDA content, SOD, XOD and MPO activities and expression of HIF-1 α are shown in Figures 2 and 4. MDA content, XOD and MPO activities increased, and SOD activity decreased. Consequently, the expression of HIF-1 α significantly increased in the I/R group; and when MDA content, XOD and MPO activities decreased and SOD activity increased, the expression of HIF-1 α was also decreased in the IpostC group.

DISCUSSION

The ischemia of lower extremities is one of the most common clinical problem. The ischemia reperfusion injury (IRI) of extensive muscle tissue mass and the sensitive vascular tissues and endothelium often leads to systemic complications with distant organ damage (e.g. lung, liver, gastrointestinal mucosa and kidney), and even systemic inflammatory response syndrome (SIRS) and multiple or-

gan dysfunction syndrome (MODS)^[17,18]. Therefore, limb ischemia can result in IRI of the gastric mucosa, which is an important clinical condition with undesirable outcomes concerning patients' morbidity and mortality. In recent years, studies on GI-RI have revealed that reactive oxygen species (ROS), microvascular dysfunction, polymorphonuclear leukocyte (PMN) infiltration and gastric acid secretion during reperfusion may play a role in the pathogenesis of gastric mucosal injury induced by IR. Additionally, oxidative stress, due to free radicals and/or ROS, is known to cause organ injury and plays an important role during ischemia reperfusion injury in the gastrointestinal tract^[1]. HIF-1 α is a nuclear transcription factor and is critical for initiating cellular response to hypoxia. Many studies demonstrated that there was a very close relationship between the expression of HIF-1 α and the formation of ROS in cancer and ischemia^[19]. This is the first study to investigate the relationship between the formation of ROS and the expression of HIF-1 α of gastric tissue following GI-RI. In this study, we investigated the oxidative stress and HIF-1 α expression of gastric tissue resulting from LI-RI, and found that IpostC can reduce post-ischemic oxidative stress and HIF-1 α expression of gastric tissue, and that ROS can modulate HIF-1 α expression under gastric ischemic condition.

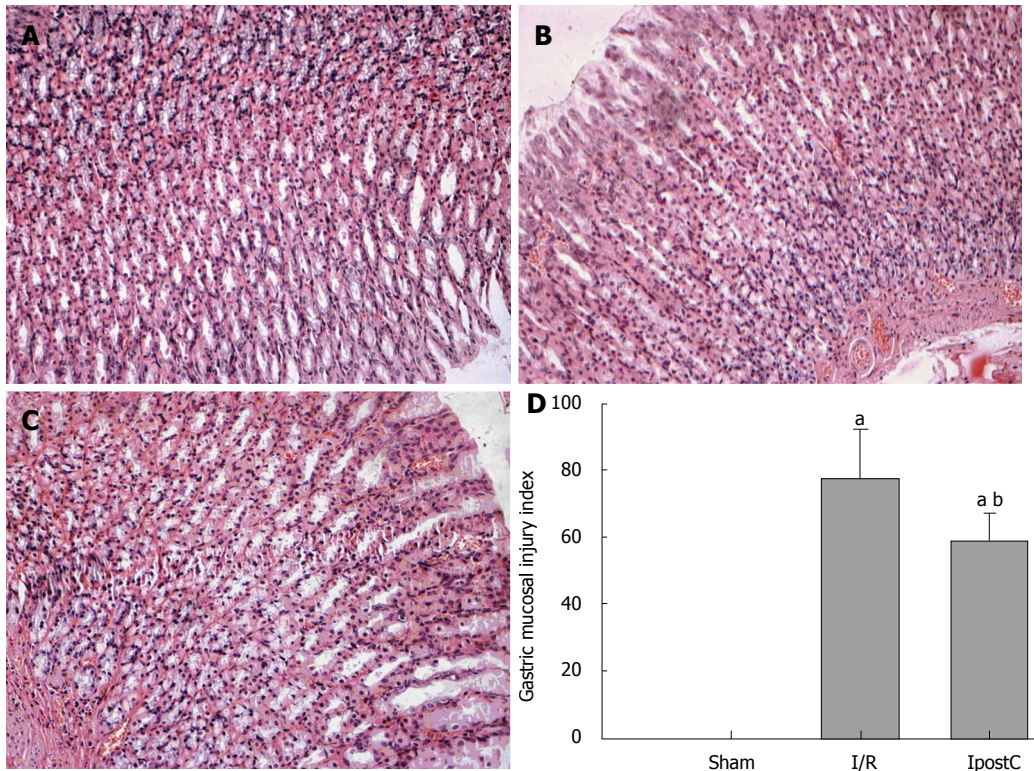


Figure 3 Histological evaluations of gastric tissue. Representative gastric sections were obtained 6 h after sham-operated surgery or ischemia/reperfusion (I/R). A: Section from sham-operated group; B: Section from I/R group; C: Section from IpostC group. All of the sections stained with hema-toxylin and eosin $\times 200$; D: Yong-Mei Zhang scores for acute gastric lesions from sham, I/R and ischemic post-conditioning (IpostC) groups (mean \pm SD; $n = 6$); ^a $P < 0.01$ vs sham-operated group; ^b $P < 0.01$ vs I/R group.

In the present study, ischemia-reperfusion (3 + 24 h) insult was sufficient to attain a considerable degree of gastric injury. IpostC prevented this deleterious effect. Some studies suggested that IpostC may reduce the post-ischemic oxidative damage through its antioxidant action^[20]. MDA and XOD are regarded as indexes for mucosal injuries from ROS. Scarcity of MDA and low activity of XOD were detected in normal mucosa. MDA is an important product of lipid peroxidation that causes cell injury and death^[21]. XOD exists in nonischemic tissue predominantly as xanthine dehydrogenase (XDH) and converts to oxygen radical-producing XOD with ischemia^[22], which is derived from XDH and is capable of generating ROS. Oxygen radicals derived from XOD are important mediators of the cellular injury associated with reperfusion of ischemic intestine, stomach, liver, kidney, and pancreas. Our study showed that gastric MDA content and XOD activity were also significantly increased, whereas administration of IpostC reversed this change. These data indicated that IpostC against GI-RI may be related to the decreased lipid peroxidation caused by oxidative stress.

The primary ROS produced in aerobic organisms is superoxide, which is a highly reactive cytotoxic agent. Superoxide is converted to H₂O₂ by SOD. H₂O₂, in turn, is converted to water and molecular oxygen by either catalase (CAT) or glutathione peroxidase (GSH-Px). Accordingly, their deficiencies can cause oxidative stress. The overproduction of oxygen-derived free radicals (OFRs) during IRI brings about a consumption and depletion of these en-

dogenous scavenging antioxidants. Concurrently, a member of endogenous antioxidant system SOD was found to be attenuated in I/R group, reflecting the over-production of OFRs. SOD is an enzyme that exists in cells removing oxyradicals, whose activity variation may represent the degree of tissue injury. In this study, compared with the I/R group, IpostC showed significantly increased antioxidase activities as well as the activities of SOD. MPO is an enzyme located mainly in the primary granules of neutrophils, thus tissue MPO levels may suggest neutrophil accumulation in the site of inflammation and generate reactive oxygen and nitrogen species and proteases^[23,24]. Polymorphonuclear neutrophil infiltration is characteristic of acute inflammation and has the collective action of chemotactic mediators. Once neutrophils migrate into the ischemic area, they release ROS, proteases, elastase, MPO, cytokines, and various other mediators, all of which are involved in tissue injury. According to our findings, MPO activity, an index of tissue neutrophil infiltration, was increased by GI-RI, whereas IpostC inhibited neutrophil infiltration and protected the tissue against further injuries. These results indicated that the protective effects of IpostC against GI-RI may be related to the improvement in the endogenous antioxidant system and anti-inflammatory action.

It is apparent that gastric tissue is vulnerable to remote organs or tissue IRI. Some studies found that generation of ROS through xanthine oxidase, lipid peroxidation and Ca⁺⁺-dyshomeostasis trigger secondary release of leukotriene (LTB4) and platelet activating factor (PAF) and pro-

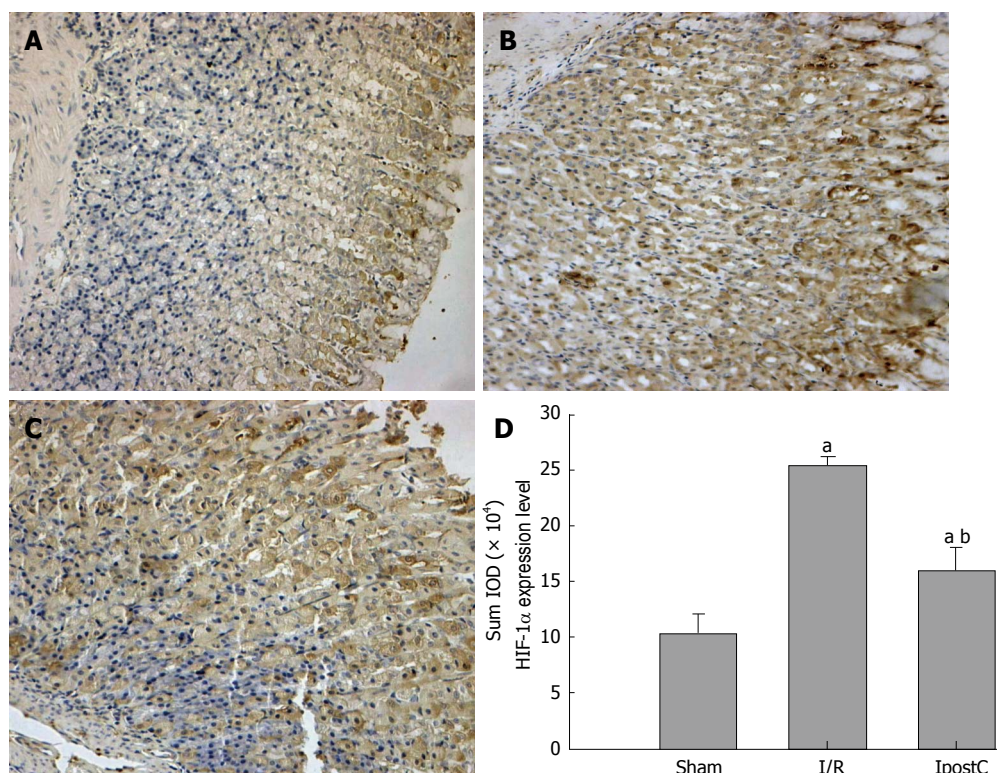


Figure 4 Photomicrographs of hypoxia-induced factor 1 α (HIF-1 α) immunohistochemistry in the gastric tissue (magnification 20 \times). The thickness of gastric containing HIF-1 α -positive gastric cells was determined at a 6-h time point. A: Weak the glandular epithelial cytoplasm and the vascular endothelial cytoplasm staining in Sham-operated group gastric; B: Strong the glandular epithelial cytoplasm, and the vascular endothelial cytoplasm and nuclear staining in ischemia/reperfusion (I/R) group gastric; C: Moderate the glandular epithelial cytoplasm, and the vascular endothelial cytoplasm and nuclear staining in ischemic post-conditioning (IpostC) group gastric; D: SUM IOD of HIF-1 α expression level in sham-operated, I/R and IpostC groups of gastric tissue (mean \pm SD; $n < 8$); ^a $P < 0.01$ vs sham-operated group; ^b $P < 0.01$ vs I/R group.

mote PMN sequestration within stomach that initiates an amplification loop via further liberation of ROS (oxidative burst) and proteolytic enzymes^[23,25]. In our study, MDA, SOD, XOD and MPO were used as indexes to mucosal injuries from ROS, and ROS was found to cause direct damage to cellular membranes as well as proteins and induce lipid peroxidation, leading to GI-RI that may be complicated by mucosal edema, microcirculation disturbance, epithelia hemorrhagic erosions and impaired function.

We also found that the expression of HIF-1 α protein significantly increased in I/R group, and when ROS decreased, the expression of HIF-1 α also decreased in IpostC group. Besides, hypoxia obviously induced the expression of HIF-1 α in the gastric epithelial cells (GECs) and the vascular endothelial cells (VECs) of gastric tissue. Thus, we suggested that ROS might contribute to the initiation and progression of gastric ischemic injury, and oxidative stress resulting from gastric oxidative damage can induce the expression of HIF-1 α in gastric ischemia induced by LI-RI.

HIF-1 α is a nuclear transcription factor that mediates adaptive responses to hypoxia in mammalian cells. It is now recognized that HIF-1 α is central to the regulation of genes involved in angiogenesis, vasomotor regulation and regulations of cell proliferation. HIF-1 α plays a critical role in limb ischemia^[26]. Some studies demonstrated that HIF-1 α can stimulate neovascularization of vessels of

ischemia tissue that respond to vascular endothelial growth factor (VEGF) and placental growth factor (PLGF)^[27], and stimulate the recovery of blood flow in operative models of hindlimb ischemia^[28]. ROS is generated from a number of sources including the mitochondrial electron transport system, xanthine oxidase, the cytochrome p450, the NADPH oxidase, uncoupled NOS and MPO, including superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical. They are closely related to the oxygen content in cells and extensive biologic activities. Exogenous ROS stimulate induction of VEGF by various cell types, and promote cell proliferation and migration, cytoskeletal reorganization and tubular morphogenesis in endothelial cells (ECs). Some studies demonstrated that ROS was also involved in physiological repair processes such as ischemia-induced angiogenesis and wound healing *in vivo*^[29,30]. Therefore, ROS also play an important role in neovascularization during limb ischemia.

According to some studies, ROS can regulate the expression of HIF-1 α via some pathways. Firstly, HIF-1 α expression is regulated in terms of not only its stability but also its transcriptional and translational activities. The extracellular signal regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB) signaling pathways are involved in the transcription and translation of HIF-1 α and can be activated by ROS. The PI3K/PKB signaling pathway phosphorylates the components that

regulate translation, and provokes the accumulation of HIF-1 α in response to growth factors, hormones, and cytokines^[31]. Secondly, HIF-1 α is a highly phosphorylated protein *in vivo* and this phosphorylation of HIF-1 α induces strong changes in the HIF-1 α 's migration pattern. Activation of the p42 and p44 mitogen-activated protein kinase (MAPK) pathway in quiescent cells induced the phosphorylation and shift of HIF-1 α , which was abrogated in presence of the MEK inhibitor. This interaction between HIF-1 α and p42/p44 MAPK suggests a cooperation between hypoxic and growth factor signals that ultimately leads to the increase in HIF-1-mediated gene expression^[32]. Some studies suggested that ROS induced HIF-1 α expression by decreasing the activity of prolyl hydroxylases (PHDs) in cancer and ischemia^[19] at high concentrations, and ROS would upregulate HIF-1 α independently and then PHD2 would be upregulated by ROS, leading to HIF-1 α down-regulation^[33]. So, ROS as a signaling molecule, can stimulate HIF-1 α protein synthesis via activation of the PI3K/AKT and p42/p44MAPK pathways, and ROS may also have the potential to interfere with prolyl hydroxylase activity to regulate HIF-1 α expression.

Some reports^[34,35] found that a variety of non-hypoxic stimuli including growth factors, hormones, vasoactive peptides and metal ions can induce HIF-1 α in normoxia, and many of these factors can stimulate ROS production as part of their signaling cascades. These indicate that ROS regulates HIF stability and transcriptional activity in well oxygenated cells, as well as under hypoxic conditions.

Our study showed that the expression of HIF-1 α protein significantly increased in gastric ischemia, and when ROS was reduced, the expression of HIF-1 α also decreased. It is shown that, at least in GECs and VECs, ROS may control the levels of HIF-1 α expression and the proliferation of GECs and VECs through regulating HIF-1 α expression. The effects of ROS on HIF-1 α can be attributed to three factors: the degree of hypoxia, the form and intracellular location of ROS produced, and the molecular microenvironment of the cell.

In conclusion, our findings suggest that LI-RI can markedly induce oxidative stress and the expression of gastric tissue HIF-1 α , and ROS controls the expression of HIF-1 α probably as a signaling molecule and has the potential to interfere with prolyl hydroxylase activity under gastric ischemic condition. Thus, ROS plays an important role in the regulation of HIF-1 α expression in gastric ischemia. The possible mechanisms of how ROS interacts with the HIF-1 pathway and alters HIF-1 α expression might be related to the activation of the PI3K/AKT, p42/p44MAPK pathways. Further understanding of these mechanisms will be undoubtedly a major contribution to the studies in the pathogenesis of GI-RI.

COMMENTS

Background

The transcription factor hypoxia-induced factor (HIF) plays a critical role in the mammalian response to oxygen (O₂) levels. HIF-1 transcriptionally activates hundreds of genes associated with angiogenesis in cancer, ischemia, as well as energy metabolism, nutrient transport, cell cycle, and cell migration. HIF-1 α

and HIF-1 β make up the HIF-1 heterodimer. The intracellular HIF-1 α rapidly accumulates in the cell nucleus and triggers gene expression under hypoxia conditions. Concomitantly, the study of reactive oxygen species (ROS) and the interest in antioxidants as potential dietary supplements for prevention of cancer, cardiac dysfunction, and neurodegeneration has grown rapidly. Oxidant stress, due to free radicals and/or reactive oxygen species, is known to cause organ injury. Although, some studies have shown that increased HIF-1 α expression contributed to mitochondrial activity, and especially the ROS formation in hypoxia. However, other studies have demonstrated a decrease of HIF-1 α with increasing ROS, and related observations seem to be conflicting.

Research frontiers

HIF-1 α is a key determinant of oxygen-dependent gene regulation in angiogenesis. HIF-1 α overexpression may be beneficial in cell therapy of hypoxia-induced pathophysiological processes, such as ischemic heart disease. Oxidative stress plays an important role in the pathogenesis of many clinical conditions involving cardiovascular diseases, liver diseases, lung disease, gastrointestinal disorders, neurological disorders, muscle damage, diabetes, aging and ischemia reperfusion (I/R). These clinical evidence focuses on the role of oxidant stress in the mechanism of I/R injury and the use of antioxidant agents for its treatment.

Innovations and breakthroughs

The authors used a rat model of hindlimb ischemic reperfusion to observe oxidative stress and the expression of HIF-1 α in the gastric injury induced by limb ischemic reperfusion. This study demonstrated that limb ischemia reperfusion injury markedly induced oxidative damage resulting from ROS and the expression of HIF-1 α of gastric tissue, and suggested that ROS controls the expression of HIF-1 α under ischemic conditions.

Applications

HIF-1 α overexpression may be beneficial in cell therapy of hypoxia-induced pathophysiological processes of many clinical conditions. The use of antioxidant agents for oxidant stress treatment in pathophysiological processes of many clinical diseases.

Peer review

The authors examined oxidative stress and HIF-1 α protein expression, and the relationship between ROS and HIF-1 α in gastric ischemia induced by limb ischemia reperfusion. It revealed that limb ischemia reperfusion injury markedly induced oxidative damage and the expression of HIF-1 α of gastric tissue, and ROS controls the expression of HIF-1 α under ischemic conditions. The paper describes a very careful biochemical and histological documentation.

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Intraductal papillary neoplasm of the bile duct in liver cirrhosis with hepatocellular carcinoma

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Abstract

A case of intraductal papillary neoplasm of the bile duct (IPNB) arising in a patient with hepatitis B-related liver cirrhosis with hepatocellular carcinoma (HCC) is reported. A 76-year-old man was admitted to our hospital with recurrent HCC. Laboratory data showed that levels of carcinoembryonic antigen and carbohydrate antigen 19-9 were elevated. He died of progressive hepatic failure. At autopsy, in addition to HCCs, an intraductal papillary proliferation of malignant cholangiocytes with fibrovascular cores was found in the dilated large bile ducts in the left lobe, and this papillary carcinoma was associated with an invasive mucinous carcinoma (invasive IPNB). Interestingly, extensive intraductal spread of the cholangiocarcinoma was found

from the reactive bile ductular level to the interlobular bile ducts and septal bile ducts and to the large bile ducts in the left lobe. Neural cell adhesion molecule, a hepatic progenitor cell marker, was detected in IPNB cells. It seems possible in this case that hepatic progenitor cells located in reactive bile ductules in liver cirrhosis may have been responsible for the development of the cholangiocarcinoma and HCC, and that the former could have spread in the intrahepatic bile ducts and eventually formed grossly visible IPNB.

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Key words: Papillary carcinoma; Bile duct neoplasms; Liver cirrhosis; Progenitor cells; Hepatocellular carcinoma; Neural cell adhesion molecules

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INTRODUCTION

Intraductal papillary neoplasms of the bile duct (IPNB) are characterized by a grossly visible, exophytic proliferation of neoplastic cholangiocytes with delicate fibrovascular cores in large bile ducts^[1-3]. IPNB not infrequently presents with mucin hypersecretion and prominent cysts in the affected bile ducts^[4,5], and is regarded as a precursor of invasive cholangiocarcinoma^[1-3]. IPNB has been reported in association with chronic biliary diseases, including hepatolithiasis,

primary sclerosing cholangitis, and parasitic biliary diseases, as well as apparently normal bile ducts, though the exact histogenesis of IPNB remains unclear.

Here, we report a case of IPNB arising in a patient with hepatitis B virus (HBV)-related liver cirrhosis associated with hepatocellular carcinoma (HCC). In this case, the malignant transformation of hepatic progenitor cells (HPCs) may be responsible for the development of IPNB and also HCC.

CASE REPORT

A 76-year-old man was admitted to hospital with recurrent HCC in the right liver lobe for transcatheter arterial chemoembolization (TACE). He had been found to have hepatitis B surface antigen 17 years ago and had suffered from HBV-related cirrhosis for nearly 10 years. HCC was found in the right lobe, and a partial hepatectomy was performed, 4 years ago. On admission, the laboratory values of tumor markers were as follows: the serum α -fetoprotein (AFP) level was within the normal range, the carcinoembryonic antigen level was 114.6 ng/mL (normal range: < 22 ng/mL) and the carbohydrate antigen 19-9 level was 52000 U/mL (normal range: < 37 U/mL). Contrast-enhanced computerized tomography revealed mild dilatation of the left intrahepatic bile duct and HCC after TACE in segment 6 of the liver. The patient died of progressive hepatic failure after hospitalization for 4 mo.

At autopsy, the liver weighed 615 g, and the majority of the liver showed cirrhotic nodules measuring less than 5 mm in diameter. Two distinct tumorous nodules (1.7 cm \times 1.4 cm and 1.2 cm \times 1.3 cm) were identified in the right lobe (Figure 1A), and these tumors showed largely coagulative necrosis compatible with HCC after TACE, while the remaining parts showed a trabecular growth pattern of HCC (Figure 1B). The rest of the liver was associated with mild to moderate necroinflammatory changes and ductular reactions.

The intrahepatic large bile ducts in the left lobe showed cystic or fusiform dilatation, and were filled with yellowish-tan papillary masses and hypersecreted mucin (Figures 1 and 2A). The affected bile duct lumen was obstructed, and the left liver was atrophic due to the obstruction. Within the left dilated intrahepatic bile ducts, the neoplastic cholangiocytes showed exophytic and papillary proliferation with delicate fibrovascular stalks (Figure 2B). The neoplastic cholangiocytes showed multilayered nuclei, an increased nucleo-cytoplasmic ratio and nuclear hyperchromasia, and these papillary tumors were diagnosed as cholangiocarcinomas. Such cholangiocarcinoma cells spread along the luminal surface of the bile ducts, with a flat and micropapillary configuration. In the subepithelial stroma, no ovarian-like mesenchymal stroma was observed. Near the large-sized bile duct with IPNB, stromal invasion of cholangiocarcinoma accompanied by mucinous carcinoma in the left lobe was observed. Mucin was positive by Alcian blue (pH 2.5) and diastase-periodic acid Schiff stains. Taken together, these carcinomas were regarded as invasive IPNB. Immunohistochemical staining revealed that cytokeratin-7 (CK7)

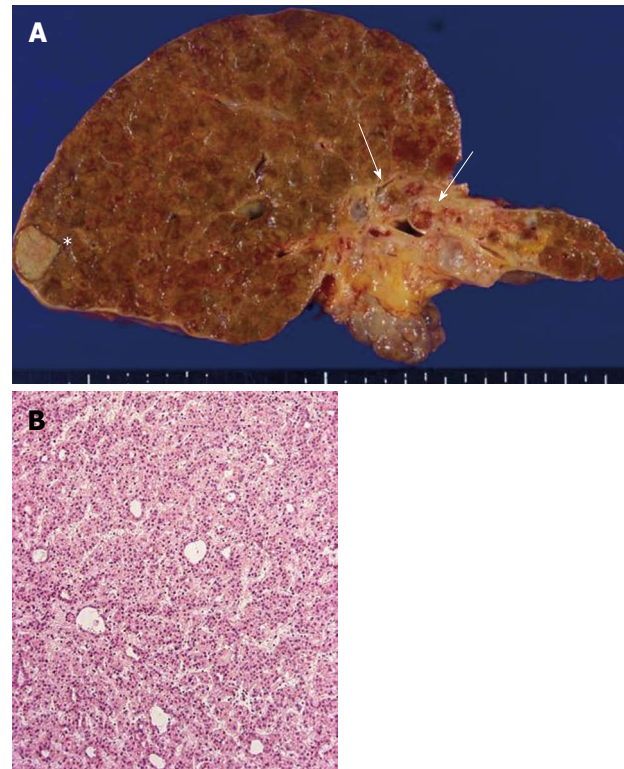


Figure 1 Liver pathology. A: The autopsied liver tissue shows cirrhosis with a solid whitish lesion (*) in segment 6 of the right lobe (hepatocellular carcinoma after transcatheter arterial chemoembolization) and cystic dilatation in the left intrahepatic bile ducts filling with yellowish-tan papillary masses and mucin production (arrows). The left liver appears atrophied; B: Well-differentiated hepatocellular carcinoma. Hematoxylin and eosin staining (H and E).

and CK19 were diffusely expressed, mucin (MUC) 5AC was moderately expressed, and CK20, CDX2, NCAM and MUC1 were focally expressed in IPNB. However, MUC2, MUC6, HepPar-1 and AFP were not observed.

Interestingly, in the left lobe including the areas near or adjacent to the left hepatic bile duct, carcinomatous cholangiocytes partially replaced the normal biliary epithelia of some septal intrahepatic bile ducts and totally replaced the epithelia of the interlobular bile ducts (Figure 3A). They showed similar mucinous and immunohistochemical profiles as seen in IPNB, and NCAM was frequently expressed in spreading neoplastic cells (Figure 3B). Interestingly, some foci of the proliferated bile ductules located in the fibrous stroma surrounding regenerative nodules showed cellular and nuclear atypia (Figure 4A) and were positive for MUC1 and focal NCAM (Figure 4B). They were considered to represent an extensive intra-bile duct spread of cholangiocarcinoma cells from reactive bile ductules, interlobular bile ducts and septal bile ducts to the large bile ducts. In contrast, the hilar bile ducts and bile ducts in the right lobe were not affected.

DISCUSSION

HCC grows occasionally in the bile duct as a tumor cast and presents clinical symptoms related to biliary obstruction^[6]. Here, we report a case of papillary cholangiocar-

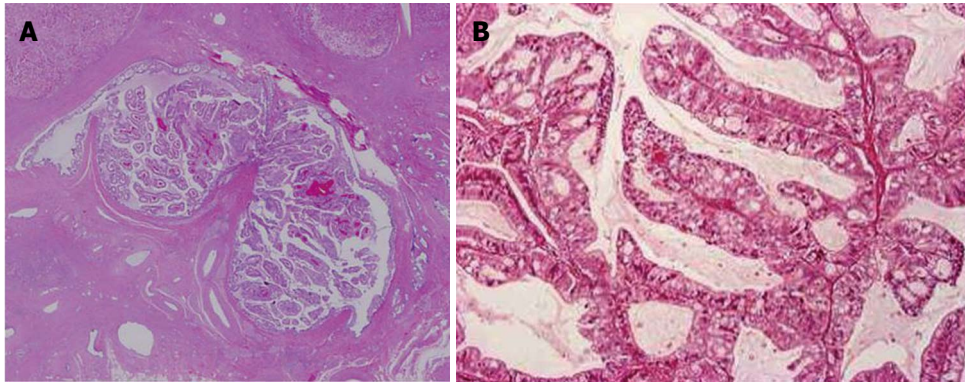


Figure 2 Histological features of the papillary tumor of the left lobe. A: Neoplastic biliary epithelia show intraductal papillary growth in the dilated bile duct lumen. Hematoxylin and eosin staining (H and E); B: The atypical biliary epithelium with a fine fibrovascular core is spreading. H and E.

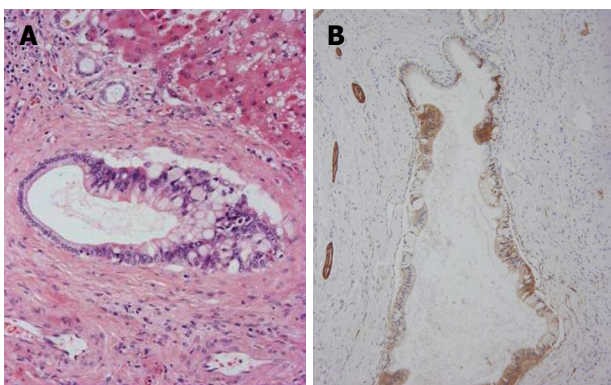


Figure 3 Carcinomatous cholangiocytes. A: Atypical biliary epithelial cells (cholangiocarcinoma) partially replace the epithelia of septal bile ducts. Hematoxylin and eosin staining (H and E); B: Neoplastic biliary epithelial cells spreading on the luminal surface of the septal bile ducts are positive for neural cell adhesion molecule (NCAM). Nerve fibers around the bile ducts are also positive. Immunostaining for NCAM and hematoxylin.

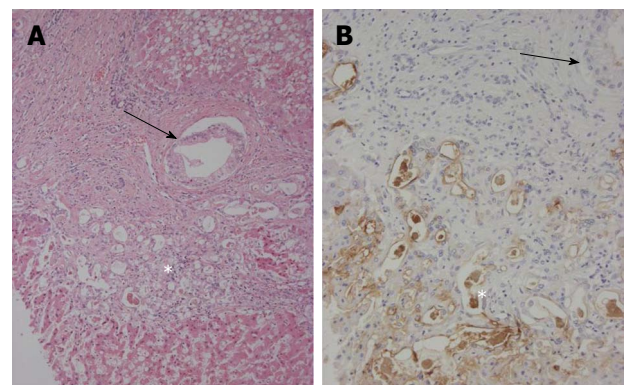


Figure 4 Bile ductular cell proliferation. A: Clusters of atypical bile ductular cells (*) are seen, and are regarded as malignant cells. An arrow shows the involvement of carcinoma cells of interlobular bile ducts. Hematoxylin and eosin staining (H and E); B: Atypical bile ductular cells (*) are positive for mucin (MUC)1. Neoplastic epithelial cells replacing interlobular bile ducts (arrow) are also focally positive. Immunostaining for MUC1 and hematoxylin.

cinoma secreting much mucin in the dilated intrahepatic large bile duct of the left lobe and showing invasion as a mucinous carcinoma. Immunohistochemically, the papillary carcinoma was strongly and diffusely positive for CK7 and CK19, and also expressed MUC5AC. CDX2, CK20, and MUC1 were also focally positive. This phenotypic profile suggests that the neoplastic cells retain a biliary phenotype (CK7 and CK19) and a gastric phenotype (MUC5AC), and intestinal metaplasia (CK20, CDX2) was also focally expressed. Carcinoma cells in the invasive area showed an apical membranous pattern for MUC1, while MUC2 (intestinal-type mucin) and MUC6 (pyloric gland-type mucin) were negative in the present case. These features are compatible with invasive IPNB. Compared with previous reports of IPNB^[1-3], the present case is unique in 2 points: (1) atypical biliary cells regarded as cholangiocarcinoma cells spread not only in the large bile ducts but also in the small bile ducts and the reactive bile ductules; and (2) IPNB was found in HBV-related liver cirrhosis associated with HCC, which was separate from the cholangiocarcinomatous components.

Papillary cholangiocarcinoma arising from extrahepatic and/or intrahepatic bile ducts occasionally shows

extensive intraductal luminal spread^[7-12]. However, the extensive intraluminal spread of atypical biliary epithelial cells extending to the reactive bile ductules found in our case, has not been reported in the literature. Recently, Ashima *et al*^[13] demonstrated 2 cases of biliary intraepithelial neoplasia (BilIN), a flat biliary epithelial neoplastic lesion, with extensive intraductal spread associated with liver cirrhosis. Rougemont *et al*^[14] also reported a case of extensive BilIN and multifocal intrahepatic cholangiocarcinoma associated with non-biliary cirrhosis. A review of the literature revealed 3 cases of biliary papillomatosis, corresponding to IPNB, associated with liver cirrhosis^[3,15-18]. However, in these cases, the involvement of interlobular bile ducts and reactive bile ductules was not shown.

It has long been controversial whether hepatic malignancies arise from stem cells or HPCs undergoing a malignant transformation^[19,20]. According to recent studies, some primary hepatic malignancies, including combined hepatocellular cholangiocarcinoma (HC-CC), originate from the transformation of HPCs^[7]. Theise *et al*^[17] demonstrated one case of biliary papillomatosis (in the left liver lobe) associated with HC-CC (in the right liver lobe)

on a background of liver cirrhosis secondary to hepatitis B, and speculated that the HC-CC was derived from neoplastic transformation of HPCs. However, they did not refer to the relationship of HC-CC with biliary papillomatosis.

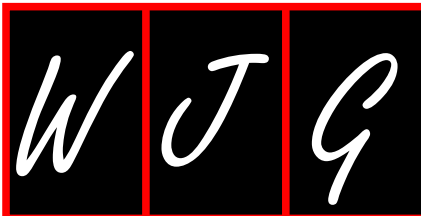
It is possible that activated HPCs located in reactive bile ductules underwent neoplastic transformation leading to hepatocellular and biliary differentiation. The former may have been followed by the development of HCC at the first step, while the latter may have been followed by superficial spread from the neoplastic foci in reactive bile ductules to the interlobular bile duct, the septal intrahepatic bile ducts, and, eventually, the left hepatic bile ducts, where the cholangiocarcinoma developed as multiple papillary growths and then an invasive mucinous adenocarcinoma. Interestingly, NCAM, a HPC marker, was also focally expressed in invasive IPNB and in the small bile ducts replaced by neoplastic cholangiocytes (Figure 3), suggesting that the cholangiocarcinoma spreading into the intrahepatic bile ducts retained a HPC phenotype.

In conclusion, a rare case of IPNB arising in a patient with liver cirrhosis associated with HCC was reported. Neoplastic transformation of bile ductules containing HPCs may have been responsible for the HCC as well as the IPNB showing extensive bile duct spread.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011
Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich,
Germany

February 4-5, 2011
13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011
2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011
International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British
Columbia, Canada

March 21-March 1, 2011
Childhood & Adolescent Obesity:
A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011
Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011
British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011
Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011
UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011
MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine:
Detection, Diagnosis and Developing
Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011
9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011
4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011
2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011
4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference

on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano
de Pediatría 'Monterrey 2011',
Monterrey, Mexico

September 2-3, 2011 Falk Symposium
178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011
ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011
Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011
19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296
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 - 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450
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- Electronic journal** (list all authors)
- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>
- Patent** (list all authors)
- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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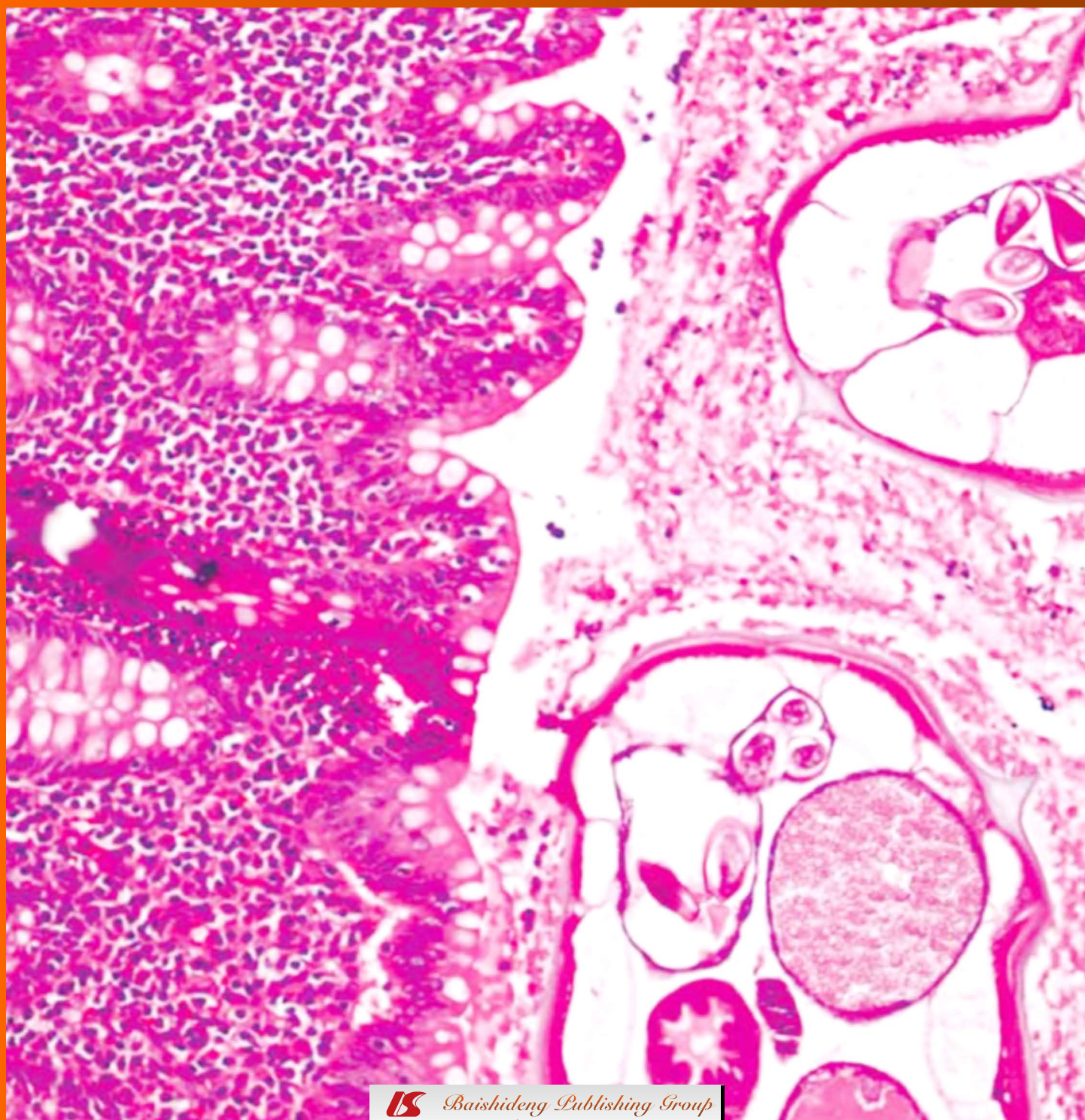
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- 1939 Perianal Crohn's disease: Is there something new?
Ruffolo C, Citton M, Scarpa M, Angriman I, Massani M, Caratozzolo E, Bassi N

ORIGINAL ARTICLE

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BRIEF ARTICLE

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Du CZ, Li J, Cai Y, Sun YS, Xue WC, Gu J
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Yuan XL, Chen L, Zhang TT, Ma YH, Zhou YL, Zhao Y, Wang WW, Dong P, Yu L, Zhang YY, Shen LS
- 2028 SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are prognosis-related in colorectal cancer
Yu SJ, Yu JK, Ge WT, Hu HG, Yuan Y, Zheng S
- 2037 DEC1 nuclear expression: A marker of differentiation grade in hepatocellular carcinoma
Shi XH, Zheng Y, Sun Q, Cui J, Liu QH, Qü F, Wang YS
- 2044 Apoptotic bone marrow CD34⁺ cells in cirrhotic patients
Dang SS, Wang WJ, Gao N, Wang SD, Li M, Liu LY, Sun MZ, Dong T
- 2049 A case-control study on the relationship between salt intake and salty taste and risk of gastric cancer
Yang WG, Chen CB, Wang ZX, Liu YP, Wen XY, Zhang SF, Sun TW

CASE REPORT

- 2054 Laparoscopic repair of hiatal hernia with mesenterioaxial volvulus of the stomach
Inaba K, Sakurai Y, Isogaki J, Komori Y, Uyama I
- 2058 Treatment of advanced rectal cancer after renal transplantation
Liu HY, Liang XB, Li YP, Feng Y, Liu DB, Wang WD

LETTERS TO THE EDITOR 2061

- Pancreatic hyperechogenicity on endoscopic ultrasound examination
Ustundag Y, Ceylan G, Hekimoglu K

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APPENDIX I Meetings
I-VI Instructions to authors

ABOUT COVER Akbulut S, Tas M, Sogutcu N, Arikanoğlu Z, Basbug M, Ulku A, Semur H, Yagmur Y. Unusual histopathological findings in appendectomy specimens: A retrospective analysis and literature review.
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An overview of occult hepatitis B virus infection

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Abstract

Occult hepatitis B virus (HBV) infection (OBI), alternatively defined as occult hepatitis B (OHB), is a challenging clinical entity. It is recognized by two main characteristics: absence of HBsAg, and low viral replication. The previous two decades have witnessed a remarkable progress in our understanding of OBI and its clinical implications. Appropriate diagnostic techniques must be adopted. Sensitive HBV DNA amplification assay is the gold standard assay for detection of OBI. Viral as well as host factors are implicated in the pathogenesis of OBI. However, published data reporting the infectivity of OBI by transfusion are limited. Several aspects including OBI transmission, infectivity and its relation to the development of chronic liver diseases and hepatocellular carcinoma have to be resolved. The aim of the present review is to highlight recent data on OBI with a focus on its virological diagnosis and clinical outcome.

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Key words: Hepatitis B virus; Occult infection; Occult hepatitis B virus infection; Occult hepatitis B; Chronic liver disease; Hepatocellular carcinoma; Hepatitis B surface antigen

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INTRODUCTION

Hepatitis B virus (HBV) remains a major public health problem worldwide^[1]. Among many transmission routes, transfusion is the one that should be prevented. Implementation of hepatitis B surface antigen (HBsAg) in routine screening of blood donors in the early 1970s has greatly enhanced transfusion safety. The incidence of transfusion-transmitted hepatitis B has been steadily reduced over the last four decades^[2]. However, it was demonstrated that HBV transmission by blood components negative for HBsAg can still occur^[3] and HBV transmission remains the most frequent transfusion-transmitted viral infection^[4-6]; thus, the term occult hepatitis B virus infection (OBI) was introduced. OBI is simply defined as serologically undetectable hepatitis B surface antigen (HBsAg-ve), despite the presence of circulating HBV DNA^[7,8]. OBI was reported for the first time almost 30 years ago in a case report of HBV infection through blood transfusion by an antibody to hepatitis B core antigen (anti-HBc) only positive donor^[9]. The residual risk of HBV transfusion transmission is mainly related to blood donations negative for HBsAg that have been collected either during the pre-seroconversion "window period" (WP), defined as the time between infection and detection of a viral antigen or antibody marker, or during the late stages of infection^[1]. Additionally, OBI has high significance in management of bone marrow and organ transplantations^[10-13]. Implementation of HBV DNA screening has the potential to significantly reduce the WP and to reveal OBI or HBV carriage^[14].

Allain^[15] reported OBI in several clinical contexts including: (1) recovery from past infection indicated by the presence of hepatitis B surface antibody (anti-HBs); (2) chronic hepatitis with surface gene escape mutants that are not recognized by current assays; (3) chronic carriage without any marker of HBV infection other than HBV DNA (referred to as “seronegative”); and (4) most commonly in endemic areas, chronic carriage stage with HBsAg too low to be detected and recognized by the presence of anti-HBc as the only serological marker (referred to as “anti-HBc alone” or “isolated anti-HBc”)^[15].

DEFINITION OF OCCULT HEPATITIS B INFECTION

Several definitions for OBI have been proposed by many authors. Bremer *et al*^[16] emphasized that the term “occult hepatitis B virus infection” has been introduced to describe a pattern with the presence of replication-competent HBV DNA in the liver but without detectable HBsAg in the serum. This often occurs after progressive disappearance of HBsAg in the years after infection^[17] and persists in low-level carriers^[14]. Early phase of HBV infection before appearance of HBsAg is not considered OBI, as the infection becomes eventually non-occult^[18].

A more specific definition was provided by Allain^[15] in 2004, who defined OBI as the presence of HBV DNA without HBsAg, with or without the presence of HBV antibodies outside the acute phase window period. This is in accordance with findings by Gerlich *et al*^[19], who identified two blood donors whose donations tested HBsAg- and HBV DNA-negative, but transmitted HBV. Both subsequently developed HBsAg and acute hepatitis. It was confirmed that such cases are transient OBI and should not be considered as true OBI. A true OBI remains HBsAg-negative during the entire course^[19]. Nevertheless, a 2008 international workshop on occult hepatitis B virus (HBV) infection (OBI), endorsed by the European Association for the Study of the Liver (EASL)^[20], as well as The Taormina Consensus Conference in 2008, defined “OBI” as the “presence of HBV DNA in the liver of individuals testing HBsAg-negative with currently available assays”^[10] and introduced a cutoff value for serum HBV DNA (< 200 IU/mL). Therefore, cases whose serum HBV DNA levels are comparable to those with different serologically evident (overt) HBV infection are generally due to infection with HBV escape mutants and should be labeled as “false” OBI^[10]. As confirmed by Hollinger *et al*^[21], this definition implies that infectious viral clones may be present. However, the detection of HBV DNA does not always correspond to infectivity or to the number of HBV progeny viruses released from hepatocytes; therefore, the authors suggested a more comprehensive term “occult hepatitis B (OHB)”^[21] rather than OBI. Moreover, nosocomial sources should be carefully excluded before speculating that blood donors with OBI were involved in HBV viral disease transmission^[22].

POSSIBLE MECHANISMS OF OBI

Several possible mechanisms have been hypothesized for the pathogenesis of OBI and the condition is probably multifactorial. Both host and viral factors are important in suppressing viral replication and keeping the infection under control^[21,23,24]. The majority of OBI cases are secondary to overt HBV infection and represent a residual low viremia level suppressed by strong immune response together with histological derangements occurring during acute or chronic HBV infection^[25]. It was previously suggested that long-term maintenance of an active anti-viral T cell response several years after clinical recovery from acute hepatitis B could be important, not only for protection against reinfection, but also for keeping the persisting virus under tight control where detection of minute amounts of virus in some recovered subjects was confirmed^[26]. Also, in a study to characterize the features of the HBV-specific T-cell response in patients with OBI, 2 different profiles were defined. Anti-HBc-positive patients showed a T-cell response typical of protective memory, suggesting that this condition represents a resolved infection with immune-mediated virus control. In contrast, HBV-specific T cells in anti-HBc-negative patients did not readily expand, suggesting the possibility of a low-dose infection insufficient to allow maturation of protective memory^[27]. Additional mechanisms not related to the host response were also extensively studied by many authors, where it was shown that the low level of viral replication was a result of the presence of defective interfering particles or of mutations in transcription control regions or the polymerase domain leading to decrease in HBV DNA replication and HBsAg expression^[21,24,28-30].

Humoral and cellular immune pressure on the HBV envelope proteins are major mechanisms generating OBI. Amino acid substitutions are significantly concentrated in the immunologically active parts of the Pre-S/S proteins affecting both cellular CD8 T-cell epitopes and B-cell neutralizing major hydrophilic region epitopes^[31]. Escape mutation is one mechanism which also leads to decreased reactivity in HBsAg detection assays^[32]. This is confirmed by Gerlich *et al*^[19]. van Hemert *et al*^[33] in 2008 proposed an evolutionary scenario for occult HBV infection. They identified a novel RNA splicing event (deleting nucleotides 2986-202) that abolishes surface protein gene expression without affecting polymerase, core or X-protein related functions. This 2986-202 splicing generates intracellular virus particles devoid of surface protein, which subsequently accumulate mutations due to relaxation of coding constraints. Such viruses are deficient in autonomous propagation and cannot leave the host cell until it is lysed^[33].

Masking of HbsAg by HbsAg-anti-HBs immune complexes is another postulated mechanism for the development of OBI^[34,35]. Also, coinfection with hepatitis delta virus or hepatitis C virus (HCV) which results in down-regulation of HBV replication and a reduction in HBsAg synthesis has been reported^[21]. Sagnelli *et al*^[36] showed an inhibitory effect of HCV on HBV replication. This inhibitory activity of HCV on HBV replication has also

been reported by other investigators in a follow-up study of 6 years duration, where it was shown that the rate of HBsAg clearance is 2.5 times higher in HBsAg/anti-HCV-positive cases than in those with HBV infection alone; it was suggested that HCV is the most important hepatotropic virus that enhances HBsAg clearance in chronic hepatitis B^[37]. The underlining molecular mechanism responsible for this suppressive effect has been extensively studied both *in vitro*^[38] and *in vivo* studies^[39]. Indirect mechanisms mediated by innate and/or adaptive host immune responses have also been postulated as being involved^[40]. In this regard, we have recently studied the prevalence of occult HBV among children and adolescents with hematological diseases with or without HCV in an area of high endemicity of HCV infection. It was shown that HCV RNA was a significant predictor for OBI ($P < 0.05$), with an increased frequency of HBV DNA in those who were HBsAg-negative and HCV RNA positive (63.2%) compared with patients negative for HCV RNA (25%) ($P = 0.009$)^[41].

Additional mechanisms for OBI have been thoroughly investigated, emphasizing that integration of viral sequence may alter HBsAg expression and decrease HBV replication^[42]. Meanwhile, reduced HBV viremia may result from extra-hepatic HBV replication such as that takes place in peripheral blood mononuclear cells (PBMCs)^[42]. Patients with long-standing abnormal results of liver function tests with unknown etiology may have HCV RNA or HBV DNA in their PBMCs in the absence of anti-HCV antibodies, HBV markers, serum HBV DNA and serum HCV RNA^[43].

EVALUATION OF DIFFERENT OBI DIAGNOSTIC TECHNIQUES

Most OBIs are asymptomatic and would only be detected by systematic screening of large populations^[7]. No published guidelines are provided up till now, categorizing those who should be screened for OBI. However, such investigations should be considered in the following situations: (1) HCV-infected patients with flares in viral replication and liver damage^[44]; (2) infected patients becoming immune deficient mainly by receiving immunosuppressive regimens for various clinical conditions^[7]; (3) screening of blood donations for immunocompromised recipients^[41]; and (4) subjects with unexplained liver diseases. Candotti *et al*^[1] further clarified that OBIs are mainly found in older donors, nearly 100% carry anti-HBc, and approximately 50% also carry anti-HBs, suggesting that OBIs occur largely in individuals having recovered from the infection but unable to develop a totally effective immune control^[31].

Liver biopsy

Detection of HBV DNA in liver biopsy is the best way for diagnosis of OBI. However, liver biopsy tissue is not always available, and standardized and valid assays for detection of HBV DNA in liver tissue are not FDA ap-

proved^[21]. A recent Italian study investigated the prevalence of occult HBV in the general population by examining 98 liver specimens from liver disease-free individuals who were HBsAg-negative, and detected HBV DNA in sixteen of them (16.3%); 10/16 (62.5%) were anti-HBc positive^[20].

HBsAg testing

The main target for antibodies used in diagnostic tests is the major hydrophilic loop (MHL, amino acids 100-160) that contains the "a" determinant (amino acids 124-147) and is coded by the envelope (S) gene. The existence of mutations in this region could cause diagnostic failure^[45]. Current HBsAg screening assays are enzyme immunoassays (EIAs), including enzyme-linked immunosorbent assays (ELISAs), and chemiluminescence immunoassays (CLIAs)^[1]. These different assays have sensitivity ranging between < 0.1 and 0.62 ng of HBsAg per mL (1 ng/mL corresponds to approximately 2 IU/mL)^[1,46,47]. Performance of commercial assays would be improved by the incorporation of OBI mutants in reagent development^[32].

The course of HBV markers during the early phase of true OBI is not well known, where, in spite of transient strong HBV replication, much less HBsAg in the serum than the normal courses is shown^[16]. This has been previously confirmed in a Japanese study by Yoshikawa *et al*^[48], where 17 million donations were tested for occult infection, and 328 HBV DNA-positive donations were found. From 26 of these donors, sequential samples were examined for the dynamics of viral markers in acute HBV infection. Six of the 26 donors were infected with mutant viruses, and 3 of these 6 donors did not develop detectable HBsAg during the entire observation period, despite a moderately high viral load of 10^4 to 10^5 HBV DNA copies per mL. The authors concluded that HBV nucleic acid amplification test (NAT), even in minipool (MP) configuration, is more effective than HBsAg testing and capable of excluding infected donors in the pre- and post-HBsAg window periods^[48].

A novel immunoassay that detects simultaneously HBV PreS1 and/or core-related antigens was developed and evaluated for its potential value for detecting HBsAg variants. The detection limits of the assay were 10 (2.9 ± 0.5) copies/mL (mean \pm SD) for HBsAg-positive sera with different genotypes, and 10 (3.5 ± 1.2) copies/mL for HBsAg variants containing sera. The specificity of the assay was 99.9% (95% CI: 99.7-99.9, 4551 healthy individuals). The sensitivities were 93.9% (95% CI: 92.8-94.9), 59.3% (95% CI: 38.7-77.6) and 80% (95% CI: 44.4-97.5) in three independent groups which included: 2065 hepatitis patients, 27 patients with OBI and 10 HBsAg variants, respectively. In addition, a novel premature stop code mutation at position 112 of HBsAg was observed in two patients with chronic hepatitis B with different genotypes^[49].

Anti-HBc testing

Serological profiling of HBV infection showed that OBI may be antibody (anti-HBc alone or together with anti-HBs) positive (seropositive OBI) or antibody negative

(seronegative OBI)^[13]. The HBV DNA detection rate is highest in subjects who are anti-HBc-positive but anti-HBs-negative, and these individuals are more likely to be infectious^[21].

Recently, Urbani *et al*^[50] illustrated that the serological assay for the long-lasting antibody response to the highly immunogenic HBV core antigen (anti-HBc) represents a qualified candidate as a surrogate for DNA amplification, or for increasing overall sensitivity when assessing the risk of occult hepatitis in peripheral blood. The risk of occult hepatitis associated with anti-HBc seropositivity has been demonstrated extensively, and the presence of antibody response to HBc can be considered a sentinel marker of occult HBV infection^[50].

In a recent review conducted by Candotti *et al*^[11] in 2009^[11], it was emphasized that approximately 90% of blood donors carrying anti-HBc also carry anti-HBs, indicating recovered HBV infection^[51]. The remaining 10% are either false-positive anti-HBc due to poor assay specificity and the lack of confirmatory assays, or true anti-HBc (anti-core antigen alone)^[1,52,53]. Anti-HBc only samples may originate either from recovered infections having lost detectable anti-HBs or from late stage chronic infections having lost detectable HBsAg^[1]. Recent studies have confirmed the existence of occult HBV infection in samples with anti-HBc alone^[54,55]. Nevertheless, low levels of HBV DNA were reported not only in anti-HBc alone positive blood donations but also in some blood units carrying low-level anti-HBs^[1]. A serologic testing algorithm with anti-HBc followed by anti-HBs (anti-HBs \geq 100 IU/L probably non-infectious) or implementation of highly sensitive HBV DNA screening are adopted in different countries; however, this is still an area of debate by many authors. In our recent study, OBI was detected in blood units from healthy volunteer blood donors showing adequate level of anti-HBs (under publication).

OBI is observed in anti-HBc-positive patients with chronic HBV infection following the decline of HBsAg to an undetectable level that is sometimes associated with the appearance of anti-HBs. This serological pattern occurs at a rate of 0.7%-1.3% per year and is associated with older age and hepatitis B e antibody (anti-HBe) reactivity^[21,56-58]. In an experimental study to determine the relationship between anticore detection and the molecular status of virus replication in a primary woodchuck hepatitis virus (WHV) surface antigen (WHsAg)-negative infection or long after resolution of WHV hepatitis, it was shown that the long-term presence of anticore antibodies alone is a consequence of sustained restimulation of the immune system by virus nucleocapsid produced during low-level hepadnaviral assembly^[59]. On the other hand, it was shown that about 20% of OHB sera are negative for all serological markers of HBV infection except HBV DNA^[21].

HBV nucleic acid (DNA) testing

The gold standard test for detection of OBI is the amplification of HBV DNA^[50]. At present, the optimal standard

for diagnosis is the analysis of HBV DNA extracts from plasma performed by real-time, nested polymerase chain reaction (PCR) techniques^[21]. False results of these assays could be avoided by choosing PCR primers that span at least three genomic regions of the HBV genome such as the S, X and core genes, and validation should require detection from at least two regions of the genome^[20]. Unfortunately, this suggestion is not usually fulfilled, and only one segment of a region is amplified. The preferred lower limit of detection (LLOD) for HBV DNA is 5 IU/mL^[21]. Some investigators prefer to repeat extraction and testing under the assumption that according to Poisson distribution, repeated testing increases the chances of detecting a low number of template sequences^[7]. Nucleic acid testing (NAT) for HBV DNA detection that combines simultaneous detection of human immunodeficiency virus (HIV) RNA, HCV RNA, and HBV DNA ("multiplex" NAT assays) and use of an automated testing platforms have made HBV NAT blood screening feasible^[1]. In order to standardize these newly developed assays, the World Health Organization International Standard for hepatitis B virus DNA (NAT)-based assays was created (code 97/750) with a potency of 10^6 IU/mL (500000 IU/vial)^[60].

Biswas *et al*^[46] showed that pooled-sample NAT would reduce the WP by 9 to 11 days; and single-sample NAT would reduce the WP by 25 to 36 d, compared to currently licensed HBsAg tests^[46]. This leaves WPs of 40-50 d and 15-34 d with minipool (MP) and individual donor (ID) HBV NAT, respectively^[1]. As emphasized by Candotti *et al*^[1], the ability of NAT to reduce the WP depends not only on the sensitivity of both the molecular and serological tests, but also on the sample volume (200 or 500 μ L) as well as the dilution factor introduced by pooling samples, the prevalent HBV genotype at the location and the level of HBV endemicity^[7,46,61-63]. Beyond shortening the WP, NAT screening, particularly in individual units, has uncovered a relatively large number of HBsAg-negative "occult" HBV infection or carriage^[11,14].

OBI is usually characterized by very low HBV DNA load in plasma (< 200 IU/mL)^[1]. Detection of OBI requires assays of the highest sensitivity and specificity with a lower limit of HBV DNA detection of less than 10 IU/mL and < 0.1 ng/mL for hepatitis B surface antigen (HBsAg)^[21].

Regarding estimation of HBV residual transfusion transmission risk, Candotti *et al*^[1] in their recent review clarified that HBV DNA yield appears directly related not only to the analytical sensitivity and serum pool size used for the HBV NAT assay, but also to the analytical sensitivity of the HBsAg test used for screening and to the general HBV prevalence in the donor population. They further added that HBV NAT yields reported from countries with low, moderate, and high HBsAg prevalence range between 1:4000 and 1:730000^[52,64-69], 1:4000 and 1:20300^[70-74], and 1:192 and 1:5200^[51,75-80], respectively^[1].

Role of Anti S

It is believed that occult HBV carriers without detectable

antibodies to the surface antigen could be infectious^[45]. Indeed, Candotti *et al*^[1] emphasized that the presence of anti-HBs following natural infection, vaccination, or passive immunoprophylaxis prevents *de novo* HBV infection in transplanted patients receiving anti-HBc positive livers^[81-84]. Experiments in chimpanzees showed no HBV infection in animals transfused with blood from three anti-HBs positive human plasma samples, despite exposure to an HBV DNA dose known to be infectious in the absence of anti-HBs^[85]. However, it has been reported by many authors that among individuals positive for anti-HBs, 0.5%-15% still tested positive for serum HBV DNA, though at a very low titer^[3,86]. Countries such as Germany, Austria and Japan allow transfusion of units with anti-HBs titers higher than 100 IU/L^[87].

CLINICAL SIGNIFICANCE

Continuous progress in molecular biology techniques has led to greater recognition and diagnosis of OBI. It has been reported in healthy blood donors, patients with chronic liver disease and patients with hepatocellular carcinoma (HCC)^[21], in viral reactivation following immunosuppression, accidental transmission through transplantation, transfusion or experimental transmission to chimpanzees^[42]. Therapy should be considered during reactivation and in cirrhotic settings^[25].

As illustrated by Shi *et al*^[88], a dynamic balance between viral replication and host immune response is pivotal to the pathogenesis of liver disease. Most HBV infections are spontaneously resolved in immunocompetent adults, whereas they become chronic in most neonates and infants who are at great risk of developing complications such as cirrhosis, chronic liver disease (CLD) and HCC. Those with chronic HBV infection may present in one of the four phases of infection: immune tolerance, immune clearance (HBeAg-positive chronic hepatitis B), inactive carrier state, and reactivation (HBeAg-negative chronic hepatitis B)^[88].

OBI is a complex biological entity with possible relevant clinical implications, mainly related to the intrahepatic persistence of viral covalently closed circular DNA (cccDNA) and to a strong suppression of viral replication and gene expression^[13]. Detection of virus-specific nucleic acid does not always translate into infectivity, and the occurrence of primer-generated HBV DNA that is of partial genomic length in immunocompetent individuals who have significant levels of anti-HBs may not be biologically relevant^[21]. Several authors concluded that as a general rule, immune individuals who have recovered from acute hepatitis B have no clinical evidence of liver disease despite the detection of traces of HBV DNA in their blood, PBMC and/or liver decades later^[20,21,23,26].

Cross-sectional studies across the spectrum of HBV infection have revealed a marked increase in OBI prevalence towards patients with cirrhosis or HCC^[25,42,89]. However, data collected in Poland indicated that approximately 50% of OBIs occur in asymptomatic, apparently healthy

blood donors carrying anti-HBs^[70]. Levels of DNA and anti-HBs are variable^[90].

OBI infectivity by transfusion

It is well known, and recently confirmed by Candotti *et al*^[1], that the estimated residual risk of HBV transfusion transmission remains significantly higher than the risk of either HIV-1 or HCV. Whether residual risk estimates translate into true rate of infection is largely unknown since estimates are generally based on the simplification that all HBV DNA-containing donations are infectious^[1].

All forms have been shown to be infectious in immunocompromised individuals, such as organ- or bone marrow-transplant recipients. In immunocompetent recipients, there is no evidence that anti-HBs-containing components (even at low titer) are infectious. Anti-HBc only, with HBV DNA, can be associated with infectivity, as can rare cases of HBV DNA without any serological HBV marker^[14].

HBV transmission was previously reported from OBI donors who had circulating HBV DNA at a low level^[74,91,92]. However, as reported by Candotti *et al*^[1], in some cases units from WP and OBI donors were not infectious even though viral load ranging between < 20 and > 500 IU/mL (< 100 and > 2500 geq/mL) was transfused^[86,91,93]. These authors emphasized that the lack of a clear relationship between infectivity and viral load in blood components may be related to immune factors affecting the susceptibility to infection in recipients. In addition, HBV infectivity is related to the amount of plasma transfused and the viral load in the product^[1].

Few data regarding the infectivity of blood components or donated organs containing both anti-HBc and anti-HBs are available. Theoretically, if HBV particles are present in the peripheral blood of subjects with high-titer anti-HBs, the anti-HBs may neutralize the infectivity of the viral particles^[3]. Nevertheless, an OBI carrier with anti-HBs was found to have transmitted HBV to two immunocompetent transfusion recipients^[90]. Gerlich *et al*^[94] reported five donors (4 genotype D, one genotype A2) with OBI, also carrying only anti-HBc, transmitting HBV to recipients. Candotti *et al*^[1] examined the infectivity of HBV-containing blood products according to the immune status of recipients and concluded that: (1) WP and anti-HBs-positive and negative OBI units can transmit HBV; (2) the confirmed HBV transmission rate of WP-derived donations is higher than by occult carriers (81% *versus* 19%) but may be biased by the large number of Japanese cases identified, with a peculiar set of anti-HBc and DNA screening protocols^[91]; (3) viral transmission can be associated with extremely low levels of HBV DNA in anti-HBc-positive only units (< 20 IU/mL) or blood collected during the very early phase of acute infection (eclipse phase) in which neither HBsAg nor HBV DNA is detectable^[86,95]; (4) HBV DNA load is similar in infectious and non-infectious anti-HBc-positive donations, suggesting that viral load is not the only factor for infectivity; and (5) the presence of anti-HBs seems to largely protect from transmission^[91,94], except in rare cases^[1,90].

No transmission of HBV has ever been demonstrated in blood donors who developed anti-HBc and anti-HBs following acute hepatitis B^[24]. Satake *et al*^[91] in Japan found that no HBV infections occurred in 22 recipients of HBsAg-negative, HBV DNA-positive blood that contained anti-HBs compared to 10 HBV infections that occurred among 37 recipients (27%) of OHB units that were devoid of anti-HBs^[21].

OBI in blood donors

It is generally admitted that pre-seroconversion WP infections are most likely to transmit HBV but transmission from occult HBV infection remains a debated subject^[11]. Occult HBV is transmissible through blood transfusion in HBV-naïve recipients^[96]. Post-transfusion hepatitis B virus (HBV) infection still occurs, although its incidence has been found to be substantially reduced since the introduction of screening for HBsAg in blood donors^[97]. A similar study was recently conducted in India and showed that a considerable number of HBV-infected donors remain undetected, if only HBsAg is used for screening^[98].

Occult HBV in blood donors has a wide range of potential origin within the natural history of the infection. It may originate from previous infections with development of anti-HBs, but be accompanied by persistent, low-level, viral replication and/or escape mutants undetected by the HBsAg assays or healthy chronic carriage. The latter situation is mostly found with anti-HBc only. Over time, antibody markers may become undetectable leaving HBV DNA as the only marker of the infection^[15].

A European study conducted by Candotti *et al*^[31] confirmed that 91% of 77 donor samples of European origin were HBV DNA-positive/HBsAg-negative. Viral load ranged between unquantifiable and 5640 IU/mL (median 25 IU/mL).

A recent study conducted in Taiwan showed that in HBV hyperendemic areas, occult hepatitis B transfusion might not lead to HBsAg carriage or post-transfusion hepatitis. The risk of transfusion-transmitted HBV infection was probably lower than that in non-endemic areas because most recipients had already experienced HBV infection^[96]. Infection of vaccinated individuals favors development of OBI, as was observed in 6 blood donors. HB vaccination may solve the problem of overt HBV infection but may favor OBI^[19].

Addition of anti-HBc testing for donor screening, although leading to rejection of a large number of donor units, will definitely eliminate HBV-infected donations and help in reducing HBV transmission with its potential consequences, especially among the immunocompromised population^[98].

OBI blood donors have very low HBV replication, and normal liver biochemistry and histology, conferring a favorable prognosis^[99].

Donations carrying anti-HBc only and HBV DNA can be infectious and this is a threat where anti-HBc is not screened. Anti-HBc screening identifies most OBI but not all. HBV NAT needs either extreme sensitivity or to be performed on individual donations to eliminate HBV

DNA-containing units^[15]. Reduction of HBV residual risk depends upon developing more sensitive HBsAg tests, adopting anti-HBc screening when appropriate, and implementing HBV NAT, either in minipools or more efficiently in individual samples^[1].

Liu *et al*^[3] emphasized that anti-HBc screening has the potential to exclude the vast majority of OHBs, leaving only the probably rare cases with HBV DNA alone undetected. This approach, however, has two main drawbacks: it does not detect the seronegative WP infections; and most importantly, it would not be practical in most parts of the world where the prevalence of anti-HBc is > 10%, as too many otherwise healthy donors will be ineligible^[3].

The transmission risk of OBIs is not well defined, although some cases of OBIs with anti-HBc only which were infectious by transfusion have been described^[91,94]. HBV transmission by blood components from a single anti-HBs-positive OBI donation to two recipients was recognized and it was clearly illustrated that the neutralizing capacity of low-level anti-HBs is limited, reinforcing the validity of considering anti-HBs below 100 IU/L to be poorly protective from infectivity when HBV DNA is present^[90]. Authors further emphasized that even in the presence of higher levels of anti-HBs in a severely immunodeficient recipient, HBV DNA-containing blood might be infectious and the clinical expression severe.

However, as emphasized by Candotti *et al*^[1], iatrogenic sources of infection should be systematically investigated before concluding that HBV-infected blood donors are involved in viral transmission^[22,100,101]. They further added that adequate donor follow-up and laboratory testing have to be performed, and more importantly, pre- and post-transfusion testing of recipients has to be completed^[1]. Definitive evidence of transfusion transmission can be obtained by genomic analysis of the viral strains present in both donor and recipient^[1]. In addition, sequencing, which might be informative, becomes very difficult to perform at levels of viremia below 200 IU/mL^[7]. Limited but convincing evidence that OBIs can be infectious and can be detected by HBV DNA screening should be carefully considered by the health authorities of countries where neither anti-HBc nor HBV NAT are implemented^[90].

Occult infection may have impact in several different clinical situations. Extensive studies have evaluated the risk of acquiring OBI in several clinical entities including the following.

OBI and chronic liver diseases

The long-lasting persistence of the virus in the liver may provoke a very mild but continuing necro-inflammation that (if other causes of liver damage coexist) may contribute over time to the progression of the chronic liver damage towards cirrhosis^[13].

In studying the situation of OBI and HCV coinfection, Hollinger *et al*^[21] reviewed several cross-sectional studies where it was suggested that HBV replication accounts for many of the ALT flares that occur in patients with HCV^[40]. OHB is also known to decrease the

response to interferon therapy when employed in patients with chronic hepatitis C^[102] and to accelerate the progression of cirrhosis, hepatic decompensation and HCC^[40,103]. A strong association was noted between the presence of OHB in 204 patients with chronic hepatitis C and the development of HCC when compared to HCV mono-infected patients^[21,104].

Chemin *et al*^[42] previously put forward the theory that estimating the percentage of OBI among non-A-E hepatitis cases depends on several parameters including: (1) the method of detection, including PCR primer selection; (2) patient recruitment; (3) patients from countries highly endemic for HBV are more likely to develop occult HBV infections; and (4) prevalence may also vary depending on the nature of biological material tested, with a higher proportion for liver compared to serum specimen^[42].

Occult hepatitis B and fulminant hepatic failure

The state of suppression of viral replication and gene expression may be discontinued when an immunosuppressive status occurs, leading to typical hepatitis B with severe - and sometimes fulminant-course^[13,105]. Gerlich *et al*^[19] studied 5 blood donors with OBI and 55 of their recipients. In 22 recipients, transmission was probable, but they remained healthy. However, in 3 recipients, who were immunosuppressed at the time of transfusion, fatal fulminant hepatitis B developed. The majority of anti-HBc-positive healthy individuals have HBV DNA in the liver which may start replication under severe immunosuppression.

Occult hepatitis B and hepatocellular carcinoma

OBI is supposed to be an important risk factor for HCC development since it maintains the pro-oncogenic properties typical of the overt infection^[13]. It has been suggested that the occult viral strains, maintaining the transcriptional activity and the pro-oncogenic assets of the clear HBV infection (HBsAg+), may harbor a potential risk for liver cancer development^[8]. A recent study conducted in Japan confirmed the existence of serum HBV DNA in OBI as a predictor of a high hepatocellular carcinogenesis rate in a cohort of patients with non-B, non-C cirrhosis. Eighty-two consecutive Japanese patients with cirrhosis, who showed negative HBsAg and negative anti-hepatitis C virus, were observed for a median of 5.8 years. The carcinogenesis rates in the patients of the positive HBV DNA group and negative DNA group were 27.0% and 11.8% at the end of the 5th year, and 100% and 17.6% at the 10th year, respectively ($P = 0.0078$)^[106]. The mechanisms leading to HCC in OBI seem similar to those in overt HBV-infected patients with low-grade but diagnosable HBV replication that retains its pro-oncogenic properties^[13,42].

Occult hepatitis B infection and immune suppression

Patients with an OBI undergoing immunosuppression are at risk of HBV reactivation. As emphasized by Allain^[7], the severity of the immunosuppression and its duration play a considerable role in triggering reactivation of HBV infection. Reactivation during relatively mild and

short immunosuppression for homologous bone marrow transplantation or solid tumor chemotherapy elicits lower frequency of reactivation than more severe regimens such as employed in allogeneic bone marrow or organ transplantation^[7]. The reactivation of OBI in hematological malignancies (< 5%), although at a lower rate than that of HBsAg-positive cases, carries a significant risk of mortality and morbidity^[107], which is much higher in the setting of stem cell transplantation^[108].

Occult HBV infection harbors potential risk of HBV transmission through hemodialysis. A recent study conducted in Italy showed that occult HBV infection is frequent among hemodialysis patients, particularly correlated to the presence of isolated anti-HBcAg and anti-HCV antibodies. The authors recommended that the presence of isolated anti-HBcAg should prompt the clinician to evaluate a possible occult HBV infection, especially if anti-HCV antibodies are also detectable^[109].

Furthermore, another recent Iranian study assessed OBI in 289 hemodialysis patients with isolated hepatitis B core antibody (18 subjects). HBV DNA was detected quantitatively in 9 of 18 patients (50%) where plasma HBV DNA load was less than 50 IU/mL^[110]. Meanwhile, a recent study conducted in Brazil found that OBI was not observed in hemodialysis patients and immunosuppression in HIV-positive patients was not a determining factor for occult HBV infection^[111].

On the other hand, Demir *et al*^[112] showed that the prevalence of occult HBV infection is higher in diabetics compared with healthy controls, which may contribute to the increased prevalence of primary HCC in diabetics^[112].

OBI and organ transplantation

OBI often leads to HBV transmission and subsequent infection during organ transplantation^[21]. In studying occult HBV infection in HBsAg-negative patients undergoing liver transplantation, Ghisetti *et al*^[113] found that OBI is not associated with increased episodes of acute rejection, coinfection with hepatotropic viruses, different responses to HBV vaccination, or the development of *de novo* hepatitis B. In OBI, a particular virus-host interaction can explain the low intrahepatic HBV content and the lack of extrahepatic HBV replication, thus justifying the low risk of hepatitis B reactivation, in absence of specific prophylaxis, once the recipient liver is removed^[113]. On the other hand, Hollinger *et al*^[21] emphasized that liver transplant recipients with serological evidence of past infection with hepatitis B (anti-HBc-positive) may have reactivation of OHB under immunosuppression in the post-transplant period^[21]. A recent systematic review by Cholongitas *et al*^[114] covering the last 15 years, identified 39 studies including 903 recipients of anti-HBc-positive liver grafts. They found that liver grafts from anti-HBc-positive donors can be safely used, preferentially in HBsAg-positive or anti-HBc/anti-HBs-positive recipients. HBsAg-negative recipients should receive prophylaxis with lamivudine, while both anti-HBc- and anti-HBs-positive recipients may need no prophylaxis at all^[114].

Transmission of HBV after kidney and heart transpla-

ntation from an anti-HBc-reactive donor occurs at a much lower rate^[21]. A study in the USA which included 1067 cadaveric kidneys, 38 of them from HBsAg(-)/HBcAb(+) donors, showed that recipients of kidneys from HBsAg(-)/HBcAb(+) donors are at a small risk of hepatitis B seroconversion and are at no excess risk of graft failure or short-term morbidity or mortality^[115]. This low viral transmission risk was also confirmed in transplantation of hearts from donors with hepatitis-B core antibodies^[116].

Currently, the critical issue in transfusion safety is to identify blood or tissue donors with OHB, and then to block this transmission route. Liu *et al*^[3] concluded that the strategy to prevent transmission of HBV by OHB carriers will be different in endemic and nonendemic areas; in low-endemic areas it is still a subject of debate whether anti-HBc screening should be implemented^[117]. Whether ID-NAT would eventually be able to replace HBsAg or anti-HBc testing also remains to be studied^[3]. On the other hand, in HBV endemic areas, the priority is to examine the prevalence of OHB in blood donors on a large scale, and so establish the cost-effectiveness of implementing sensitive ID-HBV NAT blood screening technology in order to reduce the risk of HBV transmission^[3].

PREVALENCE OF OCCULT HEPATITIS B

The prevalence of occult HBV is unclear and depends in part on the sensitivity of the HBsAg and DNA assays used as well as the prevalence of HBV infection in the study population^[117]. OHB varies significantly between different geographical regions^[11]. Studies have shown that the prevalence of occult HBV infection is closely related to the endemicity of HBV infection^[118,119]. Patients from countries highly endemic for HBV are more likely to develop occult HBV infections^[42]. As in highly endemic countries, the majority of infections are contracted perinatally or in early childhood; a higher proportion of the infected adults have late chronic HBV with undetectable HBsAg. This may account for the higher rate of OHB in anti-HBc-positive populations in these areas^[3]. Prevalence may also vary depending on the nature of biological material tested, with a higher proportion for liver compared to serum specimens^[42].

Occult HBV infection has been reported in 0.1%-2.4% of HBsAg-negative, anti-HBc-positive (\pm anti-HBs) blood donors in Western countries such as the United States, where only 5% of the population has prior exposure to HBV, and in up to 6% of a similar cohort of donors who reside in endemic areas where 70%-90% of the population has been exposed to HBV^[11,21]. When anti-HBc only data is evaluated, the rates range from 0% to 15% (median of 1.1%)^[11]. In this regard, our recent unpublished data show that OBI is present among 15% of HBsAg-negative, anti-HBc-positive (\pm anti-HBs) healthy blood donors in an area of intermediate prevalence for HBV.

CONCLUSION

OBI is defined as the presence of HBV DNA in liver/

serum with undetectable HBsAg. Advanced progress in molecular biology techniques helps in early detection of OBI and paves the way for implementing a detecting strategy to eliminate post-transfusion occult HBV infection, with consideration for the immune status of blood recipients. Evidence is accumulating supporting the prevalence of OBI among blood donors and in CLD patients. However, current data emphasize the low prevalence of OBI, implying a low impact on transfusion services. Detection of HBV DNA does not always indicate infectivity. Available data encourage testing for OBI in HCV-infected patients, in patients under immunosuppression, in people with unexplained liver diseases and in blood units for immunocompromised recipients where proper recruitment and selection of donors are highly recommended. Further work is needed to clarify the clinical significance of OBI, infectivity, possible transmission and its pathogenic consequences, reactivation and progression to chronic liver disease or hepatocellular carcinoma.

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Perianal Crohn's disease: Is there something new?

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Abstract

Perianal lesions are common in patients with Crohn's disease, and display aggressive behavior in some cases. An accurate diagnosis is necessary for the optimal management of perianal lesions. Treatment of perianal Crohn's disease includes medical and/or surgical options. Recent discoveries in the pathogenesis of this disease have led to advances in medical and surgical therapy with good results. Perianal lesions in Crohn's disease remain a challenging aspect for both gastroenterologists and surgeons and lead to a greatly impaired quality of life for all patients affected by this disease. A multidisciplinary approach is mandatory to obtain the best results.

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Key words: Crohn disease; Diagnosis; Biologic therapy; Surgery; Rectal fistula

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INTRODUCTION

Perianal lesions are common in patients with Crohn's disease (CD); these may consist of anal skin tags, hemorrhoids, anal fissures and ulcers, anorectal strictures, perianal fistulas and abscesses, rectovaginal fistulas or ultimately carcinoma^[1].

In the literature, the incidence of perianal inflammation in patients with CD ranges from 25% to 80%^[2]. Risk factors for the development of disabling disease in CD patients are an initial need for steroids, an age below 40 years, and the presence of perianal disease^[3]. Perianal lesions show a more aggressive CD phenotype, especially if perianal disease is present at the initial diagnosis^[3-5].

In approximately 10% of patients perianal fistulization is the initial manifestation, usually preceding the diagnosis by several years^[6]; less than 5% of patients have perianal disease as a unique manifestation of disease^[7]. In a population-based study of fistulizing CD the incidence of perianal fistulas was 26%^[8]. Perianal fistulizing CD should be considered as a distinct disease phenotype from luminal fistulizing disease, and it has a greater association with colonic and upper gastrointestinal rather than small bowel disease^[9].

The pathogenesis of perianal fistulas, despite the prevalence of fistulas in CD, is poorly understood. There are 2 theories: the first suggests that fistulas begin as deep penetrating ulcers, and the second that fistulas result from an anal gland abscess^[10], but it is believed that the etiology of perianal CD involves microbiological, genetic (susceptibil-

ity locus on chromosome 5) and immunological factors^[11]. This could explain the aggressive and chronic behavior of perianal lesions.

CYTOKINES

The success of antibodies towards tumor necrosis factor (TNF)- α has led to recent studies investigating other cytokines in perianal CD. In one study^[12], the serum levels of TNF- α , interleukin (IL)-12, IL-1 β , and IL-6 were analyzed in 12 patients with chronic perianal CD and a CD activity index (CDAI) score < 150 to exclude active intestinal disease, in 7 patients with indeterminate colitis (IC) after restorative proctocolectomy with perianal complications, in 7 patients with active intestinal CD without perianal manifestations, and in 19 healthy controls. Serum TNF- α levels were significantly higher in patients with IC than perianal CD patients and healthy controls. Serum TNF- α levels significantly correlated with perianal CDAI score and with the presence of anal fistulas. Serum IL-12 levels correlated with the presence of anal strictures and were similar in all groups. Serum IL-6 levels were significantly higher in the presence of perianal fistulas and lower in the presence of anal strictures. This study found that the efficacy of anti-IL-12 antibodies appeared doubtful in chronic perianal CD or IC without anal strictures while the role of IL-6 as a systemic mediator for active chronic inflammation was confirmed.

In a subsequent study^[13], the cytokine profile was assessed in the rectal mucosa of patients affected by perianal CD in order to understand its relations with the systemic cytokine profile and inflammatory parameters and the need for surgery. Seventeen patients affected by perianal CD, 7 affected by CD without perianal involvement, and 17 healthy controls were enrolled and underwent blood sampling and endoscopy. During endoscopy rectal mucosal samples were taken and the expression of TNF- α , IL-6, IL-1 β , IL-12, and transforming growth factor (TGF)-1 was quantified by enzyme-linked immunosorbent assay. Local cytokine levels were compared and correlated with diagnosis, therapy, phenotype (fistulizing and stenosing), and disease activity parameters. In the group with perianal CD, rectal mucosal IL-1 β , IL-6, and serum IL-6 and TNF- α were higher than in patients with small bowel CD and healthy controls. IL-12 and TGF-1 mucosal levels did not show any differences among the 3 groups. Mucosal IL-6 significantly correlated with the perianal disease activity index (PDAI) and mucosal TNF- α and IL-1. Mucosal TNF- α and IL-1 β showed a direct correlation with the histological grade of disease activity. Furthermore, mucosal levels of IL-6 and IL-12 seemed to be predictors of recurrence and of need for surgery in perianal CD patients.

Further prospective and randomized studies are necessary to evaluate the use of these cytokines in this complex disease.

CLASSIFICATION

In 1998, the Vienna classification categorized CD phe-

notypes, considering age at onset, location and behavior^[14], but only in the Montreal modification (2005) of this classification was perianal disease added as a subclassification of behavior; perianal fistulizing disease is not necessarily associated with intestinal fistulizing disease, and it was felt that perianal disease alone required separate subclassification^[15].

At the present time, there are different classification systems for perianal CD, but no one has achieved a widespread agreement. In 1976 Parks *et al.*^[16] proposed a classification of perianal fistulas that uses the external sphincter as a landmark, describing 5 types: inter-sphincteric, trans-sphincteric, supra-sphincteric, extra-sphincteric, and superficial. However, the value of this classification is limited because it does not consider the connection with other organs such as the bladder or the vagina. In 1978, Hughes proposed the Cardiff classification, an anatomic and pathologic classification in which each major manifestation of perianal CD (ulceration, fistula and stricture) is graded on a 2-point scale. This classification has never been globally accepted because it is considered of limited clinical relevance and difficult to use in daily practice^[17,18]. In 2003, the American Gastroenterological Association (AGA) technical review^[1] proposed an empiric approach that included: physical examination of the perianal area, endoscopic evaluation and a classification of fistulas as simple or complex: simple fistulas are low (superficial, low inter-sphincteric or low intra-sphincteric origin) with a single external opening and are not associated with perianal abscess, rectal stenosis or macroscopic proctitis and have no connection to the vagina or bladder; complex fistulas are high (high inter-sphincteric, high trans-sphincteric, supra-sphincteric or extra-sphincteric origin) and may have several external openings associated with perianal abscess, rectovaginal fistula, anorectal stenosis or macroscopic proctitis.

In 1995, Irvine described an index to evaluate perianal disease morbidity in CD patients, the PDAI, comprised of 5 categories: presence of fistula discharge, pain, restriction of daily activity, restriction of sexual activity, type of perianal disease, and degree of induration. Each category is graded on a 5-point scale, ranging from no symptoms to severe symptoms. It is widely used but it has never been compared with a reference standard^[19,20]. Another method proposed to measure perianal disease activity is the Fistula Drainage Assessment: the presence of purulent drainage from the cutaneous opening after compression is considered an index of activity, but it does not consider the morbidity of the patient and the association with an abscess^[20].

DIAGNOSIS

An accurate diagnosis is necessary for the optimal management of perianal lesions. Recently, the goal of treatment has changed from symptomatic improvement to cessation of drainage or even fistula healing. Therefore, the priority of diagnostic tools is to define the anatomy and the number of the fistulas, their complexity, and complicating features such as abscess and anal stenosis^[6].

Besides physical examination (findings of skin tags, ulcers, fissures, abscesses, fistulas or anorectal stenoses), there are several other diagnostic modalities. Endoscopic examination is important to identify macroscopic inflammation or stenosis in the rectum; furthermore AGA and the European Crohn's and Colitis Organization (ECCO) agreed on the need to complete the study of perianal disease with other diagnostic methods such as examination under anesthesia (EUA), magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS)^[21].

EUA is considered the gold standard for assessing fistulas; it has an accuracy of up to 90 for diagnosis and classification of fistulas and abscesses^[22]. At the same time, it is possible to perform several surgical procedures to treat fistulas. However, as suggested by one author, anesthesia can produce a loss of tone and could compromise precise identification of underlying muscles^[23]. MRI, an expensive modality, has an accuracy of between 76% and 100% and, combined with EUA, can obtain additional information in 15%-21% of patients; in contrast, EUS, known to be operator-dependent, has a diagnostic accuracy of between 56% and 100% and its findings can alter the surgical approach in 10%-15% of cases^[21]. When any 2 modalities are combined, the accuracy is 100%, suggesting that EUA in combination with either EUS or pelvic MRI is the best approach for evaluating and classifying perianal fistulas^[22]. The diagnostic accuracy of conventional fistulography and computed tomography (CT) does not exceed 50%-60%, which is considered too low to be clinically useful^[1]. Even though fistulography was the first technique used to assess perianal fistula, nowadays it is rarely performed because of several weak points: extensions from the primary track may fail, the sphincter muscles are not directly imaged, the levator plane cannot be visualized, and there is dissemination of septic fistula contents and discomfort for patients^[6,21]. Since CT exposes patients to not inconsiderable amounts of ionizing radiation, it may only be used for the diagnosis of fistulas associated with pelvic abscesses if other techniques are unavailable or cannot be tolerated^[23,24].

THERAPY

Treatment of perianal CD includes medical and/or surgical options. The primary aim is to heal perianal lesions, but in many cases, because of the aggressiveness of the disease, the physician's role is to relieve symptoms and treat complications of the disease to improve the patients' quality of life. The percentage of spontaneous healing for perianal fistulas is very low, ranging from 6% to 13% in the placebo arm of 2 controlled studies^[25,26].

MEDICAL THERAPY

Drugs with definite or potential efficacy for treating perianal CD include antibiotics (metronidazole and ciprofloxacin), immunosuppressors (azathioprine and 6-mercaptopurine), calcineurin inhibitors (cyclosporine and tacrolimus) and biologic agents (infliximab, adalimumab and certolizumab)^[1,6].

Antibiotics

Antibiotics are used as first-line treatment for fistula healing, and also for abscesses and infection associated with fistulas. Despite the widespread use of antibiotics for the treatment of perianal CD, there is a lack of controlled studies in the literature and usually data consist of small sample size trials^[27,28]. In these studies, the clinical response generally occurs after 6 to 8 wk, as a decreased drainage, while fistula closure is uncommon and symptoms may recur after the end of treatment. Recently, Thia *et al.*^[29] performed a randomized, double-blind, placebo-controlled trial to evaluate ciprofloxacin and metronidazole for the treatment of perianal CD, concluding that remission and response occurred more frequently in patients treated with ciprofloxacin, but the difference between the treatment arms was not significant. The limit of this study was probably the small sample size. Antibiotics are also used as a bridge to immunosuppressive therapy with azathioprine. In a prospective open-label trial, the use of metronidazole and/or ciprofloxacin at week 8 induced fistula closure in 25% of cases^[30]. At week 20, patients treated with additional azathioprine had a better mid-term response (48% *vs* 15%). Antibiotics can be also used as an adjuvant to other drugs. In a recent placebo-controlled study, all patients received infliximab and were randomized to receive either 500 mg ciprofloxacin twice daily or a placebo for 12 wk. The response at week 18 showed a better result of ciprofloxacin in combination with infliximab compared to infliximab alone^[31]. Recently, in a randomized controlled study^[32], 74 patients with perianal CD received 0.7 g 10% metronidazole ointment or placebo ointment applied perianally 3 times daily. Metronidazole ointment was not effective in reducing the perianal DCAI score, but some secondary outcomes showed improvement, suggestive of a treatment effect and it was well tolerated, with minimal adverse effects.

Immunosuppressants

Azathioprine and 6-mercaptopurine are immunosuppressive agents that, as demonstrated in the literature, successfully treat intestinal CD inflammation^[33]. A meta-analysis of 5 randomized controlled studies, in which the closure of various fistulas was considered, showed a complete closure or decreased drainage in 54% of the patients treated with azathioprine or 6-mercaptopurine compared with 21% in the placebo group^[34]. However, in this meta-analysis the fistula response was a secondary endpoint in all of the studies considered and at the moment there are no controlled trials in which fistula closure is the primary endpoint. Azathioprine or 6-mercaptopurine could be used as a second-line treatment in patients in whom immediate surgery is not mandatory, and when other pharmacological treatments have already been initiated^[6].

Cyclosporine selectively blocks T-helper and cytotoxic lymphocytes through the inhibition of the transcription of IL-2. Several uncontrolled case series reported the use of intravenous cyclosporine in perianal CD patients resistant to traditional therapy, but the initial response was rapidly lost on drug withdrawal^[35]. The effects of tacrolimus,

which has a similar mechanism, on fistulizing CD have been evaluated in a randomized, double-blind, placebo-controlled, multicenter study: 43% of the tacrolimus-treated patients had fistula improvement compared with only 8% of the placebo group; however fistula remission was comparable in the 2 groups^[36]. More studies are warranted and at the moment the use of cyclosporine and tacrolimus for treatment of fistulizing CD is not recommended^[6].

Methotrexate is used as a third-line therapeutic agent for CD patients intolerant to azathioprine and 6-mercaptopurine. No prospective studies have investigated its use for the treatment of fistulizing CD; however, in a retrospective study, 44% of patients treated with methotrexate had partial or complete fistula closure after 6 mo^[37].

Studies evaluating therapies such as sargramostim (a granulocyte-macrophage colony-stimulating factor), mycophenolate mofetil (an antimetabolite agent) and thalidomide concluded that these could be considered as potential treatments for perianal CD^[38-40].

Biologic therapy

The use of anti-TNF- α agents has changed the approach to CD, especially in patients with severe and refractory disease; in fact TNF- α is believed to play a key role in the pathogenesis of this disease^[41].

Infliximab is a murine/human chimeric monoclonal antibody directed toward soluble and membrane-bound TNF- α ^[42]. There are 2 randomized, double-blind, placebo-controlled trials that demonstrated the efficacy of infliximab in fistulizing CD^[25,43]. Present *et al*^[30] assessed infliximab induction therapy and reported that 3 infusions of infliximab, 5 or 10 mg/kg, at weeks 0, 2, and 6 resulted in complete perianal fistula closure in 46% of patients. The median length of time the fistula remained closed was 12 wk, and the response rate was higher with the 5 mg/kg dose. The ACCENT II (Adjuvant Colon Cancer End Points) study evaluated infliximab as maintenance therapy with 5 mg/kg infliximab at weeks 0, 2 and 6^[43]. Of those patients, 64% had a response to therapy at weeks 10 and 14. At week 14, responders were randomized to receive placebo or infliximab 5 mg/kg every 8 wk for 54 wk. The time to loss of response was 40 wk in the infliximab maintenance group *versus* 14 wk in the placebo group. Cessation of drainage at week 54 was maintained in 36% of the patients in the infliximab group compared with 19% of the placebo group. The regime proven to be efficacious in clinical studies comprises induction therapy with 5 mg/kg infliximab at weeks 0, 2 and 6; maintenance therapy can then be continued at 5 mg/kg every 8 wk and the dose may be increased to 10 mg/kg if loss of response is seen at the lower dose. Adverse events of infliximab include infusion reactions, an increased rate of infections, delayed hypersensitivity reactions, formation of antibodies to infliximab, formation of anti-double-stranded DNA antibodies and drug-induced lupus^[1].

Adalimumab is a fully humanized monoclonal antibody directed toward TNF- α and has proven effectiveness and efficacy in CD^[44]. Its effects have been evaluated in 2

randomized, double-blind, placebo controlled, short-term (4 wk) induction trials. In the CLASSIC-1 trial (Clinical Assessment of Adalimumab Safety and Efficacy Studied as an Induction Therapy in Crohn's Disease), adalimumab was administered at 2 different doses during weeks 0 and 2; instead in the GAIN study (Gauging Adalimumab Efficacy in Infliximab Nonresponders), adalimumab was administered at a high dose and all participants were intolerant to infliximab or had experienced loss of response during week 4 of treatment^[45]. In both studies, fistula closure was not significantly higher in patients treated with adalimumab compared with placebo. In the CHARM (Crohn's Trial of the Fully Human Antibody Adalimumab for Remission Maintenance) study, adalimumab was associated with an increased fistula closure compared with placebo. Closure of all fistulas that were draining at baseline was achieved in 30%-33% of adalimumab-treated patients compared with 13% of placebo-treated patients^[46]. CD patients, including those with a fistula, should receive an induction dose of adalimumab (160 mg in the USA and 80 mg in Europe), with a second dose (80 mg in the USA and 40 mg in Europe) during week 2; the recommended maintenance dose in both the USA and Europe is 40 mg every other week, beginning at week 4 and the dose frequency can be increased to once weekly if there is no response^[6].

Two randomized, double-blind, placebo-controlled trials that investigated the efficacy of certolizumab on fistula closure, for comparison with infliximab and adalimumab^[47], were not sufficiently powered^[48,49], and its effects require further study. Ng *et al*^[50] evaluated CD perianal fistula closure after anti-TNF- α using MRI: even though fistulas appeared clinically healed, MRI demonstrated the persistence of the fistulous tracks as already demonstrated by previous studies^[51]; so MRI fistula resolution could be useful to determine the duration of anti-TNF- α therapy.

A recent Japanese study investigated the effects of adsorptive carbon in fistulizing CD patients^[52]. Thirty-seven percent of patients treated with an oral adsorptive carbon agent (AST-120) showed an improvement compared to 10% of the placebo group; the former group also had a significantly lower rate of remission (29.6% *vs* 6.7%). Probably adsorptive carbon reverses abnormalities in the luminal environment and gut microflora. In the ECCO consensus statement antibiotics and azathioprine or 6-mercaptopurine are considered the first-line therapy in complex perianal disease, and infliximab or adalimumab are reserved as a second-line treatment in case of failure^[53]. In the AGA technical review infliximab is recommended for the treatment of complex perianal disease along with azathioprine or 6-mercaptopurine and antibiotics for the induction phase^[1]. Maintenance is recommended with azathioprine or 6-mercaptopurine, and just in some cases in association with infliximab.

Surgical therapy

In the literature the incidence of perianal CD fistulas that require surgery ranges from 25% to 30%^[54,55]. The primary goal of surgery is fistula healing and avoidance of sphinc-

ter damage. Patients with superficial or low perianal fistulas without proctitis can be treated by fistulotomy, which has reported healing rates of up to 85%^[56,57]. Surgical treatment of complex perianal fistulizing disease requires abscess drainage and usually placement of non-cutting setons^[58] before biologic therapy. Setons can be removed after 3 mo in the presence of fistula healing or can remain if the healing process has not been established. However, patients who were assessed 10 years after placement of a seton showed that complete healing was obtained in only 20% of patients^[59]. Fistulectomy or fistulotomy are rarely indicated in complex fistulas because of the high rate of subsequent proctectomy due to closure failure or incontinence caused by the transection of both anal sphincters^[53,58]. Endorectal flaps are useful when there are severe cases of high fistulas^[58,60]. An advancement flap consists of incising a flap of tissue (mucosa, submucosa, circular muscle) around the internal opening of a fistula, excising the internal opening of the fistula tract, and pulling the flap down to cover the opening^[61].

Makowiec *et al.*^[62] reported an initial healing rate of 89% in patients treated with an advancement-flap procedure, but fistulas recurred in 34% of cases during follow-up. If a second flap fails, the failure rate of subsequent flaps increases up to 75% and a temporary stoma might be necessary^[63]. In patients with severe refractory disease, fecal diversion (loop ileostomy or end colostomy) is necessary and has an early response rate of 70%-80%^[64,65]. Quality of life in symptomatic patients is rapidly improved by fecal diversion^[53]. A recent study showed that patients with complicated perianal CD, colonic involvement, and a high rate of abdominal procedures carried a significant risk for a permanent stoma; the incidence of patients requiring a permanent stoma was 31%^[66]. In another series of 86 patients with perianal CD disease, 49% of patients finally required permanent fecal diversion^[67]. In the literature, proctocolectomy is necessary in only 18% of patients^[66].

Primary closure after extended resection can be limited by scar tissue and healing can be impaired by contamination and immunosuppressive medication. Thus, myocutaneous flaps such as the gracilis and the distally based rectus abdominis muscle are used to repair perineal and vaginal defects that are too big to be closed directly.

The use of myocutaneous flaps are well described after proctectomy for cancer and there are only a few reports focusing on CD patients undergoing proctocolectomy and primary closure with myocutaneous flaps^[68-70]. Schaden *et al.*^[69] concluded that a combined proctocolectomy and a perineal single-stage myocutaneous flap closure technique can reduce recovery time, obtain complete healing and improve patients' quality of life.

The treatment of rectovaginal fistulas in CD patients remains challenging. Rectovaginal fistulas seem to be a negative prognostic indicator for successful anti-TNF- α therapy^[71]. In a study evaluating a series of 52 CD patients undergoing surgery for a rectovaginal fistula the outcome of surgery and the effect of anti-TNF therapy on healing were assessed^[72]. Fistula closure was achieved in 81% of patients. Primary and secondary surgical suc-

cess rates were 56% and 57% respectively. The primary healing rate was similar in patients who received anti-TNF treatment before the first operation (12 of 18 patients) and those who did not (19 of 34). In univariate analysis, duration of CD and previous extended colonic resection were significantly related to failure of primary surgery, but only the latter remained significant in multivariate analysis. The authors concluded that fistula closure was achieved in most patients, but more than one operation was often required.

A recent systematic review was performed including 11 observational studies with a total of 219 flap procedures for rectovaginal fistulas in CD^[73]. The pooled primary fistula closure rate was 54.2% after rectal advancement flaps and 69.4% after vaginal advancement flaps. Four studies were eligible for direct comparison between the 2 procedures. Although limited by the small number of studies at a low clinical evidence level, no significant difference in terms of outcome between rectal and vaginal advancement flaps was observed. The risk of recurrence after rectal advancement flaps compared with vaginal advancement flaps also seemed similar.

New therapies

New therapies include laser and adhesive treatment. In an uncontrolled study in perianal CD patients carbon dioxide laser ablation is considered an alternative treatment^[74]. The injection of fibrin glue into fistulas is a simple and safe procedure^[75]. The first series studies regarding this treatment reported good healing rates (52%-60%), while recent trials have not achieved the same success^[76].

Fibrin glue variants include human granulocyte colony-stimulating factor^[77] and autologous mesenchymal adult stem cells. Adult stem cells are obtained from adipose tissue with liposuction and initial studies have shown a complete response in 75% of perianal CD patients with complex fistulas^[78,79].

More recently bioprosthetic plugs, incorporating porcine intestinal submucosa, have been used in the treatment of patients with anal fistulas^[80], but in a retrospective review the use of anal fistula plugs was associated with a lower success rate (15%) than previously reported^[81]. Finally, there are other local therapies which are under development. Tacrolimus is a macrolide compound isolated from *Streptomyces tsukubaensis*. Hart *et al.*^[82], in a randomized, double-blind, placebo-controlled trial showed that, although complete healing was not observed, improvement occurred rapidly, but there was no clear clinical indication that tacrolimus was helpful for fistulizing disease. Topical tacrolimus may have a role in patients who do not respond to infliximab. Similarly, infliximab^[83,84], and more recently adalimumab^[85], injected directly into the fistula seem to result in healing in some patients resistant to systemic therapy; the rationale of this approach is to avoid systemic toxicity.

CONCLUSION

Perianal lesions in CD remain a challenge for both gas-

troenterologists and surgeons and they lead to a greatly impaired quality of life for all affected patients. A multidisciplinary approach is mandatory to obtain the best results.

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Tetracycline-inducible protein expression in pancreatic cancer cells: Effects of CapG overexpression

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RESULTS: Use of the chicken (β -actin) promoter proved superior for both the production and maintenance of doxycycline-inducible cell lines. The system proved versatile, enabling transient inducible expression of a variety of genes, including GST-P, CYP2E1, S100A6, and the actin capping protein, CapG. To determine the physiological utility of this system in pancreatic cancer cells, stable inducible CapG expressors were established. Overexpressed CapG was localised to the cytoplasm and the nuclear membrane, but was not observed in the nucleus. High CapG levels were associated with enhanced motility, but not with changes to the cell cycle, or cellular proliferation. In CapG-overexpressing cells, the levels and phosphorylation status of other actin-modulating proteins (Cofilin and Ezrin/Radixin) were not altered. However, preliminary analyses suggest that the levels of other cellular proteins, such as ornithine aminotransferase and enolase, are altered upon CapG induction.

CONCLUSION: We have generated pancreatic-cancer derived cell lines in which gene expression is fully controllable.

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Key words: Pancreatic cancer cells; Tetracycline-inducible; CapG; Suit-2; Panc-1; MiaPaCa-2

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Abstract

AIM: To establish stable tetracycline-inducible pancreatic cancer cell lines.

METHODS: Suit-2, MiaPaca-2, and Panc-1 cells were transfected with a second generation reverse tetracycline-controlled transactivator protein (rtTA2S-M2), under the control of either a *cytomegalovirus* (CMV) or a chicken β -actin promoter, and the resulting clones were characterised.

Tonack S, Patel S, Jalali M, Nedjadi T, Jenkins RE, Goldring C, Neoptolemos J, Costello E. Tetracycline-inducible protein expression in pancreatic cancer cells: Effects of CapG overexpression. *World J Gastroenterol* 2011; 17(15): 1947-1960 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i15/1947.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i15.1947>

INTRODUCTION

The ability to precisely control the levels of specific proteins in cells, either positively through overexpression, or negatively through siRNA-mediated depletion, provides an invaluable opportunity to examine their function. The most widely used technique for stringently controlling gene expression levels is the tetracycline-regulated system, originally proposed by Gossen and Bujard^[1]. Using this approach, cells are transfected with a plasmid encoding a tetracycline-controlled transactivator protein (tTA), and subsequently transfected with a plasmid carrying a gene of interest that has a tetracycline response element (TRE) in its 5' regulatory region. The addition of a tetracycline derivative, doxycycline (dox) to the cell culture medium prevents the tTA from binding to the TRE, thus inactivating the system (tet-off).

The tet system has undergone several improvements since it was first introduced. One improvement is the creation of a reverse tTA (rtTA), which can bind to the TRE in the presence of dox (Tet-on), obviating the need for the continuous presence of dox in the cell culture medium^[2-4]. Further improvements came with the introduction of the second generation rtTA, termed rtTA2s-M2^[5], which enhanced the transactivation potential and reduced the affinity for the TRE in the absence of dox. This overcame the problem of leakiness; low level gene expression in the absence of dox, sometimes observed with the earlier generation tTA^[5]. The system continues to be modified for new applications in cell lines^[6,7] and in animals^[8].

For certain cell types, such as pancreatic cancer cells, there are remarkably few reports of dox-inducible cells^[9,10]. This reflects the difficulty in obtaining such cell lines, and efficient methods for generating them are required. In this study, we attempted to derive dox-inducible pancreatic cancer cell lines, using vectors expressing rtTA2S-M2 under the control of either a viral (CMV) promoter^[5] or a chicken (β -actin) promoter^[11]. Using the resulting tet-inducible system, we went on to generate pancreatic cancer cells stably overexpressing CapG, an actin capping protein, which was previously shown to be upregulated in pancreatic ductal adenocarcinoma^[12]. CapG is activated by calcium and caps actin filaments, a function that is inhibited by membrane polyphosphoinositides^[13]. Depletion of CapG from a variety of pancreatic cancer cell lines has been associated with decreased cell motility and wound healing capacity^[12]. The dox-inducible overexpression of CapG enabled further characterisation of its role in pancreatic cancer cells, and exemplified the utility of this model system in studying the role of specific genes in pancreatic cancer cells.

MATERIALS AND METHODS

Construction of vectors

Two Tet-on plasmids were used in this study. The rtTA2S-M2^[5], which contains a viral CMV promoter driving the tet-transactivator rtTA2S-M2 (denoted here as CMV-

rtTA2S-M2), was a kind gift from Professor W Hillen, University of Heidelberg. The pN1 β actin-rtTA2S-M2-IRES-EGFP^[11], in which the CMV promoter has been replaced with a strong chicken β -actin promoter, was a kind gift from Dr. A Welman, Paterson Institute for Cancer Research, Manchester. The full-length CapG coding sequence was amplified using a forward primer 5'-AGAACGCGT-CAGCATGTACACAGCCAATTC-3', which includes an Mlu I restriction site, highlighted in bold, and a reverse 5'-AGAGCGGCCGCCACCCTCATTTCAGTCCT-3' containing a Not I restriction site, in bold. The full size CapG amplicon was inserted directionally into the pTRE-2hyg vector (Clontech, Saint-Germain-en-Laye, France) using the Mlu I and Not I restriction sites. The full-length S100A6 sequence was amplified using the following forward 5'-TCAGCCCTTGAGGGCTTCAT-3' and reverse 5'-ATGGCATGCCCCCTGGATCA-3' primers. The amplicon was ligated into the EcoRV restricted pTRE2hyg vector. Vector inserts were verified by sequencing (Eurofins MWG Operon, London, UK) and aligned using the Basic Local Alignment Search Tool (Blast). PTRE2hygGST-P, containing the full length coding sequence of human glutathione S-transferase P (GST-P), and pTRE2hygCYP2E1, containing the full-length coding sequence of cytochrome P-450 2E1, were described previously^[14].

Cell culture

Pancreatic cancer cells, Suit-2, Panc-1, and MiaPaca-2 cells were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum, 10 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich, Gillingham, UK). Growth media for cell lines stably expressing the rtTA protein was supplemented with 50 μ g/mL G418 sulphate (Invitrogen, Paisley, UK). The media for the transgenic cell lines stably transfected with the inducible CapG was supplemented with 100 μ g/mL hygromycin B (Sigma-Aldrich, Gillingham, UK). Cells were maintained at 37°C and 5% CO₂. For the induction of CapG protein expression in clones harbouring stable inducible CapG, cells were cultured for 24 h with antibiotic-free medium, and for an additional 24 h with or without 500 ng/mL dox (Sigma-Aldrich, Gillingham, UK) in RPMI-1640 media, supplemented with 10 % FBS and L-Glutamine.

Cell transfection

Cells were transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) at a 1:3 DNA: lipofectamine ratio, following the manufacturer's protocol. Briefly, cells were plated at a density of 3×10^6 per 10 cm² dish and grown for 24 h, placed in serum- and antibiotic-free medium and transfected with 5 μ g of CMV-rtTA2S-M2 or 20 μ g pN1 β actin-rtTA2S-M2-IRES-EGFP or with pTRE2hygCapG (5 μ g or 20 μ g). Cells were selected for integration of rtTA2S-M2 coding sequences by treating with G418 (300 μ g/mL; Invitrogen, Paisley, UK) for two weeks. Single colonies were separated into 96-well

plates, and positive clones identified using a luciferase assay (described below), expanded, and cryopreserved. Stable CapG-inducible cell populations were selected by incubation with hygromycin B (200 µg/mL). After two weeks, single colonies were separated into 96-well plates, cultured until confluence, and subsequently transferred to 24- and 6-well plates, respectively. Tet-on CapG-overexpressing clones were identified by Western Blotting for CapG, using dox as an inducer (see below). The transient transfection of the tetracycline responsive vectors pTRE-2hygGST-P, pTRE2hygCYP2E1, and pTRE2hygS100A6 was undertaken as described above, and 24 h later expression was induced using 500 ng/mL dox in PBS. PBS alone was used as a vehicle control. Twenty-four hours following doxycycline induction, cells were lysed, and evidence of induction of protein expression was assessed using Western blotting, as described below.

Luciferase assay

To test CMV-rtTA2S-M2 or pN1βactin-rtTA2S-M2-IRES-EGFP-transfected clones for dox inducibility, cells were seeded into 96-well plates and transfected with 50 ng pTRE2hygluc vector (Clontech, Saint-Germain-en-Laye, France) using a 1:3 DNA: Lipofectamine ratio. Twenty-four hours later, clones were treated with 500 ng/mL dox in PBS or with PBS only as a control and incubated for 24 h. Cells were harvested in 50 µL 1 × Glo Lysis buffer (Promega, Southampton, UK) and assayed for luciferase activity according to the manufacturer's instructions, using a plate reader (PerkinElmer, Bucks, UK). Each clone was assayed in triplicate and experiments were repeated at least three times. Data were analysed statistically using Student's two-tailed paired *t*-test.

Western blotting

Cells were lysed in SDS lysis buffer (0.1 mol/L Tris/HCl pH 6.8, 2% SDS) containing protease inhibitor (Roche Diagnostics Ltd., Burgess Hill, UK). For separation into cytoplasmic and nuclear fractionations, the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL, USA) were used. For analysis of phospho-proteins, cells were lysed in RIPA buffer, supplemented with protease and phosphatase inhibitor (Roche Diagnostics Ltd., Burgess Hill, UK). Protein concentrations were determined using a Bradford assay (Bio-Rad, Hemel Hempstead, UK). For Western blotting, membranes were blocked with 5% milk (Bio-Rad, Hemel Hempstead, UK) in PBS containing 0.1% Tween 20 (PBST) for 1 h at RT. As primary antibodies, polyclonal chicken anti-CapG (1:7000, GenWay Biotech, San Diego, USA), monoclonal mouse anti-β-actin (1:200000, Sigma-Aldrich, Gillingham, UK), rabbit anti-GAPDH (1:400, Santa Cruz Biotechnology, Heidelberg, Germany), monoclonal mouse anti-α-tubulin (1:3000; Sigma Aldrich, Gillingham, UK), monoclonal mouse anti-GST-P (1:2500; Sigma Aldrich, Gillingham, UK), rabbit anti-CYP2E1 (1:2500; Sigma Aldrich, Gillingham, UK) and rabbit anti-S100A6 polyclonal

antibody (1:3000; DAKO UK Ltd., Ely, UK) were used. The primary antibodies were incubated overnight at 4°C with gentle shaking. As secondary antibodies, goat anti rabbit HRP (1:3000, Dako UK Ltd., Ely, UK), goat anti mouse HRP (1:3000, Dako UK Ltd., Ely, UK), or goat anti chicken HRP (1:14000, GenWay Biotech, San Diego, USA) were used. For Cofilin and Ezrin the Actin-Reorganization Kit (Cell Signalling Technology Inc., Danvers, Maryland, USA) was used according to the manufacturer's protocol. Bound horse radish peroxidase was detected using enhanced chemiluminescence western lightning reagent (Perkin-Elmer Life Sciences, Waltham, USA). For quantification purposes, X-ray films were scanned using a GS-800- densitometry scanner (Bio-Rad, Hemel Hempstead, UK) and evaluated using the Quantity One software (Bio-Rad, Hemel Hempstead, UK), or directly developed using a Kodak Gel Logic 1500 imaging station and analysed using Kodak Molecular Imaging software (Carestream Health UK, Hemel Hempstead, UK).

Two-dimensional electrophoresis

The dox-inducible CapG-expressing clones, Sβtet29Cap35, Sβtet29Cap43, and negative control Sβtet29 were incubated for 18 h with or without 500 ng/mL dox and solubilised in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate, 40 mmol/L Tris base and 1% dithiothreitol) by sonication. Extracted proteins (200 µg) were focused on pH 3-10 non-linear strips, 18 cm in length, as described^[15], and then separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels. Gels were stained with colloidal Coomassie Blue. Gel images were scanned using a GS-800 scanner, acquired using PDQuest software (Bio-Rad, Hemel Hempstead, Herts, UK) and analysed using Progenesis Workstation software V2002.1 (NonLinear Dynamics, Newcastle, UK).

MTS proliferation assay

The proliferation of Sβtet29Cap35, Sβtet29Cap43 and control Sβtet29 clones was determined by MTS assay (EZ4U nonradioactive cell proliferation and cytotoxicity assay, Fa. Biomedica, Vienna, Austria) following the manufacturer's protocol. This assay is based on the reduction of the nontoxic yellow tetrazolium salt (450 nm) to an intensively coloured red formazan derivate (620 nm). Reduction requires functional mitochondria, an indicator of cell viability and cell proliferation. Cells (*n* = 3000) were plated in 100 µL antibiotic free RPMI medium onto 96-well plates. Dox was added at a final concentration of 0 or 500 ng/mL. After 48 h, 100 µL substrate was added to each well, and cells incubated before absorbance readings were measured at 0, 1, 2, 3, and 4 h at 450 and 620 nm in a Multiskan EX plate reader (Thermo Scientific, Basingstoke, UK). Within each experiment, each condition was plated in twelve replicates and experiments were repeated on three independent occasions. Data were analysed statistically using Student's two-tailed paired *t*-test.

Cell cycle analysis

For cell cycle analysis, cells were plated in triplicate 12 h prior to treatment with or without 500 ng/mL doxycycline for an additional 24 h or 72 h. Cells were harvested, washed twice with ice-cold PBS, and fixed with ice-cold 70% ethanol for 1 h. Cells were centrifuged at 1000 r/min for 4 min, and cell pellets washed twice with PBST. Cell pellets were resuspended in PBS containing 100 μ L RNase A (10 mg/mL) and incubated for 5 min at 4°C. Cells were then stained with 900 μ L of a PBS solution containing propidium iodide (55 μ g/mL) and 10 000 cells analysed by flow cytometry.

In vitro wound healing assay

S β tet29Cap35, S β tet29Cap43, and, as the control, S β tet29 cells were plated into 12-well plates at 5×10^6 cells per well and incubated for 24 h with or without 500 ng/mL doxycycline. Wounds were made using a P200 plastic pipette tip and photographed ($t = 0$ h) before being returned to 37°C. Wounds were photographed again eight hours later. The distance migrated by the cell monolayer was measured and the results expressed as a migration index (i.e. the distance migrated by the experimental condition, e.g. dox stimulated CapG inducible cells, divided by the distance migrated by the control treated cells, e.g. dox stimulated control cells). Experiments were performed in triplicate and repeated on three independent occasions. Statistical significance between the different conditions was determined using Student's paired t -test, with significance set at $P \leq 0.05$.

In vitro motility assay

Motility assays were performed using 24-well cell culture inserts (modified Boyden chambers, BD Biosciences, Oxford, UK) with 8 μ m pore size. CapG-inducible S β tet29Cap35, S β tet29Cap43, and, as the control, S β tet29 cells were plated in T25 flasks in RPMI media supplemented with 10% FBS and incubated with or without 500 ng/mL dox for 24 h. Cells were counted and 5×10^4 cells were plated into 24-well Boyden chamber inserts in 500 μ L serum-free RPMI medium. The medium in the lower chamber was supplemented with 1% FBS. For cells undergoing dox induction, treatment was continued by supplementing both upper and lower chambers with 500 ng/mL dox. The Boyden chambers were incubated at 37°C, 5% CO₂ for 18 h. Cells that had migrated to the lower side of the transwell insert were fixed and stained using Diff-Quik (Siemens Healthcare Diagnostics, Deerfield, USA) and counted on a Leica CME microscope (Leica, Microsystems UK, Milton Keynes, UK) at $\times 40$ total magnification. All experimental conditions were performed in triplicate with three independent repeats. Experiments were statistically analyzed using paired Student's t -test (Stat-View, version 5.0.1, Adept Scientific Plc., Letchworth, UK).

Immunohistochemistry

For immunohistochemistry (IHC), cells were grown on glass chamber slides (LabTek II, Nunc GmbH, Langenselbold, Germany) overnight at 37°C and cultured for

an additional 24 h with or without 500 ng/mL dox. Cells were fixed using acetone which had been equilibrated at -20°C for 10 min and washed with PBST. Cells were permeabilized by incubation with 0.1% Triton X-100 in PBS for 10 min at RT. Peroxidase was blocked by a 3% H₂O₂/methanol treatment for 30 min in the dark. Slides were washed with PBST and unspecific binding blocked by incubation in 1:10 diluted goat serum in PBST for 30 min at RT. The primary antibody, chicken anti CapG antibody (1:1000; GenWay Biotech, San Diego, USA), was incubated in 1% BSA/PBST overnight at 4°C in a humidified chamber. Slides were washed with PBST and incubated with the secondary anti-chicken HRP conjugated (1:1000; GenWay Biotech, San Diego, USA) for 1 h at room temperature in 3% BSA/PBS. The staining was developed with DAB and counterstained with hematoxylin.

RESULTS

Development of dox-inducible pancreatic cells

Pancreatic cancer cells, Suit-2, Panc-1 and MiaPaca-2 were transfected with plasmids CMV-rtTA2S-M2 and pN1 β actin-rtTA2S-M2-IRES-EGFP, in which rtTA2S-M2 expression is under the control of the CMV and β -actin promoters respectively. Cells were selected for resistance to G418. Emergent clones were tested for dox inducibility by transient transfection with a luciferase expression plasmid under the control of a TRE (pTRE2hygluc). Elevated luciferase activity in the presence of dox when compared to the untreated (-dox) control was taken as evidence of expression of the rtTA protein. Of the Suit-2 cells transfected with CMV-rtTA2S-M2, 167 G418-resistant clones were screened, although only three of these clones (29, 85 and 109) showed evidence of statistically significant dox-inducible luciferase activity (Figure 1A, $P < 0.05$, Student's paired t test). Of the Suit-2 cells transfected with pN1 β actin-rtTA2S-M2-IRES-EGFP, 49 G418-resistant Suit-2 clones were screened. Nineteen of these clones showed a greater than five-fold dox-inducibility of luciferase activity compared to the untreated (-dox) control. The luciferase measurement for twelve representative clones is shown in Figure 1B. The induction of luciferase was significantly increased in all clones shown ($P < 0.05$, Student's paired t test) except for clone 18 ($P < 0.09$).

Stably-transfected Panc-1 (Figure 2A) and MiaPaca-2 (Figure 2B) cell clones harbouring the pN1 β actin-rtTA2S-M2-IRES-EGFP Tet-on plasmid were also identified. Thirteen G418-resistant CMV-rtTA2S-M2 Panc-1 clones emerged; however, none showed evidence of dox-inducible luciferase activity. No G418-resistant CMV-rtTA2S-M2 MiaPaca 2 clones were obtained despite four transfection and selection attempts.

Doxycycline-inducible pancreatic cells are suitable for the induction of a variety of genes

We next wished to determine the versatility of stable dox-inducible pancreatic cells for the expression of a variety of

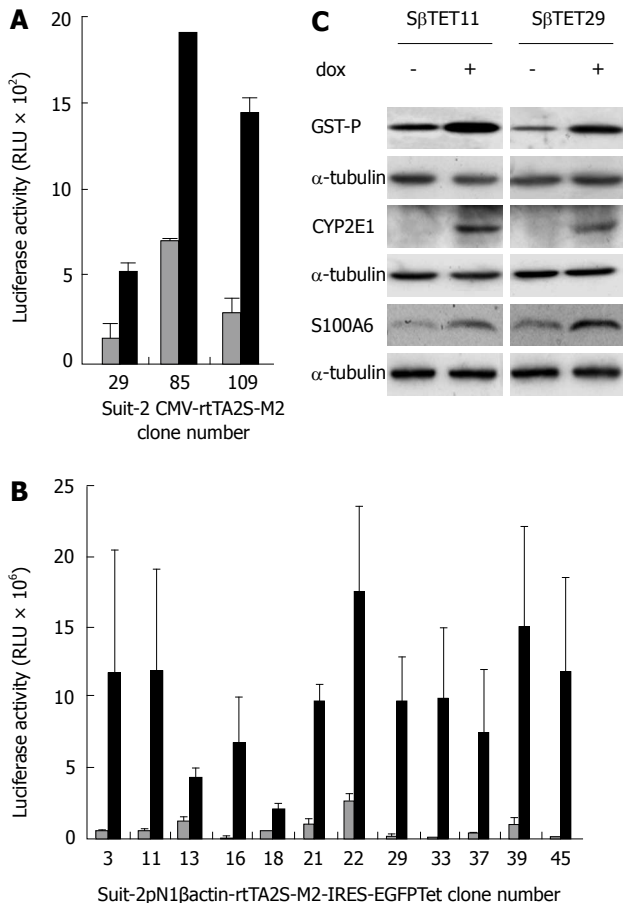


Figure 1 Identification of clones transfected with CMV-rtTA2S-M2 or pN1βactin-rtTA2S-M2-IRES-EGFP-SUIT-2 cell clones. Suit-2 cells were transfected with CMV-rtTA2S-M2 (A) or pN1βactin-rtTA2S-M2-IRES-EGFP (B) and stable clones selected using 300 μg/mL G418. Clones were isolated and transfected with pTRE2hygLuc, used as an indirect measure of rtTA activity. Cells were treated for 24 h with 500 ng/mL doxycycline (black bars) or an equivalent volume of PBS (white bars), and luciferase activity measured in 50 μg protein (1 mg/mL protein; 50 μL was assayed per sample). Error bars represent standard errors for three experiments. C: Cells were transiently transfected with pTRE2hygGST-P, pTRE2hygCYP2E1, and pTRE2hygS100A6 and treated with 500 ng/mL doxycycline or PBS as control for 24 h. Protein lysates were prepared and subjected to Western Blotting using anti-GST-P, CYP2E1 and S100A6 antibodies. α-tubulin was used as a loading control.

heterologous genes. To do this, two pN1βactin-rtTA2S-M2-IRES-EGFP-derived Suit-2 clones (Sβtet11 and Sβtet29), which showed good inducibility, were transiently transfected with plasmids containing the genes for glutathione S transferase P (GST-P), cytochrome P450 2E1 (CYP2E1), and S100A6, each under the control of a TRE. These genes were chosen because of our previous experience of their inducible expression in hepatoma cells (GST-P, CYP2E1)^[14] or because of our interest in them as genes that are overexpressed in pancreatic cancer (S100A6 and CapG)^[12,16,17]. Their inducibility was measured using dox. Western blotting (Figure 1C) revealed that dox treatment of the transiently transfected Sβtet11 and Sβtet29 clones resulted in induction of GST-P, CYP2E1, and S100A6. Two inducible pN1βactin-rtTA2S-M2-IRES-EGFP-derived Panc-1 clones and two pN1βactin-rtTA2S-M2-

IRES-EGFP-derived MiaPaca-2 clones were also evaluated by transient transfection of a plasmid carrying a pTRE-dependent *CapG* gene, and induction of expression with dox. Both Panc-1 and MiaPaca-2 clones showed increases in the CapG protein following dox treatment (Figure 2C).

Development of stable Suit-2 derived pTRE2hygCapG clones

Having observed good transient inducible gene expression, we wished to determine whether stable inducible CapG overexpressing cells could be derived. Transgenic cells stably expressing the inducible form of CapG (pTRE-2hygCapG) were constructed using Suit-2 pN1βactin-rtTA2S-M2-IRES-EGFP derived Tet-on cell clones (Sβtet11 and Sβtet29). Hygromycin B-resistant clones ($n = 49$) were screened for plasmid integration by treating with 500 ng/mL dox for 24 h and measuring CapG protein overexpression by Western blotting. The fold increase in CapG relative to expression of actin is shown in Figure 3A. Six CapG clones (clones 30-35) are shown on a representative Western blot presenting the dox-dependent CapG inducible expression (Figure 3B). From the 49 clones analysed, 20 (40%) showed no change in CapG expression, whereas 22 (44%) showed a 1.5 to 3 fold increase in CapG expression following dox treatment. For eight clones (16%), a greater than three-fold increase in CapG protein level was observed. For a proportion of clones (38%), actin levels appeared to be reproducibly induced following dox treatment/CapG induction. Since CapG is an actin-capping protein, it was decided that subsequent experiments would use alternative internal control proteins, such as tubulin or GAPDH.

Stable pTRE2CapG expressing cells show time- and dose-dependent increases in CapG expression

The time and dose-dependent inducibility of CapG protein expression was investigated. Concentration dependency was examined by inducing CapG overexpression with different concentrations of dox (0-1000 ng/mL) for 36 h (Figure 3C). CapG expression was not induced at low concentrations of dox (1-10 ng/mL), but was induced at concentrations of dox above 50 ng/mL. There was no concentration-dependent increase in the amount of CapG protein induced. For the time course study (Figure 3D) and further experiments, a concentration of 500 ng/mL dox was chosen. An increase in CapG protein level was observed after 12 h of dox treatment and was stable for at least a further 60 h (from 12 to 72 h) (Figure 3D).

Alteration in cellular protein expression associated with CapG overexpression

We next sought to determine whether artificially increasing the levels of CapG would alter the levels or activation status of other actin-modulating proteins, such as Cofilin and Ezrin/Radixin. We found that neither protein was changed in level or in phosphorylation status when CapG levels were increased (Figure 4A and B). This led us to ask

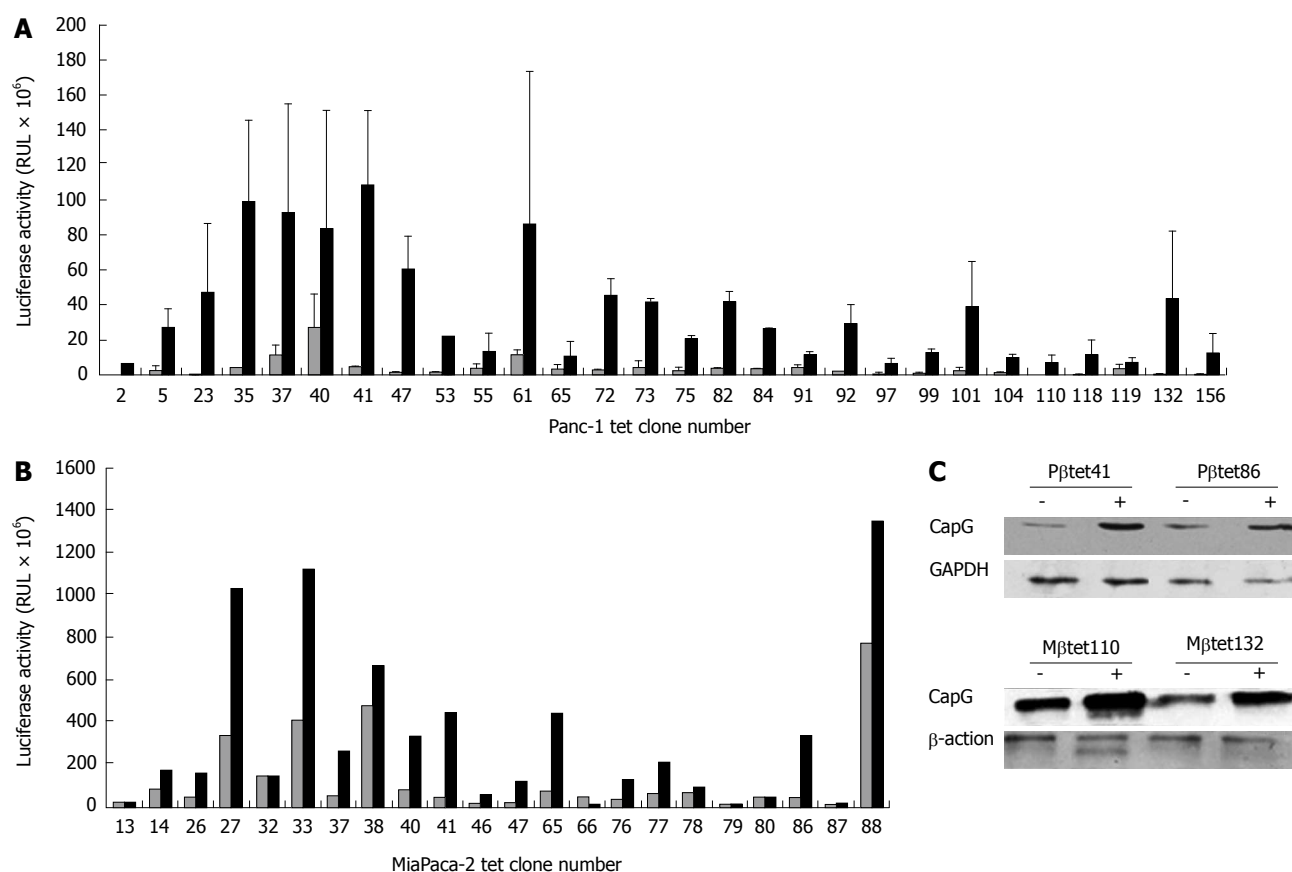


Figure 2 Identification of stable pN1βactin-rtTA2S-M2-IRES-EGFP-expressing Panc-1 (A) and MiaPaca (B) cell clones and their functionality (C). Panc-1 and MiaPaca cells were transfected with pN1βactin-rtTA2S-M2-IRES-EGFP and stable expressors selected using 300 μg/mL G418. Clones were isolated and transfected with pTRE2hygLuc, used as an indirect measure of rtTA activity. Cells were treated for 24 h with 500 ng/mL doxycycline (black bars) or an equivalent volume of PBS (white bars) and luciferase activity measured in 50 μg protein (1 mg/mL protein; 50 μL was assayed per sample). A: 90 G418 resistant clones were isolated from Panc-1 cells, of which 25% showed > 5-fold increase in luciferase activity in the stimulated (black bars in Figure 1) compared to the unstimulated (white bars in Figure 1) condition; B: Of 170 G418-resistant cells identified in MiaPaca-2 cells, 60% showed > 5-fold increase in luciferase expression; C: Two stable pN1βactin-rtTA2S-M2-IRES-EGFP Panc-1 (Pβtet41 and Pβtet86) and MiaPaca-2 (Mβtet110 and Mβtet132) cell lines were transiently transfected with the pTRE2hygCapG vector and treated for 24 h with or without 500 ng/mL doxycycline. Cell lysates were subjected to Western Blotting for the detection of CapG. β-actin and GAPDH were used as loading controls, respectively. Tet: Tetracycline.

a broader question of whether elevated CapG affected the expression of any cellular proteins. CapG overexpression was analysed by 2D-gel electrophoresis in two stable CapG inducible clones (Sβtet29Cap35 and Sβtet29Cap43), and, as the control, the rtTA protein expressing clone from which those two clones were derived (TetSβtet29). An increase in the intensity of a 38 kD protein (Figure 5A) was observed on 2-D gels following dox treatment of Sβtet29Cap35 and Sβtet29Cap43, but in not the control TetSβtet29, indicating that treatment with doxycycline alone is not sufficient to induce the expression of CapG, i.e. the cells must harbour a doxycycline-inducible CapG construct. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) confirmed the identity of this protein as CapG (Figure 5B). This finding is consistent with our previously obtained Western blot data (Figure 3). A number of other proteins also differentially expressed in cells induced to express high levels of CapG were also identified by MALDI-MS (Figure 6). HNRPH1, T-complex protein 1 subunit alpha, Adenosylhomocysteinase, and Ornithine aminotransferase were upregulated in CapG overexpressing cells. Conversely, Eno-

lase, P27BBP protein, eukaryotic translation elongation factor 1 beta 2, and human pre-mRNA splicing factor SF2p32 were downregulated in CapG overexpressing cells. As none of these proteins are associated with the cytoskeleton, they were not validated further.

Subcellular localization of capG

The localisation of CapG in the inducible clones Sβtet29Cap35, Sβtet29Cap43 and the control clone Sβtet29 was examined by immunohistochemistry. In untreated (-dox) cells, CapG was expressed homogeneously in the cytoplasm (Figure 7A), and nuclei were devoid of staining. After treatment of cells with doxycycline for 24 h, the intensity of CapG staining (brown colour) increased in the cytoplasm and around the nuclear membrane of Sβtet29Cap35 and Sβtet29Cap43 cells, suggesting CapG overexpression in the cytoplasmic compartment of these cells. As expected, the control clone Sβtet29 did not show any change in staining intensity upon treatment with dox, and no staining was observed in the nucleus. The relative levels of CapG in the cytoplasm and nucleus were quantitatively

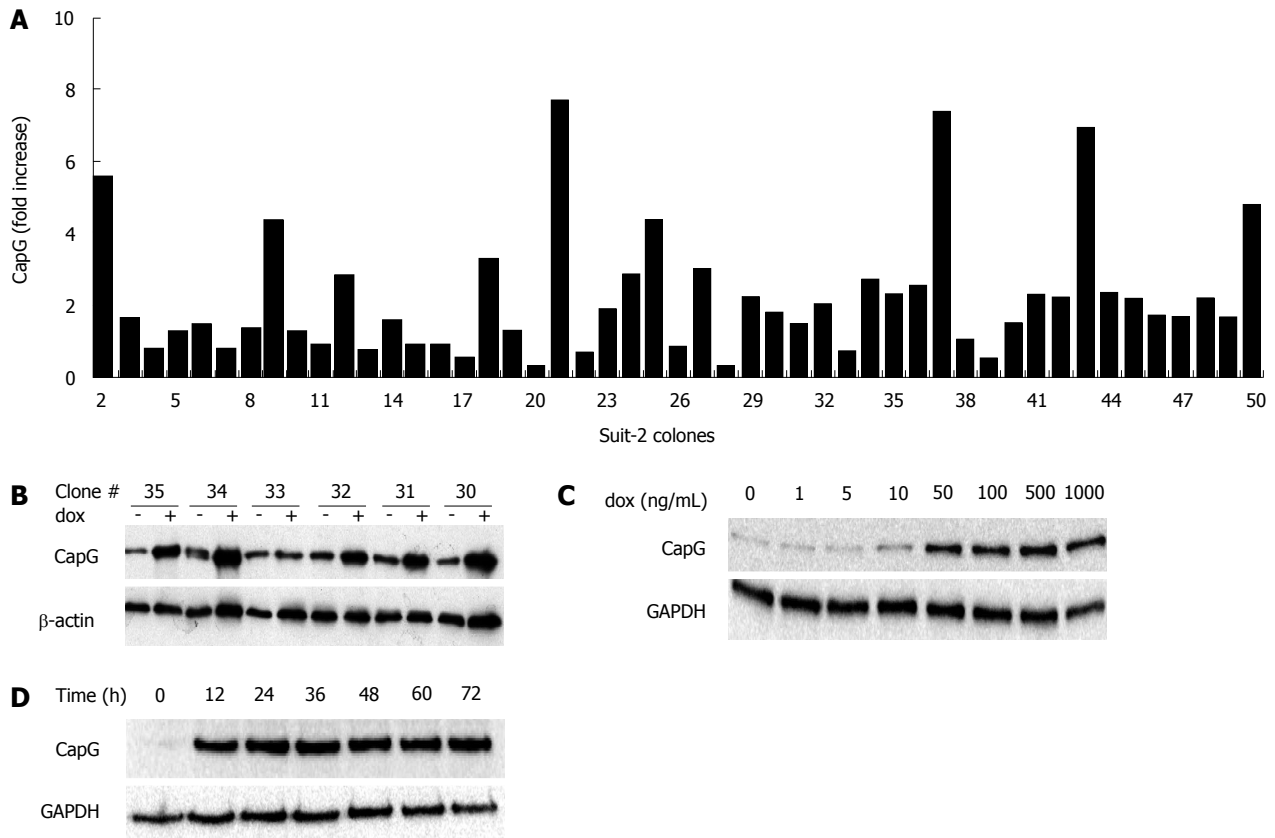


Figure 3 Selection (A, B) and inducibility (C, D) of stably-transfected Sβtet29Cap clones. The stable Sβtet29 clone was transfected with the pTRE2hygCapG full size vector and clones selected with hygromycin B (200 µg/mL). A: Individual clones ($n = 49$) were induced with 500 ng/mL doxycycline for 24 h and the protein lysate subjected to Western blotting. The CapG level was calculated for each individual clone in the non-induced and the induced state. The fold increase (normalised to actin) in CapG for each of the 49 clones is shown in the bar chart; B: A representative Western blotting for CapG is shown for pTRE2hygCapG clones 30 to 35. The indicates the basal CapG expression, + doxycycline (dox) induced; C: The inducibility of CapG protein expression was investigated by treating the Sβtet29Cap35 cells with 0, 1, 5, 10, 50, 100, 500, 1000 ng/mL dox for 24 h. The resulting Western blotting for CapG and GAPDH as a loading control is shown; D: The Western blotting represents the CapG protein level of Sβtet29Cap35 cells, induced with 500 ng/mL doxycycline for 0, 12, 24, 36 and 48 h. GAPDH was used as a loading control.

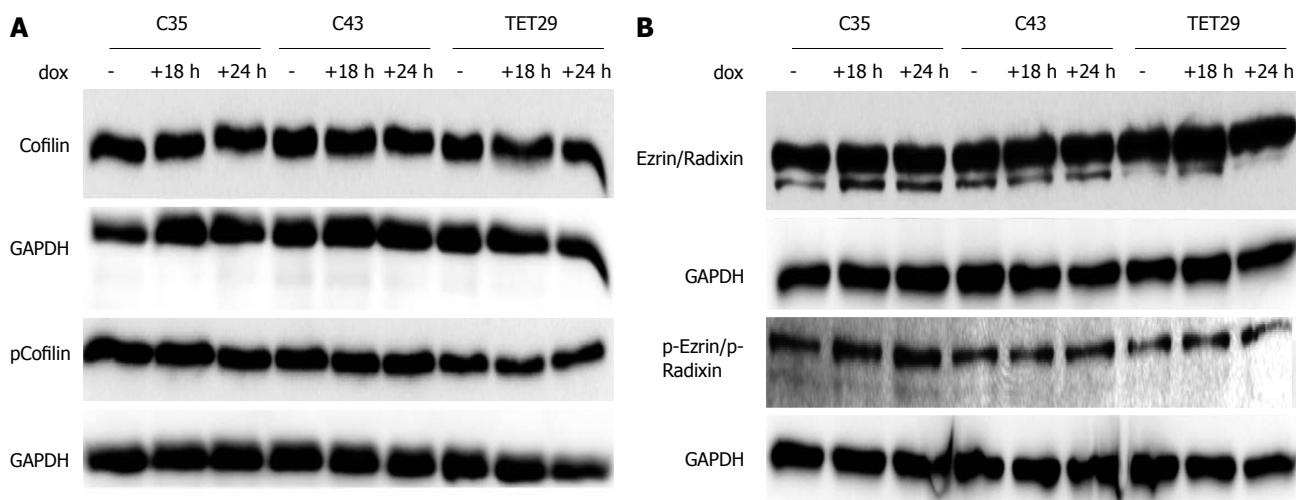


Figure 4 The effects of CapG overexpression on Cofilin and Ezrin/Radixin levels and phosphorylation status. Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and control Sβtet29 (TET29) cells were incubated with dox (500 ng/mL) for the indicated times and lysates assayed by Western blotting for changes in the levels of Cofilin (A) and Ezrin/Radixin (B) and phospho-Cofilin (A) and phosphor-Ezrin/Radixin (B).

evaluated. The nuclear and cytoplasmic fractions of Sβtet29Cap35 cells were enriched and Western blotting for CapG performed. The levels of CapG in non-

fractionated extracts in the non-induced and induced state are shown Figure 7B, lanes 1 and 2 respectively. Lanes 3, 4 and 5, 6 show nuclear and cytoplasmic CapG levels in

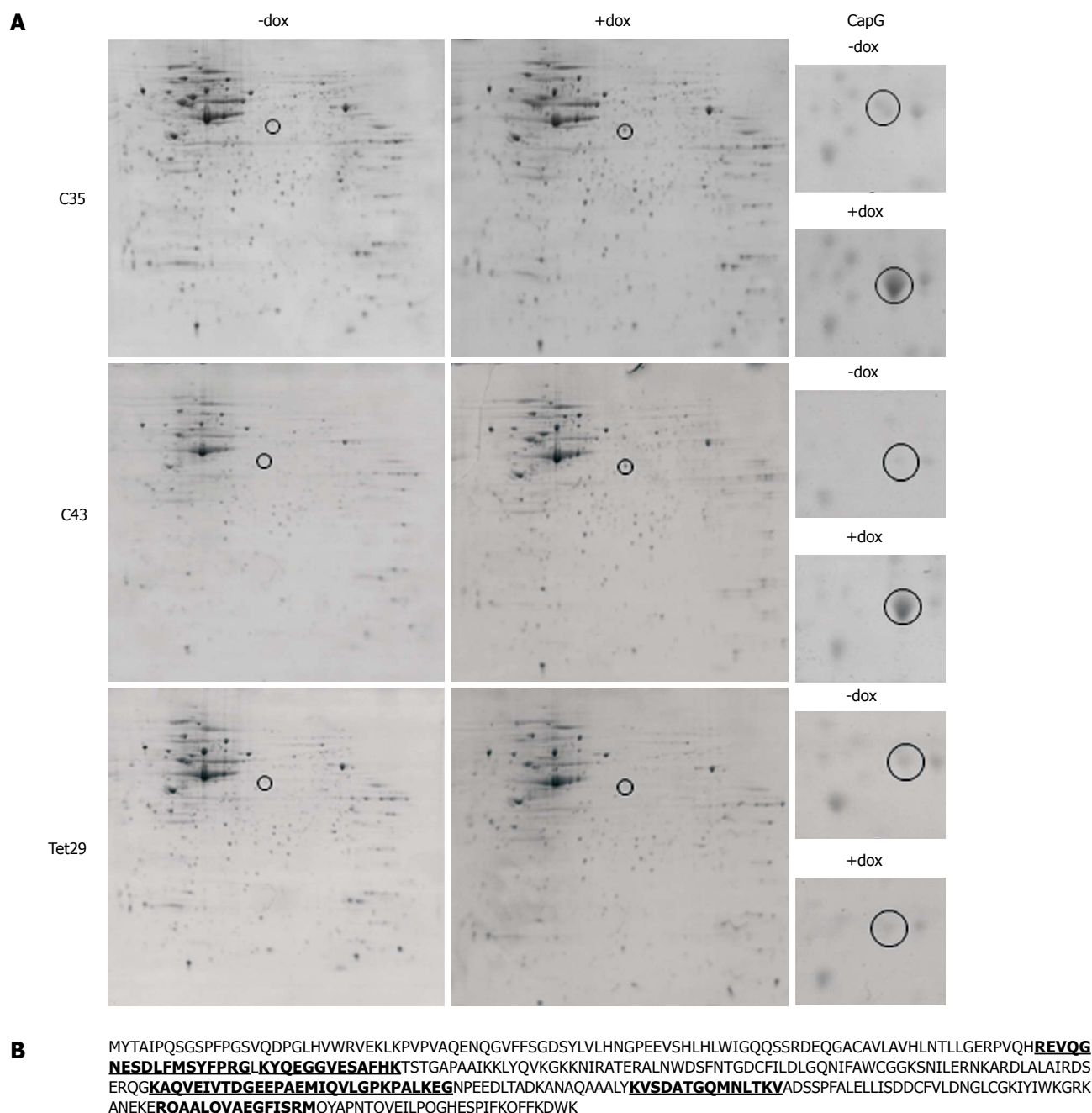


Figure 5 Two-dimensional gel analysis of stable inducible CapG overexpressing cells. A: Colloidal Coomassie Blue stained gels displaying the proteome of S β tet29Cap35 (C35), S β tet29Cap43 (C43) and control S β tet29 (TET29) cells. Cells were lysed in their uninduced state (- dox) and 18 h after treatment with 500 ng/mL doxycycline (+ dox). The area around the spot representing CapG (black circle) was magnified in the right hand column (CapG); B: The spot representing CapG was excised from the gels, trypsin digested, and analysed by MALDI-ToF. The sequence is shown and peptides identified by MALDI are bold and underlined.

the uninduced and induced states respectively. The enrichment of Lamin A is used as a marker of the nuclear fraction and GAPDH as a marker of the cytoplasmic fraction. A low level contamination of the nuclear fraction by the cytoplasmic fraction was observed. CapG protein was found exclusively in the cytoplasmic fraction in the non-induced state (lane 3). Following 24 h treatment with dox, the level of CapG was greatly increased in the cytoplasmic fraction (lane 5), with a very small proportion observed in the nuclear fraction (lane 6). The low level of CapG observed in the nuclear fraction may be due to contamination from the cytoplasmic fraction.

Stable pTRE2CapG cells show no difference in viability, proliferation or cell cycle

The viability of S β tet29Cap35, S β tet29Cap43, and S β tet29 cells was measured 48 h after plating with or without 500 ng/mL dox. The MTS solution was added ($t = 48$ h) and the absorbance measured at 0, 1, 2, 3, and 4 h later. The kinetics for the turnover of the MTS solution was similar in all cell lines, as can be seen by the slope of the lines (0.3577 ± 0.031 , Figure 8A). This indicates that 500 ng/mL dox was not toxic in any of the cell clones, including those in which dox caused induction of CapG (S β tet29Cap35, S β tet29Cap43).

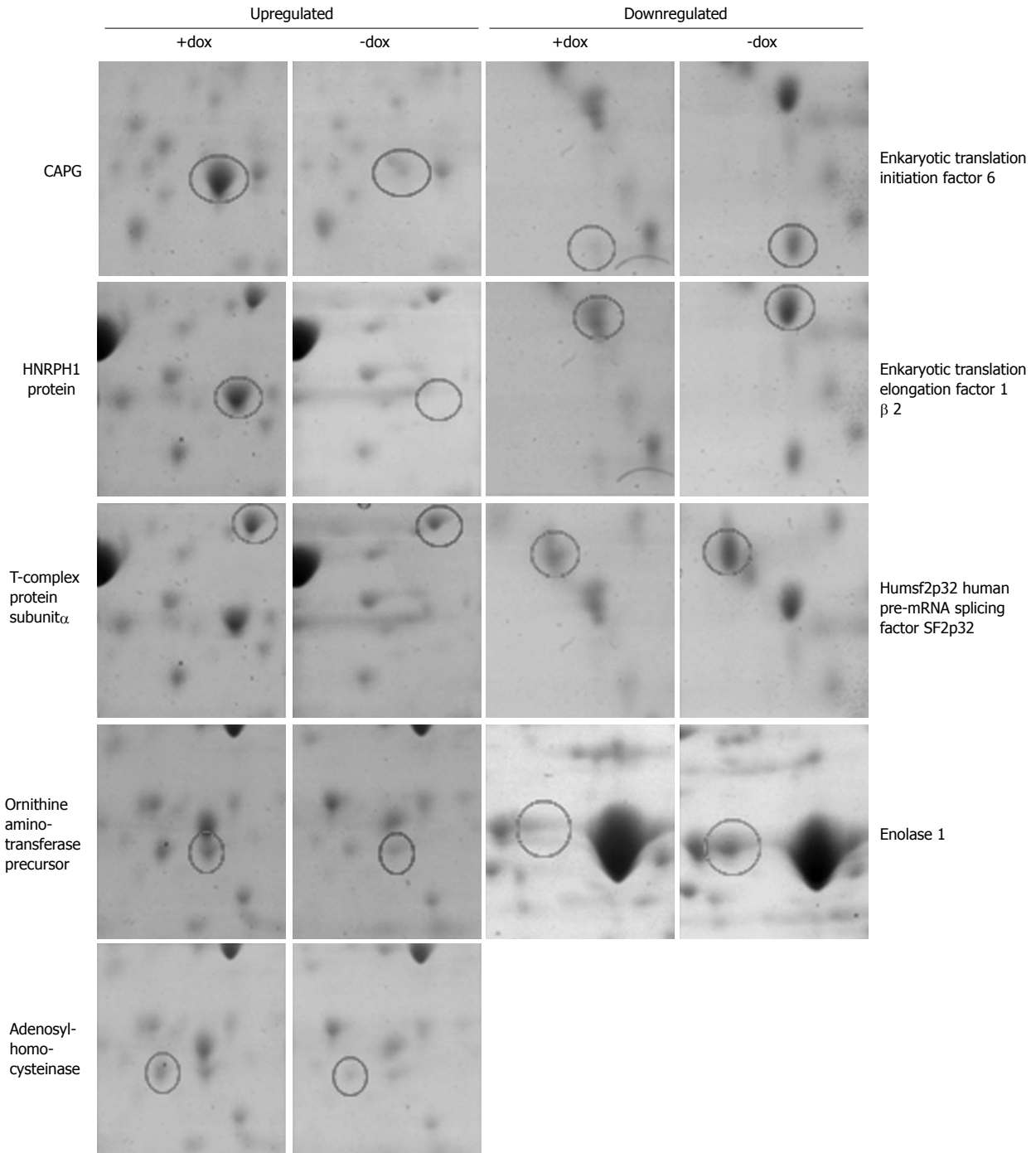


Figure 6 Two-dimensional gel analysis shows up- or downregulation of proteins associated with CapG overexpression. Colloidal Coomassie Blue stained gel insets displaying proteins found to be up- or downregulated in S β tet29Cap35 (C35), S β tet29Cap43 (C43) but not in control S β tet29 (TET29) cells, following treatment for 18 h with 500 ng/mL doxycycline (+dox). Spots were excised from the gels, trypsin digested, and analysed by MALDI-ToF, leading to the identification of proteins.

Cell cycle analysis at 24 and 72 h after dox treatment did not result in any significant change in cell cycle profile. A representative figure showing the cell cycle profile of S β tet29Cap35 (C35) and the parental cell line S β tet29 (TET29) cultured for 24 or 72 h with or without 500 ng/mL dox is presented (Figure 8B). The percentage of cells in G1, G2 and S-Phase for all three experiments is summarized in Figure 8C and Table 1. There was a considerable change in cell cycle phases between the two time points

(24 and 72 h), as cells are cultured for 48 h (24 h \pm dox) or 96 h (72 h \pm dox). There was no difference in the cell cycle phases between the groups cultured with or without dox. This shows that neither dox nor CapG overexpression affected the cell cycle of Suit-2 cells.

CapG overexpression increases Suit-2 wound healing capacity and motility

To investigate the effect of CapG overexpression on cell

Table 1 Cell cycle analysis (%)

	- doxycycline			+ 500 ng/mL doxycycline		
	G1-phase	G2-phase	S-phase	G1-phase	G2-phase	S-phase
24 h						
C35	34.8 ± 1.2	30.6 ± 5.2	34.7 ± 5.0	36.2 ± 3.2	27.3 ± 3.6	36.6 ± 1.1
C43	30.8 ± 0.9	29.8 ± 2.5	39.4 ± 3.1	30.7 ± 1.6	31.7 ± 0.9	37.6 ± 1.8
TET29	29.0 ± 1.7	38.9 ± 3.5	32.1 ± 2.4	30.2 ± 0.7	30.2 ± 1.5	39.6 ± 0.9
72 h						
C35	39.8 ± 1.1	24.7 ± 2.4	35.5 ± 2.8	41.1 ± 3.1	31.8 ± 3.6	27.1 ± 5.0
C43	42.6 ± 0.8	30.3 ± 3.5	27.1 ± 2.8	43.0 ± 4.1	24.0 ± 1.8	33.0 ± 3.0
TET29	40.4 ± 5.4	19.4 ± 3.7	40.2 ± 1.9	41.6 ± 4.3	25.9 ± 3.1	32.5 ± 5.6

10000 cells were measured, and the percentage of cells in G1, G2, and S-phase are presented with their error values. The experiment was repeated thrice.

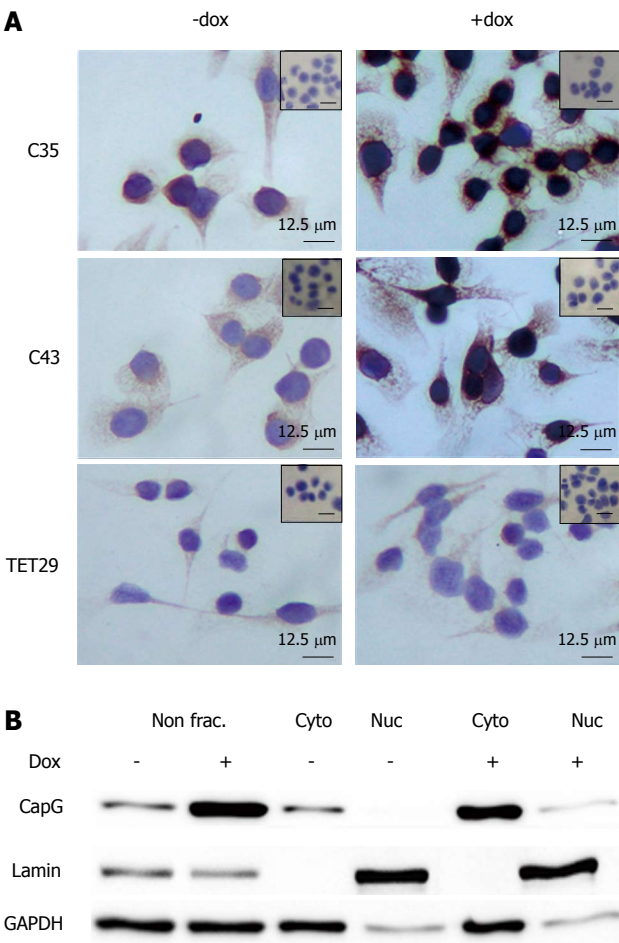


Figure 7 Subcellular localization of CapG in stable CapG inducible clones by immunohistochemistry (A) and subcellular fractionation (B). A: CapG protein expression and localization in Sβtet29Cap35 (C35), Sβtet29Cap43 (C43) and the control Sβtet29 (TET29) cells was analyzed 24 h after treatment with 500 ng/ml doxycycline (+ dox) or PBS as control (- dox). Specific antibody reaction (anti-CapG) was visualized by a peroxidase labelled secondary antibody (DAB detection, brown colour). Nuclei were counterstained with hematoxylin (blue colour). Bars = 12.5 μm. B: Whole protein lysate (non frac.) or enriched nuclear (Nuc) and cytoplasmic (Cyto) fractions of Sβtet29Cap35, Sβtet29Cap43 and the control Sβtet29 clones 24 h after treatment with 500 ng/mL doxycycline (+) or PBS as control (-) were analysed using Western blotting for the detection of CapG, Lamin (marker for nuclear fraction) and GAPDH (marker for cytoplasmic fraction). The experiment was performed three times. A representative Western blot of Sβtet29Cap35 is shown.

motility, wound healing and translocation assays were performed. When CapG was induced following dox treatment, we observed a significant increase in the distance travelled by the Sβtet29Cap35 and Sβtet29Cap43 cell clones. The distance travelled by Sβtet29Cap35 increased by 23% ± 5% and for Sβtet29Cap43 by 35% ± 7%. When treated with dox, the control clone Sβtet29 did not show any change in wound healing capacity (Figure 9A).

In Boyden chamber translocation assays, we observed a significant increase in cell migration by the Sβtet29Cap35 and Sβtet29Cap43 cell clones when CapG was induced following dox treatment (Figure 9B). The capability of Sβtet29Cap35 to cross through the membrane pores increased by 62% ± 10% and for Sβtet29Cap43 cells by 84% ± 16%. The control clone Sβtet29 did not show any change in cell migration capacity when treated with dox (Figure 9B).

DISCUSSION

In this study, we have described the generation and use of a tetracycline-inducible expression system in pancreatic cancer cell lines. Our use of both a CMV and a β-actin promoter-driven second generation reverse tetracycline transactivator protein (the rtTA2S-M2) led us to conclude that the latter was superior in terms of both production and maintenance of dox-inducible cell lines. Although we did not formally investigate mechanisms that might explain this, it is possible that the viral CMV promoter is silenced through methylation, whilst the chicken β-actin promoter remains active^[6].

Recently, Zhang *et al.*^[10], successfully used a commercially available Tet-on system with a CMV-driven tetracycline transactivator protein, in Panc-1 cells. The authors reported dox-induced overexpression of the zinc finger transcription factor INSM1, although the fold increase in INSM1 protein level, as shown by Western blotting, was modest compared to the fold increases observed in the current study. This may reflect differences in the proteins under investigation in the respective studies, although it could be due to enhanced inducibility when using the β actin-rtTA2S-M2-IRES-EGFP vector.

The pancreatic cancer cell lines Suit-2, MiaPaca-2 and

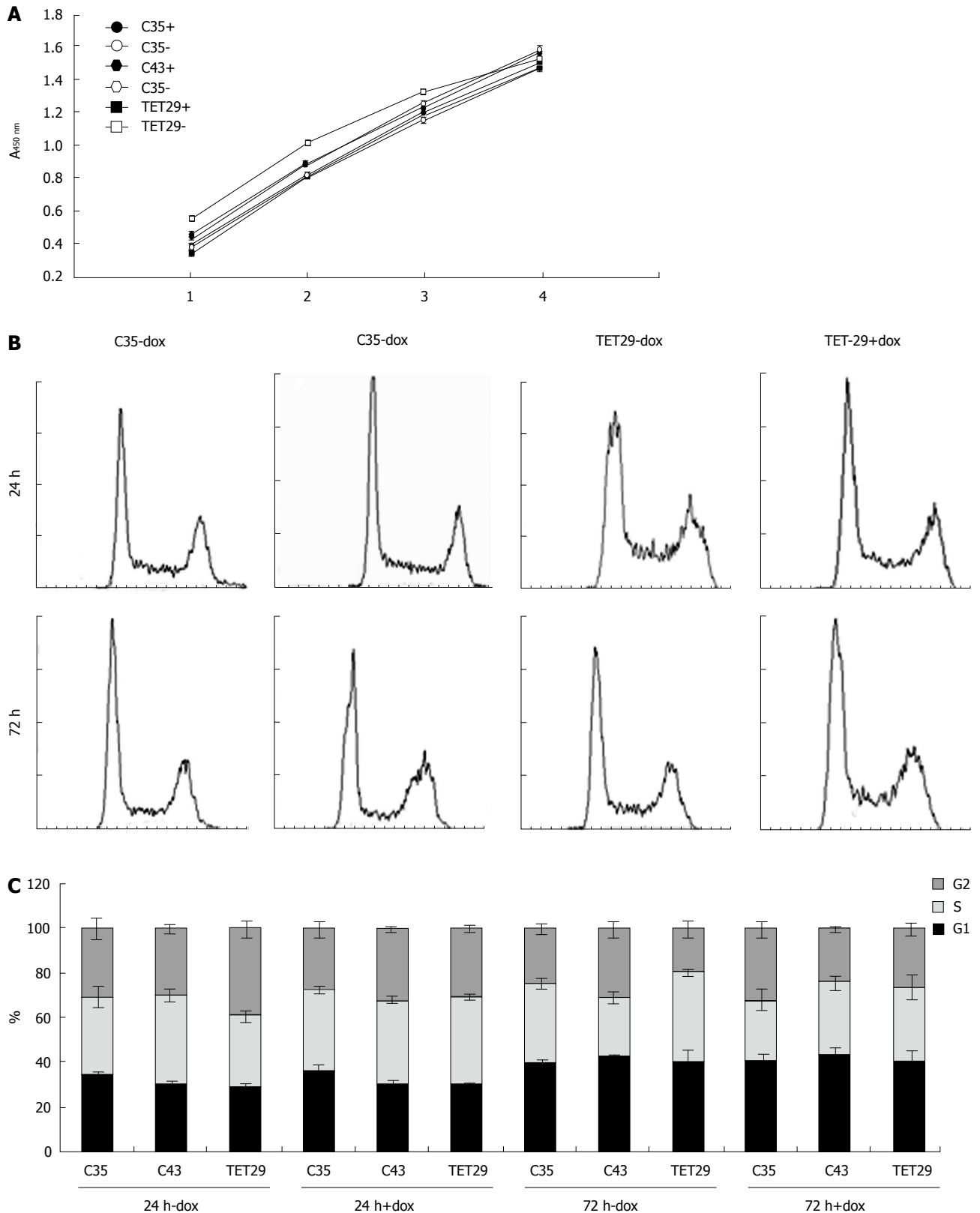


Figure 8 Cell proliferation (MTS-assay) (A) and cell cycle analysis (B) of doxycycline induced CapG overexpressing cells. A: S β tet29Cap35 (C35), S β tet29Cap43 (C43) and the control S β tet29 (TET29) cells were treated with 500 ng/mL doxycycline (+) or PBS as control (-) for 48 h. Mitochondrial activity was measured with the EZ4U assay and absorbance measured 1 h, 2 h, 3 h, and 4 h after substrate incubation. The experiment was performed three times with at least ten replicates; B: S β tet29Cap35 (C35), S β tet29Cap43 (C43) and the control S β tet29 (TET29) cells were treated with 500 ng/ml doxycycline (+) or PBS as control (-) for 24 h and 72 h. The cells were propidium iodide stained and FACS analyzed. 10 000 cells were measured and the experiment was performed in triplicate with three replicates each. A representative chart for the cell cycle of C35 and TET29 cells is presented; C: The graphics represent the average percentage (\pm SE) of cells in G1, G2, and S phase in the different experimental conditions.

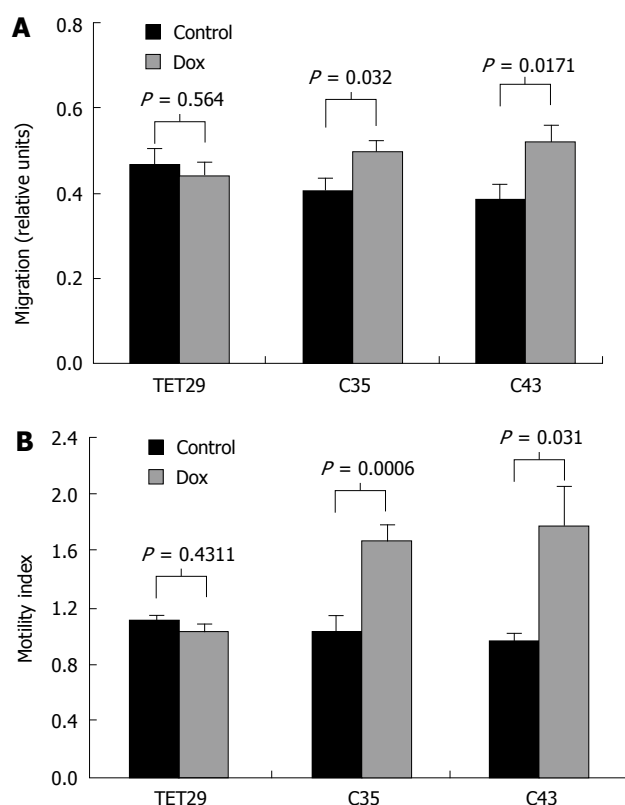


Figure 9 Wound healing capacity (A) and motility (B) of CapG-overexpressing cells. A: Bars represent the migration in relative units for Sptet29Cap35 (C35), Sptet29Cap43 (C43) and the control Sptet29 (TET29) cells treated with doxycycline (dox, gray bar) or PBS (Control, black bar). Error bars present the SE for three experiments carried out in triplicate; B: The motility investigated by a Boyden Chamber assay of Sptet29Cap35 (C35), Sptet29Cap43 (C43) and the control Sptet29 (TET29) cells treated with doxycycline (dox, gray bar) or PBS (Control, black bar) is shown. Each experiment was performed in triplicate with three replicates.

Panc-1 were all capable of stably harbouring the pN1 β actin-rtTA2S-M2-IRES-EGFP vector. Moreover, inducible gene overexpression was observed for GST-P, CYP2E1, and S100A6 in Suit-2 and for CapG in all three investigated cell lines. This indicates that the system functions for a variety of different proteins. Equally, the method was efficient with 80% of pN1 β actin-rtTA2S-M2-IRES-EGFP transfected Suit-2 clones and 60% of MiaPaca-2 clones showing greater than five-fold luciferase induction. Only Panc-1 cells showed relatively low efficiency, with only 14.4% of the selected cell clones showing a greater than five-fold luciferase increase. A clear advantage of the Tet-on system is the time and dose-dependent activation of expression of the gene of interest. Investigation of stable CapG overexpressing Suit-2 cell clones showed that CapG protein expression was dox-inducible in a time dependent manner. The level of CapG increased from 12 h after induction, and was maintained up to 72 h after induction. A similar time-dependent induction of gene expression in the liver carcinoma cells, HepG2 has been described previously^[14]. However, we did not observe a linear correlation between dox concentration and protein expression, as was reported in the HepG2 cells^[14]. Instead, we detected little increase in CapG protein concentration between 0-10 ng/mL dox, and overexpression of CapG

with concentrations of 50 ng/mL and greater (up to 1000 ng/mL). Possible reasons for these different results could be related to the particular protein overexpressed or the different promoter used to drive the rtTA2S-M2 in both studies. The HepG2 Tet-on clones harboured CMV driven rtTA2S-M2, unlike the pancreas clones in this study.

Concerns have been raised about the toxicity of dox, the agent used to activate gene expression in the Tet-on vector system^[18]. The cell growth of PC-12 cells was reduced after 96h incubation with concentrations ranging from as low as 0.2 to 100 μ g/mL. Growth perturbations were observed with WI-38 VA-13 cells, but only with concentrations of dox greater than 2 μ g/mL^[18]. It appears that the concentration of dox which mediates a toxic effect is cell-line-dependent. At the concentrations of dox used in this study, no loss of proliferation was observed 48 h after dox treatment. These data were supported by cell cycle analyses profiles, which showed no change in cell cycle following dox treatment for 24 h and 72 h.

The function of CapG in the cytoplasm is well described. CapG caps and therefore blocks rapidly growing actin filaments, promoting the elongation of shorter actin filaments. It thus contributes to cell motility^[19] and membrane ruffling^[20]. Increased levels of CapG protein have been described in several tumors, including pancreatic ductal adenocarcinoma^[12,21] and glioblastomas^[22]. Transient CapG knockdown experiments in pancreatic cancer cell lines Suit-2, MiaPaca-2, and Panc-1 cell lines led to a significant decrease in wound healing capacity and cell motility^[12]. Furthermore, Van den Abbeele *et al.*^[23] showed that downregulation of CapG in the breast cancer cells MDA-MB 231 and the prostate cancer cells PC-3 also decreased invasion and motility. Here, we show for the first time that overexpression of CapG in a pancreatic cancer cell line caused a significant increase in motility and a modest, but significant, increase in wound healing capacity. This is consistent with a previous report of an increase in invasion after CapG overexpression in Madin-Darby Canine Kidney Epithelial Cells MDCK cells^[24]. Interestingly, it has been reported that active nuclear import of CapG is necessary for CapG to promote invasion^[24]. Prevention of nuclear accumulation of CapG in MDCK cells abolished collagen invasion, whereas restoring the nuclear import also restored collagen invasion of these cells. We found no evidence for elevated nuclear levels of CapG following overexpression in Suit-2 derived cells. Immunohistochemical staining localized CapG to the cytoplasm in Suit-2 cells. Dox treatment of stable inducible CapG expressors led to an increase in cytoplasmic staining, with increased CapG around the nuclear membrane, but CapG did not accumulate in the nucleus. Subcellular fractionation further validated this finding, showing increased CapG protein levels in the cytoplasmic fraction after dox treatment. We observed increased motility, suggesting that elevated cytoplasmic CapG may be sufficient for increased cancer cell motility.

CapG has been shown to interact with microtubule-dependent organelles during the cell cycle^[25], possibly mediating cross-talk between the actin cytoskeleton and

microtubule-based organelles involved in mitosis. CapG is imported into the nucleus by NTF2, Ran GTPase and nucleoporin Nuc62^[20], a phenomenon that was observed in HEK293T, HeLa, and MDCK-AZ cells. We found no changes in the cell cycle following induction of CapG overexpression. This suggests that an excess of CapG is not sufficient to alter cell cycle dynamics, but it does not exclude a role for CapG in cell cycle processes.

In summary, we have shown that the pN1 β actin-rtTA2S-M2-IRES-EGFP Tet-on vector system can be stably transfected in a variety of pancreatic cancer cell lines, permitting the investigation of dox inducible gene overexpression in a time and dose-dependent manner. Our use of this system to overexpress CapG in Suit-2 cells resulted in accumulation of CapG protein in the cytoplasmic cellular compartment with associated increases in wound healing and cell motility, but no alterations to the cell cycle or to cellular proliferation.

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COMMENTS

Background

Pancreatic cancer cells have traditionally been recalcitrant to the tetracycline gene regulatory system, a method that allows precise control of the level of expression of specific proteins, enabling functional analysis of those proteins. In this study, the authors compare the performance, in pancreatic cancer cells, of a stable Tet-on system employing a *cytomegalovirus* (CMV) promoter with a system employing a chicken β -actin promoter. In doing so, the authors generated cells exhibiting stable inducible expression of the actin capping protein CapG, which plays a role in cell migration. This protein was recently shown to be overexpressed in pancreatic cancer tissue, and its downregulation is associated with loss of motility.

Research frontiers

Use of the chicken (β -actin) promoter proved superior to the CMV promoter for both the production and maintenance of tetracycline-inducible pancreatic cancer cell lines. The authors observed that the system was versatile, enabling transient inducible expression of a variety of genes. Moreover, with the newly developed Tet-on system, the authors showed that CapG overexpression in pancreatic cancer cells led to increased cell motility, but did not alter the cell cycle, or cellular proliferation.

Innovations and breakthroughs

The Tet-on system using a chicken (β -actin) promoter allowed the generation of stable inducible pancreatic cancer cell lines, providing an important tool for investigating doxycycline induced overexpression of proteins associated with pancreatic cancer. Furthermore, the study illustrated that cytoplasmic CapG overexpression in pancreatic cancer cells increases motility of these cells.

Applications

The stable Tet-on pancreatic cancer cell lines developed are an excellent system that will provide quantitative and temporal information on the role of given proteins in these cells.

Terminology

Panc-1, Suit-2 and MiaPaca-2 are pancreatic cancer cell lines. CapG belongs to the family of actin regulatory proteins. CapG caps the ends of barbed actin filaments and therefore contributes of the actin associated motility of cells. Nuclear CapG is involved in the cell cycle regulation.

Peer review

There are precious few published papers describing the use of stable tetracycline-inducible pancreatic cancer cells for the overexpression of proteins. This reflects the difficulty that scientists in this field have faced with the available

systems. The authors describe the successful generation of stable tetracycline-inducible pancreatic cancer cells, using a chicken β -actin promoter to express the reverse tetracycline-controlled transactivator protein. This is an important advance, illustrating an improvement to the system which other pancreatic cancer researchers could also benefit from.

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Unusual histopathological findings in appendectomy specimens: A retrospective analysis and literature review

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24 were female with ages ranging from 15 to 84 years (median, 32.2 ± 15.1 years). Final pathology revealed 37 cases of enterobiasis, five cases of carcinoids, four mucinous cystadenomas, two eosinophilic infiltrations, two mucocoeles, two tuberculosis, one goblet-cell carcinoid, and one neurogenic hyperplasia. While 52 patients underwent a standard appendectomy, two patients who were diagnosed with tuberculous appendicitis underwent a right hemicolectomy. All tumors were located at the distal part of the appendix with a mean diameter of 6.8 mm (range, 4-10 mm). All patients with tumors were alive and disease-free during a mean follow-up of 17.8 mo. A review of 1366 cases reported in the English literature is also discussed.

CONCLUSION: Although unusual pathological findings are seldom seen during an appendectomy, all appendectomy specimens should be sent for routine histopathological examination.

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Abstract

AIM: To document unusual findings in appendectomy specimens.

METHODS: The clinicopathological data of 5262 patients who underwent appendectomies for presumed acute appendicitis from January 2006 to October 2010 were reviewed retrospectively. Appendectomies performed as incidental procedures during some other operation were excluded. We focused on 54 patients who had unusual findings in their appendectomy specimens. We conducted a literature review *via* the PubMed and Google Scholar databases of English language studies published between 2000 and 2010 on unusual findings in appendectomy specimens.

RESULTS: Unusual findings were determined in 54 (1%) cases by histopathology. Thirty were male and

Key words: Appendicitis; Carcinoid; Unusual findings; Goblet cell carcinoid; Enterobius vermicularis; Mucocele

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INTRODUCTION

Appendicitis is one of most common acute surgical condi-

tions of the abdomen, and an appendectomy is one of the most frequently performed operations worldwide. The incidence of acute appendicitis roughly parallels that of lymphoid development, with peak incidence in the late teens and twenties. Obstruction of the lumen is the dominant factor in acute appendicitis, and although fecoliths and lymphoid hyperplasia are the usual cause of obstructions, some unusual factors could also be involved^[1-128]. Obstruction may be due to enterobiasis^[1,4,7,29], ascariasis^[57,92-94], balantidiasis^[2,92], taeniasis^[14,18], actinomycosis^[32-38], schistosomiasis^[2,8,42-51,57], amebiasis^[7,84-86,90], trichuriasis^[52,57], *Blastocystis hominis*^[20], tuberculosis (TB)^[8,23,53-55,57], carcinoid tumor^[11-3,5,9,12,26,28,31,95], goblet-cell carcinoid (GCC)^[5,12,21,25], primary or secondary adenocarcinoma^[16,31], cystadenocarcinoma^[31], lymphoma^[2], dysplastic changes^[2], endometriosis^[11,16,58-69], granulomatous diseases^[31,32], gastrointestinal stromal tumor (GIST)^[71,72,103], mucocoele^[11-3,32], villous adenoma^[24,39,56], tubulovillous adenoma^[24], tubular adenoma^[24,31], leiomyoma^[2], eosinophilic granuloma^[32,52], or neurogenic appendicopathy^[30].

MATERIALS AND METHODS

Between January 2006 and October 2010, 5262 patients with presumed acute appendicitis underwent surgical treatment at Diyarbakir Education and Research Hospital, Turkey. Appendectomies performed as an incidental procedure during some other operation were excluded. The data of 54 (1%) patients who were pathologically reported to have unusual appendix findings were retrospectively collected. The original pathology specimens with unusual findings were evaluated again by an experienced pathologist. The records analysis was composed of the patient's age, gender, clinical presentation, operative reports, radiological tools, pathological report, and follow-up. The length of follow-up was calculated by months from the date of diagnosis until the last clinical information available on the patient up to November 2010.

English medical language PubMed and Google Scholar database searches were conducted for case reports, retrospective and prospective studies, and literature reviews relating to "unusual causes of appendicitis". Keywords used were parasites, enterobiasis, schistosomiasis, amebiasis, yersiniosis, strongyloidiasis, actinomycosis, TB, idiopathic granulomatous appendicitis, Crohn's disease, endometriosis, appendicular adenocarcinoma, carcinoid, GCC, mucocoele, mucinous cystadenoma, lymphoma, polypoid lesion, appendectomy, and appendicitis. The search included all articles from 2000 until November 2010. Patients who had undergone an operation for presumed acute appendicitis and had "unusual findings" pathology were included in the study, whereas articles that provided inconclusive information about patients and those in which the patients could not be reached were excluded. Additionally, appendicitis cases that developed due to foreign bodies were also excluded^[1-128].

RESULTS

In total, 5262 appendectomies were performed with a

Table 1 General characteristics of the 54 patients with abnormal pathological findings

Patients' characteristics	Results	Rate (%)
Age (yr) (range)	32.2 ± 15.1 (15-84)	
Sex		
Male	30	55.50
Female	24	44.50
WBC (K/UL) (range)	11.7 ± 4.9 (4.5-26.7)	
Histopathologic findings	54	
<i>E. vermicularis</i>	37	68.50
Tuberculosis	2	3.70
Carcinoid	5	9.20
Goblet-cell carcinoid	1	
Mucocoele	2	3.70
Mucinous cystadenoma	4	7.40
Eosinophilic infiltration	2	3.70
Neurogenic hyperplasia	1	
Follow-up (mo) (range)	10.4 ± 12.4 (1-54)	
Surgical Approach		
Appendectomy	52	96.30
Right hemicolectomy	2	3.70
Recurrence	0	

diagnosis of acute appendicitis at Diyarbakir Education and Research Hospital from January 2006 through October 2010. All patients were diagnosed clinically with acute appendicitis on the basis of physical and laboratory examinations. Of all appendectomies performed, 54 (1%) specimens revealed incidental abnormal histopathological diagnoses. The general characteristics of these 54 patients are summarized in Table 1. Thirty of the patients were male and 24 were female with ages ranging from 15 to 84 years (median, 32.2 ± 15.1 years). Thirty-seven of the 54 patients revealed *Enterobius vermicularis*, five a carcinoid tumor, six a mucinous cystadenoma (two were mucocoeles), two TB, and two eosinophilic infiltration, and two each were diagnosed with GCCs and neurogenic hyperplasia (Figure 1). While 52 patients underwent a standard appendectomy, two patients, who were preoperatively diagnosed with tuberculous appendicitis, had a right hemicolectomy. All patients with malignant tumors were diagnosed clinically with acute appendicitis, and none of them had symptoms of carcinoid syndrome or were preoperatively diagnosed with an appendicular tumor. After pathological confirmation of the diagnosis, the patients were referred to our clinic for staging. Staging included abdominal ultrasonography (US), computed tomography (CT), and 24-h urinary 5-hydroxyindoleacetic acid levels. After staging, all patients were followed up at the outpatient clinic every 3 mo for the first year. All patients with tumors were alive and disease-free during a mean follow-up of 17.8 mo. The clinicopathological characteristics of six patients with tumors are summarized in Table 2.

A histopathological examination of patients with *E. vermicularis* revealed 12 with acute inflammation and 25 with no evidence of any pathological change. After obtaining the pathology reports, the patients with oxyuris were prescribed a single oral dose of 100 mg mebendazole, which was repeated 7-10 d later. All patients with oxyuris were asymptomatic on follow-up (mean, 7.2 mo;

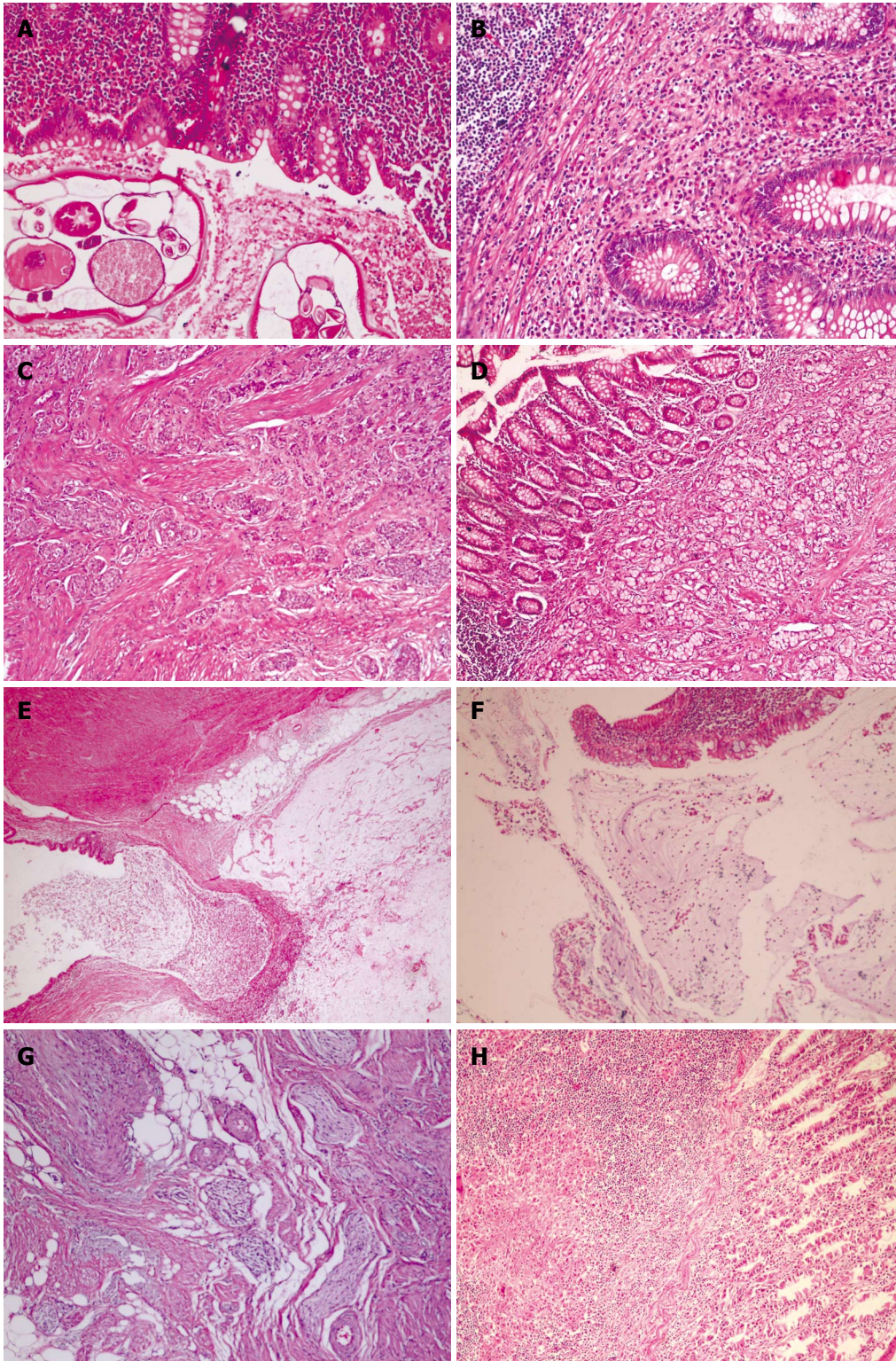


Figure 1 Unusual histopathologic findings. A: Adult of *E. Vermicularis* in appendices (HE, × 200); B: Eosinophilic appendicitis: diffuse eosinophilic infiltrate in lamina propria (HE, × 200); C: Carcinoid tumor of classic type is formed by solid nest of small monotonous cells with occasional acinar formation (HE, × 100); D: Microglandular goblet cell carcinoma. Acute appendicitis with a diffusely infiltrating goblet cell neoplasm. tumor cells infiltrated muscularis propria (HE, × 200); E: Mucosel. Dilatation of lumen by mucinous secretion, thin appendiceal wall. Mucin is protruding into surrounding fatty tissue (HE, × 40); F: Mucinous cystadenoma of appendix. Typical epithelium of a cystadenoma with pseudostratified, columnar cells containing elonged, crowded, hyperchromatic nuclei and scattered goblet cells with mucus in cavity (HE, × 100); G: Neurogenous hyperplasia of appendix. The proliferating spindle cells shown in this photography (HE, × 200); H: Tuberculous appendicitis. Granuloma which contain a caseating center surrounded by epithelioid cells, lymphocytes and histiocytes. A giant cell is present in the granuloma (HE, × 20).

range, 1-54 mo).

Two female patients (18 and 48 years old, respectively)

with tuberculous appendicitis received antitubercular therapy during the preoperative period. A right hemicolectomy was

Table 2 Clinicopathological characteristics of the six patients with primary appendicular tumors

Age	Sex	Tumor size (mm)	Location	Treatment	Pathology	Parietal spread	Follow-up (mo)
43	F	5	Distal	Appendectomy	Carcinoid	Serosa	54
42	F	10	Distal	Appendectomy	Carcinoid	Serosa	33
23	F	6	Distal	Appendectomy	Carcinoid	Subserosa	15
39	M	4	Distal	Appendectomy	Carcinoid	Submucosa	1
36	M	10	Distal	Appendectomy	Goblet cell	M.Proprio	3
26	M	6	Distal	Appendectomy	Carcinoid	Subserosa	1

Table 3 Distribution of the 1366 cases defined as “unusual findings” according to etiological causes

Total patients	1366 (1366/80698 = 1.7%)	
Unusual findings	1366	1.7%
<i>Enterobius vermicularis</i>	389	28.4%
Carcinoid	287	21.0%
Schistosomiasis	174	12.7%
Amoebic appendicitis	118	8.6%
Mucinous cystadenoma (+mucocoele)	72	5.2%
<i>Ascaris lumbricoides</i>	39	2.8%
Tuberculous appendicitis	34	2.5%
Endometriosis	41	3.0%
Goblet-cell carcinoid	28	2.0%
<i>Trichuris trichiura</i>	22	1.6%
Idiopathic granulomatous appendicitis	35	2.5%
Crohn disease	18	
Lymphoma	14	
Primary adenocarcinoma	11	
Mucinous cystadenocarcinoma	9	
Actinomycosis	8	
Melanosis	8	
Secondary adenocarcinoma	7	
Dysplastic change	7	
Villous adenoma	6	
Hyperplastic polyp	5	
Taeniasis	8	
GIST (+leiomyoma)	5	
<i>Balantidium coli</i>	3	
Tubulovillous adenoma	3	
Eosinophilic granuloma	3	
Neurogenic hyperplasia	2	
Tubular adenoma	2	
Leukaemia	4	
<i>Blastocystis hominis</i>	1	
Adenovirus	1	
<i>Strongyloides stercoralis</i>	1	
<i>Yersinia enterocolitica</i>	1	

performed in patients with an acute abdomen in the follow-up, considering the intraoperative findings. We have presented the details of these two cases in a previous article^[53].

Results of the literature review

Using the PubMed and Google Scholar databases, 128 studies published between January 2000 and November 2010 were compatible with our criteria. Fifty-one of these were written as original articles (50 retrospective and 1 prospective), 67 as case reports, eight as letters to the editor, and two as case series. When we looked at the countries in which the articles were prepared, 59 were from Europe, 40 from Asia, 19 from the Americas, six from Africa, and four were from Australia. In total, 80 698 cases

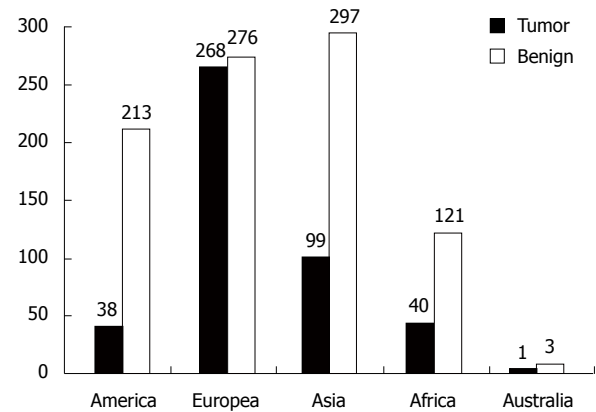


Figure 2 Worldwide distribution of the 1366 cases defined as “unusual findings”. Tumor: Carcinoid, goblet cell carcinoid, mucocoele, appendix adenocarcinoma, lymphoma, mucinous cystadenoma and adenocarcinoma, polypoid lesions, leukemia, gastrointestinal stromal tumor, dysplastic change; Benign: Non-tumoral causes.

were discussed in these articles, and all patients who were operated on had presumed acute appendicitis. Unusual findings were detected in 1366 (1.7%) of the cases with or without histopathologically acute appendicitis in their appendectomy specimens. We have summarized the causes that we qualified as “unusual findings in appendectomy specimens” in Table 3. As shown in the table, causes such as enterobiasis, schistosomiasis, amebiasis, and carcinoid tumor comprised 75.7% of all cases. The etiological (tumoral and non-tumoral causes) distribution of the 1366 patients by continent is summarized in Figure 2 to demonstrate the effects of geographic and sociocultural differences.

DISCUSSION

Acute appendicitis is the most common general surgical emergency, and obstruction of the appendiceal lumen seems to be essential for developing an appendiceal infection. Although fecaliths and lymphoid hyperplasia are the usual causes of the obstruction, some unusual factors could also be involved^[2,8,16,57].

Appendiceal tumors, occurring in less than 3% of all appendectomies, are rarely associated with clinical manifestations; they are frequently recognized either during an operation or the pathological examination. Malignant tumors of the appendix include carcinoids, GCCs, lymphomas, mucocoeles, primary adenocarcinomas, and mucinous cystadenocarcinomas. Benign tumors of the ap-

pendix consist of tubular adenomas, villous adenomas, leiomyomas, neuromas, and lipomas^[2,5,31].

An appendiceal carcinoid tumor is considered the most common type of appendiceal primary malignant lesion and accounts for almost 60% of all appendiceal tumors^[28]. An appendiceal carcinoid tumor is found in 0.3%-2.27% of patients undergoing an appendectomy. Characteristics of all appendiceal carcinoids predicting aggressive behavior include tumor size, histological subtype, and mesoappendiceal involvement. The tumors are smaller than 1 cm in 70%-95% of cases^[26,28]. The calculated risk of metastasis from tumors 1 cm or smaller is nearly zero and therefore may be managed with a simple appendectomy. An increase in metastasis risk of up to 85% occurs with a tumor of 2 cm or larger. An appendiceal carcinoid tumor larger than 2 cm should be managed with a formal right hemicolectomy^[1,3,5,6,9-13,16,26,28].

GCCs, also known as adenocarcinomas and first described by Gagne in 1969, are uncommon primary tumors of the vermiform appendix characterized by dual endocrine and glandular differentiation^[129]. Whether GCCs represent a morphological variant of appendiceal classical carcinoid or a mucin-producing adenocarcinoma is a matter of conjecture^[12]. GCCs account for 2% of primary appendiceal malignancies. Most tumors are less than 2 cm in diameter and 20% metastasize to the ovaries. Recent studies suggest that GCCs have biological and immunohistochemical profiles more similar to adenocarcinomas than to classical carcinoids, which may explain their aggressive behavior and therefore requirement for more extensive treatment^[12]. A right hemicolectomy is generally advised if any of the following features are present: tumors greater than 2 cm, involvement of resected margins greater than 2 mitoses/10 high-power fields, extension of the tumor beyond the serosa, lymphovascular invasion, or lymph node metastases^[5,12,21,25]. In our series, one patient had a GCC tumor located distally in the appendix that measured 1 cm in diameter. The patient was advised to undergo a right hemicolectomy, but he refused the procedure.

Mucinous cystadenoma is a rare tumor of the appendix associated with cystic dilatation, to which the more general term of mucocele has been applied. A mucocele of the appendix denotes an obstructive dilatation of the appendiceal lumen due to abnormal accumulation of mucus, which may be caused by a retention cyst, endometriosis, mucosal hyperplasia, cystadenoma, or a cystadenocarcinoma. The incidence of mucocele ranges from 0.2% to 0.3% of all appendectomy specimens. Mucoceles are often asymptomatic and discovered as incidental findings at appendectomy, or during laparotomy for another indication or at histological examination of an operative specimen. However, mucoceles may be diagnosed clinically from features of acute appendicitis. Appendectomy is the standard of care for mucinous cystadenoma, whereas a cystadenocarcinoma requires a right hemicolectomy. Because of the high association of mucinous cystadenoma with colon and ovarian malignancy, follow-up CT, US, and colonoscopy examinations must be performed during the postoperative period^[1-3,5,16,25,27,31,52,106,107].

Mucinous cystadenocarcinoma of the appendix, also known as a mucinous adenocarcinoma or malignant mucocele, constitutes a rare malignancy of the appendix and is often associated with a second malignancy of the gastrointestinal (GI) tract. The most common type of presentation is that of acute appendicitis. The diagnosis of mucinous adenocarcinoma of the appendix is usually given after an appendectomy, or other explorative surgical procedure, and consequent pathological evaluation of the appendiceal specimen^[5,25,31,108].

Primary adenocarcinoma of the appendix is an extraordinarily rare tumor, and its incidence was 0.01% (11 of 80 698 cases) in our literature review. Adenocarcinomas behave aggressively and in a fashion similar to that of colonic adenocarcinomas, so in the case of an appendicular adenocarcinoma, oncologic resection with right hemicolectomy is the treatment of choice^[2,16,23,31,105].

The GI tract is the most common site for extranodal lymphomas and accounts for 30%-45% of all extranodal cases. The stomach is the most commonly involved organ followed by the small intestine, colon, and esophagus. The incidence of primary appendiceal lymphoma has been estimated at 0.015%-0.022% of all appendiceal specimens. An appendiceal lymphoma usually presents in the second and third decades of life, usually manifests as acute appendicitis, and is often diagnosed postoperatively by histopathology. Therapy guidelines for primary appendiceal lymphomas are unclear because of their rarity. Our literature review revealed 14 lymphoma cases with clinical evidence of acute appendicitis; 12 of these were of B-cell origin, whereas two were of T-cell origin^[2,8,97-101,109-112].

Leukemia can involve the GI tract but rarely involves the appendix. Although appendicitis is a known complication in patients with leukemia, leukemic cell involvement in the appendix is extremely rare. When the leukemia involves soft tissue including the appendix, it is called granulocytic sarcoma. The incidence of leukemic appendicitis was 0.005% (4 of 80 698 cases) in our literature review. Surgical management of patients with leukemia and acute abdomen has not been advocated because of the high rate of operative mortality. However, some support exists for surgically managing appendicitis as the most effective method of therapy in acute leukemia cases. Systemic chemotherapy is necessary prior to additional surgery in patients with leukemia^[113-115].

GISTs, which occur most commonly in the stomach (60%) and the small bowel (30%), are the most common primary mesenchymal neoplasms of the GI tract. GISTs, known as leiomyoma or leiomyosarcoma before 1983, primary to the vermiform appendix are exceptionally rare, with only eight cases reported so far^[2,71,72,103]. Five out of eight patients were operated due to acute appendicitis symptoms. The size of the mass and degree of mitotic activity play a crucial role in tumor behavior and recurrence development. Therefore, when approaching the appendix for GIST tumors, tumor location should be evaluated along with tumor size and mitotic activity.

Enterobius vermicularis, also known as pinworm or oxyuris, is a widespread parasitic infection estimated to

affect up to 200 million people worldwide. The association of oxyuris and appendicitis was first made in the late 19 century, when Still initially documented this organism in the appendix lumen. While the reported incidence of pinworm in appendectomy specimens of patients with presumed appendicitis ranged from 0.2% to 41.8%, the reported rates of inflammation in specimens from appendices infested with pinworm ranged from 13% to 37%^[4,7,14,29]. Patients must receive antihelminthic treatment because the appendectomy treats only the consequence and not the cause of the disease. An *E. vermicularis* infestation is treated with an oral dose of mebendazole, which is repeated in 1-2 wk^[1,2,4,7,11,14,16-20,22,29,52,57,92,93].

TB may affect all tissues and organs in the body, but it most frequently involves the lungs. The GI system is ranked sixth among all extrapulmonary involvements. TB may affect all of the segments of the GI system, from the mouth to anus. However, the ileum and ileocecal region are the sites most commonly involved, followed by the colon and vermiform appendix. The appendix may be affected secondarily to ileocecal TB, but appendicular TB may occur in an even rarer primary form without any evidence of the disease elsewhere. The reported incidence of appendicular TB varies from 0.1% to 3.0% among all appendectomies performed. An accurate diagnosis is usually established after histopathological examination of a specimen. Classic histopathological analysis of an appendectomy specimen usually reveals the presence of caseating granulomas and Langhans giant cells, suggesting TB of the appendix. Although some studies have reported that treatment is not necessary for the primary disease and that appendectomy alone is sufficient, no consensus has been reached. When we reviewed the literature, 34 cases of patients undergoing an appendectomy with presumed appendicitis have been published in the last decade, including our own two cases^[23,32,53-55,57].

Actinomycosis is an uncommon chronic infectious disease. Common sites of involvement include the cervicofacial, thoracic, and abdominopelvic regions. In abdominal actinomycosis, the ileocecal region including the appendix is the most commonly involved site. A correct diagnosis can be made by culture or histopathological examination, although a definitive diagnosis of actinomycosis requires microscopic proof of either the pathogen itself or the presence of specific sulfur granules. After the diagnosis has been confirmed, the general therapeutic recommendation is to initiate treatment with intravenous antibiotic therapy for 2-12 mo. Eight cases of patients undergoing an appendectomy with presumed appendicitis have been published in the last decade^[32-38].

Taeniasis, a well-known worm infection, is characterized by the presence of the helminth in the intestine. Infection is generally recognized when a segment of the parasite appears in the stool. The occurrence of *Taenia spp.* in the appendix is so rare that the situation invites a case report. In our literature review, *Taenia* was found in only five of the cases operated on for presumed acute appendicitis. In cases of taeniasis, specific species identification is not required for treatment, as patients are treated with a

single dose of praziquantel^[14,18,93,127,128].

Amebiasis is an infection of the large intestine caused by *Entamoeba histolytica*, which affects 10% of the world population and has a worldwide distribution. This parasite is occasionally found in the appendix, usually in the lumen without accompanying inflammation, but is rarely associated with acute appendicitis. A preoperative diagnosis of amebic appendicitis is almost impossible because no clinical features or diagnostic laboratory tests distinguish amebic from bacterial appendicitis, other than a stool examination. The clinical picture presented in this report represents a typical case of amebic appendicitis with a good outcome after surgical resection and treatment with metronidazole^[84,85,87-93].

Schistosomiasis, also known as bilharziasis and most commonly caused by *Schistosoma haematobium*, only rarely leads to appendicitis, even in nations in which schistosomiasis is endemic. The pathogenesis is most probably due to a periappendicular granulomatous reaction of the host against the schistosome. Inflammation and repair causes scarring and strictural deformation of the appendiceal wall, leading to luminal obstruction and acute appendicitis. Histologically, appendices may show transmural inflammation rich in eosinophils, with a granulomatous reaction to ova. Treatment for schistosomal appendicitis consists of an appendectomy and administration of praziquantel^[2,8,13,16,32,40-52,57,93,125,126].

Ascaris lumbricoides, also known as roundworm, is one of the most common human helminthic diseases worldwide. The highest prevalence of ascariasis occurs in tropical and semitropical countries. The domain of the worm extends from the stomach to the ileocecal valve; 99% of worms inhabit the jejunum and proximal ileum, and it is rarely seen in the appendix. Appendicitis due to migration of roundworm into the appendix is still debatable because the symptoms of this migration may simulate appendicitis but rarely cause it^[57,92,94].

Because parasites such as *Balantidium coli*^[2,92], *Blastocystis hominis*^[20], *Trichuris trichiura*^[52,57,92], and *Strongyloides stercoralis*^[121] have few causative roles, interpreting their pathogenesis is difficult. A final diagnosis should be established with a histopathological evaluation of all three parasites, and antihelminthic treatment should be administered after the appendectomy.

Endometriosis is defined as the presence of ectopic endometrial tissue outside the lining of the uterine cavity. Many women of reproductive age suffer from this disease, but its occurrence in the GI tract is rare. Intestinal endometriosis is classified as external endometriosis and occurs in only about 10% of women with endometriosis. Most intestinal endometriosis occurs in the rectum and sigmoid colon but rarely in the appendix. Appendiceal endometriosis is usually asymptomatic, but it occasionally causes appendicitis, perforation, and intussusception. The diagnosis of appendiceal endometriosis is based on the histological presence of endometrial tissue in the specimen. The treatment strategy consists mainly of surgery and hormone therapy^[1,16,27,57-69,102].

The incidence of granulomatous appendicitis (GA),

a rare condition that may be discovered incidentally in a patient with a clinical presentation of acute appendicitis, ranges from 0.31% to 0.95%. Various infectious and non-infectious factors cause GA. Systemic conditions, such as Crohn's disease and sarcoidosis, may also be associated with granulomatous inflammation of the appendix. The initial belief that it represented a manifestation of Crohn's disease is incorrect in the great majority of cases, as only 5%-10% of patients with GA develop Crohn's disease elsewhere in their GI tract. Distinguishing idiopathic granulomatous appendicitis from early Crohn's disease, which affects only the appendix, is difficult. A definitive diagnosis can only be made after long-term follow-up, and sometimes further investigations are required^[31,52,116-120,122,123].

Crohn's disease is a chronic transmural inflammation characterized by epithelioid granulation formation in the intestinal wall. The clinical presentation is always variable, and patients often present with findings consistent with acute appendicitis such as right-lower quadrant pain, fever, nausea, and anorexia. The diagnosis of appendiceal Crohn's disease requires exclusion of multiple entities. Infectious causes of granulomatous appendicitis include *Yersinia*, *Mycobacterium tuberculosis*, blastomycosis, *Schistosoma*, *Actinomyces*, *Campylobacter*, *Histoplasma capsulatum*, and some parasites. An appendectomy is a routine surgical procedure when the Crohn's disease is limited to the appendix with no postoperative or intraoperative mortality and a low rate of fistula formation^[16,32,123].

In summary, although fecaliths and lymphoid hyperplasia are the usual causes of acute appendicitis, some unusual factors may also cause appendicitis. The most common unusual findings in appendectomy specimens are parasites and benign or malignant tumors. A simple appendectomy or right hemicolectomy can be performed depending on the localization, size, and histopathological structure of the tumor in the primary malignant appendiceal tumor, whereas an appendectomy alone is sufficient for benign tumors. Administering the appropriate antibacterial or antiparasitic treatment after the appendectomy is the proper approach for parasitic and bacterial infections that cause chronic inflammation. We emphasize and strongly recommend that all appendectomy specimens be examined histopathologically regardless of whether the specimens are macroscopically normal.

COMMENTS

Background

Appendicitis is one of most common acute surgical conditions of the abdomen, and an appendectomy is one of the most frequently performed operations worldwide. Obstruction of the lumen is the dominant factor in acute appendicitis, and although fecoliths and lymphoid hyperplasia are the usual causes of obstructions, some unusual factors could also be involved.

Research frontiers

The authors conducted a literature review via the PubMed and Google Scholar databases of English language studies published between 2000 and 2010 on unusual findings in appendectomy specimens. Also, we presented 54 patients who had unusual findings in their appendectomy specimens.

Innovations and breakthroughs

The authors emphasize and strongly recommend that all appendectomy

specimens be examined histopathologically regardless of whether the specimens are macroscopically normal.

Peer review

This is a very interesting paper. It will be cited many times in the future and this is good for our journal.

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Assay of ghrelin concentration in infant formulas and breast milk

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raises diverse questions regarding the uptake, absorption and metabolic effects of this hormone.

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Abstract

AIM: To test if total ghrelin is present in infant formulas.

METHODS: Using a radioimmunoassay, we measured total ghrelin concentrations in 19 samples of commercial infant formulas and in 20 samples of human milk. We also determined ghrelin concentration in the serum of infants and lactating mothers.

RESULTS: Ghrelin concentrations were significantly higher in artificial milk (2007.1 ± 1725.36 pg/mL) than in human milk (828.17 ± 323.32 pg/mL) ($P = 0.005$). The mean ghrelin concentration in infant serum ($n = 56$) was 1115.86 ± 42.89 pg/mL, and was significantly higher ($P = 0.023$) in formula-fed infants (1247.93 ± 328.07 pg/mL) than in breast-fed infants (1045.7 ± 263.38 pg/mL). The mean serum ghrelin concentration (mean \pm SD) in lactating mothers ($n = 20$) was 1319.18 ± 140.18 pg/mL.

CONCLUSION: This study provides evidence that total ghrelin is present in infant formulas. This finding

INTRODUCTION

It is now well established that early life nutrition plays an important role in long-term appetite control^[1]. Indeed, neonatal nutrition is involved in the programming of feeding regulatory mechanisms in the central nervous system, and in those mediated by factors secreted from peripheral tissues. Besides peripheral circulating factors that regulate energy balance and adiposity, gastrointestinal peptides have been demonstrated to act as hunger signals. Among the known orexigenic peptides, ghrelin has been found to be the most powerful^[2].

Ghrelin is involved in the short-term regulation of food intake, by stimulating appetite, and in the long-term regulation of weight and energy metabolism, by inducing adiposity^[3]. It is released in a pulsatile manner, with a nocturnal peak. Ghrelin responds to meals, increasing 1-2 h before eating and returning to trough levels 1-2 h after a meal^[4,5]. Ghrelin secretion increases under negative energy-balance

conditions, and decreases under positive energy-balance conditions, such as food intake and obesity^[6,7]. It is one of the most powerful orexigenic and lipogenic hormones and represents an interface between energy balance regulation, glucose homeostasis and hypothalamic neuropeptides^[8]. The amino acid sequence of ghrelin is highly conserved throughout mammalian species^[9]. Ghrelin has been found in human milk, but the source of the hormone is unclear. Aydin *et al*^[10] reported that its levels in colostrum, transitional and mature milk were lower than those found in plasma and they assumed that ghrelin present in milk probably comes from the plasma of lactating mothers. In contrast, Kierson *et al*^[11] showed that ghrelin levels in breast milk are higher than plasma levels and identified ghrelin mRNA from human mammary epithelial cells and mammary gland. Based on these findings these authors suggested that ghrelin in breast milk is probably synthesized and secreted from the breast. The identification of ghrelin in breast milk suggests that breast milk is a source of compounds critical for the metabolic development of infants^[12,13].

Early feeding mode affects growth and body composition^[14]. Breast-fed (BF) and formula-fed (FF) infants have similar weight gains in the first three months of life, however, BF infants gain weight less rapidly during the following months of the first year^[15]. BF and FF infants have different feeding behaviors: FF infants eat less frequently and consume higher amounts of food than BF infants^[16,17]. We previously reported that serum ghrelin concentrations were higher in infants exclusively FF than in infants exclusively BF^[18]. Recently, serum ghrelin values were positively correlated with fasting times only in FF infants^[19].

The aim of this study was to investigate whether infant formulas contain ghrelin.

MATERIALS AND METHODS

In this study, we enrolled 56 infants aged from 11 d to 5 mo (mean \pm SD: 81 \pm 46 d) born from a normal spontaneous vaginal delivery, consecutively referred to the Department of Paediatrics of the University of Turin, Regina Margherita Children's Hospital. The inclusion criteria for infants were gestational age between 37 and 42 wk, birth weight appropriate for gestational age (between 2500 and 4000 g), Apgar score higher than 7 at 5 min, no fetal anomaly, absence of acute or chronic gastroenteric diseases or other growth-affecting pathologies.

We collected milk samples from 20 lactating mothers of the infants enrolled. Eligibility criteria for mothers were: no maternal medical complications, non-smoking mothers, normal response to a glucose tolerance test, no mastitis, no prescribed medication, no digestive disorders.

Appropriate Ethics Committee permission was obtained and each parent signed a written informed consent. Milk samples (2 mL) were collected from the lactating women ($n = 20$) before breakfast at around 09:00 h. Samples of 19 infant formulas were collected: 9 starting formulas, 6 follow-on formulas and 4 special formulas (anti-regurgitation and hydrolyzed casein protein formulas). We

also obtained unpasteurized milk samples (2 mL) from 5 dairy cows. Breast, cow and artificial milk were separated by centrifuging the samples twice at 2000 r/min and 4°C for 20 min. After the first centrifugation, the thick fat layer at the top of the tube, which could interfere with detection of the hormone, was removed with a sterile toothpick. We collected a venous blood sample from 56 infants (37 BF infants and 19 FF infants) after a fast of 3 h. We also collected venous blood samples from 20 lactating mothers. Blood samples were immediately centrifuged at 4000 r/min and 4°C for 10 min and each sample of the resulting serum was divided in 3 tubes and these were stored at -30°C until analysed.

Hormone assays

Serum and milk total ghrelin were assayed by radioimmunoassay using a commercial kit (Ghrelin (total) RIA 3967, DRG Diagnostic, New Jersey, USA) according to the manufacturer's instructions (using a polyclonal antibody that recognizes octanoylated and non-octanoylated ghrelin with I¹²⁵ ghrelin as a tracer molecule). Measurements of total ghrelin in mature milk and serum samples have been validated as reported elsewhere^[10]. The intra-assay and inter-assay coefficients of variation of ghrelin were 5% and 7.6%, respectively. The lowest level of ghrelin that can be detected by this assay is 93 pg/mL with a 100- μ L sample size. The specificity for human ghrelin is 100%. The limit of linearity for the ghrelin assay is 6000 pg/mL (any result greater than 6000 pg/mL was repeated on dilution using Assay Buffer as a diluent). Spike and Recovery of ghrelin in human plasma are shown in Table 1. As the kit is designed for human samples, a validation process was undertaken for use with bovine milk. We obtained 3 measurements of the hormone for each blood sample and milk sample. The manufacturer's protocol was followed and standard validations, including parallelism and recovery, conducted.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

A normal distribution was verified with the Shapiro-Wilk test ($P > 0.05$). The data are expressed as arithmetic means \pm SD; $P < 0.05$ was considered statistically significant. Differences in ghrelin concentrations between human milk and formula milk were determined by the Student's *t* test.

RESULTS

As shown in Table 2, the mean \pm SD of ghrelin concentration in human milk was 828 \pm 323 pg/mL. Ghrelin levels varied widely in infant formulas (from 300 to 6110 pg/mL; mean: 2007 \pm 1725 pg/mL). The mean ghrelin concentration in starting formulas and in follow-on formulas was 2699 \pm 233.5 pg/mL and 2561 \pm 215.2 pg/mL, respectively, whereas the mean ghrelin concentration was 1160 \pm 340 pg/mL in special formulas (Table 3). Ghrelin

Table 1 Spike and recovery of ghrelin in human plasma (mean of the observed levels from 3 duplicate determinations in 3 separate assays)

Sample No.	Ghrelin added ¹ (pg/mL)	Recovery (%)
1	500	96
2	1000	90
3	2000	91

¹Different concentrations of human ghrelin were added to 3 different human plasma samples and the ghrelin content was determined by RIA. Percent recovery was calculated on the observed *vs* expected.

Table 2 Ghrelin concentrations in infant formulas, in mother's milk, in cow's milk and in serum of lactating mothers, breast-fed infants and formula-fed infants

	Ghrelin concentration (pg/mL \pm SD)
Milk	
Different kinds of artificial infant formula (<i>n</i> = 19)	2007.1 \pm 1725.36 ^a
Breast milk (<i>n</i> = 20)	828.17 \pm 323.32 ^a
Non pasteurized cow milk (<i>n</i> = 5)	2816.00 \pm 219.00
Serum	
Lactating mothers (<i>n</i> = 20)	1319.18 \pm 140.18
Breast-fed infants (<i>n</i> = 37)	1045.7 \pm 263.38 ^b
Formula-fed infants (<i>n</i> = 19)	1247.93 \pm 328.07 ^b

^a*P* = 0.005, infant formulas *vs* human milk, student's *t* test; ^b*P* = 0.023, breast-fed infants *vs* formula-fed infants, student's *t* test.

levels were significantly higher in infant formulas than in human milk (*P* = 0.005). The mean ghrelin concentration in unpasteurized cow milk was 2816 \pm 219 pg/mL. The mean ghrelin concentration in infant serum was 1115.86 \pm 42.89 pg/mL, and was significantly higher (*P* = 0.023) in FF infants (1247.93 \pm 328.07 pg/mL) than in BF infants (1045.7 \pm 263.38 pg/mL). The mean serum ghrelin concentration (mean \pm SD) in lactating mothers was 1319.18 \pm 140.18 pg/mL.

DISCUSSION

In this study, we showed that artificial milk contains the orexigenic hormone, ghrelin, and that its concentration was higher in infant formulas than in human milk. This finding might explain the higher serum levels we previously observed in FF infants *vs* BF infants, which was also confirmed in the present research^[18,19].

If FF infants receive a higher amount of ghrelin, it is conceivable that they have a greater feeding stimulus than BF infants, and a consequent increase in weight and growth rate. Our observations could explain the more appropriate growth curves of BF infants, who physiologically receive less ghrelin^[20]. Therefore, breast-feeding may protect against the development of obesity in childhood and adulthood, not only because of its nutrient composition, but also because of the presence of bioactive factors such as ghrelin, leptin and adiponectin^[12,21].

Table 3 Ghrelin concentrations in three different types of artificial infant formula

Type of infant formula	Ghrelin concentration (pg/mL \pm SD)
Starting infant formulas (<i>n</i> = 9)	2699 \pm 233.5
Follow-on infant formulas (<i>n</i> = 6)	2561 \pm 215.2
Special infant formulas (anti-regurgitation and hydrolyzed casein protein formulas) (<i>n</i> = 4)	1160 \pm 340

Several assays are available to measure human serum ghrelin, whereas there is no commercial milk ghrelin assay kit. Consequently, we carried out a validation process using a basic clinical chemistry method (linearity)^[10]. Similarly, there is no assay kit specific for bovine ghrelin; however, given the high structural homology between human ghrelin and mammalian ghrelin, the kit used in our study to detect ghrelin in infant formulas can be considered reliable^[22].

The ghrelin concentrations reported in our study refer to the final volume after centrifugation, because the RIA kit instructions indicate that samples with high lipidemia be avoided. After centrifugation, we removed the supernatant and the fat layer that could interfere with detection of the hormone. This method has been used in previous studies^[10]. Kiersen *et al*^[11] reported higher ghrelin levels in whole milk than skimmed milk, with a direct relationship between estimated milk fat content and ghrelin levels. Therefore, it is possible that ghrelin levels detected in milk after centrifugation are lower than those present in whole milk and consumed by the infants.

The mechanism by which ghrelin influences growth in early infancy is not yet completely known. Ghrelin levels are higher in small-for-gestational-age (SGA) newborns than in adequate-for-gestational age (AGA) newborns^[23]. Reduced ghrelin suppression and higher postprandial ghrelin concentrations in SGA infants could cause a sustained orexigenic drive and may contribute to catch-up growth in these infants. Kitamura *et al*^[24] reported high ghrelin levels during the early neonatal period. Onal *et al*^[25] observed that plasma ghrelin concentration was inversely associated with birth weight and body length in term newborns. We previously observed a negative correlation between ghrelin concentration and weight gain in BF infants in the first months of life, which suggests that ghrelin may play a role in body weight regulation in healthy infants^[26,27]. More recently, we found a positive correlation between circulating ghrelin concentration and fasting time in FF infants; these infants have a higher serum ghrelin concentration, longer fasting time and fewer meals than BF infants^[19]. Clearly, we need to learn more about early feeding and the mechanisms regulating satiety and feeding behavior.

Finally, it should be noted that, similar to previous studies^[11,28], we measured total milk ghrelin level. Deacyl ghrelin influences food intake, gut motility, insulin secretion and resistance and adipogenesis, whereas the acylated form of ghrelin, known as active ghrelin, is essential for binding to the growth hormone secretagogue receptor 1a^[29]. Additional studies are needed to evaluate active ghre-

lin in infant formulas.

There have been some studies conducted on the effects of ghrelin administration (i.e. in anorexia, cachexia), however, the hormone was administered intravenously and not orally^[30]. More recently, animal studies have investigated the effects of oral administration of ghrelin receptor agonist, and found that it survives the acid environment of the stomach and could exert some of its biological functions^[31,32].

In conclusion, specific research is needed to understand the origin of ghrelin found in infant formulas. Furthermore, it would be interesting to determine whether ghrelin present in infant formulas survives the acid environment of the stomach in humans, exerts biological activity through receptors present in the gastrointestinal tract of newborns and whether the higher ghrelin concentration in infant formulas result in a higher serum concentration in FF infants. Lastly, investigations are required to determine whether these higher levels of ghrelin affect feeding habits and thus obesity in later life.

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COMMENTS

Background

Current research highlights the importance of early life nutrition in long-term appetite control, with consequent programming of regulatory mechanisms. Ghrelin is a recently discovered hormone involved both in the short-term regulation of food intake, by stimulating appetite, and in the long-term regulation of weight and energy metabolism, by inducing adiposity.

Research frontiers

Ghrelin has recently been detected in breast milk, but data on ghrelin in infant formula are lacking. Using a radioimmunoassay, the authors measured ghrelin concentrations in commercial infant formulas versus concentrations in human milk. Surprisingly, ghrelin was significantly higher in artificial formulas. This finding raises diverse questions.

Innovations and breakthroughs

Little is known about ghrelin regulation, especially in early infancy. Breast milk contains ghrelin, but it was not known whether infant formulas contain ghrelin. The finding that infant formulas do indeed contain ghrelin, and at levels higher than those found in breast milk, raises questions about the uptake, absorption and metabolic effects of this feeding stimulus and growth rate of artificially fed infants. Further research is needed to determine whether the higher levels of ghrelin in formulas could affect infant feeding habits and thus obesity in later life.

Applications

The higher ghrelin levels found in artificial milk in the present study might explain the higher serum values the authors recently observed in formula-fed infants compared with breast-fed infants. Thus, if artificially fed infants receive a higher amount of ghrelin together with a higher intake of protein, it is conceivable that formula-fed infants have a greater feeding stimulus with a consequent increase in weight and growth rate.

Terminology

Ghrelin is involved in the short-term regulation of food intake, with an orexigenic action, and in the long-term regulation of weight and energy metabolism, by inducing adiposity.

Peer review

The manuscript by Savino *et al* provides original data on the presence of the

orexigenic hormone ghrelin cow's milk formulas. The authors suggest that the high levels of hormone present in formulas can influence the feeding habits of formula feed subjects in infancy and also later and can be responsible for the high risk of obesity observed in formula fed infants as compared to breast fed ones. The article could be of some general interest, both for pediatricians and for nutritionists.

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Factors associated with irritable bowel syndrome symptoms in hemodialysis patients

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METHODS: This was a cross-sectional study. A questionnaire based on the Bowel Disease Questionnaire that records gastrointestinal symptoms was given to 294 patients in 4 dialysis centers. A total of 196 (67%) subjects returned the survey. A multivariable logistic regression model was used to identify factors significantly associated with IBS symptoms.

RESULTS: Symptoms compatible with IBS were present in 27 (13.8%) subjects and independently associated with low post-dialysis serum potassium [OR = 0.258, 95% CI (0.075-0.891), $P = 0.032$], paracetamol use [OR = 3.159, 95% CI (1.214-8.220), $P = 0.018$], and Kidney Disease Quality of Life (KDQOL) cognitive function score [OR = 0.977, 95% CI (0.956-0.999), $P = 0.042$]. Univariate regressions were also performed and the reported significance is for multivariate analysis. No association was detected for age, gender, depressed mood, smoking (present or past), body mass index, albumin level, Kt/V, sodium pre- or post-dialysis level, change in potassium level during HD, proton pump inhibitor or H2 blocker use, aspirin use, residual diuresis, hepatitis B or C infection, diabetes mellitus, marital status and education level.

CONCLUSION: This study examined potential risk factors for symptoms compatible with IBS in HD patients and identified an association with paracetamol use, post-dialysis potassium level and KDQOL-cognitive function score.

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Key words: Hemodialysis; Irritable bowel syndrome; Risk factors

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Abstract

AIM: To investigate clinical characteristics associated with the presence of irritable bowel syndrome (IBS) symptoms in hemodialysis (HD) patients.

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INTRODUCTION

Chronic gastrointestinal symptoms are common in patients with chronic kidney disease (CKD). The prevalence rate is reportedly as high as 70%^[1-4], and there is an association with impaired psychological well-being^[5]. Among these gastrointestinal symptoms, irritable bowel syndrome (IBS) is also more frequent than in the general population, and is present in 11%-44% of hemodialysis (HD) patients^[2-4]. Although the pathophysiology of IBS is uncertain, altered gut reactivity (motility, secretion), visceral hypersensitivity and dysregulation of the brain-gut axis are believed to play an important role^[6]. The risk factors associated with IBS in HD patients are not known. The aim of this study was to determine the possible relationship between IBS symptoms in HD patients and their clinical characteristics.

MATERIALS AND METHODS

Patients

This was a cross-sectional study. All patients in 4 HD centers (2 state financed and 2 privately owned) were asked to complete a questionnaire. The questionnaires were given to patients during a planned hemodialysis procedure. The study was approved by the local Ethics Committee.

All of the subjects were Caucasian. They were dialyzed 3 times a week for 180 to 300 min using either polysulfone or cellulose acetate dialyzers and bicarbonate dialysis fluid containing 2 mEq/L of potassium in 3 centers and 2 or 3 mEq/L (adjusted on the basis of the knowledge of prevailing pre-dialysis serum potassium levels in a given individual) in 1 center.

Causes of ESRD were as follows: glomerulonephritis, $n = 60$ (30.6%); diabetic nephropathy, $n = 32$ (16.3%); amyloidosis, $n = 11$ (5.6%); polycystic kidneys, $n = 22$ (11.2%); hypertension/atherosclerosis, $n = 16$ (8.2%); tubulointerstitial disease, $n = 39$ (19.9%); unknown/uncertain, $n = 15$ (7.7%); nephrectomy, $n = 1$ (0.5%).

Questionnaire

A questionnaire based on the Bowel Disease Questionnaire was used^[7]. It was translated to the Polish language by 2 of the authors (BF, AR). Translations were compared and discrepancies reconciled. The resulting translation was then tested in 20 randomly selected dialysis patients, and as a result some of the expressions in the translation were altered to make them easier to understand. The question-

naire was then checked by a person who was not involved in translation (ARR), and finally evaluated by a certified gastroenterologist (GR).

IBS was defined using Manning criteria^[8] as described by Talley *et al.*^[9], as an ache or pain that occurred more than 6 times per year which was either often made better by a bowel movement or often associated with more frequent or looser bowel movements when the pain began. In addition, 2 or more of the following symptoms had to be present: fewer than 3 bowel movements per week or more than 3 bowel movements per day; loose, watery stools or hard stools; straining to have bowel movements; feelings of incomplete rectal evacuation; urgency; mucus; or bloating with distention.

Additionally, we included questions taken from the validated Polish translation of Kidney Disease Quality of Life (KDQOL) questionnaire, related to depressed mood and cognitive function^[10]. Depressed mood was measured by the following KDQOL items: How much of the time during the last 30 d have you felt so down in the dumps that nothing could cheer you up? and How much of the time during the last 30 d have you felt downhearted and blue? The six possible responses to these questions were (1) none of the time; (2) a little of the time; (3) some of the time; (4) a good bit of the time; (5) most of the time; and (6) all of the time. Patients were classified as reporting depressed mood when they indicated that they had felt down in the dumps or felt downhearted and blue a good bit of the time or more often^[11].

Cognitive function was measured by the KDQOL-CF score. Patients had to answer the following questions: During the past 4 wk, did you react slowly to things that were said or done? Did you have difficulty concentrating or thinking? Did you become confused? Responses on a six-point scale were weighted and transformed to a score ranging from 0 to 100, with higher scores indicating better self-assessed cognitive function^[12].

Relevant laboratory and clinical data were extracted from medical records. Data corresponding closest to the date of the HD session during which the questionnaire was distributed, were used. We allowed for a time span of 14 d before and after HD.

Statistical analysis

Results are expressed as means \pm SD or frequency. Variables were tested for normality of distribution using the Wilk-Shapiro test. The Fisher's exact test and χ^2 test were used for comparing categorical variables, as appropriate.

Univariate and multivariable logistic regression was used to identify patient characteristics associated with IBS compatible symptoms. Risk factors considered in this analysis included age, sex, education level, marital status, presence of diabetes mellitus, procedure, hemoglobin level, pre- and post-HD potassium level, change in potassium level during HD, use of paracetamol in the last year, KDQOL-CF score, depressed mood, smoking

Table 1 Patients' demographic and clinical data expressed as (mean \pm SD) *n* (%)

Group	IBS symptoms		All	<i>P</i> value
	(+)	(-)		
<i>n</i>	27	169	196	
Gender (M/F)	13/14	105/64	118 / 78	0.168
Age (yr)	68.1 \pm 11.5	63.2 \pm 13.4	63.9 \pm 13.2	0.073
Dialysis duration (min)	40.1 \pm 36.9	38.6 \pm 45.8	38.8 \pm 44.6	0.874
BMI (kg / m ²)	25.0 \pm 3.7	24.9 \pm 4.9	24.9 \pm 4.7	0.897
Residual diuresis (mL/24 h)	301 \pm 559	401 \pm 592	388 \pm 42	0.411
Kt/V	1.26 \pm 0.31	1.21 \pm 0.27	1.22 \pm 0.28	0.355
Hepatitis C or B infection (<i>n</i>)	7 (25.9%)	31 (18.3%)	38 (19.4%)	0.430
Hemoglobin (g/dL)	11.4 \pm 1.4	10.7 \pm 1.5	10.8 \pm 1.5	0.026 ^a
Albumin (g/dL)	3.72 \pm 0.40	3.71 \pm 0.44	3.71 \pm 0.44	0.857
Smoking (<i>n</i>)	7 (25.9%)	23 (13.6%)	30 (15.3%)	0.144

IBS: Irritable bowel syndrome; BMI: Body mass index. ^a*P* < 0.05.

(present or past), body mass index (BMI), albumin level, Kt/V, sodium pre- and post-dialysis level, proton pump inhibitor (PPI) or H2 blocker use, aspirin and paracetamol use, residual diuresis, hepatitis B or C infection. Variables were included in the multivariable logistic model if *P* < 0.10 in the univariate analysis. A *P* value less than 0.05 was considered statistically significant. The software, used for statistical computations was Stata 9.2 (StataCorp, College Station, TX, USA).

RESULTS

Patients

A total of 294 HD patients were asked to complete the questionnaire, of which 196 were returned giving a 67% response rate. All the responders completed the questionnaires by themselves. Their clinical characteristics are given in Table 1.

IBS symptoms

Symptoms compatible with IBS were present in 27 (13.8%) subjects. They were more common in women (18.0%) than in men (11.0%), but the difference was not statistically significant (*P* = 0.168). Symptoms of IBS were more frequent in patients with a post-hemodialysis potassium level \leq 3.5 mEq/L than in subjects with potassium > 3.5 mEq/L. Also pre-dialysis potassium level was related to the frequency of IBS symptoms (Figure 1).

In univariate logistic regression, pre-dialysis serum potassium [OR = 0.462, 95% CI (0.222-0.965), *P* = 0.040], post-dialysis serum potassium [OR = 0.237, 95% CI (0.084-0.666), *P* = 0.006], hemoglobin level [OR = 1.403, 95% CI (1.038-1.897), *P* = 0.028], use of paracetamol in the last year [OR = 3.541, 95% CI (1.499-8.364), *P* = 0.004], and KDQOL-CF score [OR = 0.972, 95% CI (0.954-0.991), *P* = 0.004] were associated with IBS symptoms. Age (*P* = 0.076), gender (*P* = 0.172), depressed mood (*P* = 0.118), smoking (present or past) (*P* = 0.105), BMI (*P* = 0.896),

Table 2 Multiple logistic regression analysis to identify independent predictors of irritable bowel syndrome symptoms in hemodialysis patients

Variable	<i>b</i> coefficient (SE)	<i>P</i> value	OR (95% CI)
Potassium level pre-HD	-0.325 \pm 0.437	0.457	0.723 (0.307-1.703)
Potassium level post-HD	-1.356 \pm 0.633	0.032 ^a	0.258 (0.075-0.891)
Paracetamol use	1.150 \pm 0.488	0.018 ^a	3.159 (1.214-8.220)
Cognitive function	-0.023 \pm 0.011	0.042 ^a	0.977 (0.956-0.999)
Hemoglobin	0.327 \pm 0.168	0.052	1.387 (0.998-1.928)
Age	0.026 \pm 0.021	0.213	1.027 (0.985-1.070)

HD: Hemodialysis; OR : Odds ratio. ^a*P* < 0.05.

albumin level (*P* = 0.856), Kt/V (*P* = 0.353), sodium pre- (*P* = 0.961) or post-dialysis level (*P* = 0.176), change in potassium level during HD (*P* = 0.556), PPI or H2 blocker use (*P* = 0.857), aspirin use (*P* = 0.172), residual diuresis (*P* = 0.411), hepatitis B or C infection (*P* = 0.358), diabetes mellitus (*P* = 0.822), marital status (*P* = 0.941) and education level (*P* = 0.377) were not associated with IBS symptoms.

When the risk factors for symptoms of IBS were assessed by multiple logistic regression analysis, independent predictors of IBS symptoms included: paracetamol use, post-dialysis serum potassium and KDQOL-CF score (Table 2).

DISCUSSION

The frequency of symptoms compatible with IBS in our study is somewhat higher than that reported (11%) among 105 Austrian HD patients^[3] using similar criteria, and lower than that among 148 English HD patients (21%) using Rome II criteria^[2]. In the latter study, IBS was significantly more common in HD subjects than in both hospital outpatients and community controls^[2]. In a study from Turkey, the prevalence of IBS, using Rome II criteria, was 44% among 93 HD patients, significantly more common than in healthy volunteers (21%)^[4].

The overall IBS prevalence in Europe is 11.5%^[13]. It varies, however, widely among countries, being highest in the UK and Italy^[13], depending to a large extent on the diagnostic criteria used^[14]. There is even more variability between continents^[13]. Unfortunately, there is no data on IBS prevalence in the general population in Poland.

As there is no biologic marker of the disease, the diagnosis of IBS relies heavily on symptom-based criteria. The most widely used is a consensus definition called the Rome criteria^[16,17], where IBS is defined chiefly by abdominal pain associated with defecation or a change in bowel habit and with features of disordered defecation. Some researchers have suggested that these criteria overemphasize abdominal pain and fail to emphasize postprandial urgency, abdominal pain, and/or diarrhea^[18,19]. We thought that for the HD population, with frequent comorbidities, the use of supportive symptoms that are not part of the Rome criteria would be more appropriate. The Kruis scoring system^[20], which in addition to self-reported symptoms includes: erythrocyte sedimentation

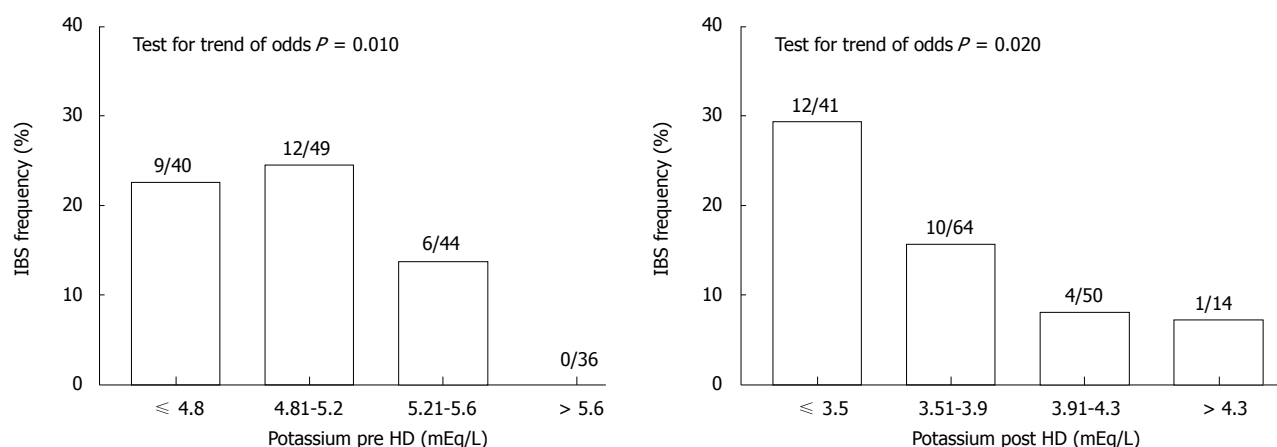


Figure 1 Frequency of irritable bowel syndrome symptoms in hemodialysis subjects stratified by pre- or post-hemodialysis potassium level. Numerator corresponds to number of irritable bowel syndrome (IBS) cases and denominator to number of all patients with given potassium level. HD: Hemodialysis.

rate, leukocytosis and anemia could not be used in HD patients for obvious reasons.

Fulfillment of IBS criteria is not unequivocally diagnostic of IBS, however, to make a positive diagnosis of IBS the use of diagnostic criteria is the recommended method, rather than exhaustive investigations to exclude an underlying organic cause^[21]. Surprisingly, however, very few studies have examined the utility of the various diagnostic criteria in differentiating IBS from organic disease. The authors of a recent systematic review were able to find only 4 studies that reported on the accuracy of the Manning criteria, 1 study that reported on the Rome I criteria, and no studies on the Rome II or Rome III criteria^[21].

In clinic-based studies, IBS has been associated with female gender, psychological distress, physical and sexual abuse, food allergies, enteric infections, and previous abdominal surgeries^[22]. In a community-based study Locke *et al.*^[23] found associations with somatic symptoms, analgesic use, and food allergies and sensitivities^[23].

The association between acetaminophen use and IBS has been previously reported, and is difficult to explain^[23]. A possibility exists that most people take paracetamol for their IBS. It is interesting that there was no association between aspirin use and IBS symptoms, similar to Locke *et al.*^[23] when the use of these drugs was reported independently. Additionally, in this country aspirin is most frequently used for cardiovascular prophylaxis.

Cognitive deficits often accompany chronic illnesses, although the underlying mechanisms are not fully understood and may differ between diseases. Cognitive impairment is common in HD patients^[24]. Risk factors include: age, race, stroke, diabetes, low education status, anemia, measures of malnutrition and an equilibrated Kt/V ≥ 1.2 ^[25,26]. Also, IBS in the general population is associated with cognitive impairment^[27,28]. An observed association between IBS symptoms and KDQOL-CF score might suggest that IBS worsens cognitive deficits in HD patients. We used the KDQOL-CF self reported score for the assessment of cognitive function. Although

the KDQOL-CF provides estimates rather than a definitive assessment of cognitive function, it was shown that the KDQOL-CF scale scores correlate with the Modified Mini-Mental State Examination, and are acceptable for estimating cognitive function in dialysis subjects^[12,29].

In the general population, IBS is associated with stress^[6]. In our study, the presence of IBS compatible symptoms was not related to the presence or absence of anxiety or depression. This is similar to the findings by Cano and colleagues^[2]. They, therefore, concluded that IBS in HD patients might be related to either the “uremic” state or the treatment method. This is in contrast to Kahvecioglu *et al.*^[4] who found that IBS in dialyzed patients was associated with depression and anxiety. In the general population, IBS seems to be more common in younger people. Our population was mostly over 50 years, and that might explain the lack of an age effect on the frequency of IBS symptoms.

Patients suffering from diabetes mellitus report a greater prevalence of gastrointestinal symptoms than controls, which is not related to glycemic control^[30]. We did not observe, however, any difference in the frequency of IBS symptoms between diabetic and non-diabetic HD patients. This is in agreement with Cano *et al.*^[2].

It is difficult to offer an explanation for the unexpected univariate association between IBS symptoms and hemoglobin level. Patients dialyzed in central and eastern European dialysis centers have lower mean hemoglobin levels and are less likely to attain target levels than those treated in western European counterparts^[31]. HD patients with higher hemoglobin levels report higher quality of life, and IBS patients in the general population have lower health-related quality of life^[32]. Additionally, gastrointestinal symptoms in patients with chronic kidney disease are associated with impaired general psychological well-being^[5]. However, IBS patients have a propensity to report pain and label negatively expected adverse sensations, so it is conceivable that IBS specific symptoms are “unmasked” in patients who have an overall higher quality of life.

To our knowledge, the association between serum potassium level and the frequency of symptoms compatible with IBS has not been reported before. This finding, however, has to be treated with caution. Although gastrointestinal motility is impaired in chronic pre-dialysis kidney disease^[33], it is alleviated by hemodialysis^[34]. Thus, another mechanism may be responsible. Hypokalemia may cause decreased motility and propulsive activity of the intestine, and even lead to ileus. We recorded potassium levels before and after a single HD session, whereas a level prevailing over a specific time period might be more appropriate. It may be, however, that episodes of hypokalemia, which are likely just after hemodialysis, are responsible for the appearance of IBS symptoms. A consistent trend of a higher prevalence of IBS compatible symptoms with lower potassium concentration, also suggests a causative role for hypokalemia. During conventional HD, large amounts of potassium are removed, approximately 40% of which originates from the extra- and the remainder from the intracellular space^[35]. A change in the plasma potassium concentration during hemodialysis, however, is difficult to predict, due to the concomitant movement of the ion into cells due to correction of metabolic acidosis. After HD, plasma potassium concentration increases rapidly during the first hour and steadily thereafter. The post-dialysis rise in potassium concentration is not correlated with pre- or post-dialysis plasma K^[35].

In CAPD patients, it has been reported that episodes of hypokalemia are a risk factor for developing peritonitis and bacterial overgrowth, possibly due to altered intestinal motility^[36,37].

In our study, neither the dialysis session time nor the change in potassium level influenced the prevalence of IBS symptoms, what suggests that the between dialyses level rather than the intradialytic change is important. In line with this electrophysiological mechanism reasoning, is the observation that pre-dialysis potassium < 4.0 or > 5.6 mEq/L is associated with increased mortality in HD patients, most probably due to cardiac arrhythmias^[38]. Despite the plausible electrophysiological mechanism, the association between potassium level and IBS symptoms might be confounded by other factors. It has been suggested for both HD and PD patients, that hypokalemia could be a surrogate marker for poor nutrition and associated comorbidities^[38,39].

This study has a number of shortcomings: firstly the Bowel Disease Questionnaire was not formally validated. To that end we ensured that the Polish translation was faithful and easy to understand. Additionally, the number of subjects was rather low, the study was observational and could not prove causality in relationships. Finally, the study potentially lacked generalizability due to cross-cultural differences in the symptomatology of functional gastrointestinal disorders^[15].

In summary, this study examined potential risk factors for symptoms compatible with IBS in HD patients and identified an association with acetaminophen use, serum potassium level, and KDQOL-cognitive function score.

The role of hypokalemia requires further well designed and controlled clinical studies.

COMMENTS

Background

Chronic gastrointestinal symptoms are very common in patients with chronic kidney disease (CKD) treated by hemodialysis (HD) including irritable bowel syndrome (IBS). Risk factors associated with IBS in HD patients are not known.

Research frontiers

Risk factors that are associated with IBS in the general population include: somatic symptoms, female gender, psychological distress, physical and sexual abuse, food allergies, enteric infections, previous abdominal surgeries, analgesic use, and food allergies and sensitivities. Of the 196 HD patients included in this study, symptoms compatible with IBS were present in 27 (13.8%) subjects and were independently associated with low post-dialysis serum potassium, paracetamol use, and KDQOL cognitive function score.

Innovations and breakthroughs

This study showed that low post-dialysis serum potassium, paracetamol use, and KDQOL cognitive function score are independently associated with increased risk of IBS compatible symptoms.

Applications

This analysis of the risk factors for IBS may be helpful in reducing the risk of abdominal symptoms in HD patients.

Peer review

The authors report an observational study looking at various factors associated with IBS as defined by Manning criteria in haemodialysis patients.

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Viscosity of food boluses affects the axial force in the esophagus

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Abstract

AIM: To study the effect of viscosity on axial force in the esophagus during primary peristalsis using a newly validated impedance-based axial force recording technique.

METHODS: A probe able to simultaneously measure both axial force and manometry was positioned above the lower esophageal sphincter. Potable tap water and three thickened fluids were used to create boluses of different viscosities. Water has a viscosity of 1 mPa·s. The three thickened fluids were made with different concentrations of Clinutren Instant thickener. The viscous fluids were in appearance comparable to pudding (2 kPa·s), yogurt (6 kPa·s) and slush ice (10 kPa·s). Six healthy volunteers swallowed 5 and 10 mL of boluses multiple times.

RESULTS: The pressure amplitude did not increase with the bolus viscosity nor with the bolus volume whereas the axial force increased marginally with bolus volume ($0.1 > P > 0.05$). Both techniques showed that contraction duration increased with bolus viscosity ($P < 0.01$). Association was found between axial force and pressure but the association became weaker with

increasing viscosity. The pressure amplitude did not increase with the viscosity or bolus volume whereas the axial force increased marginally with the bolus size.

CONCLUSION: This indicates a discrepancy between the physiological functions that can be recorded with axial force measurements and pressure measurements.

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Key words: Axial force; Manometry; Esophagus; Primary peristalsis; Viscosity

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INTRODUCTION

The primary function of the esophagus is to transport swallowed material from the pharynx to the stomach. A voluntary swallow initiates coordinated neuro-motor activity resulting in an aborally propagating contraction termed primary peristalsis. The primary effect of peristalsis is to develop force in the axial direction to pass the food into the stomach. Esophageal peristalsis depends on several factors such as body position, gravity, bolus size, and bolus viscosity^[1,2].

The most common method to study esophageal peristalsis is manometry using low-compliance perfused catheters or more recently high-resolution manometry using multiple solid state transducers mounted on the catheter.

It has been shown that bolus volume affects the peristaltic contraction velocity and duration^[3,4]. The interval between swallows^[5,6], body position^[2,4] and temperature of the bolus^[7] also affect peristalsis. However, the pressure amplitude is not affected by increasing bolus viscosity whereas the duration and velocity is reduced^[7,8].

Since the swallowed material is transported in the axial (longitudinal) direction of the esophagus, axial force measurements from a theoretical and practical standpoint better reflect esophageal function than pressure recordings do. Manometry measures the radial pressure which merely is an indirect measurement of the radial force (perpendicular to the axial force direction). Video-fluoroscopy is used to visually assess esophageal motor flow but it does not provide quantitative information on force in either radial or axial directions^[9,10]. Several attempts have been made to develop techniques to measure the axial force in the esophagus^[11-14]. Despite promising initial results based on strain gauge technology, this method has never been thoroughly tested or never made a breakthrough in clinical studies. Thus, only scarce data exist on the axial force in the esophagus. To the best of our knowledge, no studies have been published on the effect of bolus viscosity with axial force recordings.

The aim of this paper was to study the effect of bolus viscosity on axial force in the esophagus during primary peristalsis using a newly validated impedance-based force recording technique^[15,16] and to compare these results with manometric recordings.

MATERIALS AND METHODS

Probe design and hardware setup

The probe was custom-made to measure axial force and pressure simultaneously at different positions on the probe (Ditens A/S, Aalborg, Denmark). The probe was 60 cm long and constructed from three different catheters. The proximal catheter of the probe was used for manometry, the middle catheter contained the transducer for axial force measurement, and the distal catheter was 2.5 cm long and contained a small bag (Figure 1).

The proximal catheter of the probe was made from an 8-lumen polyurethane catheter with an outer diameter of 4.6 mm. The 8 channels had different diameters. Three channels (diameters of 0.5 mm) were used for manometric measurements using a low compliance perfusion system. The side holes for manometric measurements were placed 6, 8 and 10 cm proximal to the tip of the catheter. Steel threads were placed in a 1.0 mm lumen to avoid elongation of the proximal catheter. One 0.5 mm lumen contained two wires connected to the force transducer electrodes in the middle catheter. Two channels with diameters of 2.0 mm were used to re-circulate saline (0.09%) in the middle catheter and to inflate the bag. The last lumen with a diameter of 1.3 mm contained a temperature sensor (TC Ltd., Uxbridge, England) for measurement 0.5 cm proximal to the force transducer. It was used to temperature compensate the impedance signal.

The middle catheter was 2 cm long and consisted

of three single-lumen catheters/cylinders inside each other^[15,17]. The innermost catheter was 3 mm in diameter and made of elastic Natvar catheter (Colorite Polymers, Belfast, Ireland). Two electrodes for electrical impedance measurement were placed inside this catheter. One electrode was mounted on the distal catheter and the other on the proximal catheter. Two overlapping rigid cylinders (outer diameters of 4 and 5 mm) surrounded the inner catheter. They protected the elastic catheter from radial forces and from bending, factors that would introduce errors.

The distal catheter was a non-stretchable catheter with an outer diameter of 1.5 mm through which inflation of the bag was done. The cylindrical shaped bag was made of 25 μ m thick polyurethane (Ditens A/S, Aalborg, Denmark) and contained up to 13 mL. It was mounted on the outer rigid cylinder and the distal catheter. Tensile axial force applied to the bag made the rigid cylinders and electrodes move apart, resulting in increased electrical impedance. The electrical impedance, measured as the electrical potential difference (voltage), was calibrated to axial force [g] by applying precision weights in the range of 0-200 g.

Subjects

Six healthy men were included in the study (mean 38.3 years, range 25-61). Oral and written informed consent was obtained from all subjects. Ethics approval for the study was obtained from the Local Ethics Committee (Protocol No. VN 2003/120 mch).

Protocol

The catheter was first inserted through the mouth and esophagus into the stomach. The lower esophageal sphincter (LES) was located by the proximal pressure recordings. The probe was further retracted, placing the middle of the bag 5 cm proximal to the LES. Pressure was recorded 8, 10 and 12 cm proximal to the LES. Axial force was recorded 6.5 cm proximal to the LES.

Potable tap water and three thickened fluids were used to create boluses of different viscosities. Water has a viscosity of 1 mPa·s. The three thickened fluids (TF1-TF3) were made by mixing 100 mL tap water with 13.8, 16.5 and 19.3 g Clinutren Instant thickener (Nestlé, Vevey, Switzerland), respectively. An analysis of the instant thickener product was generated prior to the study by Vysera Biomedical Ltd., showing the viscosity as a function of the concentration in water (Figure 2). The selected viscosities of the thickened fluids were 2 kPa·s, 6 kPa·s and 10 kPa·s. The viscous fluids were in appearance comparable to pudding (2 kPa·s), yogurt (6 kPa·s) and slush ice (10 kPa·s). The bag was inflated with 2 mL of fluid throughout the experiments to enable peristalsis to grip the bag. The protocol included four series of five dry swallows, five 5 mL swallows and five 10 mL swallows. Each series tested a different fluid. At the end of each series the fluid used for the next series was mixed. All boluses were given at room temperature (23-26°C). The interval between swallows was at least 45 s. All subjects were studied in upright position with the upper body tilted

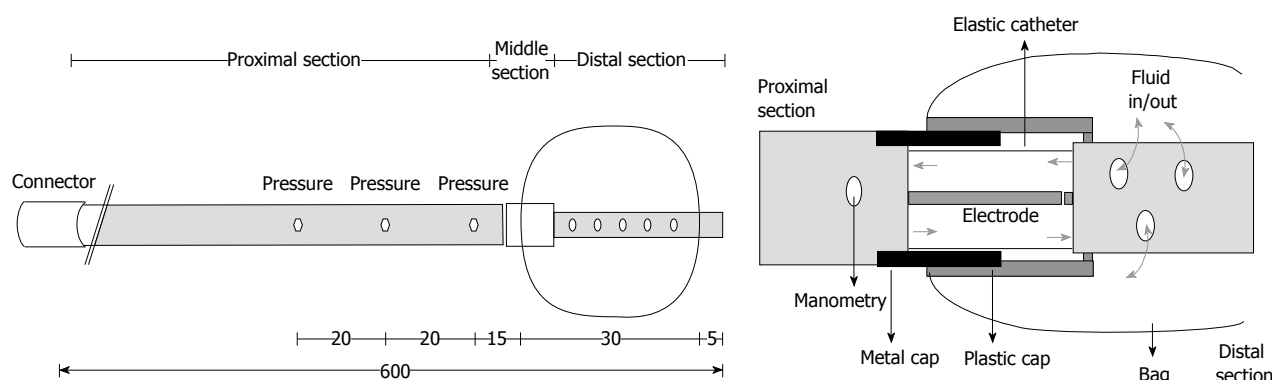


Figure 1 A sketch of the probe capable of measuring axial force and manometry simultaneously (see text for detailed explanation).

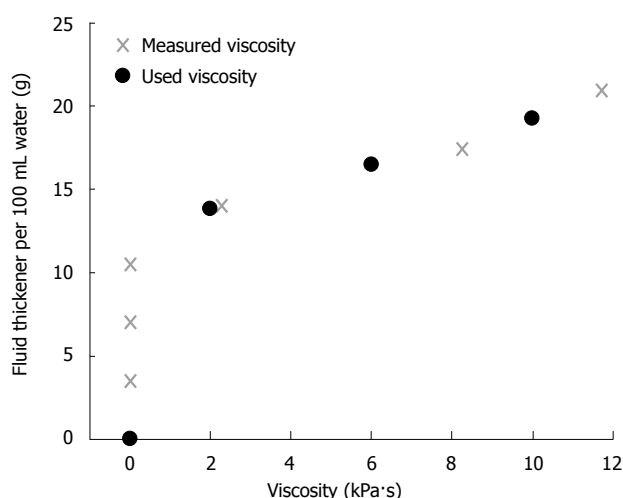


Figure 2 Viscosity recordings of the thickening powder in water. The light grey marks were measured while the black marks are used in the study.

30 degrees posterior and instructed to swallow as normally as possible. The volunteers drank 65 mL water between each series (after the five consecutive swallows) to clear the esophagus from any excess fluids of high viscosity.

Analysis

Contraction amplitude and duration were analyzed for both force and pressure measurements. The start of a contraction was defined as the interception with the x-axis for the linear fit of the steep incline of a contraction wave. The end of a contraction was defined as the interception with the x-axis for the linear fit to the steep decline of a contraction. This definition was used because the bolus itself affected the measures before the arrival of a peristaltic contraction. This definition has previously been verified by video fluoroscopy^[18] and used in different manometric studies^[8,19]. The linear fit was calculated using a semi-automatic program custom made for the purpose using MatLab® version 7 (Mathworks, Natick, MA, USA).

Complete absence of motor activity (manometry < 15 mmHg^[20,21], force < 10 g) at a given site was termed “failure of contraction”. Double-peaked, triple-peaked and repetitive waves were quantified manually during the semi-automatic analysis.

Statistics analysis

The results are given as grand mean \pm SD. The correlation coefficient ρ between the force and pressure measurements was computed with Pearson's correlation test for duration and amplitude at individual bolus size. Two-way analysis of variance (ANOVA) was used for the analysis of axial force and pressure. ANOVA was also used to analyze differences between bolus size. The axial force and pressure amplitude cannot be compared directly. Therefore, normalization was done by division with the overall mean of the axial force amplitude and pressure amplitude, respectively. The overall mean was computed from all the contraction amplitudes. $P < 0.05$ was considered significant.

RESULTS

The study was conducted without adverse events for the subjects. The age of the subjects was 27.7 ± 4.2 years. Representative recordings obtained in a subject swallowing 5 mL of water and 10 mL of thickened fluid (10 kPa·s) are shown in Figure 3. The arrival of the bolus in front of the peristaltic wave can be seen in the recording from the 10 mL swallow (marked with an arrow). The number of peristaltic contractions was higher during 5 and 10 mL swallows when compared to dry swallows; that is fewer wet swallows failed to induce contraction. The number of contractions was lower for multi-peaked contractions for both manometry and axial force during 5 mL swallows compared to dry swallows. Manometry and axial force showed an equal number of contractions. Only contractions, but not the events that did not fulfill the contraction criteria, were included in the subsequent analysis. No qualitative changes in the shape of the peristaltic contraction were found in association with the quantitative changes described. No increase or decrease in contractile amplitude or duration was found during the five subsequent swallows in a series. Thus, in the following analysis the averages were used.

Contraction amplitude

The most distal pressure recording site had the biggest amplitude for both 5 mL swallows ($F = 22.5$, $P < 0.001$) and 10 mL swallows ($F = 26.3$, $P < 0.001$). Thus, the manometric amplitude increased distally when comparing

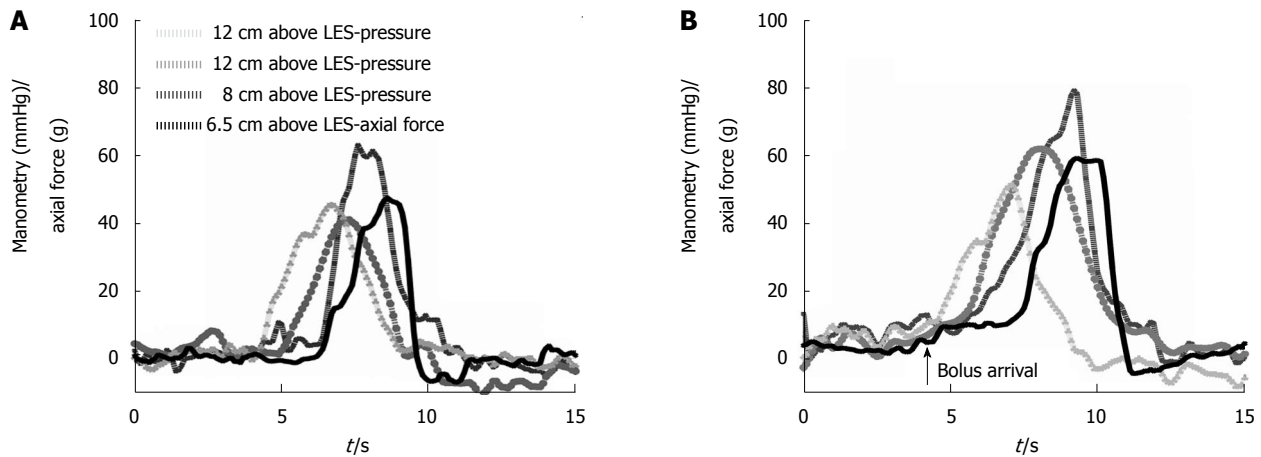


Figure 3 Two swallows (initiated at time = 0) from one subject. One swallow of 5 mL water (A) and one swallow of 10 mL thickened fluid (B). The arrival of the fluid before the actual peristaltic wave was clear during swallowing of 10 mL thickened fluid. LES: Lower esophageal sphincter.

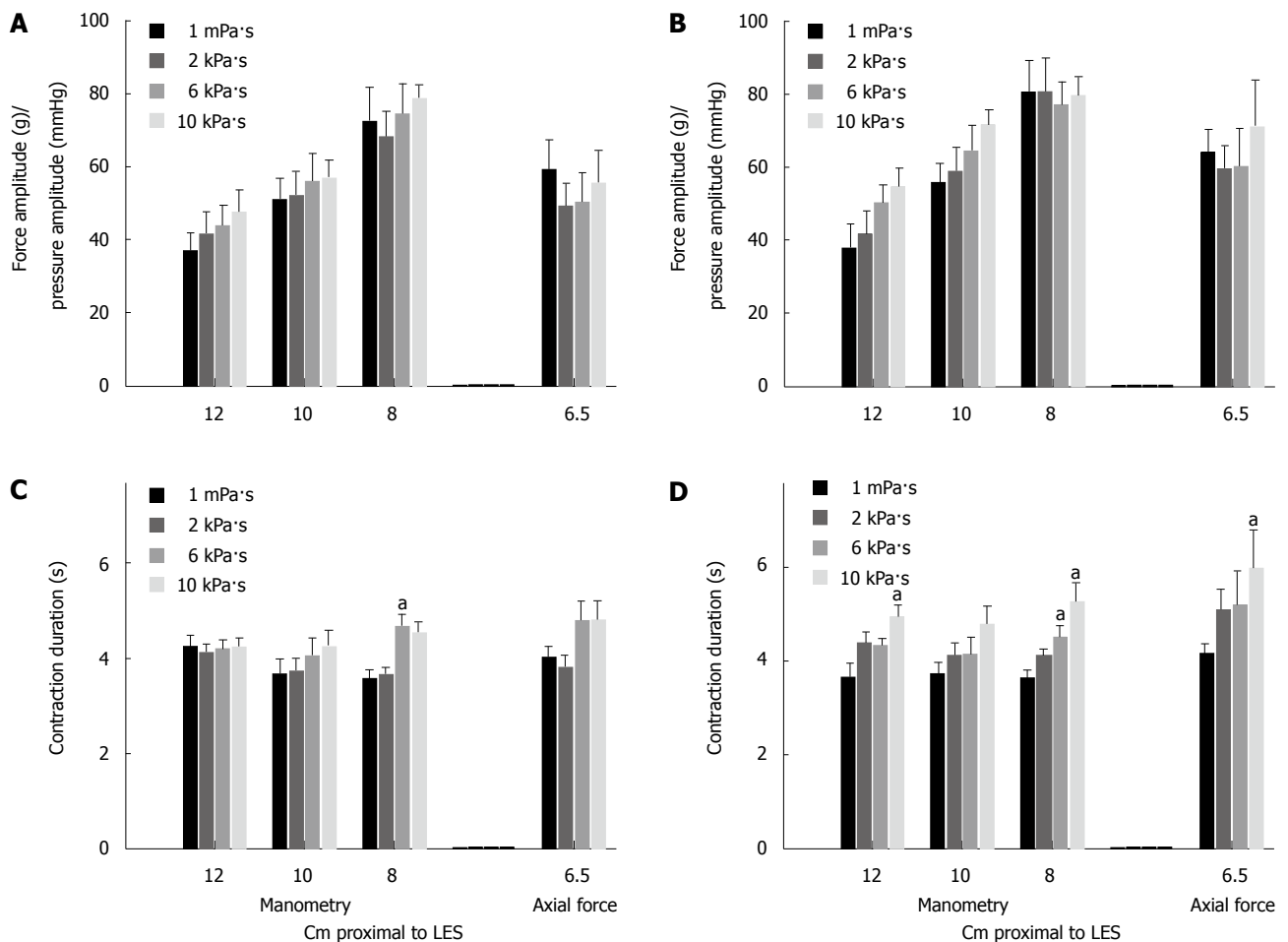


Figure 4 The mean \pm SE amplitude (A, B) and duration (C, D) for manometry and axial force recordings during 5 mL swallows (A, C) and 10 mL swallows (B, D). The color of the columns represents the viscosity from 1 to 10 kPa·s. The axial force amplitude was marginally affected by bolus size ($F = 3.5$, $P = 0.069$) while pressure recordings were unaffected. The duration of the contraction increased as the viscosity increased. ^a $P < 0.05$ vs water. LES: Lower esophageal sphincter.

recordings at different levels.

The pressure amplitude did not depend on viscosity or bolus size at any recordings site (Figure 4). The axial force amplitude was 38.7 ± 17.2 g during dry swallows (not shown). The axial force amplitude was marginally

influenced by bolus size ($F = 3.5$, $P = 0.069$) but did not increase with the bolus viscosity (Figure 4). Using 2-way ANOVA no difference was found when normalized amplitudes for pressure recorded 8 cm proximal to LES was compared to normalized axial force amplitudes.

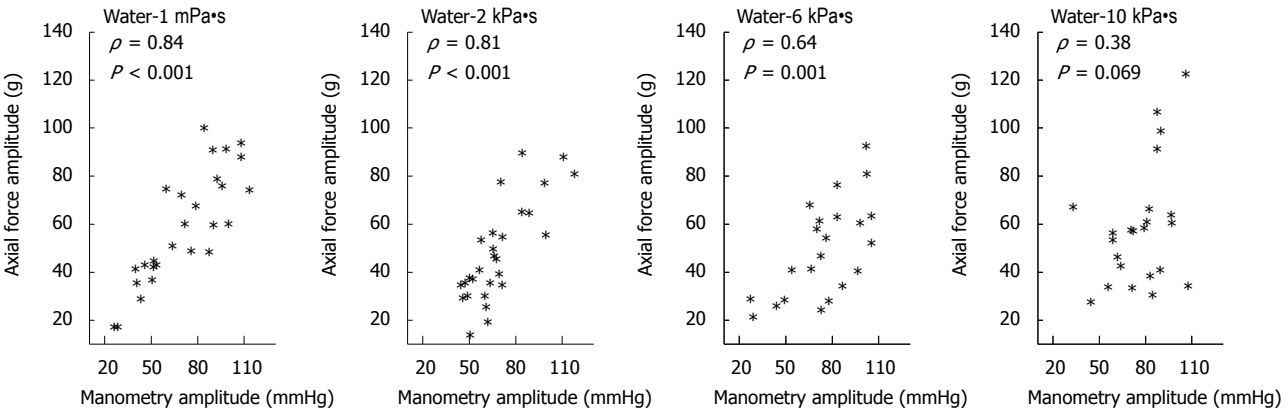


Figure 5 The correlation between manometry amplitude recorded 8 cm proximal to LES and axial force recorded 6.5 cm proximal to LES. The graphs from left to right are swallows of fluids with viscosities of 1 mPa·s, 2 kPa·s, 6 kPa·s and 10 kPa·s, respectively. LES : Lower esophageal sphincter.

Table 1 Axial force and manometry association									
Volume		5 mL	5 mL	5 mL	5 mL	10 mL	10 mL	10 mL	10 mL
Viscosity (Pa·s)		1 m	2 k	6 k	10 k	1 m	2 k	6 k	10 k
Amplitude									
8 cm	ρ =	0.84 ^a	0.81 ^a	0.64 ^a	0.38	0.7 ^a	0.52 ^a	0.46 ^a	0.7 ^a
	P =	< 0.001 ^a	< 0.001 ^a	0.001 ^a	0.069	< 0.001 ^a	0.008 ^a	0.02 ^a	< 0.001 ^a
10 cm	ρ =	0.49 ^a	0.68 ^a	0.57 ^a	0.41	0.27	0.08	-0.7	0.26
	P =	0.016 ^a	< 0.001 ^a	0.021 ^a	0.063	0.163	0.695	0.749	0.29
12 cm	ρ =	0.41	0.39	0.48	-0.32	-0.25	-0.06	-0.15	0.27
	P =	0.06	0.058	0.062	0.157	0.244	0.785	0.494	0.255
Duration									
8 cm	ρ =	0.36	0.34	0.47 ^a	0.32	0.55 ^a	0.4 ^a	0.58 ^a	0.53 ^a
	P =	0.061	0.08	0.027 ^a	0.133	0.002 ^a	0.048 ^a	0.002 ^a	0.014 ^a
10 cm	ρ =	-0.23	0.17	0.48	0.33	0.34	0.29	0.32	0.25
	P =	0.292	0.433	0.062	0.138	0.075	0.159	0.122	0.307
12 cm	ρ =	-0.32	0.31	-0.28	0	-0.35	0.03	-0.07	-0.25
	P =	0.14	0.136	0.287	0.997	0.104	0.908	0.766	0.293

The association between axial force [6.5 cm proximal to the lower esophageal sphincter (LES)] and manometry recorded 8, 10 and 12 cm proximal to the LES for both duration and amplitude. ^aIndicate a significant association ($P < 0.05$).

Contraction duration

The contraction duration recorded with manometry was not influenced by the recording site (Figure 4). The duration increased with viscosity for pressure recorded 8 cm above LES ($F = 12.3$, $P < 0.01$) (Figure 4).

The contraction duration measured with axial force increased with increasing viscosity ($F = 4.3$, $P = 0.01$) and bolus size (5 mL versus 10 mL) ($F = 4.9$, $P = 0.03$). The pressure duration recorded 8 cm proximal to the LES was lower than that for axial force for 10 mL swallows ($F = 4.9$, $P = 0.033$). Pressure recorded 8 cm proximal to the LES showed a change with viscosity ($F = 12.3$, $P < 0.001$) and the post hoc analysis showed difference between water and 10 kPa·s fluid ($P < 0.001$) and between water and 6 kPa·s fluid ($P = 0.001$). Contrary to the amplitude the duration did not change when comparing the different manometric recording sites.

Association between pressure and axial force

The association between the pressure amplitude measured 8 cm proximal to the LES and force amplitude recorded

during 5 mL swallows decreased with increasing bolus viscosity (Figure 5 and Table 1). The correlation coefficients for amplitudes during 10 mL swallows were lower compared to 5 mL swallows. Association was not found at any viscosity or bolus volume when comparing axial force amplitudes to pressure amplitudes recorded 12 cm proximal to the LES (all $P > 0.05$). With regard to contraction duration no association was found for 5 mL swallows except in one case (Table 1). A weak association between axial force and manometry 8 cm proximal to LES was found for 10 mL swallows ($P < 0.58$).

DISCUSSION

The major results were that the pressure and force amplitude did not increase with the viscosity. The axial force amplitude depended on the bolus size. This was not the case for the pressure amplitude. The association between pressure and force amplitudes was weak at large bolus size (10 mL), indicating that pressure will be a less exact measure of the esophageal function.

Methodological considerations

The swallow sequence was not randomized or blinded since pilot experiments showed that the subjects could easily tell the difference between dry swallows, 5 mL and 10 mL volumes in the mouth. The same accounted for the fluids of different viscosity. However, the lack of blinding is of minor importance as a previous study did not show a learning effect regarding the swallowed bolus size^[22].

The viscous fluids did not influence subsequent recordings by accumulating around the probe or bag between swallows since the parameters for dry swallows did not change and the number of contractions remained constant. Individual successive contractions showed no increase in amplitude or duration in five succeeding swallows.

In the current study the viscosity ranged from 1 mPa·s to 10 kPa·s. A previous study used somewhat lower viscosities between 1 mPa·s to 860 mPa·s^[8]. Since the viscosity curve is highly non-linear this may not affect the appearance of the fluid to the same degree. However, it is important to emphasize that the exact viscosity should be given in scientific publications.

The association between manometric data recorded 8 cm proximal to the LES and axial force recorded 6.5 cm proximal to the LES was calculated. However, the distance between recording sites is of minor importance because the amplitude and duration were calculated for each curve individually. One may argue that it is important to evaluate intrabolus pressure since it will be influenced by viscosity^[18]. However, we believe that the contraction pressure is more important.

Amplitudes as function of viscosity

This study confirms the results of Dooley and coworkers^[7] that the contraction pressure amplitude is not affected by changes in bolus viscosity. We further show that the axial force amplitude does not depend on the viscosity.

Previous studies found a poor correlation between the axial force and manometry^[23-25]. Pope and Horton found that swallowing 10 mL of salad oil reduced the axial force amplitude by 50% in the subsequent swallows^[13]. This shows the frictional force is an important factor and the esophagus ability to “grip” the bolus is of great importance to a powerful forward-moving peristaltic wave. In the present study the pressure amplitude was not affected in the same way as axial force amplitude when the frictional force is changed. We believe that one of the reasons that the association between pressure amplitudes and axial force amplitudes became weaker as viscosity increased was due to a change in the frictional resistance (the frictional force resisting fluid movements). It is suspected that the range in frictional resistance between water and the viscous fluids was too low to reveal any difference when comparing axial force amplitudes and manometry amplitudes. Frictional resistance is a complex mechanism and will change depending on e.g. the bolus content, amount of mixed saliva, bolus velocity^[26]. Thus the axial force amplitude (or function) generated by the esophagus may be affected.

Duration in relation to viscosity

The duration of the peristaltic wave was affected by bolus viscosity, especially for 10 mL swallows. This confirms previous manometric studies^[7,8] showing a difference in the whole range of viscosities compared to water. It has been suggested that the increased duration is due to that the bolus reside longer time at the recording site^[7]. However, this is not likely to happen even with very viscous fluids. As seen in the raw tracings the bolus reached the axial force transducer before the actual wave arrived. This implies that the bolus has passed the pressure recordings sites.

In conclusion, the study provided information about the pressure and axial force during peristaltic contractions when exposed to change in bolus viscosity and volume. Though the contractile patterns appeared the same for pressure and force measurements, clear differences were found between the recordings, especially at high volumes and high viscosities. It is expected that profound differences between manometry and axial force will be found between patient groups with esophageal diseases. For example it is well known that some achalasia patients show a “common cavity phenomenon” with aperistalsis. This will create a radial pressure without changes in axial force. The same may account for other esophageal diseases. Thus, it is expected that axial force measurements will have clinical relevance. This will be a subject for subsequent studies.

COMMENTS

Background

Development of diagnostic tools within esophageal motility diseases have for a long time been based on manometry. The development has mainly focused on use of multiple pressure sensors (high resolution manometry). Several authors have previously shown that relying on manometry alone can lead to erroneous conclusions.

Research frontiers

Axial force measurements, also known as traction force, provide additional information not currently available through manometry examinations alone. Axial force provides a more physiological measurement. However few studies have compared simultaneous manometry and axial force.

Innovations and breakthroughs

This is the first study of its kind to examine axial force in relation to viscosity. The data suggests that axial force can provide additional information in relation to motility.

Applications

These data are in accordance with other studies relying on axial force measurements though the areas of interest have been bolus size, temperature etc. The data, together with other papers, suggests that using axial force to assist manometry in motility examinations will provide additional information important to provide a valid diagnosis.

Peer review

This is a very interesting experimental study with important clinical implications in the diagnosis of esophageal disease.

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Pancreatic duct guidewire placement for biliary cannulation in a single-session therapeutic ERCP

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lation success and post-endoscopic retrograde cholangiopancreatography (ERCP) complication rate.

RESULTS: SGT (92.3% success rate) was characterized by statistically significant enhanced patient outcome compared to either the DGT (43.8%, $P < 0.001$), pre-cut failed DGT (73%, $P < 0.001$) or pre-cut as first step method (80.6%, $P = 0.002$). Pre-cut as first step method offered a statistically significantly more favorable outcome compared to the DGT ($P < 0.001$). The incidence of post-ERCP pancreatitis did not differ in a statistically significant manner between either method (SGT: 5.3%, DGT: 6.1%, Pre-cut failed DGT: 7.9%, Pre-cut as first step: 7.5%) or with patients' gender.

CONCLUSION: Although DGT success rate proved not to be superior to SGT or pre-cut papillotomy, it is considered highly satisfactory in terms of safety in order to avoid the risk of a pre-cut when biliary therapy is necessary in difficult-to-cannulate cases.

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Key words: Endoscopic retrograde cholangiopancreatography; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Pre-cut papillotomy; Pancreatic duct

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Abstract

AIM: To investigate the technical success and clinical complication rate of a cannulated pancreatic duct with guidewire for biliary access.

METHODS: During a five-year study period, a total of 2843 patients were included in this retrospective analysis. Initial biliary cannulation method consisted of single-guidewire technique (SGT) for up to 5 attempts, followed by double-guidewire technique (DGT) when repeated unintentional pancreatic duct cannulation had taken place. Pre-cut papillotomy technique was reserved for when DGT had failed or no pancreatic duct cannulation had been previously achieved. Main outcome measurements were defined as biliary cannu-

Xinopoulos D, Bassioulas SP, Kypreos D, Korkolis D, Scorilas A, Mavridis K, Dimitroulopoulos D, Paraskevas E. Pancreatic duct guidewire placement for biliary cannulation in a single-session therapeutic ERCP. *World J Gastroenterol* 2011; 17(15): 1989-1995 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i15/1989.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i15.1989>

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is widely considered as the most demanding endoscopic interventional procedure offering the least invasive way for biliary manipulations. In terms of procedure-related safety, atraumatic biliary cannulation is the fundamental prerequisite to secure a successful therapeutic-intended ERCP^[1]. Nowadays, non-invasive imaging modalities [such as magnetic resonance cholangiopancreatography (MRCP) and endoscopic ultrasound (EUS)] have superseded diagnostic ERCP, reducing the potential complication rate and giving better selection criteria for patients who would benefit from therapeutic biliary manipulations^[2].

Long discussions and reports from ERCP experts have proposed a plethora of different ways to provide an uncomplicated biliary cannulation^[1]. Several factors during ERCP may lead an endoscopist to use the appropriate technique according to his experience and training. Many reports have shown that selective cannulation of the common bile duct (CBD) by insertion of a hydrophilic guidewire through a papillotome may minimize procedure-related complications (particularly post-ERCP pancreatitis-PEP), as opposed to standard CBD access method with direct injection of contrast media^[3-7].

In difficult-to-cannulate cases, pre-cut papillotomy has been established as the alternative method to gain CBD access when biliary therapy is strongly indicated^[8,9]. However, pre-cut technique predisposes to a higher rate of post-ERCP complications including hemorrhage, pancreatitis and perforation, even in the most experienced hands^[10-14].

Furthermore, several studies have documented that a pancreatic duct (PD) previously cannulated with a guidewire may facilitate selective CBD cannulation with a second wire preloaded into a papillotome (double guidewire technique - DGT)^[15-19]. Placement of such a guidewire into the pancreatic duct may act as an endoscopic road map for the CBD, open a stenotic papillary orifice, stabilize the papilla or straighten the common channel when dealing with a tortuous intraduodenal segment^[1].

In view of the above, we retrospectively analyzed our data concerning the use of this method (DGT) in terms of procedure-related efficacy, safety and complication rate.

MATERIALS AND METHODS

Patients

During a 5-year period (from June 2003 through July 2008), 2843 therapeutic ERCPs were performed in our hospital. A retrospective database review was conducted in order to identify all cases involving the use of DGT. Inclusion criteria were the existence of an intact papilla (no prior ERCP attempts) in patients with clinical, laboratory and radiological (transabdominal US, abdominal CT scans, MRCP) findings of pancreatobiliary pathology. Patients with previous gastrointestinal surgical operation, use of needle-knife fistulotomy for papilla impacted stones and suspected sphincter of oddi dysfunction (SOD) were excluded from our study. Endoscopic sphincterotomy (EST) was performed in all patients.

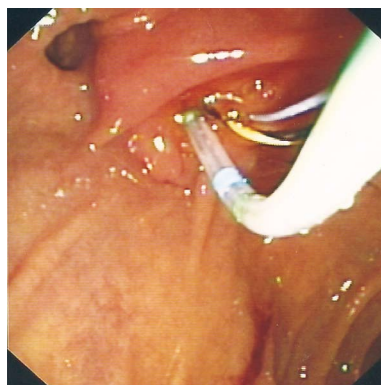


Figure 1 Endoscopic view of the papilla with a hydrophilic wire advanced into the pancreatic duct. A sphincterotome is advanced alongside the pancreatic wire with its tip oriented in the anticipated bile duct position.

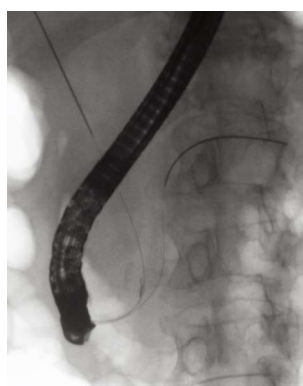


Figure 2 Biliary cannulation with the use of double-guidewire technique. One guidewire has been inserted into the distal part of the pancreatic duct and another is being moved in the direction of common bile duct through the sphincterotome inserted into the ampulla.

Methods and definitions

Our department protocol for biliary cannulation consists of a 3-step procedure undertaken in a single session. In the first step, attempts to cannulate the CBD consist of the use of a sphincterotome preloaded with a hydrophilic guidewire (single guidewire technique-SGT). Initial cannulation using SGT is both time- and cost-efficient when sphincterotomy is anticipated. The use of a guidewire seems to reduce the possibility of chemical-and pressure-related pancreatic injury by avoiding unintentional injection of contrast medium into the main PD or the papilla itself (submucosal injection). Up to 5 attempts within a 15-min period are considered adequate to provide safe cannulation without significant injury of the papillary area (i.e. trauma, edema, bleeding). If these attempts fail and repeated deep PD insertion has resulted, then the guidewire is left distally in the main PD and DGT technique is performed up to 3 times. The tip of the papillotome is positioned against the first wire placed in the PD (Figure 1) and its curve is altered in the anticipated CBD axis. The guidewire into the PD acts as a radiological marker for the PD and facilitates endoscopic location of the biliary orifice (Figure 2). Pre-cut technique with needle-knife is reserved as the 3rd step in cases when DGT has failed or no PD cannulation has

Table 1 Patient characteristics and indications *n* (%)

Dermographics	
Patient number	2332
Age (year, mean)	68.4
Gender (male/female)	1236/1096
Indications	
Choledocholithiasis	1732 (74.3)
Malignant stricture	545 (23.4)
Bile leak after cholecystectomy	42 (1.8)
Primary sclerosing cholangitis	11 (0.5)

been achieved in the first place.

Procedural success was defined as the insertion of the guidewire into the CBD. To determine safety and complication rate, all patients underwent measurement of serum amylase before and 24 h after ERCP. Asymptomatic hyperamylasemia was defined as a threefold rise in serum amylase without epigastric pain at 24 h after ERCP. Definition of PEP was the incidence of epigastric pain associated with serum amylase elevation to greater than 3 times normal values at 24 h after the procedure. Bleeding, perforation and other post-ERCP-related adverse events were recorded in detail.

Statistical analysis

Due to the fact that the distribution of the variables under study was not Gaussian, analysis of differences between these parameters, in each patient group, was performed with the non-parametric Mann-Whitney, χ^2 , or Fischer's exact statistical tests, where applicable. The correlation between the employment of different cannulation methods and either final patient outcome, or presence of asymptomatic hyperamylasemia or clinical pancreatitis, was studied using univariate logistic regression analysis.

RESULTS

Of the 2843 ERCPs performed in the study period, 511 were excluded as patients had previously undergone endoscopic manipulations (EST, endoscopic papillary balloon dilation), or altered anatomy was observed due to gastrectomy, or SOD was suspected on the basis of clinical and ERCP findings. A total of 2332 patients met selection criteria. Indications for therapeutic ERCP varied, with suspected common bile duct stones and suspected malignant biliary strictures being the most common (Table 1).

Using SGT as the first step method, CBD cannulation was achieved in 2153 patients (92.3%). Unintentional PD guidewire insertion after 5 attempts was documented in 112 patients. In these cases, DGT was performed after the last effort and selective CBD access was successful in 49 out of 112 patients (43.8%). In the 63 failed DGT cases pre-cut papillotomy gained CBD access in 46 patients (73%). In 67 patients no CBD or PD cannulation was possible with the initial 5 attempts. Fifty-four of them underwent successful pre-cut papillotomy at the same session (80.6%).

SGT was characterized by statistically significant enhanced patient outcome compared to either the DGT ($P < 0.001$), pre-cut failed DGT ($P < 0.001$) or pre-cut as first

step method ($P = 0.002$) (Tables 2 and 3). In addition, pre-cut as first step method offered a statistically significantly ($P < 0.001$) more favorable outcome compared to the DGT method. These observations were further confirmed using univariate logistic regression analysis (Table 4).

Thirty patients in whom therapeutic ERCP failed were referred to surgical or interventional radiological treatment. The total success rate of CBD cannulation in the study population was 98.7% (2302/2332).

The development of asymptomatic hyperamylasemia was significantly more frequent in the DGT and pre-cut-failed DGT group of patients, compared to the SGT patients (Table 2). Employing regression analysis, patients who underwent DGT were 2.15 times [HR] more likely (95% CI 1.39-4.60, $P = 0.002$) to develop asymptomatic hyperamylasemia than the SGT patients.

The rates of PEP did not differ in a statistically significant manner between groups of patients who underwent different types of cannulation (Tables 2 and 3). The presence of PEP in the DGT group of patients was statistically significantly ($P = 0.010$) more evident in younger individuals (Median = 42.00 years) than in older ones (63.00 years), without statistical correlation with the initial pathology (Table 4).

In the group of patients who underwent SGT, a statistically significant difference ($P = 0.005$) between age and outcome was observed, as younger patients (Median = 64.00 years) were more likely to be attributed with a successful outcome than older ones (Median = 67.00 years). However, PEP was more frequently present ($P < 0.001$) in younger (Median = 62.00 years) patients than older ones (Median = 67.00 years). Patients who suffered from choledocholithiasis were more likely to present PEP than patients who suffered from malignancy, without reaching statistically significant difference (Table 5).

In the pre-cut failed DGT group of patients, PEP was more frequently present ($P = 0.021$) in younger (Median = 45.00 years) patients than older ones (Median = 63.50 years). As far as the pre-cut as first step method is concerned, the presence of PEP was found more frequently ($P = 0.017$) in younger patients (Median = 52.00 years) than older patients (Median = 68.00 years). The presence of PEP was statistically more evident in patients with choledocholithiasis compared to patients with malignancy in both groups of patients in which pre-cut technique was performed (Table 5).

Patients' gender did not seem to relate in any statistically significant way with the presence of asymptomatic hyperamylasemia and PEP within patients who underwent pre-cut first step, DGT or pre-cut failed DGT method. On the contrary, female patients who underwent SGT showed a statistically significant elevated presence of asymptomatic hyperamylasemia (14.0%) compared to male patients (Table 6).

One case of retroperitoneal perforation was recorded using SGT, and 2 patients developed massive bleeding using pre-cut as first step and sphincterotomy after successful SGT, respectively. These patients underwent immediate surgical treatment without further complications.

Table 2 Comparison between single-guidewire technique/double-guidewire technique, single-guidewire technique/pre-cut failed double-guidewire technique and single-guidewire technique/pre-cut first step methods in terms of patient outcome, the development of asymptomatic hyperamylasemia or post-endoscopic retrograde cholangiopancreatography pancreatitis *n* (%)

Variables	SGT	DGT	SGT	Pre-cut failed DGT	SGT	Pre-cut first step
Outcome (success)	2153 (92.3)	49 (43.8)	2153 (92.3)	46 (73.0)	2153 (92.3)	54 (80.6)
<i>P</i> value ¹	< 0.001 ^a		< 0.001 ^a		0.002 ^a	
Asymptomatic hyperamylasemia	258 (12.0)	11 (22.4)	258 (12.0)	15 (23.8)	258 (12.0)	10 (14.9)
<i>P</i> value ¹	0.046 ^a		0.008 ^a		0.591	
PEP	115 (5.3)	3 (6.1)	115 (5.3)	5 (7.9)	115 (5.3)	5 (7.5)
<i>P</i> value ¹	0.935		0.538		0.629	

^a*P* < 0.05. ¹Calculated by Fisher's exact test; SGT: Single-guidewire technique; DGT: Double-guidewire technique; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis.

Table 3 Comparison between double-guidewire technique/pre-cut failed double-guidewire technique, pre-cut failed double-guidewire technique/pre-cut first step and double-guidewire technique/pre-cut first step methods in terms of patient outcome, the development of asymptomatic hyperamylasemia or post-endoscopic retrograde cholangiopancreatography pancreatitis *n* (%)

Variables	DGT	Pre-cut failed DGT	Pre-cut failed DGT	Pre-cut first step	DGT	Pre-cut first step
Outcome (success)	49 (43.8)	46 (73.0)	46 (73.0)	54 (80.6)	49 (43.8)	54 (80.6)
<i>P</i> value ¹	< 0.001 ^a		0.405		< 0.001 ^a	
Asymptomatic hyperamylasemia	11 (22.4)	15 (23.8)	15 (23.8)	10 (14.9)	11 (22.4)	10 (14.9)
<i>P</i> value ¹	0.955		0.266		0.426	
PEP	3 (6.1)	5 (7.9)	5 (7.9)	5 (7.5)	3 (6.1)	5 (7.5)
<i>P</i> value ¹	0.999		0.588		0.928	

^a*P* < 0.05. ¹Calculated by Fisher's exact test. SGT: Single-guidewire technique; DGT: Double-guidewire technique; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis.

Table 4 Logistic regression analysis for every possible comparison of the cannulation method used, for the prediction of outcome (failure)

Method	HR ¹	95% CI	<i>P</i> value ²
SGT	1		
Pre-cut first step	1.43	1.16-1.76	0.001
SGT	1		
DGT	3.93	3.21-4.81	< 0.001
Pre-cut first step	1		
DGT	1.23	1.13-1.35	< 0.001
Pre-cut first step	1		
Pre-cut failed DGT	1.063	0.95-1.20	0.307

¹Hazard ratio; ²Test for trend. SGT: Single-guidewire technique; DGT: Double-guidewire technique.

DISCUSSION

In the era of modern non-invasive imaging modalities, the role of ERCP has been focused in the therapeutic management of pancreatobiliary diseases. Several techniques and accessories have been used in order to achieve selective CBD cannulation and to decrease the rate of post-ERCP complications.

In the present study, SGT proved to be an effective and safe CBD cannulation approach. The ability to manually control the angle of orientation of the sphincterotome has been shown to be advantageous when dealing with unusually oriented or distorted papillas caused by either diverticula or surgically altered anatomy^[20]. Moreover, the use of hydrophilic coated-tip guidewires seems to

Table 5 Associations between the development of post-endoscopic retrograde cholangiopancreatography pancreatitis and the initial pathology within groups of patients who underwent each cannulation method *n* (%)

Method	Choledocholithiasis	Malignancy
Pre-cut first step		
PEP	5 (19.2)	0 (0.0)
<i>P</i> value ¹	0.007 ^a	
DGT		
PEP	3 (8.3)	0 (0.0)
<i>P</i> value ¹	0.556	
Pre-cut failed DGT		
PEP	5 (16.1)	0 (0.0)
<i>P</i> value ¹	< 0.001 ^a	
SGT		
PEP	97 (5.8)	17 (3.9)
<i>P</i> value ¹	0.146	

^a*P* < 0.05. ¹Calculated by Fisher's exact test. SGT: Single-guidewire technique; DGT: Double-guidewire technique; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis.

offer a direct way of CBD cannulation under fluoroscopy with less papillary trauma compared to the rather blind method of contrast media injection^[4].

Three large prospective randomized studies comparing post-ERCP complications between the use of a papillotomy with a guidewire (SGT) and papillotomy with contrast media injection as cannulation methods, demonstrated significantly lower incidence of PEP in the SGT group (0%-8.6% *vs* 4.1%-16.6%)^[4-6]. In addition, PEP rate was

Table 6 Associations between patient gender and the presence of PEP and asymptomatic hyperamylasemia, within groups of patients who underwent each cannulation method *n* (%)

Method	PEP	Asymptomatic hyperamylasemia
Pre-cut first step		
Male	2 (5.1)	3 (7.7)
Female	3 (10.7)	7 (25.0)
<i>P</i> value ¹	0.642	0.081
DGT		
Male	1 (3)	6 (18.2)
Female	2 (12.5)	5 (31.3)
<i>P</i> value ¹	0.086	0.467
Pre-cut failed DGT		
Male	1 (2.9)	9 (26.5)
Female	4 (13.8)	6 (20.7)
<i>P</i> value ¹	0.171	0.768
SGT		
Male	62 (5.5)	115 (10.2)
Female	53 (5.2)	143 (14.0)
<i>P</i> value ¹	0.77	0.008 ^a

^a*P* < 0.05. ¹Calculated by Fisher's exact test. SGT: Single-guidewire technique; DGT: Double-guidewire technique.

lower if unintentional guidewire cannulation of the PD occurred compared to that of unintentional PD opacification by contrast injection (0%-5.1% *vs* 4.4%-19%). In a SGT group, Lee *et al*^[6] reported a total of 3 cases of PEP, two in patients with SOD diagnosis, a well-known risk factor for PEP. In these studies, the authors concluded that the reduction in PEP in SGT groups of patients was mainly the result of avoiding elevation of hydrostatic pressure into the PD, partly attributed to the contrast media injection.

Dumonceau *et al*^[21] first described the successful use of PD guidewire placement as an adjunct to cannulate CBD in a case of Billroth I gastrectomy with a distorted prepapillary segment of CBD. Later on, Gyökeres *et al*^[15] reported successful use of DGT in 24 difficult CBD cannulation cases with no significant difference in PEP rate when compared to conventional cannulation. Maeda *et al*^[16], in a prospective study, documented a superior CBD cannulation rate (93%) with a modified technique, using a PD wire placement and a cannula instead of a papillotomy, as opposed to the conventional method with cannula and contrast media (58% success rate). No episodes of pancreatitis with either method were noted, but a significantly higher incidence of hyperamylasemia occurred in the PD wire group. It should be mentioned, however, that patients of the latter group were submitted to pancreatography prior to PD wire insertion, thus increasing the risk of overinjection into the PD with possible acinarization.

In our study, using DGT, selective CBD cannulation was achieved in 43.8% of patients (49/112) with previously failed SGT. Three patients of this group developed pancreatitis (6.1%) and 11 patients asymptomatic amylasemia (22.4%), showing an acceptable safety profile comparable to SGT (5.3% and 12%, respectively). Recently published data from a prospective randomized study assessing clinical efficacy and safety of DGT *versus* conventional cannulation method reported almost equivalent CBD cannulation

success in difficult cases (47.3% *vs* 54%). The DGT group showed an increased incidence of clinical pancreatitis compared to the SGT group, without, however, reaching a statistically significant difference (17% *vs* 8%)^[22].

According to our results, PD reaction (i.e. PEP) seems to be affected by both iatrogenic and patient-related characteristics. Young patients suffering from choledocholithiasis proved to be statistically more prone to develop pancreatitis when pre-cut access papillotomy as first step was used, or after a failed DGT technique was performed. On the other hand, elderly patients, despite repeated PD wire insertion and use of pre-cut, demonstrated hyperamylasemia as part of a benign post-ERCP clinical course. In addition, 12.5% and 13.8% of female patients who underwent a successful DGT and pre-cut failed DGT developed PEP, respectively. Although these observations were not found to be statistically significant, patients' gender seems to carry an independent risk factor concerning pancreatic injury following PD manipulations.

Age, sex, prior PEP or recurrent history of acute pancreatitis and SOD are well known risk factors for PEP^[23,24] and many experts have already proposed the use of a temporary prophylactic pancreatic stent in these situations^[1,25]. Several uncertainties remain regarding patients' selection criteria for pancreatic stent use and the appropriate diameter. These may be attributed to the lack of long-term data on changes in PD anatomy and stent-induced complications in failed stent placement cases^[26]. Goldberg *et al*^[27] documented the aid of a 5F pancreatic stent placement for CBD cannulation in 39 patients with 97.4% success rate. To achieve biliary cannulation, 59% of patients underwent pre-cut sphincterotomy over the pancreatic stent with only 5% PEP rate. The authors, however, do not mention the existence of SOD indication, nor do they report in how many cases pancreatic sphincterotomy was performed to facilitate stent insertion.

No pancreatic stent insertion was performed in our series, as our initial aim was to investigate the contribution of a simple and non-time-consuming technique involving the use rather than abuse of PD. In addition, patients with suspected SOD were excluded due to the lack of manometry which would imply a clear therapeutic indication and the need for a pancreatic stent use. What seems technically more important in DGT technique, regarding success and complication rate, is the gentle straight deep passage of the wire into the PD. Insertion of the wire in angulated mode into a normal PD may potentially increase side-branch acinarization associated with the occurrence of PEP.

It should always be kept in mind that basic CBD cannulation techniques cannot be substituted by any available trick and only proper expertise in ERCP secures periprocedural efficacy. Based on this knowledge, the wire into the PD seems to be helpful for cases of difficult cannulation, particularly when the papilla is mobile with small orifice or disoriented due to a diverticulum or malignancy. In these cases, the anatomical axis of the common channel is stabilized by the inserted wire into the PD, offering the possibility of a new, less traumatic, wire cannulation attempt than pre-cut access papillotomy may cause.

The special incisional technique of pre-cut has been estimated to enable biliary access in about 5% to 10% of hard-to-cannulate cases, according to several reports^[28]. However, pre-cut has been described to be an independent risk factor for post-ERCP complications (variation between 8% and 35.3%) and only recommended as an expert's alternative method^[14]. In full agreement with these reports, 5.6% of patients in the period of our study underwent pre-cut papillotomy (77% overall success rate) and 10 patients experienced PEP (7.7%). Data analysis showed no statistically increased possibility of PEP in patients with previously failed DGT followed by pre-cut (7.9% of cases), compared to patients who underwent pre-cut as initial cannulation technique (7.5% of cases).

The decision as to the appropriate CBD cannulation method in specialized situations should to be dependent on a patient's individual clinical and anatomical basis as well as on an endoscopist's experience in the available techniques. In the present study, while proposing the SGT as the standard technique for achieving CBD cannulation, DGT offered a remarkable alternative in difficult cases before proceeding to pre-cut papillotomy. DGT success and low complication rate is considered highly satisfactory in order to avoid the risk of a pre-cut when biliary therapy is necessary. Further randomized prospective trials comparing various pre-cut techniques and pancreatic duct cannulation approaches, as rescue methods to facilitate biliary access, will eventually offer an evidence-based approach.

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COMMENTS

Background

Biliary cannulation is the fundamental prerequisite to secure a successful therapeutic-intended magnetic resonance cholangiopancreatography (MRCP). Selective biliary cannulation by insertion of a hydrophilic guidewire tends to be the standard method of choice in terms of efficacy and safety [single guidewire technique (SGT)]. In difficult-to-cannulate cases, pre-cut papillotomy has been established as the alternative method to gain biliary access. However, pre-cut technique predisposes to a higher rate of post-ERCP complications, even in the most experienced hands.

Research frontiers

Several studies have documented that a pancreatic duct previously cannulated with a guidewire may facilitate selective biliary cannulation with a second wire (double guidewire technique-DGT).

Innovations and breakthroughs

In a single-center, retrospective study using DGT, selective biliary cannulation was achieved in 43.8% of patients with previously failed SGT. Pre-cut papillotomy gained biliary access in 73% of failed DGT cases and 80.6% of cases as first step method, respectively. Univariate analyses revealed no statistically significant difference in terms of complication rate between patients with different types of cannulation.

Applications

Although the DGT success rate proved not to be superior to that of SGT or pre-cut papillotomy, it is considered a highly satisfactory technique in terms of safety in order to avoid the risk of a pre-cut when biliary therapy is necessary.

Peer review

In this study, the authors conclude that the DGT may be safely performed before proceeding to pre-cut if repeated pancreatic duct cannulation occurs.

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Microscopic colitis as a missed cause of chronic diarrhea

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Abstract

AIM: To determine the prevalence of increased intraepithelial lymphocytes, using immunohistochemistry in patients with normal colonoscopy and near normal biopsy.

METHODS: We retrospectively reviewed all non-malignant colon mucosal biopsies between 2005 and 2007, reported as normal, chronic inflammation or melanosis coli in patients who were undergoing routine colonoscopy. Immunohistochemistry using CD3 was performed on all mucosal biopsies and an intraepithelial lymphocyte count (IEL) was determined. Cases with an IEL count of ≥ 20 IELs per 100 surface epithelial cells were correlated with demographic, clinical and follow-up data. A further subgroup was evaluated for lymphocytic colitis.

RESULTS: Twenty (8.3%) of 241 cases revealed an IEL count ≥ 20 . Six (2.5%) patients were identified as having lymphocytic colitis ($P < 0.001$), of whom, five were missed on initial evaluation ($P = 0.01$). Four of these five patients were labeled with diarrhea-predominant irritable bowel syndrome (IBS). On follow-up, three of the remaining 20 cases were diagnosed with malignancy (renal cell carcinoma and myelodysplastic syndrome) and one had an unknown primary tumor with multiple liver metastases. Two cases of collagenous colitis with an IEL count < 10 were included in this study. Increased IELs were not confined to patients with diarrhea as a primary presenting symptom, but were also present in patients with abdominal pain ($n = 7$), constipation ($n = 3$) and loss of weight ($n = 1$).

CONCLUSION: Immunohistochemistry using CD3 is of value in identifying and quantifying IELs for the presence of microscopic colitis in patients with diarrhea-predominant IBS.

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Key words: Microscopic colitis; Lymphocytic colitis; Collagenous colitis; CD3 immunohistochemistry; Intraepithelial lymphocytes

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INTRODUCTION

Microscopic colitis is regarded as a common cause of chronic watery diarrhea, accounting for approximately

4%-13% of patients presenting with this symptom^[1]. By definition the colon appears normal or nearly normal on colonoscopy, with set histopathological criteria required for the diagnosis on mucosal biopsy. Lymphocytic colitis and collagenous colitis constitute the two major subtypes of microscopic colitis that share many similarities, including almost identical clinical symptoms, together with a macroscopically normal colonic mucosa. Both entities demonstrate colonic intraepithelial lymphocytosis, increased inflammatory cells within the lamina propria, and preserved crypt architecture, but are distinguished by the presence of a thickened basement membrane in collagenous colitis.

In the past, microscopic colitis was thought to be a rare disorder and very little was known about its etiology or epidemiology. It has become apparent that microscopic colitis is now regarded as common cause of diarrhea in middle-aged and elderly patients. Many recent publications have shown that the incidence of microscopic colitis is on the increase. Epidemiological data have now been reported from seven major regions^[2], with most of the reported data coming from North American and European studies. The incidence rates for collagenous colitis is 0.8-6.2/100 000 and lymphocytic colitis is 0.5-12.9/100 000^[2]. According to various studies, the prevalence of collagenous colitis and lymphocytic colitis is 10-15.7/100 000 and 14.4/100 000, respectively^[1,3-5]. There are very few data available from developing countries, with a few case series reported from India^[6], Turkey^[7] and Sri Lanka^[8].

Currently there are no data regarding this disease in South Africa, where infectious diseases are more prevalent. Isolated cases have been reported from Nigeria^[9,10]. In this region, microscopic colitis is underdiagnosed because of a lack of colonoscopic facilities and the assumption that most cases of chronic diarrhea are likely to be infective, therefore, most patients self medicate and do not present to a hospital^[10,11].

At Tygerberg Hospital, a tertiary referral center, approximately 1700 colonoscopies are performed each year, which include colonoscopies for non-infective diarrhea-related causes. Colonic biopsies at our institution are often reported as chronic inflammation, indeterminate colitis, chronic colitis or normal in the investigation of diarrheal disease. It is possible that the diagnosis of LC may have been missed in a proportion of these cases, because under-reporting of cases is common and has been documented in other studies^[12]. According to a Swedish study, in a third of cases the diagnosis was missed in the primary histological examination^[13,14]. The important role of the pathologist was clearly illustrated in this study that showed the difficulties in diagnosing microscopic colitis, especially the lymphocytic subtype^[14]. According to Nielson, terms such as "unspecific chronic inflammation" or "signs of chronic inflammatory bowel disease but not diagnostic" should be avoided^[14].

Immunostaining does not seem to play a major role in the diagnosis of LC. According to Tysk *et al*^[2] and

Chang *et al*^[15], in some uncertain cases, immunostaining may facilitate the assessment of intraepithelial counts. There have been no studies to validate the benefit of performing immunohistochemistry in uncertain cases. Currently, the histological diagnosis is based on hematoxylin and eosin (H and E) assessment of criteria: (1) increase in intraepithelial lymphocytes (IELs > 20/100 surface epithelial cells); (2) surface epithelial damage; and (3) infiltration of lymphocytes and plasma cells into the lamina propria, with no subepithelial collagen deposition, as identified in collagenous colitis^[16,17]. The diagnosis of lymphocytic colitis remains a challenge because it is often difficult to identify and quantify lymphocytes due to orientation of the biopsy, or nuclei having similar cytological features to those of columnar cell nuclei. Immunohistochemistry staining with CD3 has been shown to play a role in the counting and identification of IELs in celiac disease; incidentally, there is also an association between microscopic colitis and celiac disease^[18].

Only one recent study has examined the reproducibility of histological diagnosis in microscopic colitis. That study found excellent correlation in distinguishing microscopic colitis and non-microscopic colitis amongst pathologists using H and E- stained slides. That study claimed κ values of 0.90 and 0.83 for inter-observer agreement and 0.89 for intra-observer agreement^[19]. However, the authors have stated that their high rate of concordance was due to their particular expertise within the field of gastroenterology. In their study, immunohistochemistry was not performed to assess whether it allows easier identification of IELs.

In the present study, we aimed to determine the prevalence of increased IELs with the use of immunohistochemistry and described the spectrum of disease in all non-malignant biopsies reported as normal or chronic inflammation over a 3-year period. By facilitating counting of IELs, we hoped to identify cases that may have the lymphocytic colitis subtype.

MATERIALS AND METHODS

Study design and population

The study design was a retrospective analysis of all non-malignant colonoscopic biopsies diagnosed as normal or chronic inflammation in patients who underwent colonoscopy at the Tygerberg Hospital Gastroenterology Unit, for the period 2005-2007. Cases were retrieved from the Department of Pathology, Division of Anatomical Pathology, DisaLAB database for the 3-year period. Of the 1212 cases identified, only 247 met the criteria necessary for our analysis: (1) normal or chronic colitis on histology; (2) melanosis coli; or (3) microscopic colitis including collagenous and lymphocytic colitis. The melanosis coli category was incorporated as it is possible that reported cases of diarrhea might not be due to laxative abuse. Cases that were excluded were: (1) known cases of inflammatory bowel disease, malignancy, radiotherapy, infective diarrhea, rectal bleeding, and an abnormal colonoscopy.

Table 1 Microscopic colitis cases identified by IHC using CD3

	No. of cases	¹ P value
Cases marked as normal, chronic inflammation or melanosis coli	241	
IELs ≥ 20	20	< 0.001
Known case of LC	1	0.158
Missed LC	5	< 0.001
Collagenous colitis	2	0.078
Microscopic colitis (Total No. of cases)	8	< 0.001

¹P value for comparing whether the proportion was different from 0. LC: Lymphocytic colitis.

Immunohistochemistry

Immunohistochemistry using antibodies against CD3 was performed on all cases as the primary evaluation method for IELs. The staining was performed on 4- μ m thick, formalin-fixed, paraffin-embedded tissue sections, using the Bond max autoimmune stainer with the Bond Polymer Refine Detection system (DS9800). Antibodies against CD3 (Leica Biosystems, Newcastle, UK; NCL-L-CD2-565, dilution 1:300) were applied to each case. For epitope retrieval ER2 (Leica Biosystems) was used for 20 min.

All immunohistochemical slides were randomly assigned a study number and an IEL count was performed. For a lymphocyte to be counted, the nucleus had to be visible with cytoplasmic and membrane staining. Inter-cryptal areas were counted and areas overlying lymphoid follicles were avoided. Only cases with ≥ 20 per 100 IELs were further investigated. In addition, all H and E-stained sections were re-evaluated for basement membrane thickening. In suspected cases, a Masson Trichrome stain was performed and the basement membrane measured with an Olympus ocular micrometer. Poorly orientated biopsies were excluded from evaluation.

Data regarding presenting history, microscopic diagnosis, patient age, sex and follow-up of patients with ≥ 20 IELs were recorded. A subcategory of patients presenting with chronic diarrhea was identified and further evaluated for microscopic colitis. The results were correlated with clinical findings.

Histological criteria

Histological diagnosis of lymphocytic colitis was confirmed with ≥ 20 IELs per 100 surface epithelial cells, with normal being < 5 ^[16,19,20]. In addition, a mixed inflammatory infiltrate in the lamina propria that consisted of lymphocytes and plasma cells with surface epithelial damage was noted^[3,16]. Diagnosis of collagenous colitis was established with a subepithelial collagen layer reaching or exceeding 10 μ m^[16,21,22].

Ethics

The study protocol was approved by the University of Stellenbosch Ethics committee.

Statistical analysis

Microsoft Excel was used to capture the data and STA-

TISTICA version 9 was used to analyze the data. Summary statistics were used to describe the demographic variables and certain laboratory parameters. Medians and means were used as the measures of central location and SDs and quartiles as indicators of spread. For demographic variables such as laboratory parameters, 95% CIs were calculated. Incidence rates in the population studied were determined as proportions and these were compared to determine whether they were significantly different from zero. $P < 0.05$ represented statistical significance in hypothesis testing.

RESULTS

Clinical and immunohistochemical findings

Immunohistochemical evaluation of 241 cases revealed a mean lymphocyte count of 7.7 (95% CI = 6.4-8.9). Twenty cases (8.3%) were identified as having an IEL count of ≥ 20 per 100 surface epithelial cells ($P < 0.001$) (Table 1).

These 20 patients were further categorized and the clinicopathological features summarized in Table 2. Six (2.5%) of the 241 patients were identified as having lymphocytic colitis ($P < 0.001$). Five (2%) of these patients were only diagnosed in this review and were therefore missed on initial evaluation ($P = 0.01$). Four of the five patients were labeled with irritable bowel syndrome (IBS). On follow-up, 3/5 patients had persistent diarrhea, despite ongoing investigations. On review, their diagnosis was changed to microscopic colitis. The remaining 2/5 patients were lost to follow-up. Clinical symptoms that were not in keeping with irritable bowel syndrome in this group included increase stool frequency of up to three times per day and abdominal pain that woke the patient at night. These patients were not evaluated for response to treatment.

We included two patients who were later diagnosed with malignant disease (myelodysplastic syndrome and renal cell carcinoma) after a 2-year follow-up. A third patient developed multiple liver lesions with an unknown primary tumor. In addition, three patients initially reported as having normal colonoscopy were diagnosed with diverticular disease ($n = 2$) and ulcerative colitis ($n = 1$). Subsequent review of the surgical notes indicated an error in documentation.

Among the remaining eight patients, the primary presenting symptom resolved in four. Two patients with abdominal pain were later diagnosed with pancreatitis and Behcet's disease. It is not clear if the latter patient's abdominal pain was related to her condition. Two patients were lost to follow-up.

The two (0.8%) cases of collagenous colitis that were included in the total study population of 241 ($P = 0.07$) had an IEL count of 7 and 8, respectively. No additional cases of collagenous colitis were identified by selective staining with the Masson Trichrome technique.

The most common presenting complaint was chronic diarrhea in 9/20 cases, abdominal pain in 7/20, and constipation in 3/20, followed by loss of weight in 1/20. Seventeen cases were originally reported as normal on histology; one of lymphocytic colitis and two of melanosis coli (Table 2).

Table 2 Clinicopathological features and follow-up data in patients with > 20 intra-epithelial lymphocytes

Age	Sex	IEL count	Presenting symptom	Original biopsy diagnosis	Follow-up period for 2 yr
32	F	22	Chronic diarrhea	Normal	Lymphocytic colitis ^{1,2}
32	F	27	Chronic diarrhea	Normal	Lymphocytic colitis ²
55	F	27	Constipation	Normal	Resolve
65	M	26	Abdominal pain	Normal	Myelodysplastic syndrome
22	F	31	Constipation	Normal	Lost to follow up
35	M	38	Chronic diarrhea	Normal	Lymphocytic colitis ¹
42	F	30	Constipation	Normal	Lost to follow up
59	F	28	Abdominal pain	Normal	Resolve
59	F	40	Chronic diarrhea	Normal	Multiple liver lesions
56	M	31	Chronic diarrhea	Normal	Ulcerative colitis
79	F	20	Abdominal pain	Melanosis coli	Diverticulitis
19	F	28	Abdominal pain	Normal	Behcet's disease
24	F	24	Chronic diarrhea	Normal	Lymphocytic colitis ²
51	M	48	Chronic diarrhea	Normal	Metastatic renal cell carcinoma
56	F	30	Chronic diarrhea	Normal	Lymphocytic colitis ²
34	F	30	Abdominal pain	Normal	Resolved
33	F	30	Chronic diarrhea	Lymphocytic colitis	Lymphocytic colitis ³
71	M	20	Loss of weight	Normal	Diverticulitis
59	F	25	Abdominal pain	Normal	Resolve
35	F	35	Abdominal pain	Melanosis coli	Pancreatitis

¹Lost to follow-up; ²On review diagnosis changed from irritable bowel syndrome to lymphocytic colitis; ³Known patient included in the study. IEL: Intra-epithelial lymphocyte.

Table 3 Clinicopathological features and follow-up data on patients with chronic diarrhea and 10-19 intra-epithelial lymphocytes

Age	Sex	IEL count	Original diagnosis	Comorbid disease	Follow-up period for 2 yr	
					Diagnosis	Diarrhea
54	F	10	Chronic inflammation	Diabetic hypertension	Lactose intolerant	Persistent
70	F	12	Melanosis coli	Diabetic	Autonomic neuropathy	Persistent
22	M	13	Chronic inflammation		Lost to FU	Lost to FU
64	M	15	Melanosis coli	Diabetic, asthmatic	Autonomic neuropathy	Persistent
59	F	16	Melanosis coli	Schistosomiasis contact	Irritable bowel syndrome	Persistent
64	F	18	Normal	Diabetic, asthmatic, previous sigmoidectomy for benign stricture	Hypothyroid	Persistent

IEL: Intra-epithelial lymphocyte; FU: Follow-up.

In addition, patients with an IEL > 10 and < 19 who presented with chronic diarrhea were documented to identify possible cases of paucicellular lymphocytic colitis (Table 3). Although no patients could be confidently diagnosed in this subgroup, comorbid disease such as diabetes accounted for several cases of diarrhea within this subgroup.

Histological findings

The histological findings among the different groups were very similar with IELs apparent in all 20 cases by H and E staining. However, the lymphocytes were more easily identified with the aid of CD3 (Figure 1). Poor staining quality and tangential biopsies accounted for misidentification of lymphocytes by H and E staining (Figure 2). Chronic inflammation was mild to moderate within the lamina propria.

In the lymphocytic colitis group, chronic inflammation was regarded as moderate in the lamina propria and 3/6 cases showed surface epithelial damage. Crypt branching was absent. Again, lymphocytes were more easily counted and identified with the immunohistochemical stain compared to H and E.

The two cases of collagenous colitis had a thick collagen band that measured 10 and 11 μ m with visible entrapment of capillaries and mild to moderate chronic inflammation in the lamina propria.

Pigment was confirmed in two cases of melanosis coli but was subtle. The site of most biopsies could not be verified because it was not documented. None of the patients with microscopic colitis was evaluated for response to medical treatment.

DISCUSSION

An increase in the awareness of the entity of microscopic colitis has resulted in it being recognized as a known cause of chronic watery diarrhea^[2,13,23]. Although the incidence of this disease seems to be rising, there has been very little documentation of this entity from our hospital. The importance of recognizing this condition is crucial, firstly, because chronic diarrhea is a debilitating illness, and secondly, treatment of this condition is no longer empirically based, as several recent randomized, double-blind, placebo-controlled trials have shown budesonide to be ef-

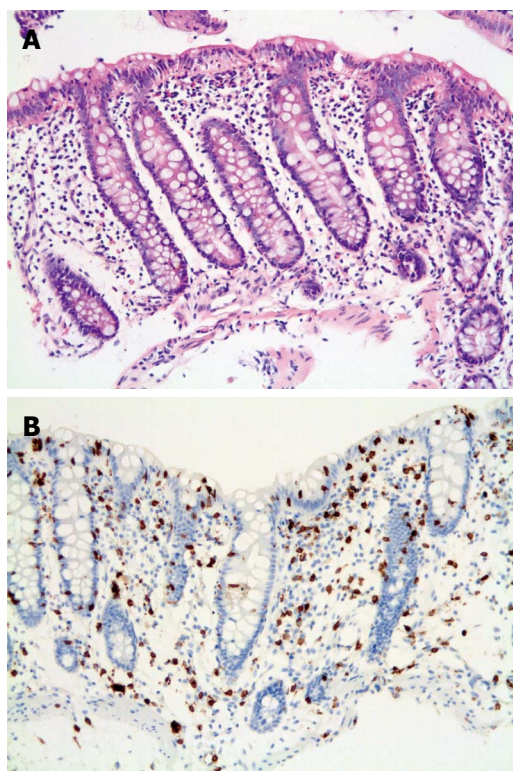


Figure 1 Lymphocytic colitis. A: Classic form. Colonic biopsy showing typical findings of diffuse increase in intraepithelial lymphocytes, mild inflammation with surface epithelial damage (H and E stain $\times 200$); B: CD3 immunohistochemistry highlighting lymphocytes ($\times 200$).

fective in the treatment of this disorder^[24-27].

Our study is novel in the sense that we reviewed all our non-malignant colon biopsies reported as normal or chronic inflammation, to identify patients with chronic diarrhea that might have had microscopic colitis. Using this bottom up approach, we focused on lymphocytic colitis. Neither the incidence nor the prevalence of this disease could be estimated using this approach, because our sample population did not consist of patients presenting exclusively with chronic diarrhea. Instead, we identified secondary causes of intraepithelial lymphocytosis that included diverticular disease, ulcerative colitis, and malignancy. These secondary causes need to be excluded before making a diagnosis of microscopic colitis^[14]. Other secondary causes of intraepithelial lymphocytosis, not identified in this study but described by Nielson, include Crohn's disease, colonic infections and amyloidosis^[14]. According to Fenoglio-Preiser^[28], there have also been reports of lymphocytic-colitis-like histology in patients with constipation, which is similar to our findings. We have also identified patients with abdominal pain as another group presenting with lymphocytosis. Although the clinical symptoms of constipation and abdominal pain resolved in a few cases, others were later identified as having significant pathology. Our results suggest that any normal colonoscopy with a finding of intraepithelial lymphocytosis should be carefully monitored for future disease.

In the present study, the diagnosis of lymphocytic

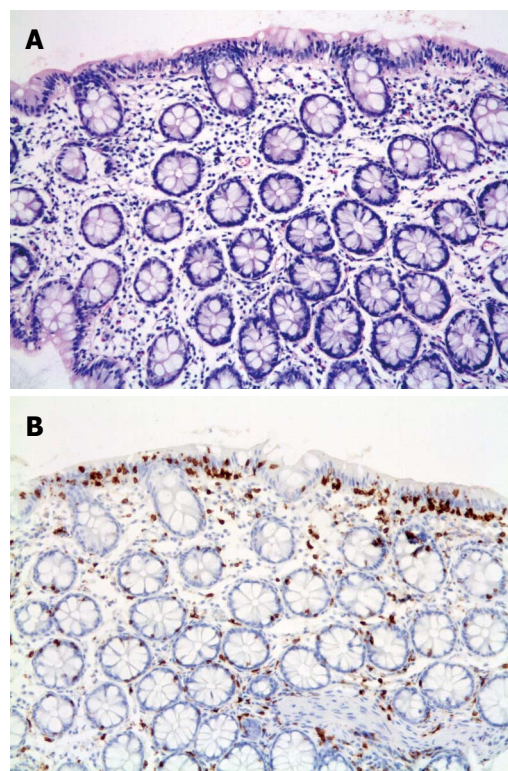


Figure 2 Lymphocytic colitis. A: Tangential colonic biopsy showing possible intraepithelial lymphocytes (H and E, original magnification $\times 200$); B: The intraepithelial lymphocytes are more prominent with CD3 immunostaining ($\times 200$).

colitis was missed in five patients at the initial histological evaluation. It is particularly interesting to note that four of these patients were labeled as having IBS, in view of the biopsy being reported as normal. In a population-based cohort from Olmsted County, approximately one half of patients with microscopic colitis met the symptom-based criteria for IBS^[29]. It is therefore not surprising that there is symptomatic overlap between these two entities. The recommendations from the Olmsted County study are that patients with diarrhea-predominant IBS should undergo colonoscopy to exclude microscopic colitis^[29]. Similarly, Madisch *et al*^[30] have shown that 30% of patients with microscopic colitis had clinical symptoms that overlap with IBS. We can therefore conclude that patients with microscopic colitis can be misdiagnosed with IBS.

Even though there is very little inter- and intra-observer variability in the histological diagnosis^[19], the diagnosis of microscopic colitis can be challenging at times, especially due to the morphological heterogeneity described in microscopic colitis. Since the initial description of lymphocytic colitis in 1989, there have been several atypical forms of microscopic colitis described^[15], including a paucicellular variant^[31]. In this variant, patients still have the same clinical symptoms, but the IEL count is less, with only 10-12 IELs/100 enterocytes cited^[32]. We feel that, in these cases, immunostaining might be of more diagnostic value in determining a low IEL that is not so apparent by H and E staining. A recent study has challenged the notion of regarding paucicellular lymphocytic colitis as a variant of classical lymphocytic colitis, based on the dem-

onstration of a distinct immunological difference^[33]. This group also has indirectly claimed that immunostaining displays a clear contrast between immunoreactive lymphocytes and negative epithelial cells. However, the comparison between H and E staining and immunohistochemistry was not directly evaluated in their study^[33]. Our study was not designed to identify cases of paucicellular lymphocytic colitis, but it is an area that requires further study.

As stated earlier, the prevalence of microscopic colitis is difficult to estimate from our study due to our selection criteria and referral bias. However, this study does indicate that microscopic colitis, especially the lymphocytic colitis subtype, is underdiagnosed at our institution ($P < 0.05$). For a true estimate of the prevalence of this disorder, further studies are needed, combining data from all referral centers in the region. Other factors not taken into account in this study are the site of the biopsy and drug history. It is well known that lymphocytic colitis and collagenous colitis can be patchy in distribution, and the topographic gradient of IELs decreases from the right colon to the rectum^[34]. Therefore, representative biopsies should be taken from each part of the colon and submitted in a separate container. Concomitant drug use can cause or worsen drug-induced microscopic colitis. It is important to recognize these drugs because drug withdrawal may improve symptoms. Among the more common drugs implicated are non-steroidal anti-inflammatory drugs, lansoprazole, clozapine, ranitidine, ticlopidine, carbose and flutamide^[32]. Future studies at our institution need to take these factors into account.

We identified that intraepithelial lymphocytosis may be an early manifestation of a disease other than microscopic colitis within our defined population. IEL count alone is not specific for microscopic colitis and the biopsy findings need to be correlated with clinical information for a more specific diagnosis. In cases in which there is a history of chronic watery diarrhea, the use of CD3 immunohistochemistry may be of additional value in making the diagnosis of lymphocytic colitis. We suggest that patients with diarrhea-predominant IBS should have a routine colonoscopy and be evaluated for microscopic colitis.

COMMENTS

Background

Microscopic colitis was previously considered a rare disorder, but it now accounts for approximately 10% of cases of chronic watery diarrhea. Cases are often under-recognized despite there being well-established histopathological criteria. It is suspected that colon mucosal biopsies are often under-reported as chronic inflammation, normal or colitis, not otherwise specified.

Research frontiers

Intra-epithelial lymphocytes (IELs) are crucial to the histological diagnosis of the lymphocytic colitis subtype. Immunohistochemistry has been shown to be of value in the quantification of IELs in celiac disease, but not in the identification and quantification of IELs in lymphocytic colitis.

Innovations and breakthroughs

A recent randomized, double-blind, placebo-controlled study has confirmed that budesonide is effective in the treatment of lymphocytic colitis. It has therefore become increasingly important to recognize this condition. This is believed to be the first time that normal colon biopsies were retrospectively reviewed and evaluated for IELs. The authors demonstrated that a subset of patients with chronic diarrhea was identified as having lymphocytic colitis using this approach.

Application

The value of this study demonstrates that immunohistochemistry is a useful adjunct to hematoxylin and eosin staining in the evaluation of IELs required for the diagnosis of lymphocytic colitis.

Terminology

Microscopic colitis is an umbrella term that comprises lymphocytic and collagenous subtypes. Although the latter is distinguished histologically by a thickened membrane, the clinical symptoms and colonoscopy findings are identical.

Peer review

This study illustrates well that patients with diarrhea-predominant irritable bowel syndrome should have a colon biopsy with close scrutiny of mucosal lymphocytes to exclude microscopic colitis.

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Extracapsular invasion as a risk factor for disease recurrence in colorectal cancer

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CONCLUSION: Our results suggest that ECI at metastatic nodes can identify which cases are at high risk of short-term disease recurrence in colorectal cancer.

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Key words: Extracapsular invasion; Lymph node; Metastasis; Colorectal cancer; Risk factor; Adjuvant therapy

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Abstract

AIM: To evaluate the presence of extracapsular invasion (ECI) in positive nodes as a predictor of disease recurrence in colorectal cancer.

METHODS: Two hundred and twenty-eight consecutive patients who underwent colorectal resection were identified for inclusion in this study, of which 46 had positive lymph nodes. Among 46 cases with stage III colorectal cancer, 16 had ECI at positive nodes and 8 had disease recurrence. The clinical and pathological features of these cases were reviewed.

RESULTS: In the univariate analysis, the number of positive lymph nodes and depth of tumor invasion were significantly associated with the presence of ECI at positive nodes. Multivariate analysis demonstrated that only ECI was a predictor of recurrence. The recurrence-free interval differed significantly among patients with ECI at positive nodes.

INTRODUCTION

The role of systemic adjuvant chemotherapy in colorectal cancer patients with lymph node involvement has been established in a large number of clinical trials^[1-3]. Lymph node status is one of the most important prognostic factors for colorectal carcinoma. However, patients with TNM stage III colorectal cancer are a heterogeneous group. Some patients with stage III colorectal cancer have good prognoses, similar to that of patients with stage II disease, whereas others develop disease recurrence. It is of utmost importance to develop markers that can predict which patients are at high risk for disease recurrence.

Previous studies have demonstrated and confirmed that the presence of extracapsular invasion (ECI) at metastatic lymph nodes is significantly related to prognosis in various types of carcinoma including colorectal cancer^[4-11]. We have also recently demonstrated that ECI at metastatic nodes in breast and colorectal cancer was strongly associated with

further regional nodes metastasis^[12]. The purpose of this study was to investigate the correlation between the presence of ECI in positive lymph nodes and disease recurrence in cases of colorectal cancer undergoing curative operation. It will be advantageous to be able to tailor therapy individually, using ECI as an indicator of the risk of recurrence.

MATERIALS AND METHODS

Two hundred and twenty-eight consecutive patients who underwent colorectal resection in the Department of General Surgical Science, Graduate School of Medicine, Gunma University, from January 2007 to December 2009 were identified for inclusion in this study. Patients with recurrence or metastasis at operation, neo-adjuvant chemotherapy, radiation, or incomplete clinical information were excluded. Of the eligible cases, 46 (20.2%) with positive lymph nodes, identified as TNM stage III colorectal, were analyzed in this study. The clinical features of these cases were reviewed according to the presence or not of ECI at positive lymph nodes, and statistical analysis was performed. ECI was defined as extracapsular growth of tumor cells, invasion into perinodal fat or extranodal location of tumor cells^[12]. Informed consent was obtained from all patients.

Age, sex, primary tumor size, location, depth of tumor invasion, histological type, lymphovascular invasion at the primary tumor site, number of metastatic lymph nodes, ECI at positive lymph nodes, administration of adjuvant therapy and serum tumor markers (carcinoembryonic antigen) were tested as possible predictors of disease recurrence. Recurrence-free interval was defined as the interval from surgery to the time disease recurrence was diagnosed. The overall median follow-up period was 1.7 years and none of the patients died of surgical complications. Fisher's exact test, the Chi-squared test, and the Student t-test were used to compare the 2 groups. Multivariate analysis was performed with logistic regression analysis to select covariates (primary tumor size and ECI at positive lymph nodes). The recurrence-free interval was calculated by the Kaplan-Meier method. The log-rank test was used to evaluate differences between recurrence-free intervals. Differences were considered to be significant at $P < 0.05$.

RESULTS

Table 1 summarizes the characteristics of the patients who underwent colorectal resection with TNM stage III colorectal cancer. The series consisted of 16 cases with ECI at positive nodes and 30 with no ECI at positive nodes. Table 1 also summarizes the results of the univariate analysis conducted to determine the relationship between the clinicopathologic variables and the presence of ECI at positive nodes. The number of positive lymph nodes and the depth of tumor invasion were significantly associated with the presence of ECI at positive nodes.

The 46 cases with metastatic lymph nodes were divided into 2 groups based on the presence of disease recurrence. Among 46 cases with stage III colorectal cancer, 8 (17.4%) had disease recurrence. Table 2 summarizes the results of

Table 1 Patients characteristics and clinicopathological features associated with the presence of extracapsular invasion at lymph node metastases

	ECI	Positive <i>n</i> = 16	Negative <i>n</i> = 30	<i>P</i> value
Age (yr)		65.3 ± 16.1	66.5 ± 13.8	0.798
Sex				
Male		7	21	0.082
Female		9	9	
Location				
Colon		13	20	0.295
Rectum		3	10	
Histological type				
Tub		15	28	0.957
Muc		1	2	
pT category				
T 1,2		1	10	0.040
T 3,4		15	20	
Tumor size (mm)		47.3 ± 15.1	40.4 ± 21.2	0.270
Number of positive LNs		3.63 ± 2.29	1.70 ± 1.27	0.001
Lymphovascular invasion		16	29	0.460
CEA ≥ 3.0		3	3	0.041
Adjuvant treatment	13	0.720	23	0.720

LN: Lymph node; ECI: Extracapsular invasion; CEA: Carcinoembryonic antigen.

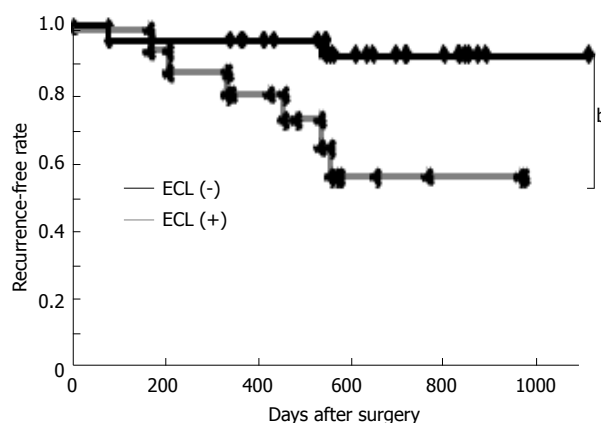


Figure 1 Impact of the presence of extracapsular invasion at positive nodes on postoperative recurrence-free interval. Recurrence-free interval by Kaplan-Meier curves differed significantly among patients with and without extracapsular invasion at positive nodes. ^b $P < 0.01$.

the univariate analysis conducted to determine the relationship between the clinicopathologic variables and disease recurrence. In the univariate analysis ECI at positive nodes and the depth of tumor invasion were the factors significantly associated with disease recurrence. Among those, multivariate analysis demonstrated that only ECI was a predictor of the recurrence ($P = 0.016$). Time to tumor recurrence by Kaplan-Meier curves was significantly shorter among patients with ECI at positive nodes (Figure 1).

DISCUSSION

The key observations made in this study can be summarized as follows: (1) the presence of ECI at positive nodes

Table 2 Patient characteristics and clinicopathological features associated with recurrent disease

Recurrent disease	Positive 8	Negative 38	P value
Age (yr)	68.0 ± 17.2	65.7 ± 14.0	0.780
Sex			
Male	3	25	0.278
Female	5	13	
Location			
Colon	6	27	0.795
Rectum	2	11	
Histological type			
Tub	7	36	0.451
Muc	1	2	
PT category			
T 1, 2	0	11	< 0.001
T 3, 4	8	27	
Tumor size (mm)	44.9 ± 11.6	42.5 ± 20.8	0.679
Number of positive LNs	2.38 ± 1.41	2.37 ± 2.02	0.906
ECI	6	10	0.009
Lymphovascular invasion	8	37	0.643
CEA	≥ 3.0	3	0.093
Adjuvant treatment	5	31	0.473

ECI: Extracapsular invasion; CEA: Carcinoembryonic antigen.

was significantly associated with the number of positive lymph nodes and depth of tumor invasion; (2) multivariate analysis demonstrated that only ECI was a predictor of recurrence; and (3) the recurrence-free interval by Kaplan-Meier curves was significantly shorter among patients with ECI at positive nodes. These findings suggest that the presence of ECI at positive lymph nodes is a strong predictor for short-term recurrence in cases with colorectal cancer undergoing curative surgery.

The surgical stage remains the most accurate predictor of survival for colorectal cancer^[13]. Pathologic prognostic factors of primary tumor invasion and regional node involvement predict the risk of relapse of cases with colorectal cancer undergoing curative operation. In the current study, both the number of positive lymph nodes and the depth of tumor invasion were significantly associated with the presence of ECI at positive nodes. Lymph node metastasis is one of the most important prognostic factors in patients with colorectal cancer, and many studies have indicated that the location and number of metastatic nodes affect prognosis^[5,14-16]. Regarding ECI, previous studies have demonstrated and confirmed that the presence of ECI at metastatic lymph nodes is significantly associated with prognoses in various types of carcinoma including colorectal carcinoma^[4-11]. The ability of metastatic nodes to recruit degradation factors that permit cancer cells to break through the lymph node capsule is indicative of a very aggressive cancer. We previously demonstrated that the presence of ECI in positive lymph nodes is significantly related to the nodal spread of tumor cells in colorectal and breast cancer patients^[12]. These studies imply that ECI is a biologic marker of aggressive cancer and essentially support our findings. Tumor cells are thought to invade the lymphovascular vessels, which enables tumor cells to spread metastatic or recurrent disease.

Adjuvant therapy is systemic treatment administered

with the intent of reducing the risk of recurrence. The benefit of adjuvant therapy in patients with lymph node involvement (stage III) has been well established in large prospective randomized trials^[1-3]. In Japan, the oxaliplatin plus 5-fluorouracil/leucovorin (LV) (FOLFOX) regimen has not been approved for adjuvant therapy for patients with stage III colorectal cancer at this time. In this Japanese population study, oral chemotherapeutic agents, including capecitabine or UFT (tegafur plus uracil) with oral LV, were used for adjuvant therapy for stage III colorectal cancer. Oral chemotherapeutic agents are advantageous because of their ease of administration. However, in the current series, 13 (81.3%) of the 16 cases with ECI in positive nodes had oral adjuvant therapy but 6 of 16 cases (37.5%) had disease recurrence. These results imply that high-risk patients with ECI at positive nodes should receive stronger adjuvant chemotherapy, including FOLFOX with or without monoclonal antibody.

This study has several potential limitations. The major limitation of our study is that it used retrospective methods of data collection. In addition, the number of cases in our study was relatively small and the follow-up periods were relatively short. However, the clinical implications of this data are very important, and these findings serve to emphasize that ECI at metastatic lymph nodes is an important prognostic factor for stage III colorectal carcinoma and will be advantageous in tailoring therapy to the individual case. In patients treated in phase III adjuvant clinical trials, disease-free survival and overall survival have been highly correlated, both within studies and across trials^[17]; however, in a number of patients, improving the quality of life and the length of recurrence-free intervals may be more important statistical parameters than median overall survival. Additional research is needed to explore this putative association between the presence of ECI and the risk of recurrence.

In conclusion, we have demonstrated that ECI at met-

astatic lymph nodes may predict which cases are at high risk of short-term disease recurrence in colorectal cancer. Thus, it will be possible to tailor therapy individually, using ECI as an indicator of the risk of recurrence. Analyses from large randomized trials or experimental data are warranted to evaluate this relationship between the presence of ECI and disease recurrence.

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COMMENTS

Background

The authors have demonstrated that extracapsular invasion (ECI) at metastatic nodes in breast and colorectal cancer was strongly associated with further regional nodes metastasis. The purpose of this study was to evaluate the presence of ECI in positive nodes as a predictor of disease recurrence disease in colorectal cancer.

Research frontiers

Lymph node status is one of the most important prognostic factors for colorectal carcinoma. However, patients with TNM stage III colorectal cancer are a heterogeneous group. It is of utmost importance to develop markers that can predict which patients are at high risk for disease recurrence.

Innovations and breakthroughs

This study suggests that the presence of ECI at positive lymph nodes is a strong predictor for short-term recurrent-free interval in cases with colorectal cancer undergoing curative operation.

Applications

It will be possible to tailor therapy individually, using ECI as an indicator of the risk of recurrence.

Terminology

ECI was defined as extracapsular growth of tumor cells, invasion into perinodal fat or extranodal location of tumor cells.

Peer review

The key observations made in this study suggest that the presence of ECI at positive lymph nodes is a strong predictor of short-term recurrent-free interval in cases with colorectal cancer undergoing curative operation.

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Impact of disease severity on gastric residual volume in critical patients

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tween mean daily GRV and SOFA scores and the correlation between mean daily GRV and mean APACHE II score of all patients were evaluated and compared.

RESULTS: Of the 61 patients, 43 patients were survivors and 18 patients were non-survivors. The mean daily GRV increased as SOFA scores increased ($P < 0.001$, analysis of variance). Mean APACHE II scores of all patients correlated with mean daily GRV ($P = 0.011$, Pearson correlation) during the study period. Patients with decreasing GRV in the first 2 d had better survival than patients without decreasing GRV ($P = 0.017$, log rank test).

CONCLUSION: GRV is higher in more severely ill medical ICU patients. Patients with decreasing GRV had lower ICU mortality than patients without decreasing GRV.

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Key words: Critical care; Outcome; Residual volume; Severity of illness index; Tube feeding

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Abstract

AIM: To investigate whether illness severity has an impact on gastric residual volume (GRV) in medical critically ill patients.

METHODS: Medical intensive care unit (ICU) patients requiring nasogastric feeding were enrolled. Sequential Organ Failure Assessment (SOFA) score was assessed immediately preceding the start of the study. Acute Physiology and Chronic Health Evaluation (APACHE) II scores were recorded on the first, fourth, seventh, and fourteenth day of the study period. GRV was measured every 4 h during enteral feeding. The relationship be-

INTRODUCTION

Malnutrition is prevalent in intensive care unit (ICU) patients and is associated with increased morbidity and mortality^[1]. Early administration of enteral nutrition to

critically ill patients has been associated with a significantly lower incidence of infections and a reduced length of hospital stay^[2,3]. However, intragastric enteral nutrition often is complicated by intolerance, as indicated by elevated volumes of aspirated gastric residuals^[4]. Disordered upper gastrointestinal (GI) tract motility occurs frequently in ICU patients. Intolerance to nasogastric delivery of feeding is the most important consequence of the abnormal upper GI motility that occurs in critically ill patients^[5]. Several factors related to critical illness have been reported to be associated with gastric dysmotility and feeding intolerance including age, admission diagnosis, hyperglycemia, the nature of the acute illness, mechanical ventilation, sedatives, cytokine release and splanchnic hypoperfusion due to shock and sepsis^[6]. Two studies have shown that “upper digestive intolerance” and “enteral feeding intolerance” are linked to adverse outcomes, suggesting that decreased gastric emptying (GE) is related to clinical deterioration and worsening of patient outcomes^[7,8]. Direct measurement of GE is usually inconvenient and impractical in routine clinical practice. Clinically, gastric residual volumes (GRVs) are easier to measure than GE, and GRV measurements are by far the most frequently recommended assessment for GE^[9]. They are used as a surrogate marker to determine the success or failure of nutrition delivered *via* a nasogastric route. However, the relationship between GRV and disease severity is not clear. The aim of this study is to investigate whether disease severity has an impact on GRV and whether GRV is a predictor of ICU mortality.

MATERIALS AND METHODS

This prospective, observational study was conducted during a 2-year period from January 2005 to December 2006 in a medical ICU of a tertiary medical center. Patients who required enteral feeding were enrolled. Criteria for exclusion included abdominal surgery, acute pancreatitis, GI bleeding, intestinal obstruction, and patients with subtotal or total gastrectomy. The protocol was approved by the Human Investigation and Research Committee of the hospital.

After informed consent was obtained, the following demographic data were collected: primary ICU admission diagnosis, age, gender, body mass index (BMI), use of mechanical ventilation, Sequential Organ Failure Assessment (SOFA) score^[10], Acute Physiology and Chronic Health Evaluation (APACHE) II score^[11], blood glucose level, number of ICU days, ventilator days, hospital days, and Glasgow Coma Scale score. A standard 12 French enteral feeding tube (Abbott, Chicago, IL, USA) for general patients was placed into the stomach. The correct position of the nasogastric tube was confirmed by injecting 50 mL of air with a syringe into the tube and auscultating the epigastric area, or by radiograph if necessary. We checked the tube position by measuring the exposed portion of the tube and compared the length with previous measurements. The patients were fed in a semi-recumbent position, and the patient's position and tube length were kept the same in each measurement. As soon

as the feeding tube was inserted, continuous tube feeding using enteral feeding pumps (Abbott) was started. Enteral feeding was initiated at 20 mL/h. The rate was increased by 20 mL/h every 4 h until the volume required to meet the patient's optimum caloric support was achieved. The rate of continuous enteral feeding was controlled by the pumps. GRV was measured by aspirating with a 50-mL syringe every 4 h until the end of enteral feeding. Feeding was stopped for 30 min before GRV was measured. After measurement, the nurses stopped enteral tube feedings if residual volume was higher than 500 mL or residual volume was between 200 to 500 mL and patients had abdominal distension, absence of bowel sounds, or presence of nausea or vomiting^[12]. Feeding re-started immediately at original rate if GRV < 200 mL and there was low risk of aspiration. Daily GRV was calculated by summation of each GRV measurement. Serum glucose was controlled by an intensive insulin control protocol in order to reach the target glucose level of 140 mg/dL.

APACHE II scores^[11] were recorded on the first, fourth, seventh, and fourteenth day of the study period. Study observations continued from start of enteral feeding until one of the following events occurred: the enteral tube was removed, the patient was discharged from the ICU, or he/she expired. SOFA scores^[10] were assessed within a 24-h period preceding the start of study as the presence or absence of prospectively defined cardiovascular, respiratory, renal, hepatic and hematologic dysfunction, as well as level of consciousness. Dysfunction of cardiovascular, respiratory, renal, hepatic, hematologic and central nervous systems was determined based on laboratory data, vasopressor dosage, Glasgow Coma Scale score, and PaO₂/FiO₂.

Patients were classified as diabetic on the basis of their medical history. Survivors were defined as patients who were alive when discharged from the ICU or transferred to a general medical ward; this was determined at time of ICU discharge.

Statistical analysis

All the statistical analyses were done with the SPSS (Inc., Chicago, IL, USA) version 12.0. mean \pm SE were recorded for all continuous variables. For discrete variables, the frequencies were reported. One way analysis of variance (ANOVA) was used to compare differences among more than 2 groups. Pearson correlation was used to compare correlation between daily GRV and APACHE II score. Kaplan-Meier curves were used to estimate the probability of survival. Log-rank test was used to compare the difference between the patients with decreasing daily GRV and patients without decreasing daily GRV in the first 2 d. Cox model was used to construct the relative risk among the percentage change of daily GRV in the first 2 d. All *P* values were two-tailed. A *P* value < 0.05 was considered as significant.

RESULTS

Demographics

Sixty-one patients were enrolled in this study. Patient char-

Table 1 Demographic data of the 61 study subjects (mean \pm SE) *n* (%)

Average age (yr)	67.9 \pm 2.0
Gender (male)	43/61 (70.1)
Body mass index (kg/m ²)	23.1 \pm 0.5
Diabetic patients	22/61 (36.1)
Mechanically ventilated patients	61/61 (100)
Study days	11.7 \pm 0.8
ICU days	19.1 \pm 1.6
Ventilator days	23.8 \pm 2.3
Hospital days	31.7 \pm 2.7
SOFA score	7.5 \pm 0.6
APACHE II score	20.2 \pm 0.9
Blood glucose level (mg/dL)	184.7 \pm 8.6
ICU mortality rate	18/61 (29.5)
Admission diagnosis	
Pneumonia	19/61 (31.1)
Sepsis	18/61 (29.5)
CHF	6/61 (9.8)
ARDS	6/61 (9.8)
Stroke	5/61 (8.2)
COPD or asthma	5/61 (8.2)
Myocardial infarction	1/61 (1.6)
Toxic overdose	1/61 (1.6)

ICU: Intensive care unit; SOFA: Sequential organ failure assessment; APACHE: Acute physiology and chronic health evaluation; CHF: Congestive heart failure; ARDS: Acute respiratory distress syndrome; COPD: Chronic obstructive pulmonary disease.

acteristics are summarized in Table 1. The mean patient age was 67.9 ± 2.0 years, and 70.1% of the patients were male. The mean BMI was 23.1 ± 0.5 kg/m². Thirty-six percent of patients had diabetes and all patients were mechanically ventilated. The mean number of study days was 11.7 ± 0.8 d, mean number of ICU days was 19.1 ± 1.6 d, mean number of ventilator days was 23.8 ± 2.3 d, mean number of hospital days was 31.7 ± 2.7 d, mean SOFA score was 7.5 ± 0.6 , mean APACHE II score was 20.2 ± 0.9 , and mean blood glucose was 184.7 ± 8.6 mg/dL. The ICU mortality rate was 29.5%. The 4 most common admission diagnoses were pneumonia ($n = 19$), sepsis ($n = 18$), congestive heart failure ($n = 6$) and acute respiratory distress syndrome ($n = 6$).

Relationship between mean daily GRV and SOFA score

We stratified study patients into 4 groups by SOFA score. There were 27 patients with SOFA scores below 6; 18 patients with scores in the range of 6-10; 10 patients with scores in the range of 11-15; and 6 patients with scores above 15. Figure 1 demonstrates that the mean daily GRV was 7.8 ± 1.3 mL in the patients with a SOFA score below 6, 26.6 ± 4.8 mL in the patients with SOFA scores in the range of 6-10, 59.8 ± 4.5 mL in the patients with SOFA scores in the range of 11-15, and 133.2 ± 40.0 mL in the patients with a SOFA score above 15. Patient with higher SOFA scores had significantly higher daily GRV ($P < 0.001$, ANOVA).

Relationship between daily GRV and APACHE II score during study period

During the study period, the mean APACHE II scores

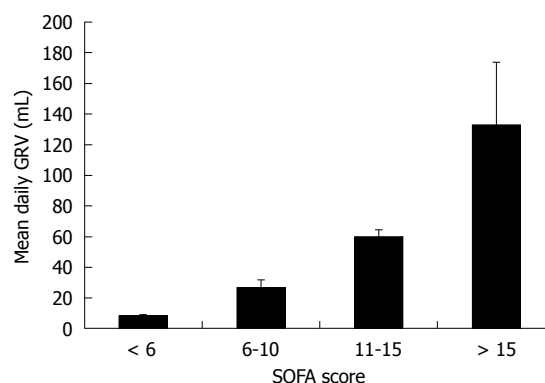


Figure 1 Mean daily gastric residual volume of patients with different Sequential Organ Failure Assessment scores ($P < 0.001$). SOFA: Sequential Organ Failure Assessment.

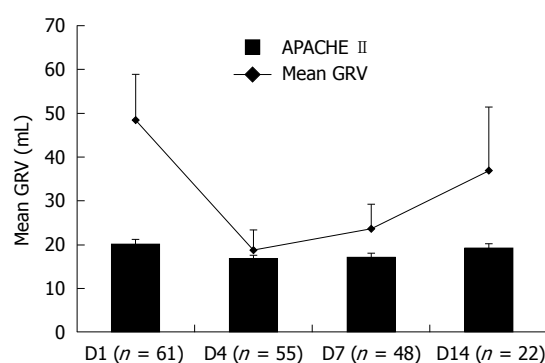


Figure 2 The relationship of mean daily Acute Physiology and Chronic Health Evaluation II score to mean daily gastric residual volume on different study dates ($P = 0.011$).

and mean daily GRV of all patients on the first, fourth, seventh, and fourteenth day are shown in Figure 2. The mean APACHE II scores of all patients on the first, fourth, seventh, and fourteenth day were 20.2 ± 0.9 , 16.8 ± 0.7 , 17.0 ± 0.9 and 19.1 ± 1.1 , respectively. The mean daily GRVs of all patients on the first, fourth, seventh, and fourteenth day were 48.4 ± 10.4 , 18.6 ± 4.6 , 23.6 ± 5.5 and 36.9 ± 14.4 mL, respectively. The mean daily GRV fluctuated simultaneously with the mean APACHE II scores during the study period. There was a significant correlation between daily GRV and APACHE II score ($P = 0.011$, Pearson correlation = 0.338).

Difference of mean daily GRV between survivors and non-survivors

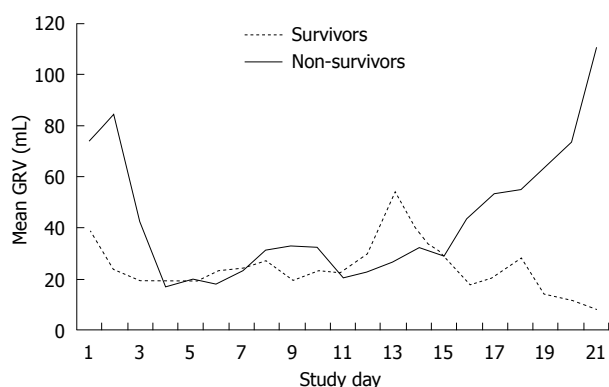
We divided study patients into survivors and non-survivors. There were 43 patients in the survivor group and 18 patients in the non-survivor group. Figure 3 shows the mean daily GRV of survivors and non-survivors during the study period. The mean daily GRV of non-survivors was higher than that of survivors in the early and late stages of the study period. Non-survivors had a trend of increasing mean daily GRV in the first 2 d, while survivors had a decreasing trend of GRV.

Table 2 demonstrates that the mean GRV1 (daily GRV on the first day) was 74.4 ± 31.0 mL in non-survivors

Table 2 Difference of GRV1 and PDay12 between survivors and non-survivors (mean \pm SE)

	Non-survivors (<i>n</i> = 18)	Survivors (<i>n</i> = 43)
GRV1 (mL)	74.4 \pm 31.0	38.0 \pm 6.9
PDay12	-1.1 \pm 0.6	0.3 \pm 0.1

GRV1: Gastric residual volume on the first day; GRV2: Gastric residual volume on the second day. PDay12 = (GRV1 - GRV2)/GRV1.

**Figure 3** Mean daily gastric residual volume change of survivors and non-survivors during the study period.

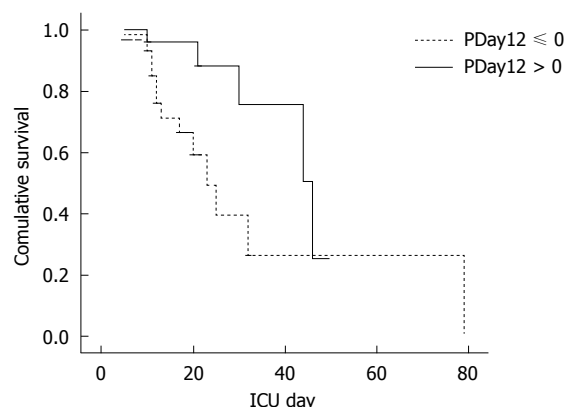
and 38.0 \pm 6.9 mL in survivors. Additionally, the mean [GRV1-GRV2 (daily GRV on the second day)]/GRV1 was -1.1 \pm 0.6 mL in non-survivors and 0.3 \pm 0.1 mL in survivors. If we define the percentage of daily GRV change from day 1 to day 2 (PDay12) as (GRV1-GRV2)/GRV1, non-survivors had a negative PDay12 while survivors had a positive PDay12.

Change of daily GRV in first 2 d and ICU mortality

We categorized patients into 2 groups; one group was patients without a decreasing daily GRV (PDay12 \leq 0) in the first 2 d, and the other group was patients with a decreasing daily GRV in the first 2 d (PDay12 > 0). Thirty patients did not have a decreasing daily GRV (PDay12 \leq 0) and 31 patients had a decreasing daily GRV (PDay12 > 0). Patients with PDay12 > 0 had a significantly higher ICU survival rate than patients with PDay12 \leq 0 (P = 0.017) (Figure 4). Relative risk among the percentage change of daily GRV in the first 2 d was constructed by Cox model. The relative risk is equal to $\exp \{-1.211x\}$ where x represents the percentage change of daily GRV in the first 2 d. If the daily GRV decreased by 10% in the second day, the relative risk is equal to 0.886 ($= \exp \{-0.1211\}$). If the daily GRV increased by 10% in the second day, the relative risk is equal to 1.129 ($= \exp \{0.1211\}$). There was no evidence indicating that the proportional hazards assumption was not fixed in the data set.

DISCUSSION

In this prospective study, we found disease severity was associated with GRV in these medical ICU patients. This

**Figure 4** Kaplan-Meier estimates of the probability of survival between patients with decreasing change of daily gastric residual volume (PDay12 > 0) and patients without decreasing change of daily gastric residual volume (PDay12 \leq 0) (P = 0.017).

study showed patients with higher SOFA scores had higher GRV, i.e. GRV tended to be higher in more severely ill patients. These results are similar to those of the study by Mentec *et al.*^[8], in which patients with high gastric aspirate volume had a higher ICU mortality rate. Slow GE may partly explain why patients with more severe illness had higher GRV. GRV is determined by the balance between the amount of infused formula plus endogenous secretions (saliva and gastric secretions), and the amount of fluid emptied from the stomach^[9]. GE is influenced by many factors, including admission diagnosis, nature of illness, age, medications and mechanical ventilation^[5,6,8,9]. Nguyen *et al.*^[6] reported that slow GE was more common in patients who were older, had higher admission APACHE II scores, admission blood glucose and bilirubin concentrations, and were ventilated with synchronized intermittent mandatory ventilation. Of these, APACHE II scores correlated best with GE, suggesting that illness severity is an important determinant of GE in critically ill patients. Patients with severe illnesses have high levels of circulating catecholamines which are likely to have an impact on GI motor function. Adrenaline reduces GE *via* a β -adrenergic effect^[13].

During the study period, we also found a significant correlation between mean APACHE II scores and mean GRV. There was a trend that patients with higher APACHE II scores had higher daily GRV. Our results also demonstrated that illness severity was related to GRV, and illness severity varied during the ICU course. Concurrent day-to-day variation in illness severity and daily GRV were seen in this study. GRV was the greatest in the first few days of tube feedings. Some of the severely ill patients with high APACHE II scores expired in the first few days, thus the overall illness severity of patients declined from the first day to the fourth day, so mean APACHE II score also decreased from the first day to the fourth day. Mean daily GRV decreased as mean APACHE II score decreased. As the study went on, some patients improved, they were extubated, and their enteral feeding stopped; therefore the number of

study patients decreased gradually. The rest of the study patients were more complicated or severely ill and they had higher APACHE II scores, thus the mean APACHE II score increased gradually after the seventh day of the study. From that time on, mean daily GRV also increased gradually. Mean daily GRV exhibited a good correlation with APACHE II scores ($P < 0.05$).

In the interim analysis, we also found that patients with the same SOFA score had widely different daily GRV. This suggests that there was wide variability of daily GRV among patients with the same disease severity, since GRV is not only influenced by disease severity but also by other factors such as GE, medications, size of the feeding tube and timing of measurement.

GE itself is influenced by many factors. Admission diagnosis had modest impact on GE in critically ill patients^[6]. Most patients with increased intracranial pressure after a head injury have been found to have slow GE, and elevated intracranial pressure is thought to be the main mediator of impaired gastric motility and emptying^[14]. Other diseases associated with delayed GE include burns, multiple trauma, sepsis, chronic liver disease, and renal disease^[6,15]. Patients with myocardial injury and non-intestinal post-operative respiratory failure have the lowest incidence of delayed GE^[6]. Hyperglycemia has also been shown to result in delayed GE^[16].

Medications used routinely in ICU patients are also likely to have clinically important effects on GI motor function. Opioids and benzodiazepines can impair gastric motility and reduce GE^[17,18]. High gastric aspirate volume was more frequently seen in patients who received at least 1 d of sedation^[8]. Both endogenous and administered opiates, acting *via* μ receptors, may contribute to abnormal upper GI motor function^[19,20]. Dopamine slows GE by reducing antral contractions^[21]. Its negative effect on GI motility can be seen at doses as low as 5 μ g/kg per minute, and the effect increases with increasing rates of infusion^[22]. Proton pump inhibitors^[23] and cimetidine^[24] can delay GE. Other medications such as phenothiazines, diltiazem, verapamil and anticholinergic drugs also cause GI hypomotility^[5]. Use of promotility agents is associated with reduced GRV^[25], e.g. erythromycin is a powerful stimulator of gastric contractions^[26].

The size of enteral feeding tube has also been shown to influence the measurement of GRV. Higher GRVs are found in patients with larger enteral feeding tubes^[27]. Timing of measurement of GRV also affects the value of GRV. GRV typically increases and then plateaus in the first 3 to 6 h after feeding. The highest GRV tends to occur in the 2 to 4 h after initiation of feedings. Additionally, GRV may vary with different infusion rate. To avoid this confounding factor, we stopped feeding for 30 min prior to measurement of GRV. This is the reason why the measured GRV values in this study are lower than in previous studies^[28,29].

As discussed above, there are many factors that influence GRV. This results in wide variability of daily GRV in patients with the same illness severity. For this reason,

we used daily GRV change rather than a single daily GRV to predict a patient's ICU outcome. This may avoid some of the confounding factors such as admission diagnosis, nature of illness, medications and age, as it reflects the change of illness severity more accurately. In addition, we also found that there was a different trend of GRV change between survivors and non-survivors in the first 2 d. Daily GRV tended to increase in non-survivors and decrease in survivors. We found that patients with a decreasing change of daily GRV had better ICU survival than those without a decreasing change of daily GRV in the first 2 study days. Because daily GRV correlated with illness severity, decreasing changes of daily GRV in the earlier ICU days represented decreasing illness severity; thus could indicate a positive sign for the medical ICU patients.

There are limitations in this study. Firstly, this was not a double-blind study. However, nurses who measured GRV were not aware of the study purpose. Secondly, all patients in this study were mechanically ventilated and enrolled from a medical ICU; thus, the results may not be applicable to all critically ill patients. Further larger studies including all critically ill patients are needed. Thirdly, we did not know how many patients had diabetes with gastroparesis which is characterized by severely slow GE; such patients may have increased GRVs secondary to gastroparesis^[30].

In conclusion, illness severity has an impact on daily GRV. There was a trend that more severely ill medical ICU patients had higher daily GRV. Patients with decreasing change of daily GRV in the earlier ICU days had better ICU survival than patients without decreasing change of daily GRV.

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COMMENTS

Background

Disordered upper gastrointestinal tract motility occurs frequently in critical patients, resulting in intolerance to nasogastric delivery of feeding. Upper digestive intolerance and enteral feeding intolerance are linked to adverse outcomes, suggesting that decreased gastric emptying (GE) is related to clinical deterioration. It is easier to measure gastric residual volume (GRV) than GE, but it is not clear whether illness severity has an impact on GRV.

Research frontiers

GE was influenced by age, Acute Physiology and Chronic Health Evaluation (APACHE) II score, nature of illness, medication and mechanical ventilation. Of these, APACHE II score correlated best with GE. This study found disease severity had an impact on GRV. Concurrent day-to-day variation in illness severity and daily GRV were seen during hospitalization course. There is a trend that GRV increases when disease severity increases, and *vice versa*.

Innovations and breakthroughs

Patients with decreasing change of GRV in the earlier intensive care unit (ICU) had better ICU outcome than patients without decreasing change. We emphasized that change of daily GRV, not a single daily GRV, can predict ICU

outcome. Since GRV was influenced by many factors, change of daily GRV can avoid some confounding factors other than illness severity.

Applications

Increasing change of daily GRV is a negative sign for critical patients. In the clinical situation, we should be careful if a patient has increasing change of daily GRV day by day as it may indicate the patient getting gradually worse.

Terminology

GRV is determined by the balance between the amount of infused formula plus endogenous secretion and the amount of fluid emptied from the stomach. APACHE II score, a system for classifying disease severity, is widely used to predict hospital mortality based on a number of laboratory values and patient characteristics. Sequential Organ Failure Assessment scores were assessed as presence or absence of cardiovascular, respiratory, renal, hepatic, hematologic and central nervous systems dysfunction. A higher score means more severe illness.

Peer review

The manuscript is of good quality.

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Effect of multidisciplinary team treatment on outcomes of patients with gastrointestinal malignancy

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Abstract

AIM: To evaluate the effect of multidisciplinary team (MDT) treatment modality on outcomes of patients with gastrointestinal malignancy in China.

METHODS: Data about patients with gastric and colorectal cancer treated in our center during the past 10 years were collected and divided into two parts. Part 1 consisted of the data collected from 516 consecutive complicated cases discussed at MDT meetings in Peking University School of Oncology (PKUSO) from December 2005 to July 2009. Part 2 consisted of the data collected from 263 consecutive cases of resect-

able locally advanced rectal cancer from January 2001 to January 2005. These 263 patients were divided into neoadjuvant therapy (NT) group and control group. Patients in NT group received MDT treatment, namely neoadjuvant therapy + surgery + postoperative adjuvant therapy. Patients in control group underwent direct surgery + postoperative adjuvant therapy. The outcomes in two groups were compared.

RESULTS: The treatment strategy was altered after discussed at MDT meeting in 76.81% of gastric cancer patients and in 58.33% of colorectal cancer patients before operation. The sphincter-preservation and local control of tumor were better in NT group than in control group. The 5-year overall survival rate was also higher in NT group than in control group (77.23% vs 69.75%, $P = 0.049$).

CONCLUSION: MDT treatment modality can significantly improve the outcomes of patients with gastrointestinal malignancy in China.

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Key words: Multidisciplinary team; Rectal cancer; Neoadjuvant radiotherapy; Prognosis

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INTRODUCTION

Treatment of cancer has evolved toward a multidisciplinary

team (MDT) approach^[1-3]. The effect of MDT treatment modality on cancer is significantly better than that of conventional treatment modalities^[4-6]. Although the MDT treatment modality has been successfully implemented in Western countries for decades, no report is available on its application in China. We conducted a study on the MDT treatment modality in a representative cancer center of China to evaluate its effect on outcomes of patients with gastrointestinal malignancy in China.

MATERIALS AND METHODS

Clinical data

Data about patients with gastric and colorectal cancer were collected and divided into two parts. Part 1 consisted of the data collected from 516 consecutive complicated cases discussed at MDT meetings in Peking University School of Oncology (PKUSO) from December 2005 to July 2009. Complicated cases were defined as those with synchronic distant metastasis, marginally resectable or unresectable lesions, postoperative progression, and other conditions leading to difficulty in making treatment strategy. Records and treatment plans or recommendations for MDT treatment were used to investigate the effect of MDT treatment modality on clinical decision making and outcomes of patients with gastrointestinal malignancy (Table 1).

Part 2 consisted of the data collected from 263 consecutive cases of resectable locally advanced rectal cancer from January 2001 to January 2005. Patients included in this study were those with resectable rectal cancer located 12 cm or less from the anal verge, histologically identified primary carcinoma of the rectum, no clinical evidence of preoperative distant metastasis, transabdominal radical resection based on the principle of total mesorectal excision (TME)^[7], and R0 resection. Finally, 263 eligible patients included in this study (Table 2) were divided into neoadjuvant therapy (NT) group and control group according to whether they underwent neoadjuvant radiotherapy.

Treatment strategy

Patients in NT group received neoadjuvant therapy + surgery + postoperative adjuvant therapy. The total preoperative radiation dose was 30 Gy (30 Gy/10 fractions, bioequivalent dose 36 Gy) recommended by the Chinese Anti-Cancer Association (CACA)^[8], and 5-FU or capecitabine was used in postoperative chemotherapy.

Contrast to MDT treatment, the conventional treatment strategy for locally advanced rectal cancer in China is surgery followed by postoperative chemoradiotherapy which was commonly used in China 5 years ago. Patients in NT group were evaluated before operation by special MDT members while those in control group were not evaluated.

Follow-up

Patients were followed-up every three months for the first 2 years after surgery followed by every six months for 5 years. Serum carcinoembryonic antigen (CEA) level was

Table 1 Complicated cases of different types of cancer discussed at multidisciplinary team meetings *n* (%)

Variables	Rectal cancer	Colon cancer	Gastric cancer
Pre-operation	68 (33.10)	40 (30.77)	69 (38.12)
Postoperative progression ¹	121 (59.02)	62 (47.69)	101 (55.80)
Other condition	16 (7.80)	28 (21.54)	11 (6.08)
Total	205 (100)	130 (100)	181 (100)

¹Including patients with both local recurrence and distant metastasis.

Table 2 Baseline characteristics of 263 patients with locally advanced rectal cancer *n* (%)

Baseline characteristics	NT group (<i>n</i> = 101)	Control group (<i>n</i> = 162)	<i>P</i> value
Sex			
Male	57	88	0.737
Female	44	74	
Age (yr) ¹	55 (51-59)	55 (50-60)	0.664
Distance of tumor from anal verge			
< 5 cm	35 (34.7)	37 (22.8)	0.051
5-12 cm	66 (65.3)	125 (77.2)	
Surgery			
APR	25	32	0.422
LAR	76	130	
Preoperative serum CEA level			
Normal	52 (51.5)	82 (50.6)	0.745
Abnormal	35 (34.7)	52 (32.1)	
Unknown	14 (13.9)	28 (17.3)	
Pretreatment staging tools			
MRI	61 (60.4)	66 (40.7)	< 0.001
ERUS	28 (27.7)	34 (21.0)	
CT	12 (11.9)	62 (38.3)	
Pretreatment TNM stage			
II A (T3 N0)	24 (23.8)	54 (34.0)	0.278
II B (T4 N0)	4 (4.0)	4 (2.5)	
III A (T1-2 N1)	3 (3.0)	8 (4.9)	
III B (T3-4 N1)	32 (31.7)	37 (22.8)	
III C (AnyT N2)	38 (37.6)	58 (35.8)	
Pathologic TNM stage			
I (T1-2 N0)	35 (34.7)	12 (7.4)	< 0.01
II A (T3 N0)	26 (25.7)	54 (33.3)	
II B (T4 N0)	1 (1.0)	0 (0)	
III A (T1-2 N1)	6 (5.9)	7 (4.3)	
III B (T3-4 N1)	17 (16.8)	40 (24.7)	
III C (AnyT N2)	16 (15.8)	49 (30.2)	
Histologic differentiation			
High	2 (2.0)	20 (12.3)	0.013
Moderate	70 (69.3)	110 (67.9)	
Poor	24 (23.8)	24 (14.8)	
Mucinous and signet	5 (5.0)	8 (4.9)	
Lymphovascular invasion			
Present	21 (20.8)	50 (30.9)	0.074
Absent	80 (79.2)	112 (69.1)	

¹Values are medians (interquartile ranges). NT: Neoadjuvant therapy; APR: Abdominal-perineal resection; LAR: Low anterior resection; MRI: Magnetic resonance imaging; ERUS: Endorectal ultrasonography; CT: computed tomography; CEA: Carcinoembryonic antigen.

measured and abdominal ultrasound, pelvic MRI, chest radiograph were performed every six months, and colonoscopy was performed annually during the follow-up. The follow-up time ranged from six to ninety-six months, with a median time of seventy-two months. The outcomes of

patients with gastrointestinal malignancy were evaluated at the end of 5-year follow-up with a follow-up rate of 87.8% (231/263).

Statistical analysis

Demographic and clinicopathologic data were analyzed by χ^2 test. Kaplan-Meier life table and log-rank test were used to compare the disease-free survival (DFS) and overall survival (OS) rates. Cox proportional hazards regression was used in multivariate analysis. Statistical analysis was performed using the SPSS version 16.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

MDT treatment modality

The working model of MDT in our center includes two major components: weekly MDT meetings to discuss complicated clinical cases and interdisciplinary consultations for preoperative and postoperative evaluation and therapy. Most patients receive MDT therapy according to interdisciplinary consultations while only complicated cases are discussed at MDT meetings. Although the MDT team modalities are different, the treatment strategies for patients are made by the same team in our center. The key members of MDT team include a surgeon, a medical oncologist, a radiation oncologist, a radiologist, a pathologist, and specialized nurses. Attendance of the key members at MDT meetings is not compulsory but enhanced by a special coordinator who is responsible for organizing and recording the MDT meetings. The discussion processes and conclusions for each patient are recorded in special tables.

Effect of MDT meetings on clinical decision making and outcomes of cancer patients

Complicated cases of gastric cancer ($n = 181$), colon cancer ($n = 130$), and rectal cancer ($n = 205$) were discussed at MDT meetings during the last 5 years (Table 1). Among the discussed cases, outpatients accounted for 84.69% ($n = 437$) and inpatients accounted for 15.31% ($n = 79$), respectively. For each disease classification, patients with postoperative recurrence or metastasis accounted for 48%-59%, suggesting that such patients are needed to be discussed at MDT meetings.

The MDT team modality directly influenced the clinical decision making. Of the 69 preoperative patients with gastric cancer discussed at MDT meetings, 53 (76.81%) underwent neoadjuvant chemotherapy instead of direct surgery. Of the 63 preoperative patients with extensive lesions or synchronous distant metastasis of colorectal cancer who underwent MDT treatment, including chemotherapy, chemoradiotherapy, or target therapy, 7 with initially inoperable liver metastasis underwent radical resection after MDT treatment.

Effect of MDT treatment on clinical outcomes of rectal cancer patients

To verify the comparability of outcomes in NT and con-

Table 3 Clinical outcome of patients in two groups n (%)

Clinical outcome	NT group ($n = 101$)	Control group ($n = 162$)	Odds ratio (95% CI)	P value
Sphincter preservation ¹	13 (37.14)	5 (13.51)	3.78 (1.18-12.13)	0.041
Local recurrence	4 (3.96)	18 (11.11)	0.33 (0.11-1.00)	0.042
Distant metastasis	22 (21.78)	36 (22.22)	0.87 (0.48-1.57)	0.933
5-yr disease-free survival rate	77 (76.24)	109 (67.28)	-	0.039
5-yr overall survival rate	78 (77.23)	113 (69.75)	-	0.049

¹Within the patients whose distance of tumor from anal verge were less than 5 cm, $n = 72$ (Table 1). NT: Neoadjuvant therapy.

trol groups, the major demographic and tumor variables were analyzed (Table 2). No difference was found in gender and age of the patients, tumor location, preoperative serum carcinoembryonic antigen (CEA) level, pretreatment clinical stage and lymphovascular invasion (LVI) of tumor between the two groups. The histological differentiation of tumor appeared poorer in control group than in NT group, implying that the prognosis of patients with gastrointestinal malignancy is potentially better in control group than in NT group. However, multivariate analysis demonstrated that it was not a major factor for the clinical outcome of such patients, indicating that the outcomes of patients in the two groups are comparable.

Different pretreatment evaluation strategies for the outcomes of patients in two groups

The staging tools used for pretreatment evaluation of the two groups differed significantly. Magnetic resonance imaging (MRI) was more frequently used in NT group than in control group (60.4% *vs* 40.7%, $P < 0.05$), while computed tomography (CT) was more commonly used in control group than in NT group.

Effect of MDT treatment on the clinical outcomes of patients in two groups

Although no significant difference was found in pretreatment stage between the two groups, the proportion of pathologic stage I was higher in NT group than in control group (34.7% *vs* 7.4%), while that of stage III was higher in control group than in NT group (Table 2).

Among the patients with low rectal cancer less than 5 cm from the anal verge ($n = 72$), the sphincter preservation rate was 37.14% (13/35) and 13.51% (5/37), respectively, for the NT group and control group ($P < 0.05$, Table 3).

The local recurrence rate was 3.96% (4/101) and 11.11% (18/162), the 5-year DFS rate was 76.24% and 67.28% ($P < 0.05$, Figure 1), and the 5-year OS rate was 77.23% and 69.75% (Figure 1, Table 3), for the NT group and control group, respectively.

Multivariate analysis demonstrated that the pretreat-

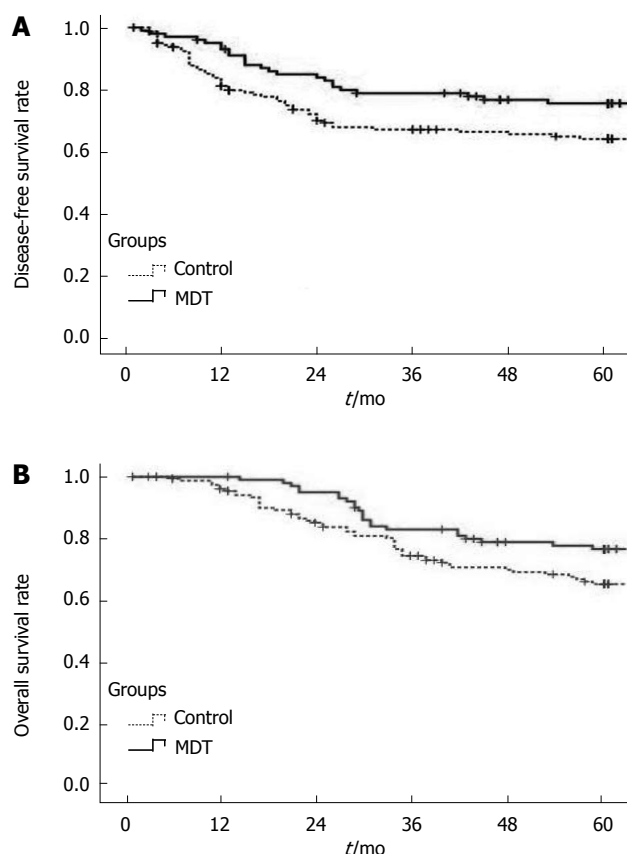


Figure 1 Disease-free survival rate (A) and overall survival rate (B) for patients in two groups. MDT: Multidisciplinary team.

ment serum CEA level, pathologic TNM stage, and LVI were the major factors for the long-term survival rate of patients with gastrointestinal malignancy (Table 4). Other variables, including neoadjuvant radiotherapy, were not the independent factors for the OS rate.

DISCUSSION

Treatment of cancer increasingly requires the cooperation of specialists from various disciplines^[9], although surgery still plays a critical role in cancer treatment. Currently, most doctors around the world have recognized the effect of MDT approach^[10,11] and endorse it as a principal treatment modality for cancer^[1,2]. Although the composition of MDT in China is similar to that in Western countries, there are many distinct differences in working models of China. First, no special rules or guidelines are available on MDT in China, thus it is not compulsory for all cancer patients to receive MDT treatment. Second, not all but some big cancer centers adopt MDT treatment modality without consistent indications for discussion at MDT meetings in different hospitals. In general, MDT is still under development in China^[12,13].

Our cancer center is one of the earliest hospitals adopting MDT approach in China. It is difficult to quantify improvement in outcomes of cancer patients, especially those with complicated clinical conditions, after MDT treatment. In this study, data on cases of locally advanced

Table 4 Multivariate analysis of overall survival rate by COX model (enter method)

Variables	Hazard ratio	95% CI	P value
Pretreatment CEA level	1.429	1.044-1.956	0.026
Pathologic TNM stage	1.440	1.137-1.825	0.002
Lymphovascular invasion	0.468	0.286-0.765	0.002
Sex	1.164	0.726-1.867	0.529
Age	0.700	0.424-1.156	0.163
Distance of tumor from anal verge	0.994	0.854-1.157	0.934
Pretreatment TNM stage	0.949	0.727-1.239	0.703
Surgery form (LAR or APR)	0.853	0.575-1.264	0.427
Histologic differentiation	0.969	0.822-1.142	0.706
NT	0.878	0.519-1.483	0.626

CEA: Carcinoembryonic antigen; LAR: Low anterior resection; APR: Abdominal-perineal resection; NT: Neoadjuvant therapy.

rectal cancer, which is considered the most successful and mature model of MDT approach^[14-16], were collected to evaluate the effect of MDT treatment on the clinical outcomes of patients with gastrointestinal malignancy. Cases discussed at MDT meeting were reviewed to assess the influence of MDT treatment modality on the treatment strategy for patients with gastrointestinal malignancy. The data included in the two parts were completely independent without any overlap.

Several studies demonstrated that MDT approach can optimize the decision making, enhance the quality of cancer care, and improve the clinical outcomes of cancer patients^[1,11,17,18]. Our data indicate that MDT meetings change a considerable proportion of treatment strategies, including neoadjuvant therapy for preoperative patients and MDT treatment modality for patients with tumor recurrence and metastasis. In this study, 7 patients with inoperable liver metastasis of colorectal cancer underwent R0 resection after MDT treatment. However, the limited time of MDT meetings and the large number of patients who need to be discussed at MDT meetings made it impossible to discuss and evaluate all patients, thus the vast majority of patients were evaluated before operation and neoadjuvant therapy was evaluated according to the interdisciplinary consultations.

It is widely believed that accurate and integrative evaluation before operation, as well as active strategies for adjuvant therapy used by MDT members, are the primary factors for improving the clinical outcomes of cancer patients^[2,3,16,19,20]. The meticulous and reliable assessment of patients with locally advanced rectal cancer before operation by MDT members is closely associated with the treatment strategy. It was reported that MRI is more accurate in clinical staging of tumor and in predicting of circumferential resection margin (CRM) when it is used in evaluation of rectal cancer^[21-23]. In this study, the strategy for preoperative evaluation of the two groups differed significantly. MRI was used more frequently in NT group than in control group (60.4% *vs* 40.7%, *P* < 0.01), suggesting that MRI can improve the accuracy of clinical tumor staging in NT group.

It has been shown that neoadjuvant radiotherapy for

rectal cancer can improve the local control of cancer before operation^[24-27], which constituted the major difference between MDT and traditional treatment modalities in this study. Neoadjuvant radiotherapy can decrease the size or stage of low rectal cancer, thus preserving the anus^[28-30]. In this study, the sphincter preservation, the local control of cancer, and the 5-year OS rate were better in NT group than in control group, suggesting that patients may benefit from MDT treatment.

Finally, although our study showed the advantages of MDT treatment modality for gastrointestinal cancer, its widespread use in China is still problematic. First, administrative support is insufficient in some places, leading to organizational problems and even its discontinuation. Second, MDT meetings are time-consuming and incomplete attendance is a barrier to success. However, these problems will not hinder the popularity and application of MDT treatment modality in China.

In conclusion, MDT treatment modality can significantly improve the clinical strategies for the treatment of gastrointestinal malignancy, and Chinese patients can benefit from it.

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COMMENTS

Background

Treatment of cancer has evolved toward a multidisciplinary team (MDT) approach. Although the MDT treatment modality has been successfully implemented in Western countries for decades, it is not widely applied in China. The authors conducted a study on the MDT treatment modality in a representative cancer center in China to evaluate its effect on clinical decision making and outcomes in patients with gastrointestinal malignancy.

Research frontiers

MDT treatment modality is the major concern in cancer treatment, and this study addressed it for cancer in China.

Innovations and breakthroughs

MDT treatment modality, systematically introduced in this study, can significantly improve the clinical strategies for the treatment of gastrointestinal malignancy, and Chinese patients can benefit from it.

Applications

MDT treatment modality is of high values for patients with gastrointestinal malignancy and can be commonly used in hospitals of China in treatment of cancer patients.

Peer review

This paper is very good and shows that neoadjuvant therapy can improve the outcomes of patients with gastrointestinal malignancy, thus providing a novel therapy for gastrointestinal cancer.

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Gastric cancer cells induce human CD4⁺Foxp3⁺ regulatory T cells through the production of TGF-β1

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flow cytometry and enzyme-linked immunosorbent assay respectively. *Foxp3* gene expression was analyzed by real-time polymerase chain reaction. The gastric cancer microenvironment was modeled by establishing the co-culture of gastric cancer cell line, MGC-803, with sorting CD4⁺ T cells. The normal gastric mucosa cell line, GES-1, was used as the control. The production of TGF-β1 was detected in supernatant of MGC and GES-1. The carboxyfluorescein diacetatesuccinimidyl ester (CFSE) dilution assay was performed to evaluate the proliferation characteristics of induced Tregs. Neutralizing anti-TGF-β1 antibody was added to the co-culture system for neutralization experiments.

RESULTS: The level of serum TGF-β1 in gastric cancer patients (15.1 ± 5.5 ng/mL) was significantly higher than that of the gender- and age-matched healthy controls (10.3 ± 3.4 ng/mL) ($P < 0.05$). Furthermore, the higher TGF-β1 level correlated with the increased population of CD4⁺Foxp3⁺ Tregs in advanced gastric cancer ($r = 0.576$, $P < 0.05$). A significant higher frequency of CD4⁺Foxp3⁺ Tregs was observed in PBMCs cultured with the supernatant of MGC than GES-1 (10.6% ± 0.6% vs 8.7% ± 0.7%, $P < 0.05$). Moreover, using the purified CD4⁺CD25⁻ T cells, we confirmed that the increased Tregs were mainly induced from the conversation of CD4⁺CD25⁻ naive T cells, and induced Tregs were functional and able to suppress the proliferation of effector T cells. Finally, we demonstrated that gastric cancer cells induced the increased CD4⁺Foxp3⁺ Tregs *via* producing TGF-β1. Gastric cancer cells upregulated the production of TGF-β1 and blockade of TGF-β1 partly abrogated Tregs phenotype.

CONCLUSION: Gastric cancer cell can induce Tregs development *via* producing TGF-β1, by which the existence of cross-talk between the tumor and immune cells might regulate anti-tumor immune responses.

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Key words: Transforming growth factor-β1; Regulatory

Abstract

AIM: To elucidate the molecular and cellular features responsible for the increase of regulatory T cells (Tregs) in gastric cancer.

METHODS: The frequencies of CD4⁺Foxp3⁺ Tregs and the level of transforming growth factor-β1 (TGF-β1) were analyzed from 56 patients with gastric cancer by

T cells; Gastric cancer; Immune suppression

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INTRODUCTION

Gastric cancer (GC) is a common fatal malignancy from cancer worldwide^[1,2]. Although the incidence of GC is declining in most developed countries, it remains one of the most common causes of cancer-related death in many Asian countries, such as China, Japan, and Korea^[3,4]. Certain tumors, including GC, have developed the capacity to escape immune surveillance or to inhibit immune functions. Recently, emerging evidence suggests that CD4⁺ regulatory T cells (Tregs) play an important role in tumor escape from immunological control by suppressing the activation and proliferation of T cells, B cells, and natural killer (NK) cells^[5,6].

Our recent results have showed that the existence of Tregs maintained immune tolerance in gastric tumor micro-environments^[7]. In addition, we found increased expression of Foxp3 protein per cell in tumor-infiltrating Tregs and Tregs can mediate immune suppression *via* COX-2 production^[8]. Interestingly, our and others data showed that after patients received curative resection for GC, the increased proportion of Tregs was significantly restored to normal levels^[7,9,10]. These results strongly suggest that gastric cancer-related factors induce and/or expand the accumulation of Tregs. However, the detailed mechanism underlying the induction of Tregs during GC progress remains undefined.

Transforming growth factor- β 1 (TGF- β 1), as well as other mediators such as prostaglandin E2 and H-ferritin, has been reported to induce Treg cells^[11]. In vitro, studies have shown that TGF- β 1 can impose a regulatory phenotype on CD4⁺CD25⁻ T cells through the induction of Foxp3 expression^[12,13]. In contrast, other studies have shown that the development and functional capacity of CD4⁺CD25⁺ Tregs is normal in TGF- β 1 deficient mice^[14], questioning a role for TGF- β 1 in mediating Treg development and function. Over the past few years, significant progress has been made in defining the cellular and molecular basis for these protumorigenic effects of TGF- β 1 within tumor microenvironment^[15]. The mechanism of TGF- β 1 function in gastric cancer is believed to be mediated primarily by increasing the deposition of extracellular

matrix and immunosuppression. However, the underlying mechanism of TGF- β 1 responsible for regulating gastric cancer immunosuppression has not been fully elucidated yet.

In this study, we examined the serum level of TGF- β 1 in gastric cancer patients and analyzed the correlation of TGF- β 1 with the prevalence of Tregs. We confirmed that serum level of TGF- β 1 was elevated in GC and correlated with increased CD4⁺Foxp3⁺ Treg cells. By the co-culture system in vitro, we evaluated the contribution of GC cell supernatant to CD4⁺ T cell dysfunction. Our results indicated that MGC supernatant can induce the increase of Tregs, which especially from the conversation of CD4⁺CD25⁻ naive T cell and blockade of TGF- β 1 production partly impaired the development of Tregs. These results suggested that the gastric cancer cells played a pivotal role in impairing the antitumor T cell response by induction of Tregs.

MATERIALS AND METHODS

Patients

Fifty six patients with gastric cancer, who underwent surgery at Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine, China, were included in this study. Prior to the sample collection, appropriate permission was granted from the research ethical committee of Xinhua hospital, Shanghai Jiao Tong University School of Medicine. Peripheral bloods were collected from each patient and from 20 healthy volunteers as previously described^[7]. Sera were frozen at -80°C immediately after centrifugation for later determination of concentrations of TGF- β 1. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient. All patients were diagnosed by pathological analyses based on the UICC (International Union Against Cancer) criteria. At the time of sample collection, none of the patients had suffered other cancer, acute and chronic infections, autoimmune diseases, inflammatory diseases and none were receiving concomitant medications. The laboratory characteristics of patients were as follow: WBC $4.2-10.6 \times 10^9/L$; RBC $3.9-5.7 \times 10^{12}/L$; platelets $181-350 \times 10^9/L$; neutrophils 47.8%-73.9%; lymphocytes 15.2%-44.9%; monocytes 2.9%-10.2%. The clinicopathologic characteristics of the tumors are summarized in Table 1.

Cell culture and supernatant collection

Human GC cell lines (MGC-803, SGC-7901) were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and normal gastric mucosa cell line (GES-1), derived from a human fetal gastric mucosa epithelium, was obtained from Beijing Institute for Cancer Research. The cells were routinely cultured in DMEM media (GIBCO, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco) in 5% CO₂ at 37°C. MGC, SGC and GES cells were washed twice with PBS when they grew to 60%-80% confluence and then

Table 1 Serum levels of transforming growth factor- β 1 and the population of CD4⁺Foxp3⁺ Tregs in patients with gastric cancer according to clinicopathological findings

Variables	n	TGF- β 1 (ng/mL)	P	Tregs (%)	P
Gender			> 0.05		> 0.05
Male	38	16.1 \pm 6.8		8.0 \pm 3.2	
Female	18	14.0 \pm 5.1		7.6 \pm 2.5	
Age			> 0.05		> 0.05
< 55	25	14.1 \pm 4.1		8.2 \pm 4.1	
> 55	31	15.9 \pm 4.6		7.7 \pm 3.5	
TNM stage			< 0.05		< 0.05
Early stage (I / II)	22	12.4 \pm 5.0		6.3 \pm 1.2	
Advanced stage (III / IV)	34	18.1 \pm 7.8		8.8 \pm 2.4	
Histological type			> 0.05		> 0.05
Well and Moderately	20	14.1 \pm 4.9		7.0 \pm 3.7	
Poor	36	16.8 \pm 7.9		8.6 \pm 4.5	
Lymph node metastasis			< 0.05		< 0.05
Negative	18	11.2 \pm 5.2		6.5 \pm 2.4	
Positive	38	17.4 \pm 7.2		8.6 \pm 2.9	

TGF: Transforming growth factor; Tregs: Regulatory T cells.

kept in serum-free culture medium for an additional 48 h. Supernatant was collected and debris was removed by centrifugation at 1500 *g* for 10 min, and then passed through a 0.45 mm filter (BD, USA). 100 μ L supernatants were stored at -80°C for later determination of concentrations of TGF- β 1. For co-culture assay, all the remaining supernatants were further concentrated 20-fold with a Microcon Ultracel YM-10 filter (Millipore, USA) according to the manufacturer's instructions. In the induction experiments, different volumes of supernatant protein concentrate from MGC or GES were added to sorted naive T cell culture system.

TGF- β 1 measurement

The cell supernatants of MGC, GES-1 and GC patients' sera previously stored at -80°C were thawed, and measured for TGF- β 1 concentration by enzyme-linked immunosorbent assay using human TGF- β 1 immunoassay kit (R&D, USA) in triplicate following the manufacturer's protocol. The minimum detectable dose of this assay is 30.0 pg/mL. The intra-assay coefficient of variation (CV) was 5.7% and the interassay CV was 10.6%.

Treg cells analysis and sorting by FCM

Phenotype analysis of regulatory T cells (Tregs) and cell sorting were performed by BD FACS Aria flow cytometer (BD, USA) as previously described^[8]. Briefly, the cells were labeled with CD3-PC7, CD127-PE, CD4-APC, and CD25 PerCP. Intracellular staining for Foxp3 was performed using Alexa Fluor® 488 anti-human Foxp3 Antibody and Foxp3 Fix/Perm Buffer Set (BioLegend, USA) following the manufacturer's protocol. In the preliminary experiments, we found that CD25 expression was nonspecific and was higher in CD4⁺ T cells after coculturing with GC cells. Therefore, for consistency, the gating strategy for Tregs was based on the expression of CD4 and Foxp3. To analyze the prevalence of Treg cells, CD4⁺Foxp3⁺ Treg

cells were evaluated after gating on CD3⁺CD4⁺ T cells and expressed as a percentage of the total CD4⁺ T cells. The FACS Aria was adjusted with Accudrop Fluorescent Beads (BD bioscience, USA) for optimum sorting conditions which allowed CD4⁺CD25⁺CD127^{low/-} T cells and CD4⁺CD25⁺CD127⁺ T cells to be sorted. The purity of the isolated T cells was greater than 95%.

Induction and neutralization experiments

The purified CD4⁺CD25⁺CD127⁺ T cells (1×10^5) were cultured with conditioned medium described above in 96-well plates at 37°C and 5% CO₂ in the presence or absence of soluble anti-human CD3 (10 μ g/mL; eBioscience) plus anti-CD28 (10 μ g/mL; eBioscience) and IL-2 (100 U/mL; Sigma, USA). After 72 h of cultivation, the proportion of CD4⁺Foxp3⁺ T cells was detected by FCM, and Foxp3 gene expression was analyzed by real-time PCR. For anti-TGF- β 1 antibody neutralization experiments, neutralizing mouse anti-TGF- β 1 antibody (500 μ g/mL; Clone: 27235; R&D Systems, USA) and normal mouse IgG1 (500 μ g/mL; Clone: 11711; R&D Systems, USA) were added to the culture medium with a final concentration of 0.1 μ g/mL at the beginning of the culture.

Real-time quantitative RT-PCR

Foxp3 mRNA expression was performed using the SYBR Premix Ex Taq™ (Takara) according to the manufacturer's instructions. Amplification reactions were performed by primers specific for Foxp3 (forward, 5'-CAGCACATTCCCAGAGTTCCTC-3'; reverse, 5'-GCGTGTGAACCAGTGGTAGATC-3'). The relative quantity of the Foxp3 mRNA was normalized to the level of the internal control GAPDH mRNA level.

CFSE-based suppression assay in vitro

For the proliferation inhibition assay, the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay was performed per standard technique. Briefly, the sorted CD4⁺CD25⁺CD127⁺ T cells were co-cultured with MGC-803, GES-1 or medium only for 2 d. As suppressor cells, equal numbers of cells were removed and placed in co-culture with CFSE-labeled CD4⁺CD25⁺ T cells at a ratio of 1:1 in the presence of soluble 10 μ g/mL mouse anti-human CD3 and 10 μ g/mL mouse anti-human CD28 antibodies (eBioscience, USA). After 4 d, the cells were harvested and proliferation was measured by loss of CFSE dye with flow cytometry. Cell proliferation indices were calculated with Modfit software (Topsham, USA) based on the reduction of CFSE positive cells.

Statistical analysis

Data were expressed as mean \pm SD. The statistical significance of the difference between the two means was assessed using Student's *t*-test, and the one-way ANOVA with Tukey's post test was performed for multiple comparisons. Correlation between variables was evaluated by Pearson's rank correlation coefficients. All the statistical analyses were performed using GraphPad Prism version 5.0

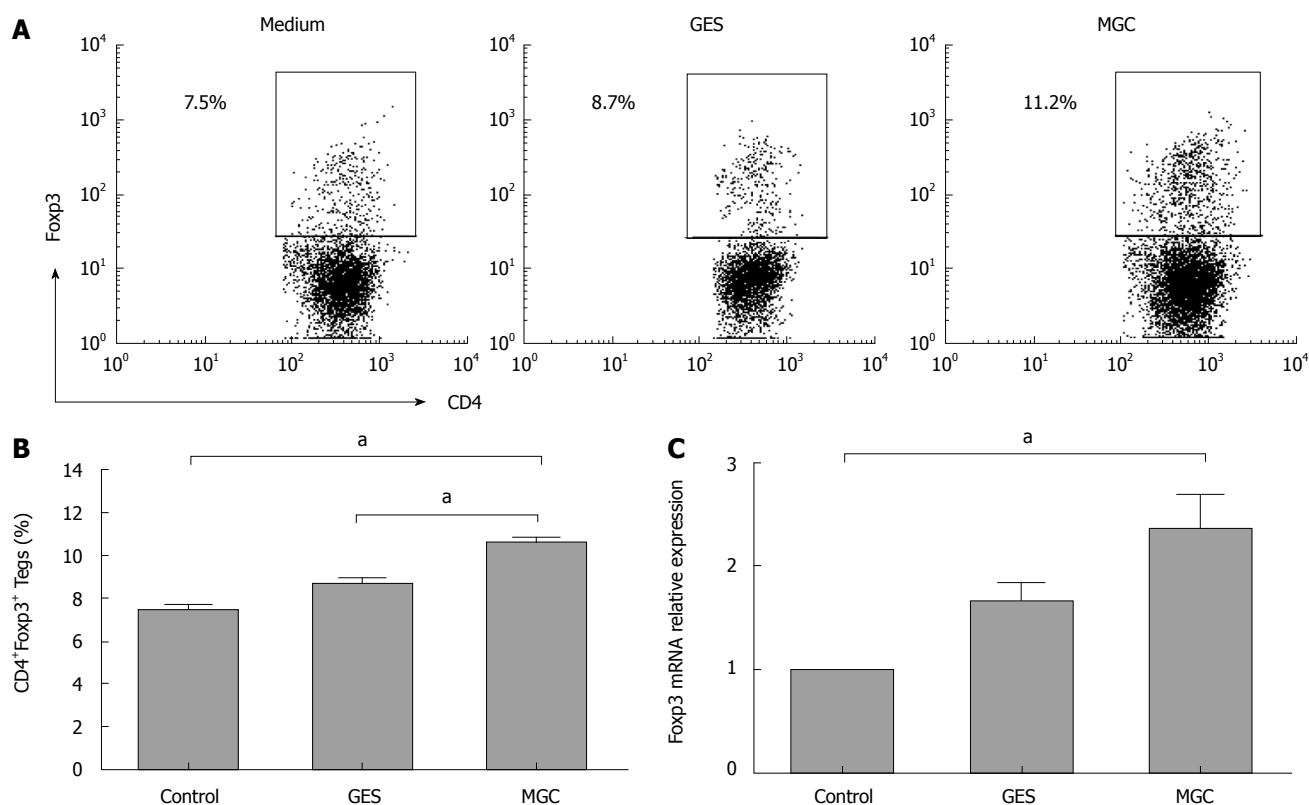


Figure 1 Gastric cancer cell supernatant induces the increased Tregs in coculture with peripheral blood mononuclear cells. A: Representative flow cytometry analysis of CD4⁺Foxp3⁺ Tregs frequency in CD4⁺ T cells population following peripheral blood mononuclear cells co-culture with medium, GES, or MGC supernatants. Rectangles show double positive gating and numbers reflect percentage of cells in that gate; B: Summarized data from all subjects showed that CD4⁺Foxp3⁺ Tregs increased in coculture with MGC supernatants ($^aP < 0.05$); C: Relative quantity of Foxp3 mRNA was measured by real-time polymerase chain reaction before and after culture with medium, GES, or MGC supernatants.

for Windows (GraphPad Software, USA) and a significant difference was considered as $P < 0.05$.

RESULTS

Elevated serum level of TGF- β 1 in gastric cancer correlated with increased CD4⁺Foxp3⁺ Treg cells

To determine whether serum TGF- β 1 correlated with the clinicopathological findings, we summarized the mean values of TGF- β 1 in patients with GC according to clinical variables as shown in Table 1. The mean level of serum TGF- β 1 in GC patients (15.1 ± 5.5 ng/mL) was significantly higher than that of the gender- and age-matched healthy controls (10.3 ± 3.4 ng/mL) ($P < 0.05$), which was consistent with previous reports^[16,17]. Furthermore, the serum TGF- β 1 levels increased as GC stage progressed. Compared to those with early stage disease, patients with advanced stage disease had significantly elevated serum TGF- β 1 ($P < 0.05$). As shown in Table 1, no significant differences in serum TGF- β 1 levels were found in GC patients with different age, genders, and histological types ($P > 0.05$). However, the serum concentration of TGF- β 1 was positively correlated with lymph node metastasis ($P < 0.05$). The results also showed that the population of CD4⁺Foxp3⁺ Tregs in the peripheral blood of advanced stage GC patients

was significantly higher than that in healthy controls or early stage GC patients ($P < 0.05$) (Table 1).

The consistency of Tregs and serum TGF- β 1 level in patients with GC encouraged us to perform a correlation study, and the results showed that the increased TGF- β 1 was correlated with the Treg cells ($r = 0.576$, $P < 0.05$) in advanced stage patients, but not in early stage patients ($r = 0.248$, $P < 0.05$). The present results indirectly suggested the relationship of TGF- β 1 and Tregs in gastric cancer.

GC cell supernatant induces the increase in CD4⁺Foxp3⁺ Treg cells

Based on the above results, we hypothesized that gastric cancer-derived stimulators may contribute to increased Tregs. To address this hypothesis, we established a co-culture system with human GC cells and PBMCs from healthy donors to model the gastric cancer microenvironment *in vitro*. After 3 d of culture, our data showed that a higher frequency of Tregs was observed in PBMCs cultured with the supernatant of MGC. However, the frequency of Tregs had almost no significant difference in PBMCs cultured with GES-1 cell supernatants and medium control (Figure 1A and B). When cocultured with MGC cell culture supernatant, Foxp3 mRNA expression level was higher than that with GES-1 and medium (Figure 1C).

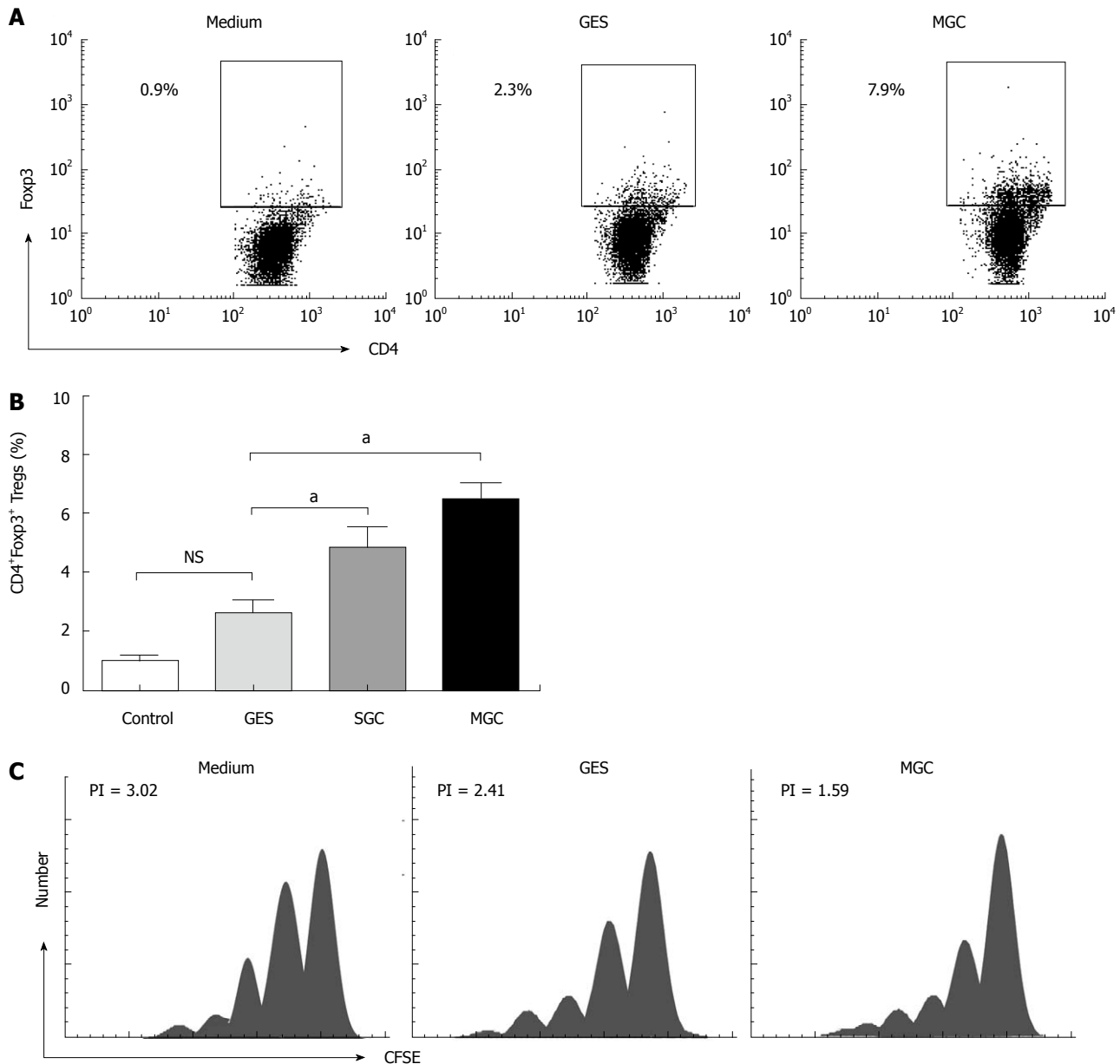


Figure 2 Gastric cancer cell supernatant mediates the conversion of CD4⁺CD25⁺ T cell to CD4⁺Foxp3⁺ Tregs. A: Representative flow cytometry analysis of CD4⁺Foxp3⁺ Tregs frequency in CD4⁺ T cells population following sorted CD4⁺CD25⁺CD127⁺ T cells co-culture with medium, GES, or MGC supernatants; B: Summarized data showed that both MGC and SGC supernatants induced higher CD4⁺Foxp3⁺ Tregs (^a*P* < 0.05); C: After co-culture with MGC, GES or medium, CD4⁺CD25⁺ T cells were placed in coculture with CFSE-labeled CD4⁺CD25⁺CD127⁺ T cells at a ratio of 1:1 in the presence of soluble anti-CD3/CD28 as well as IL-2. The representative data from three independent experiments are shown.

GC cell supernatants induce the conversion of CD4⁺CD25⁺ naive T cells to CD4⁺Foxp3⁺ Tregs

To investigate whether the supernatant of GC cell culture induced the increased Tregs from the conversion of natural CD4⁺CD25⁺ T cells, we performed co-culture experiments with the sorted natural CD4⁺CD25⁺ T cells using our previous method^[7]. Because naive CD4⁺ T cells were more susceptible to the induction of Foxp3 by TGF-β1^[18] and to minimize any potential contaminating CD25⁺Foxp3⁺ nTregs, we used the CD4⁺CD25⁺CD127^{low/-} population for all of our coculture experiments. To ensure consistency, the same cell culture supernatants were used for conversion experiments. Our results showed that

MGC cell supernatant can induce a higher population of CD4⁺Foxp3⁺ Tregs than GES-1 and medium (*P* < 0.05) (Figure 2A and B) and, the CD4⁺CD25⁺ cells decreased respectively in coculture system. More interesting is that the induced Tregs correlated with the TGF-β1 level in different repeated experiments (*r* = 0.635, *P* < 0.05).

To further confirm that the supernatant of GC cell culture could increase Tregs population and Foxp3 expression, the cell culture supernatant from another GC cell strain, SGC-7901, was collected. As observed in the culture with supernatant of MGC cell culture, the supernatant of SGC cell cultures also increased Foxp3 expression in naive T cells (Figure 2B). Collectively, our results suggested

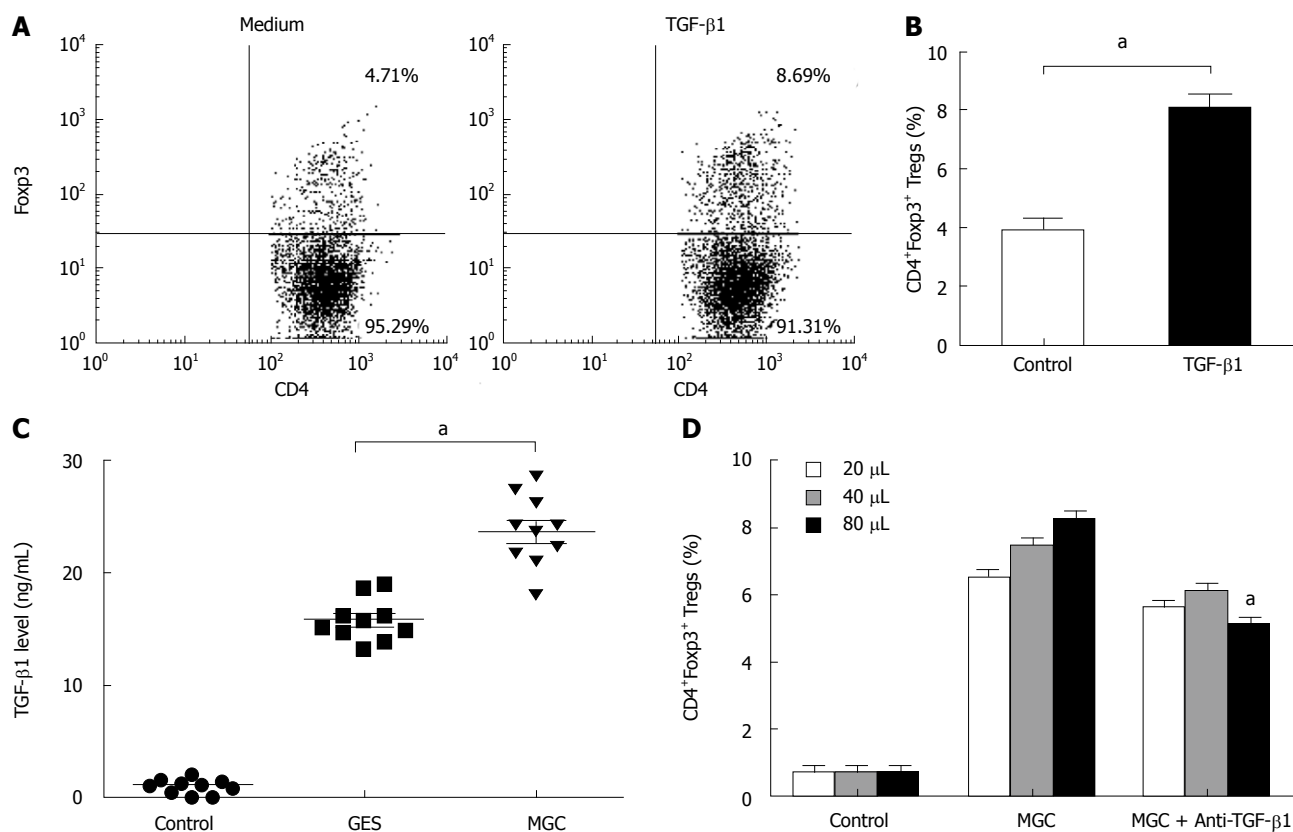


Figure 3 Gastric cancer cells producing transforming growth factor-β1 can partially mediate the conversion of CD4⁺CD25⁻ T cells to CD4⁺Foxp3⁺ Treg cells. A: Representative flow cytometry analysis of CD4⁺Foxp3⁺ Tregs frequency in the presence or absence of transforming growth factor-β1 (TGF-β1); B: Summarized data showed that CD4⁺Foxp3⁺ Tregs frequency increased in presence of TGF-β1 in comparison with that in absence of TGF-β1 ($^aP < 0.05$); C: Gastric cancer cell TGF-β1 production was assessed by enzyme-linked immunosorbent assay in supernatants. ($^aP < 0.05$); D: TGF-β1 blocking antibody or control IgG1 antibody was added to the co-culture in order to monitor impairment of Tregs development within the coculture system. Different volumes of supernatant protein concentrate (20, 40, and 80 μL) from MGC were added to the co-culture system. ($^aP < 0.05$).

that the GC supernatant can induce the increased Tregs, which was mainly because of the conversion of natural CD4⁺CD25⁻ T cells.

Gastric cancer cell induced CD4⁺Foxp3⁺ Treg cells that can suppress T cell activation

In order to understand whether GC cell supernatant induced CD4⁺Foxp3⁺ Tregs can inhibit effector T cells, we analyzed the suppressive function of GC cell induced Tregs. To eliminate the influence of cell culture related factors, the induced co-cultured supernatants were removed after coculture with MGC, GES-1, or medium. Then the CFSE dilution assay was used to evaluate the proliferation characteristics of T cells. Compared with medium, a significant decrease in the proliferative response of responder CD4⁺CD25⁻ T cells could be found with MGC after being cocultured (Figure 2C). These results demonstrated that GC cell induced Treg cells displayed the suppressive activity *in vitro*.

Gastric cancer cells induce conversion of naive T cells into Treg through TGF-β1

To further elucidate the possible mechanism of the conversion, we presumed that TGF-β1 from gastric cancer cells can serve as a key factor in the induction of Foxp3

expression of natural CD4⁺CD25⁻ T cells. To address this possibility, we firstly performed the inducing experiments for TGF-β1. Consistent with other studies, compared with control Ab, TGF-β1 can induce an increase in Tregs (Figure 3A and B). MGC cells secreted significant higher TGF-β1 into supernatant than that GES-1 and medium (Figure 3C).

To test the role of TGF-β1 in GC cell mediated increasing Tregs, we compared the effects of neutralizing monoclonal antibody with activity against TGF-β1 and isotypic control antibody. To some extent, blocking TGF-β1 activity in MGC supernatant with anti-TGF-β1 mAb, instead of the isotypic control antibody, reduced the frequency of induced CD4⁺FOXP3⁺ T cells. But our results also showed that this blocking effect was not complete because the converted numbers of CD4⁺Foxp3⁺ T cells in MGC were still higher than that of the control (Figure 3D). Our data indicated that gastric cancer-derived TGF-β1 played a certain role in the conversion of natural CD4⁺CD25⁻ T cells to CD4⁺Foxp3⁺ Treg cells.

DISCUSSION

In the current study, we showed that higher levels of TGF-β1 in gastric cancer patients have been correlated with the frequency of CD4⁺Foxp3⁺ regulatory T cell. Nu-

merous studies have respectively reported an increased frequency of circulating Tregs and higher level of TGF- β 1 during GC progression^[7,9,16,19]. However, to date, there has been no report to directly demonstrate the relationship of higher TGF- β 1 levels and increased frequency of Tregs in GC. Given that TGF- β 1 is a key factor for Foxp3 expression maintenance, regulatory function, and homeostasis in peripheral CD4⁺CD25⁺ Treg cells^[20], tumor-derived TGF- β 1 may contribute to the development of Tregs during GC progression. Indeed, our work supports this possibility by demonstrating a mechanism of CD4⁺Foxp3⁺ Tregs development mediated through TGF- β 1 production by GC cells.

TGF- β 1 is a tumor suppressor growth factor, anti-inflammatory cytokine, and immunosuppressant. Therefore, the levels of TGF- β 1 were different according to the carcinogenic process, the stage of carcinogenesis, and organ. It has been reported that high levels of TGF- β 1 are produced by many types of tumors, including melanomas and cancers of the colon, stomach, liver, and prostate, as well as other malignancies^[16,21,22]. Generally defective TGF- β 1 signaling seems to be essential in the carcinogenic process, but the level of TGF- β 1 is increased in advanced cases or some types of cancer. TGF- β 1 levels were significantly increased in gastric cancer tissue compared with adjacent normal tissues^[17]. In this study, our data confirmed the higher level of serum TGF- β 1 in patients with gastric cancer. Furthermore, compared to early stage patients, elevated serum TGF- β 1 was observed in patients with advanced stages. However, the role of TGF- β 1 varied in different tumor stages, in which TGF- β 1 seems to act as a tumor suppressor in early stages of tumorigenesis and during later stages of tumorigenesis, TGF- β can foster tumor progression, and metastasis^[23,24]. Our results showed that the serum concentration of TGF- β 1 was positively correlated with lymph node metastasis in GC. This study reinforced the role of TGF- β 1 in promoting GC progression. An increase in the Treg population has been observed in both the periphery and tumor microenvironment in patients with cancer^[25]. We find a positive correlation between TGF- β 1 and Tregs in advanced stage GC patients. To our knowledge, this is the first report to show the correlation of TGF- β 1 level with increased Treg cells in GC.

In this report, an *in vitro* co-culture system was used to understand the underlying mechanisms responsible for the upregulation of Tregs observed in our clinical cohorts. After co-culture with GC cell supernatants, an increased population of CD4⁺Foxp3⁺ T cells was found in PBMCs. Of note, upregulation of Foxp3 mRNA expression supported that Tregs increased in the culture system. This increase was observed using a different GC cell line, suggesting that the induction of Tregs is a feature common to GC cells. Multiple mechanisms have been involved in production of increased Treg cells in the tumor microenvironment including expansion, conversion and recruitment. Mizukami *et al.*^[26] found that CCL17 and CCL22 are related to the increased population of Foxp3⁺ Tregs in early GC^[26]. Using the optimized conditions for sorting effector and Treg cells, we

provided evidence that the conditioned medium obtained from GC supernatant was capable of inducing the conversion of CD4⁺CD25⁻ T cells to CD4⁺Foxp3⁺ Tregs, which is different from the effects of chemokines on Treg infiltration in GC microenvironment. Moreover, the induced Tregs were functional and inhibited the proliferative response of CD4⁺CD25⁻ effector T cells. Although a prior study showed that increased Treg frequency was derived from natural Treg self expansion by factors secreted by hepatocellular carcinoma cell^[27], our data clearly demonstrated that the conversion of natural CD4⁺CD25⁻ T cells may be an important pathway of Treg cell maintenance in GC. Of course, we cannot discount the important role of chemokines in inducing Tregs migration to the microenvironment of GC.

Tumor cell supernatants include a complex protein component. Based on the fact that GC cells could produce a higher level of TGF- β 1 and TGF- β 1 correlated with Tregs from our data, we questioned if gastric cancer derived TGF- β 1 induced Treg increases in the coculture system. Although TGF- β 1 can promote the generation of Tregs *in vitro*, it has been controversial whether TGF- β 1 is involved in the generation or maintenance of Tregs under pathologic conditions, especially in tumor environments^[28]. Some studies showed that tumor-derived factors such as TGF- β 1 may contribute to CD4⁺CD25⁺ Treg cell expansion^[29], but also enhance their suppressor ability^[27]. In contrast to them, Zhao *et al.*^[30] recently found that neutralization of TGF- β 1 did not affect Foxp3 expression in ovarian carcinoma cells. The role of TGF- β 1 needs to be elucidated in GC. Our results demonstrated that a higher TGF- β 1 level was found in GC cell supernatant and TGF- β 1 can induce increased Treg cells. Surprisingly, although blocking TGF- β 1 could decrease the conversion activity in our study, it was not completely abrogated. Accordingly, we could not rule out that a fraction of converting Treg cells may be generated in the presence of other unknown tumor-derived soluble factors besides TGF- β 1. It is likely that multiple cytokines are involved in the induction of Foxp3 expression^[30]. TGF- β 1, together with other factors, seemed to account for the induction of Treg cells in the GC microenvironment. Further research is needed to elucidate the potential mechanism of GC derived other factors for induction of Tregs. Additionally, what should be noted is that TGF- β 1 derived from GC can also account for immunosuppression of other cell types, and besides GC cells, macrophages and stromal cell also can secrete TGF- β 1 in tumor environments^[31]. It is documented that TGF- β 1 is important for the inhibition of CD8⁺ CTL and NK cells, which play a critical role in the prevention and clearance of tumors^[32]. A better understanding of the mechanisms of the Treg increase in GC may allow for future immunotherapeutic and diagnostic opportunities in this population. Recent reports have shown that functional polarization of Th subsets of lymphocytes has been implicated in tumor promotion. Tumor derived TGF- β 1 in the tumor microenvironment could promote tumor eradication by influencing the polarization of Th1/Th2 and controlling Treg/Th17 cell polarization^[33]. This study

only focuses on the specific role of TGF- β 1 in the induction of Tregs. However, the polarization of other Th cells in the tumor environment by TGF- β 1 needs to be further elucidated. A complete understanding the role of TGF- β 1 in controlling T cell polarization in tumors is crucial for dissecting the beneficial use of TGF- β 1 in future immunotherapies against gastric cancer.

In conclusion, we provide evidence that a higher TGF- β 1 level is related to the increased population of CD4⁺Foxp3⁺ Tregs. GC cell supernatants can stimulate induction of human CD4⁺Foxp3⁺ Treg cells. This study suggests that gastric cancer cell supernatants can induce the conversion of Tregs from CD4⁺CD25⁻ naive T cells partly *via* mechanisms involving TGF- β 1. Our data support the existence of intercellular cross-talk between the tumor cell and Tregs that might regulate anti-tumor immune responses.

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COMMENTS

Background

Regulatory T cells (Tregs) accumulate in the tumor environment and suppress tumor-specific T-cell responses. Previous studies have suggested that Tregs were elevated in gastric cancer and increased populations of Tregs impaired anti-tumor immunity. However, the molecular and cellular features responsible for the increase and maintenance of Treg cell levels in gastric cancer remain elusive.

Research frontiers

Recently, emerging evidence suggests that Tregs play an important role in tumor escape from immunological control. Although the precise mechanism causing the increased numbers of Treg cells is unknown, transforming growth factor- β 1 (TGF- β 1), as well as other mediators, has been reported in inducing Treg cells. However, there are contradictory reports regarding the role of TGF- β 1 in the induction of Tregs in cancer. The research aimed to explore whether or not gastric cancer cells producing this cytokine would account for the increased Treg and promote tumor progression.

Innovations and breakthroughs

In this study, the data confirmed the higher level of serum TGF- β 1 in patients with gastric cancer. Moreover, the higher TGF- β 1 level correlated with the increased population of CD4⁺Foxp3⁺ Tregs in advanced gastric cancer. Gastric cancer cell induced the increase of functional CD4⁺Foxp3⁺ Tregs, mainly from the conversion of CD4⁺CD25⁻ naive T cells. Furthermore, gastric cancer cells can induce Tregs development *via* production of TGF- β 1.

Applications

The results indicated that the gastric cancer cell played a pivotal role in impairing the antitumor T cell response by induction of Tregs. This study supports the existence of intercellular cross-talk between the tumor cell and Tregs that might regulate anti-tumor immune responses. A complete understanding of the role of TGF- β 1 in tumors is crucial for dissecting the beneficial use of TGF- β 1 in future immunotherapies against gastric cancer.

Terminology

Regulatory T cell (Treg cell) is functionally defined as a T cell that inhibits an immune response by influencing the activity of another cell type. Tregs are characterized by specific expression of the forkhead transcription factor Foxp3, and make up 5%-10% of the normal peripheral CD4⁺ T cell population. Treg cells within the tumor microenvironment are a crucial component of the tumor immunosuppressive network.

Peer review

The authors examined the level of serum TGF- β 1, and found that it was higher in

patients with gastric cancer than in healthy controls. In addition, they also found that gastric cancer cells induced the increased CD4⁺Foxp3⁺ Tregs *via* production of TGF- β 1. Gastric cancer cells upregulated the production of TGF- β 1 and blockage of TGF- β 1 partly abrogated Tregs phenotype. These experiments are well-designed and results are clear.

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SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are prognosis-related in colorectal cancer

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Abstract

AIM: To investigate the expression of markers that are correlated with the prognosis of colorectal cancer (CRC) patients.

METHODS: One hundred and fifty-six CRC patients

were followed up for more than 3 years after radical surgery. Immunohistochemical (IHC) analysis was performed to detect the expression of 14 pathway-related markers (p53, APC, p21ras, E-cadherin, endothelin-B receptor, Shp2, ADCY-2, SPARCL1, neuroligin1, hsp27, mmp-9, MAPK, MSH2 and rho) in specimens from these patients. Bioinformatics analysis involving a Support Vector Machine (SVM) was used to determine the best prognostic model from combinations of these markers.

RESULTS: Seven markers (SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK) were significantly related to the prognosis and clinical pathological features of the CRC patients ($P < 0.05$). Prognostic models were established through SVM from combinations of these 7 markers and proved able to differentiate patients with dissimilar survival, especially in stage II/III patients. According to the best prognostic model, the p53/SPARCL1 model, patients having high p53 and low SPARCL1 expression had about 50% lower 3-year survival than others ($P < 0.001$).

CONCLUSION: SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are potential prognostic markers in CRC. A p53/SPARCL1 bioinformatics model may be used as a supplement to tumor-nodes-metastasis staging.

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Key words: Colorectal cancer; Prognosis; SPARCL1; p53; Bioinformatics

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INTRODUCTION

The incidence and mortality of colorectal cancer (CRC) are in the forefront of all cancers in western developed countries^[1]. In China, the incidence of CRC has also increased in recent years, with 177 000 new cases and 99 000 deaths every year and a 5-year survival rate of 63.4%^[2]. Tumor-nodes-metastasis (TNM) staging is helpful in predicting the survival of most patients. However, the heterogeneity of patients in their clinical outcome and their response to adjuvant chemotherapy calls for more useful prognostic pooled/panel molecular markers that will provide evidence for the choice of adjuvant therapy, especially for stage II and III patients.

Recently, Parsons *et al*^[3] analyzed DNA mutations in CRC patients, and found that genetic changes of tumors are based on signaling pathways. Additionally, Wood *et al*^[4] listed the number of mutations of all 140 genes included in 38 groups or pathways, which provided an impetus for the ongoing research on markers in CRC. After ranking these genes and pathways by the number of mutations listed by Wood *et al*^[4], we selected three genes (p53, APC, ras) and 11 pathways which included genes with several mutations, and 14 genes were ultimately chosen from the pathways as candidate markers for our study. The genes are p53, APC, p21ras, E-cadherin, endothelin-B receptor, Shp2, ADCY-2, SPARCL1, neuroligin1, hsp27, mmp-9, MAPK, MSH2 and rho.

To identify prognosis-related markers of CRC, 156 patients who were followed up for more than 3 years after radical surgery were included in our survey. Immunohistochemical (IHC) analysis was performed to individually detect the expression of the 14 candidate markers in the specimens. The survival status of these patients was also analyzed. We found that seven tumor markers were found to be significantly related to the prognosis and clinical pathological features of these patients.

With the rapid development of the life sciences, bioinformatics has been developed and applied to collect, deposit and analyze large datasets and screen for useful information. In order to select molecular biomarkers more intelligently, we used a bioinformatics tool, the Support Vector Machine (SVM) classifier, to discriminate patients with different prognoses. SVM is based on the principles of Structure Risk Minimization and Vapnik-Chervonenkis Dimension as statistical learning theory, and thus provides a good generalization control^[5]. SVM applications are actively used in various areas, from face recognition to genomics^[6], and SVM is also a powerful tool for analyzing multiple markers. In this study, the seven prognostic markers were randomly combined, and SVM was used to evaluate which combination model was the best for predicting the prognosis of CRC patients.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University, College of Medicine, along with the patients' informed consent.

Patients and specimens

Tumor specimens included in this study were from 156 CRC patients who underwent a radical resection operation in the Second Affiliated Hospital of Zhejiang University, College of Medicine, between 1999 and 2004, with a median age of 60 years (range 20-92 years) at diagnosis. The clinical data of all patients are presented in Table 1. Tumor specimens for IHC were from filed blocks in the histopathological department.

Living patients were all followed up for > 36 mo after the radical operation, with a median follow-up of 62 mo (range 36-108 mo). The follow-ups were performed by history and physical surveillance every 3-6 mo for 2 years, then every 6 mo up to 5 years and every year after 5 years (conforming to NCCN V.2.2010). No patient was lost during the follow-up.

IHC

All 156 specimens in paraffin blocks were made into tissue arrays using a ZM-1 tissue array machine^[7]. Sections (4-μm thick) were cut, and immunostaining for each antigen was conducted using the avidin-biotin peroxidase complex technique (MaxVision™ HRP-Polymer IHC Kit, MAIXIN-Bio), following the manufacturer's instructions. The antibodies used were p53 (monoclonal mouse, ZhongShan), APC (polyclonal rabbit, ZhongShan), p21ras (monoclonal mouse, MAIXIN), E-cadherin (monoclonal mouse, ZhongShan), endothelin-B receptor (polyclonal rabbit, CHEMICON), Shp2 (monoclonal rabbit, Abcam), ADCY-2 (monoclonal rabbit, Abcam), SPARCL1 (polyclonal goat, R & D), neuroligin (polyclonal rabbit, CHEMICON), HSP27 (monoclonal mouse, ZhongShan), mmp9 (polyclonal rabbit, ZhongShan), ERK1 + ERK2 (monoclonal mouse, ZhongShan), MSH2 (monoclonal mouse, ZhongShan) and Rho(-A,-B,-C) (monoclonal rabbit, MILLIPORE).

The IHC results were assessed using a semi-quantitative system, as previously described^[8]. According to the percentages of positive cells (0: none, 1: < 25%, 2: 25%-50%, 3: 50%-75% and 4: > 75%) and staining intensity (0: negative, 1: weak, 2: moderate and 3: strong), the expression levels of the proteins were divided into four groups by the sum of the two scores above: 0 (0, negative expression), 1 (2-3, low expression), 2 (4-5, medium expression) and 3 (6-7, high expression).

Bioinformatics analysis

Experimental data were then analyzed by the Zhejiang University ProteinChip Data Analysis System (ZUCIPDAS, www.zlzx.net). We constructed a non-linear SVM classifier (with a radial based function kernel, a parameter Gamma of

Table 1 Clinicopathologic data of patients

Terms	n (%)
Sex	
Male	85 (54.5)
Female	71 (45.5)
Location	
Right hemicolon	45 (30.1)
Transverse colon	3 (1.9)
Left hemicolon	8 (5.8)
Sigmoid colon	32 (20.5)
Rectum	67 (41.7)
Differentiation	
Well	95 (60.9)
Moderately	40 (25.6)
Poorly	17 (10.9)
Unknown	4 (2.6)
Bowel wall invasion (pT)	
T1	7 (4.5)
T2	30 (19.2)
T3	116 (74.4)
T4	3 (1.9)
Lymph node metastasis (pN)	
N0	82 (52.6)
N1	43 (27.5)
N2	31 (19.9)
Distant metastasis (pM)	
M0	144 (92.3)
M1	12 (7.7)
TNM staging	
I	29 (18.6)
II	52 (33.3)
III	63 (40.4)
IV	12 (7.7)
Post-surgery event	
Recurrence or metastasis	51 (32.7)
Survival status	
Dead	51 (32.7) ¹
Alive	105 (67.3)

¹Among patients who have died, 40 patients died from recurrence or metastasis, while 11 patients died from causes such as heart or lung failure, or reasons unknown. TNM: Tumor-nodes-metastasis.

0.6, and a cost of the constraint violation of 19) to distinguish groups with different prognoses, and validated results by a 10-fold cross validation method.

One hundred and thirty-one patients with complete data were then filtered for the ongoing bioinformatics analysis. The seven prognostic biomarkers (SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK) were combined randomly to build 127 SVM models. For each model, the expression of these markers was the input, and the 3-year survival status of each patient was the evaluation criteria. The model with the highest accuracy for predicting the 3-year survival of the patients was selected as the best prognostic model, and the accuracy of the models was then validated by 10-fold cross validation between training sets and test sets.

Of the 131 patients, 44 died within 3 years after surgery, but the other 87 were still alive after 3 years. Because the number of patients dead at 3 years was about half of the number of living ones, the model showed low sensitivity due to the unbalanced data. Obviously, sensitivity is important for a prognostic model, so we next defined an

Table 2 Expression of candidate markers in 156 colorectal cancer patients

Markers	Numbers of patients with different expression ¹			
	Negative	Low	Medium	High
P53	42	36	32	40
APC	80	37	21	10
MAPK	114	27	8	0
E-cadherin	44	47	36	22
Mmp9	109	40	6	1
Hsp27	96	33	18	6
MSH2	17	52	47	39
P21ras	102	43	8	2
ADCY-2	45	67	36	3
Shp2	108	34	13	0
ETB	59	60	30	5
Neurologin	53	52	43	4
Rho	28	68	45	9
SPARCL1	23	52	61	20

The immunohistochemical (IHC) results were assessed using a semi-quantitative system, as previously described^[8]. According to the percentages of positive cells and staining intensity, the expression levels of the proteins were divided into four groups as negative, low, medium and high expression. ¹Data were missing because some specimens were lost during sectioning and staining of tissue arrays.

adjusted accuracy [accuracy = (sensitivity+specificity)/2 + sensitivity]/2; sensitivity = true positive/(true positive + false negative), specificity = true negative/(true negative + false positive). This increased the weight of sensitivity and allowed SVM to select models with higher sensitivity.

Statistical analyses

Kaplan-Meier survival analysis (log-rank test) was used to evaluate the relationship between marker expression and the survival of patients. Kruskal-Wallis test was used to evaluate the relationship between the expression of candidate markers and some pathologic features in IHC analyses. SPSS Version 13.0 software (SPSS Inc., Chicago, IL) was used for all statistical analyses. $P < 0.05$ was considered to be statistically significant, and all P values were two-sided.

RESULTS

Association between the expression of candidate markers and the survival of CRC patients

The expression of candidate markers in CRC was investigated by IHC (listed in Table 2). It should be noted that some specimens were lost during sectioning and staining of tissue arrays, resulting in an average of 3.6 specimens per marker (2.3%). Representative examples of immunassayed slides for each marker are shown in Figure 1.

Kaplan-Meier survival analysis revealed that markers significantly related with survival were SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK. The higher protein expression of SPARCL1, Shp2, MSH2, E-cadherin, and MAPK in CRC patients was related to better survival, while the higher expression of p53 and ADCY-2 was related to worse survival. The Kaplan-Meier survival curves of these markers are shown in Figure 2.

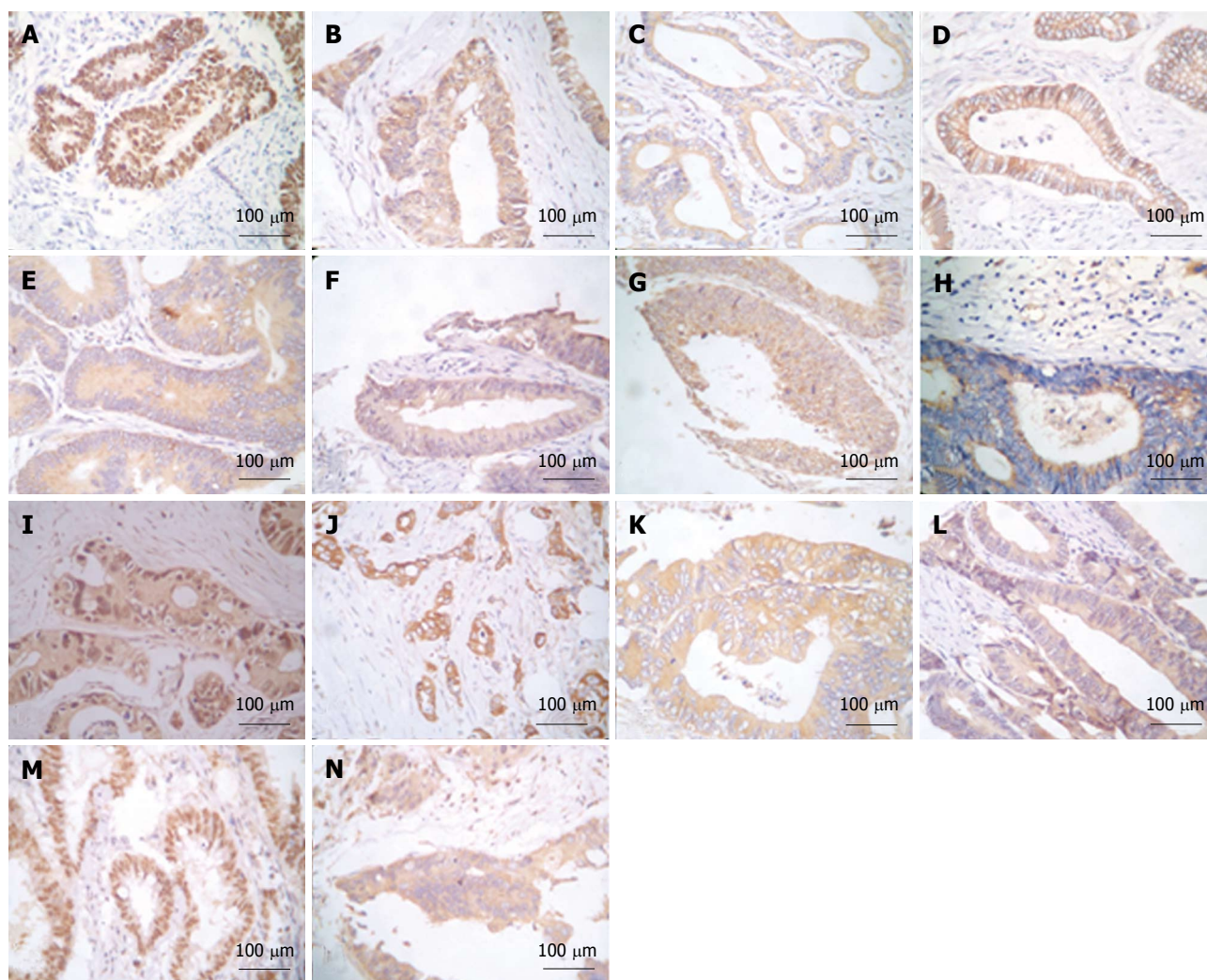


Figure 1 Immunohistochemical expression of 14 markers. The markers and the cellular location of positive staining are listed below: A: P53: nuclear; B: APC: cytoplasm; C: P21ras: cytoplasm; D: E-cadherin: membrane or cytoplasm; E: Endothelin B receptor: cytoplasm; F: Shp2: cytoplasm; G: ADCY-2: cytoplasm; H: SPARCL1: cytoplasm; I: neuroligin1: nuclear or cytoplasm; J: hsp27: nuclear or cytoplasm; K: MMP9: cytoplasm; L: MAPK: cytoplasm; M: MSH2: nuclear; N: Rho: cytoplasm. (Scale bar = 100 μ m).

Kruskal-Wallis tests revealed that among these markers, SPARCL1, Shp2 and MSH2 were noticeably associated with the most clinical pathological features of CRC patients, including differentiation, bowel wall invasion (pT), lymph node metastasis (pN), distant metastasis (pM), TNM stage, post-surgery recurrence or metastasis. P53 was mainly related to TNM staging; E-cadherin and MAPK were mainly related to post-surgery recurrence and metastasis (Table 3). However, other markers, such as endothelin B receptor, APC and rho, were just related to differentiation or stages (data not shown).

Prognostic bioinformatics model established by combining the seven markers and evaluated by survival analysis

By SVM, the seven markers can randomly form 127 combinations. After being validated by 10-fold cross validation, the model with the highest accuracy (65.3) was the p53/SPARCL1 combination among all these combinations.

According to the prediction result (PR) given by the

p53/SPARCL1 model, patients can be divided into two groups: “high risk” (PR > 0) and “low risk” (PR < 0). Three-year survival of the low risk group (88.30%) was more than twice as high as that of the high risk group (37.84%). Kaplan-Meier analysis revealed that the difference of survival was significant between these two groups ($P < 0.001$) (Figure 3A).

Prognostic value of the p53/SPARCL1 model for stage II and III CRC patients

Among these 131 patients, 99 patients were classified as stage II or III. We found that the difference in 3-year survival was not great between stage II ($n = 43$) and III ($n = 56$) patients, i.e. 88.40% *vs* 62.50% ($P = 0.039$) (Figure 3B). However, when these 99 patients were grouped by the PR of the p53/SPARCL1 model, the 3-year survival rate was very different between the low risk ($n = 70$) and high risk ($n = 29$) groups, i.e. 87.14% *vs* 37.93% ($P < 0.001$) (Figure 3C). Thus, the survival difference was much greater between low and high risk groups than between stage II and

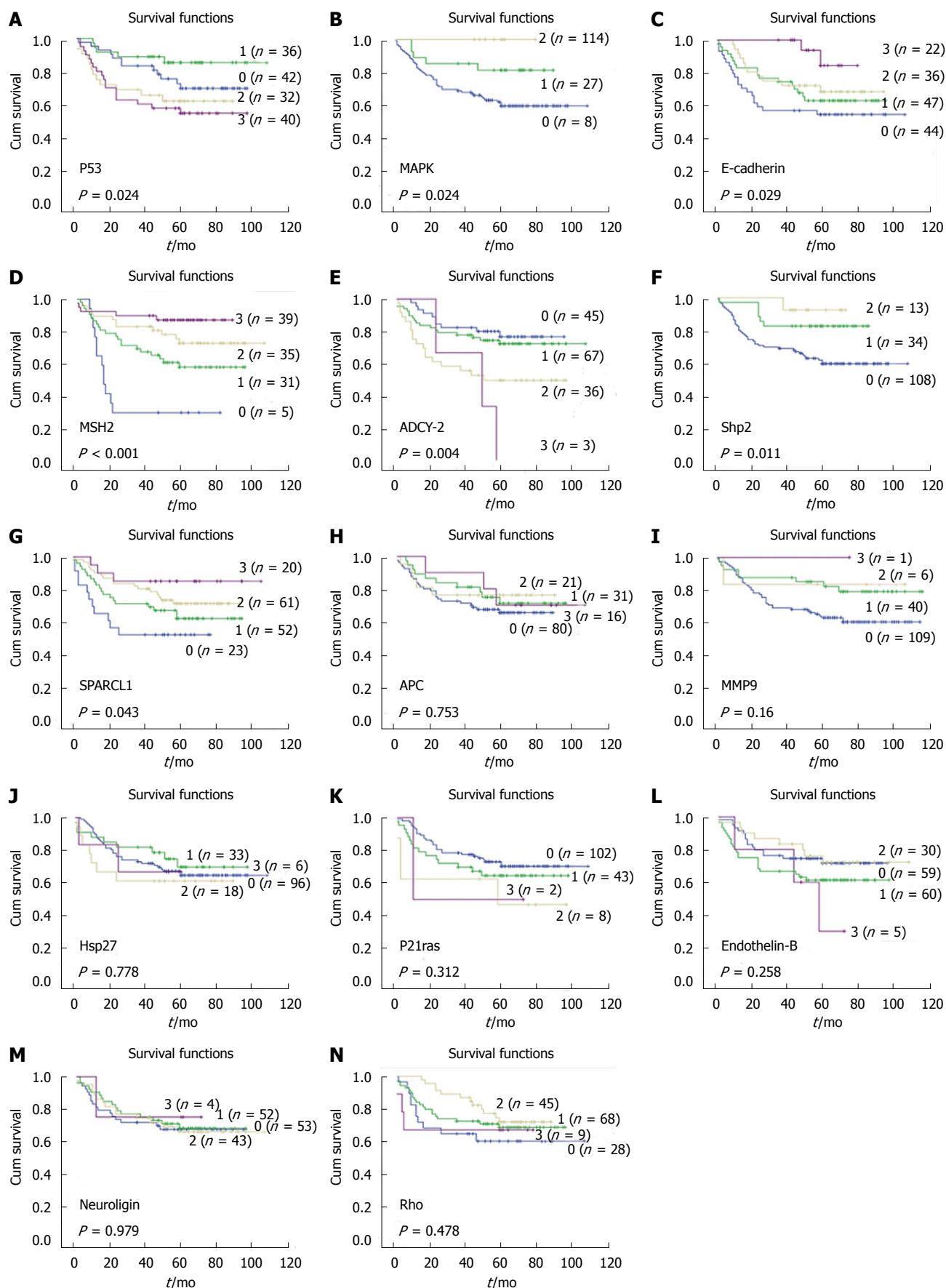


Figure 2 Kaplan-Meier curves of 14 markers. A: P53; B: MAPK; C: E-cadherin; D: MSH2; E: ADCY-2; F: Shp2; G: SPARCL1; H: APC; I: MMP9; J: Hsp27; K: P21ras; L: endothelin-B receptor; M: Neuroligin1; N: Rho.

Table 3 Relationship between marker expression and clinical features (*P* values)

Markers	<i>P</i> values					
	Differentiation	pT	pN	pM	TNM	Post-surgery
P53	0.671	0.654	0.003 ^b	0.119	0.024 ^a	0.207
MAPK	0.186	0.597	0.230	0.188	0.182	0.001 ^b
E-cadherin	0.028 ^a	0.342	0.080	0.041 ^a	0.223	0.004 ^b
MSH2	0.964	0.006 ^b	0.012 ^a	0.061	0.001 ^b	0.001 ^b
ADCY-2	0.458	0.430	0.779	0.470	0.878	0.082
Shp2	0.020 ^a	0.004 ^b	0.035 ^a	0.849	0.006 ^b	0.006 ^b
SPARCL1	0.002 ^b	0.171	0.037 ^a	0.021 ^a	0.044 ^a	0.014 ^a

The relationship between the expression of candidate markers and some pathologic features was evaluated by Kruskal-Wallis test (SPSS Version 13.0 software) in immunohistochemistry analyses. All *P* values are two-sided (^a*P* < 0.05, ^b*P* < 0.01). The pathologic features in the table were differentiation, bowel wall invasion (pT), lymph node metastasis (pN), distant metastasis (pM), TNM stage (TNM), post-surgery recurrence or metastasis (Post-surgery).

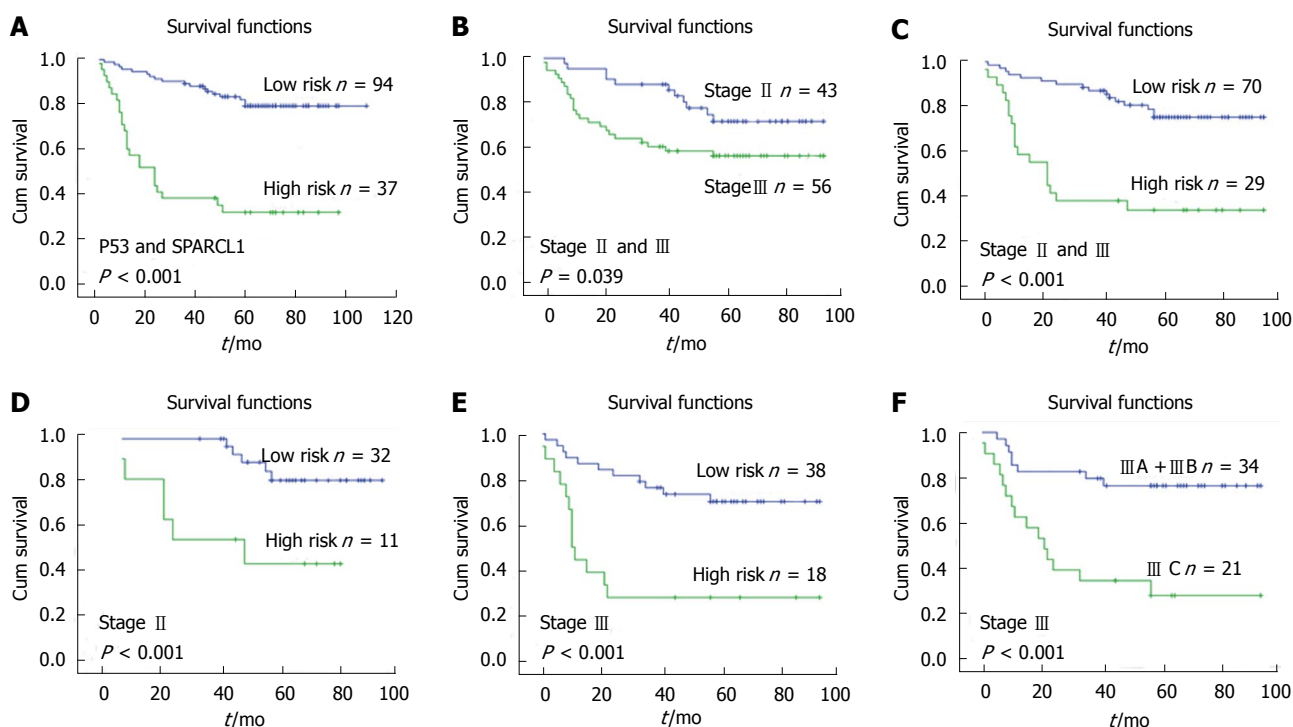


Figure 3 Prognostic value of p53/SPARCL1 model in colorectal cancer patients. According to the prediction result (PR) given by the p53/SPARCL1 model, patients could be divided into two groups: "high risk" (PR > 0) and "low risk" (PR < 0). A: 3-year survival of the "low risk" group was 88.30%, significantly higher at twice that of the "high risk" group, which was only 37.84% (*P* < 0.001). B: The 3-year survival of stage II (*n* = 43) and III (*n* = 56) patients was 88.40% vs 62.50% (*P* = 0.039), with an only 15.90% survival difference (*P* = 0.039); C: The same 99 stage II/III patients, when divided by the PR of the p53/SPARCL1 model: the 3-year survival of "low risk" (*n* = 70) and "high risk" (*n* = 29) group was 87.14% vs 37.93%, with a survival difference of 49.21% (*P* < 0.001), much more than the difference between stage II and III patients; D: According to the PR of the p53/SPARCL1 model, the 3-year survival of "low risk" and "high risk" patients at stage II was 100% and 54.55%, respectively, with a significant difference of 45.45% (*P* < 0.001); E: At stage III (*n* = 56), the 3-year survival was 78.95% of "low risk" patients and 27.78% of "high risk" patients, with a 51.17% higher survival rate (*P* < 0.001); F: At stage III (*n* = 56), the 3-year survival was different between stage IIIA/IIIB (*n* = 34) and IIIC (*n* = 22) patients: 82.36% vs 31.82% (*P* < 0.001).

III patients.

Among the 99 stage II/III patients, 43 patients were of stage II, all of whom were classified as stage II A. According to the PR of the p53/SPARCL1 model, the 3-year survival rates of low risk and high risk stage II patients were 100% and 54.55%, respectively. This 45.45% difference between the survival rates of low risk and high risk stage II patients was significant (*P* < 0.001) (Figure 3D).

Among the 56 stage III patients, the 3-year survival was 78.95% for low risk patients and 27.78% for high risk ones, and this 51.17% difference was statistically

significant (*P* < 0.001) (Figure 3E). Similar survival difference was found between stage IIIA/IIIB (*n* = 34) and IIIC (*n* = 22) patients, i.e. 82.36% vs 31.82% in 3-year survival rates (*P* < 0.001) (Figure 3F).

DISCUSSION

Which CRC patients should receive adjuvant chemotherapy after radical resection? Currently, it is a standard recommendation for stage III but not stage II patients. However, the 5-year survival rate of stage II B (T4N0M0)

patients is even lower than stage IIIA (T1-2N1M0)^[9]. One explanation for this may be due to not dissecting enough lymph nodes during surgery. Another potential cause is that stage IIB tumors penetrate to the surface of the visceral peritoneum or directly invade the adjacent organs, which indicates that the biological behavior of the tumor is poor. Further, we do not know which of the stage II patients are at high risk and should receive adjuvant chemotherapy to improve their survival. Therefore, better molecular tumor markers are urgently needed to predict which patients may potentially benefit from adjuvant chemotherapy.

In the network of cancer-related genes, pathways are the frame by which we can understand the network logically. In the present study, 14 candidate markers were selected based on the most frequently mutated genes and pathways listed in the study of Wood *et al.*^[4], and their expression levels in CRC specimens were detected by IHC, which is generally used for regular pathological detection. Among these 14 markers, seven markers (SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK) were significantly prognosis-related.

Shp2 is an essential component in several oncogene signaling pathways^[10]. Here, we surprisingly found that Shp2 is a predictive marker for good prognosis, which is in stark contrast to previous studies indicating a role for Shp2 in promoting carcinogenesis in other cancers^[11-13]. MSH2 is a vital mismatch repair gene. Patients with high MSH2 expression had better survival in CRC^[14,15], and higher gene expression of MSH2 in responders to 5-fluorouracil-based chemotherapy indicates a predictive value of MSH2 in chemotherapy^[16,17]. The MAPK signal pathway is associated with proliferation, survival and apoptosis of tumor cells and therefore plays a very important role in carcinogenesis^[18,19]. E-cadherin, a member of the cadherins, is related to invasion and metastasis in many cancers^[20]. Loss or low expression of E-cadherin is more frequent in CRC patients with liver metastasis^[21], demonstrating that loss of E-cadherin is related to poor prognosis. ADCY is involved in the G-protein system-related GnRH signal pathway. The proliferation of rat pancreatic tumoral AR4-2J cells can be stimulated by pituitary ADCY-activating peptide through the ADCY pathway^[22,23], which suggests that ADCY promotes the growth of tumor cells. Among these markers in the present study, Shp2, MSH2, MAPK and E-cadherin were significant markers for predicting good prognosis, but ADCY-2 was not.

p53 is an indispensable tumor suppressor that plays an important role in several carcinogenic processes. Previous studies suggest that p53 has an influence on the prognosis of patients in many cancers^[24] including CRC, and is associated with tumor staging, multi-drug resistance, response to chemotherapy or radiotherapy, post-surgery recurrence and metastasis^[25-30]. Recently, research has shown that mutant p53 proteins not only lose their tumor suppressive functions but may also gain new abilities that enhance tumorigenesis^[31]. Indeed, the p53 mutation is linked with chemo-resistance and trans-

formation to a more aggressive disease in many tumor types^[32]. The p53 codon 72 polymorphism causes an increased risk for liver metastases in CRC patients positive for p53 overexpression^[33]. In the present study, we found that a high expression of mutant p53 protein was associated with more frequent lymph node metastasis, advanced TNM stage and poor survival (Table 2), which is consistent with other reports.

SPARCL1, also known as hevin^[34], belongs to the matricellular protein family. SPARCL1 is down-regulated in transformed prostate epithelial cell line P69SV40T^[35,36], and tissues of metastatic prostate adenocarcinoma, non-small cell lung cancer, bladder and pancreatic ductal carcinoma, but up-regulated in liver cancer tissues^[35-39]. Additional work by our group has revealed that SPARCL1 expression is significantly different between CRC specimens with and without liver metastasis (to be published). In the present study, the expression of SPARCL1 was not only significantly associated with histological differentiation and survival but also with distant and lymph node metastasis, suggesting that SPARCL1 is likely to be an important negative regulator in the progression or metastasis of CRC.

In the present study, SVM was utilized to analyze and establish prognostic models of CRC from the combinations of the 7 prognostic biomarkers mentioned above. For SVM, the right balance is struck between the accuracy attained on a particular training set and the "capacity" of the machine, i.e. the ability of the machine to learn any training set without error to achieve the best generalization ability. The remarkably robust performance of SVM with respect to sparse and noisy data has made it the system of choice in a number of applications. When used for classification, SVM separates a given set of binary labeled training data and can work in combination with the kernels technique for cases in which no linear separation is possible. The accuracy of our models was evaluated by 10-fold cross validation.

Ultimately, the combination of p53 and SPARCL1 was found to be the best prognostic model of those tested. Survival analysis proved that the prediction result of the p53/SPARCL1 model was a statistically significant prognostic factor for CRC patients in all stages or only stage II/III (Figure 3).

Other researchers have attempted to identify biomarkers to further stratify stage II or stage III patients. Prognostic advantages were found in patients with MSI-high tumors and stage II and III CRC patients treated with 5-fluorouracil-based adjuvant therapy^[40,41]. In the PETACC-3 study, the prognostic value of MSI status was found to be more significant in patients with stage II disease than in stage III cases^[42]. However, value of MSI status as a prognostic or predictive marker may be affected by mutations in other genes involved in cancer etiology, such as the BRAF gene^[43]. Additionally, chromosome 18q loss of heterozygosity (LOH) has been associated with poor prognosis in stage II and stage III CRC patients in some studies^[40,44] but not others^[45,46]. Differences in the methodologies used possibly explained the contradictory

findings reported. In a large prospective study of patients with non-MSI-high CRC, 18q LOH was also not associated with patient survival, indicating that 18q LOH is not an independent survival predictive marker^[47].

In our study, according to the p53/SPARCL1 model, the survival rate of low risk stage II A patients was 45.45% higher than that of high risk ones. Moreover, low risk stage III patients had a 51.17% higher 3-year survival rate than high risk ones ($P < 0.001$), the same as the survival difference between stage III A/III B and III C. Therefore, the p53/SPARCL1 model established in this study can likely be used to supplement TNM staging, especially in stage II and III patients.

In conclusion, we discovered that SPARCL1, Shp2, MSH2, E-cadherin, p53, ADCY-2 and MAPK are significant prognostic markers in CRC. The p53/SPARCL1 model is of predictive use in discriminating patients with high or low risk, especially at stage II and III. Patients may benefit from accurate valuation and realistic treatment strategies for their disease with the help of potential prognostic markers. Larger scale studies and those involving multiple centers are planned to confirm clinic applicability of this prognosis model.

COMMENTS

Background

The incidence and mortality of colorectal cancer (CRC) are in the forefront of all cancers in China and western developed countries. More useful prognostic markers are urgently needed to provide evidence for the strategy of adjuvant therapy, especially for stage II and III patients.

Research frontiers

There are many ways to find useful markers in cancers. Cancer genomic research from the Vogelstein group has provided an enormous amount of information on genetic alterations in colorectal cancers. In this study, the authors set out to utilize this information to help in choosing their candidate markers. Moreover, the bioinformatics tool, which is used more and more to screen useful information from a large data set, was used here to further build prognostic models in CRC patients.

Innovations and breakthroughs

Some biomarkers have been identified as being related to the prognosis of CRC patients, including MSH2, E-cadherin and p53. However, this is the first study to report that SPARCL1, Shp2, ADCY-2 and MAPK are also potential prognostic markers in CRC. Furthermore, survival analysis proved that the p53/SPARCL1 model, established by the bioinformatics tool, could differentiate CRC patients with different prognoses in all stages or only stage II/III.

Applications

By finding significant prognostic markers in CRC, patients could be discriminated with high or low risk, especially in stage II and III. Patients may benefit from accurate valuation and realistic treatment strategies for their disease with the help of potential prognostic markers and models.

Terminology

The Support Vector Machine (SVM) classifier is a kind of bioinformatics tool, which is considered to be powerful for identifying the best discriminator from a large data set. Therefore, SVM applications are actively used in various areas from face recognition to genomics and SVM is also a powerful tool for analyzing multiple markers.

Peer review

This paper reports some novel findings on patient outcome with colorectal cancer. The array of genetic markers is extensive and worthy of publication.

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DEC1 nuclear expression: A marker of differentiation grade in hepatocellular carcinoma

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differentiated embryo chondrocyte 1 (DEC1) in hepatocellular carcinoma (HCC) and corresponding adjacent non-tumor and the normal liver tissues, the association between DEC1 expression and histopathological variables and the role of DEC1 in hepatocarcinogenesis.

METHODS: The expression of DEC1 was detected immunohistochemically in 176 paraffin-embedded sections from 63 patients with HCC and 50 subjects with normal liver tissues.

RESULTS: DEC1 protein was persistently expressed in the cytoplasm of hepatocytes in normal liver and HCC tissues. Compared with adjacent non-tumor liver tissues, HCC tissues showed high nuclear expression of DEC1 protein. However, high DEC1 nuclear expression was more frequently detected in well-differentiated (83.3%) than in moderately (27.3%) and poorly differentiated HCC (16.7%). Low DEC1 expression was associated with poor histological differentiation and malignancy progression. A correlation was found between the nuclear expression of DEC1 protein and histological differentiation ($r = 0.376$, $P = 0.024$).

CONCLUSION: DEC1 is expressed in the cytoplasm of hepatocytes and because nuclear DEC1 expression is decreased with decreasing differentiation status of HCC, nuclear DEC1 might be a marker of HCC differentiation.

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Key words: Differentiated embryo chondrocyte 1; Hepatocellular carcinoma; Differentiation; Immunohistochemistry

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Abstract

AIM: To investigate the expression patterns of human

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major global health problem, with an estimated incidence of 500 000-1 000 000 cases and 600 000 deaths annually. It is the fifth most common cancer in the world and the third most common cause of cancer-related death^[1]. The high morbidity and mortality of HCC is due to pre-existing primary chronic liver diseases, such as chronic viral hepatitis, aflatoxin B1, alcoholic liver disease and dysmetabolism, including hereditary haemochromatosis, obesity, diabetes and steatosis^[2]. Each of these scenarios has its own genetic and epigenetic alterations, chromosomal aberrations, gene mutation and altered molecular pathways in the process of hepatocarcinogenesis^[2,3]. Because of these varied background and heterogeneity, HCC is complex. Although dysregulation of signaling pathways such as Wnt/b-catenin, Ras, p14ARF/p53, p16INK4A/Rb, transforming growth factor-beta (TGF-beta) and PTEN/Akt has been reported in some HCC cases^[4], the specific gene mutation(s) and exact molecular mechanism involved in hepatocarcinogenesis is not well known.

Human differentiated embryo chondrocyte 1 (DEC1), a basic helix-loop-helix (bHLH) transcription factor, has rat and mouse orthologs, named enhancer of split and hairy-related protein-2 (SHARP-2) and stimulation of retinoic acid 13 (Stra13), respectively^[5-7]. The factors play important roles in regulation of gene expression in cell differentiation, proliferation, immune regulation and metabolism homeostatic control^[8]. DEC1 is expressed ubiquitously in both embryonic and adult tissues with human and various extracellular stimuli such as growth factors, serum starvation, hypoxia, hormones, nutrients, cytokines, UV radiation, and infection, which regulate its expression^[8,9]. The regulation of DEC1 is cell-type specific^[5,9-11].

Several studies have described various DEC1 expression patterns in different tumor tissues, which suggest that it might contribute to oncogenesis. In human breast cancer, the overexpression of DEC1 contributes to a more aggressive phenotype^[12]. The association between upregulation of DEC1 expression and differentiation of gastric cancer suggests its important role in the differentiation and progression of gastric cancer^[13]. Linked to oncogenesis, DEC1 is highly expressed in colon carcinomas but not in the adjacent normal tissues^[14]. It is involved in the UV signal transduction pathway and takes part in the process leading to skin cancer^[15]. In combination with carbonic anhydrase-IX (CAIX) and carbonic anhydrase-X II (CAX II), DEC1 may help with a more accurate classification of all renal carcinomas^[9]. However, in lung cancer, upregulated or downregulated DEC1 expression has been found^[16,17]. The expression patterns

and level of DEC1 protein in HCC have not been systematically investigated, and its potential role in hepatocarcinogenesis is unknown.

We aimed to investigate the expression of DEC1 in HCC. We evaluated the distribution and level of expression of DEC1 protein in 176 paraffin-embedded tissue sections from 63 patients with HCC and 50 subjects with normal liver tissues by immunohistochemistry. We also investigated the correlation of DEC1 expression with clinicopathological features and differentiation status of HCC to evaluate the functional characteristics of DEC1 in the development of HCC.

MATERIALS AND METHODS

Patients and samples

Three kinds of human liver sections ($n = 176$) were evaluated, including 126 HCC and adjacent non-tumor tissues from 63 patients with primary HCC, and 50 normal liver tissues from patients with hepatic hemangioma who underwent hepatectomy in Qianfoshan Hospital and Jinan Central Hospital, Shandong University, China. The 63 HCC patients included 52 males; the median age was 56 years (range, 35-77 years), and the 50 normal liver patients included 17 males. The formalin-fixed, paraffin-embedded tissue samples were retrospectively collected and randomly selected from the files of the Department of Pathology after the protocol was approved by the local research ethics committee. All HCC patients underwent surgery without prior radiotherapy or chemotherapy and other diseases such as viral hepatitis had been excluded in the hemangioma patients. All sections were reviewed independently by pathologists blinded to the clinicopathological characteristics, 63 HCC and 50 normal livers with the same pathological results were selected in our study. Among the 63 HCC samples, 18 were well differentiated, and 45 were moderately and poorly differentiated. We also collected data on sex, age, tumor size, hepatitis B virus infection, presence of cirrhosis, and α -fetoprotein (AFP) level in HCC.

Immunohistochemical staining

The tissues were fixed in 10% neutral buffered formalin for 12 h and routinely processed. Paraffin wax-embedded tissue blocks were cut into 4- μ m-thick sections. Briefly, formalin-fixed, paraffin-embedded sections were heated at 60°C for 60 min and placed into xylene to be deparaffinized and graded ethanol to be rehydrated, and then washed in phosphate-buffered saline (PBS). Antigen retrieval was performed in a prewarmed pressure cooker with a solution of antigen retrieval citrate buffer (pH 6.8) for 3 min. Following de-pressurization, cold water was poured into the cooker for 10 min, and then sections were rinsed well in warm water. Endogenous peroxide and oxidative compounds were quenched by incubation in 3% H₂O₂ in methanol for 10 min. Sections were washed 3 times with PBS, and incubated with rabbit polyclonal DEC1 antibody diluted in TBS-Tween20 (1:300 dilution)

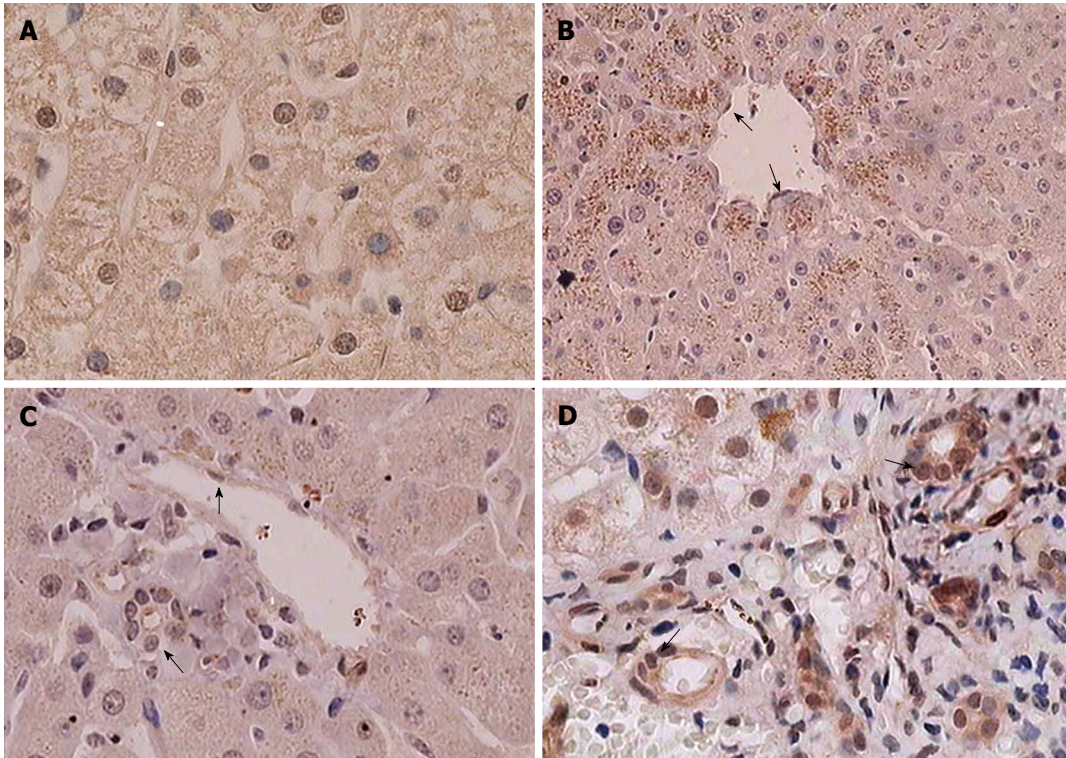


Figure 1 Differentiated embryo chondrocyte 1 expression in normal liver tissue by immunohistochemical staining. A: Normal liver tissues showing strong nuclear differentiated embryo chondrocyte 1 (DEC1) staining and diffuse cytoplasmic staining in hepatocytes, $\times 400$; B: Strong granular staining of DEC1 in cytoplasm of hepatocytes around the central vein in the intact hepatic lobule, $\times 200$; C: Absent granular staining and diffuse DEC1 staining pattern around portal area, $\times 400$; D: Liver mesenchymal tissues, $\times 400$. Endothelial cells (single arrow) and bile duct epithelial cells (double arrow) showing cytoplasm and/or nuclear DEC1 immunoreactivity.

(Bethyl Laboratories Inc, Montgomery, TX) overnight in a moist chamber at 4°C . After a final wash, secondary antibody (KIT-5010, Max Vision, Maixin.Bio, China) was applied, and TBS-Tween20 was used in all the dilutions and intervening rinses involved. Diaminobenzidine (DAB) was the chromogenic substrate. Sections were allowed to develop in DAB for 5 min, and then counterstained with hematoxylin. Slides were reviewed under microscope. Sections incubated without primary antibody were used as negative controls, and breast cancer sections were used as positive controls. Positive and negative controls were included in each run.

In hepatocytes, DEC1 protein was persistently expressed in all cytoplasms. We used a scoring standard for nuclear DEC1 expression according to our former paper^[13]: negative expression, no nuclear staining; low expression, nuclear staining $< 10\%$ of cancer cells; and high expression, nuclear staining $> 10\%$ of cancer cells. The staining was evaluated by two independent observers.

Statistical analysis

Categorical variables were compared by χ^2 test or Fisher's exact test as appropriate. Spearman analysis was used to assess the correlation between DEC1 expression and tumor differentiation status. All statistical analyses were performed using SPSS v11 for Windows (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

DEC1 expression in normal liver tissues

In normal liver tissues, DEC1 expression was diffuse in the cytoplasm of hepatocytes accompanied by a varying degree of nuclear immunoreactivity (Figure 1A). A strong granular pattern of DEC1 staining was seen within the cytoplasm of the hepatocytes around the central vein in the intact hepatic lobule, and this special staining strength became gradually weakened away from the central vein (Figure 1B). However, no granular cytoplasmic staining was seen around the portal area (Figure 1C). The two different cytoplasmic staining patterns may result from the special anatomic structure and the particular blood supply of the hepatic lobule. The specific conditions around the central vein, such as hypoxia, low nutrition and acidity, could affect DEC1 expression status and lead to the granular pattern, and the diffusive expression pattern may be hypoxia independent. In addition, endothelial and bile duct epithelial cells showed nuclear and/or cytoplasmic DEC1 immunoreactivity (Figure 1A, B, D).

DEC1 expression in HCC and adjacent non-tumor tissues

Diffuse cytoplasm expression of DEC1 protein was observed in all 126 HCC and adjacent non-tumor liver tissues, except in 2 poorly differentiated HCC samples with weak cytoplasmic staining. Nuclear DEC1 expression was

Table 1 Differentiated embryo chondrocyte 1 nuclear expression in hepatocellular carcinoma and adjacent non-tumor tissues *n* (%)

	Negative	Positive
HCC (63)	27 (42.9)	36 (57.1) ^a
Adjacent non-tumor tissues (63)	46 (73.0)	17 (27.0)

^a*P* < 0.05 *vs* adjacent non-tumor tissues. HCC: Hepatocellular carcinoma.

detected in 36 (57.1%) of 63 samples of primary HCC, with 26 (41.3%) samples showing high nuclear expression (Table 1). Nuclear DEC1 expression was observed in only 17 (27.0%) of the 63 matched adjacent non-tumor tissues. Compared with HCC tissue, adjacent non-tumor tissues showed reduced nuclear DEC1 expression (*P* = 0.001). The proportion of positive DEC1 nuclear staining was higher in normal (66.7%) than in HCC tissues, but was not significantly different (*P* = 0.650). In LO-2 cells (normal hepatocytes), nuclear DEC1 expression was higher than in HepG-2 cells (data not shown). Compared with the normal control, adjacent non-tumor tissues showed a significantly low positive staining rate (*P* = 0.002), which may result from extrusion around the tumor or the response of hepatocytes to extracellular stimulation from the tumor. In addition, in HCC tissues with negative nuclear staining, only two cases showed positive nuclear staining in the corresponding adjacent non-tumor tissues. Therefore, the specific microenvironment around the HCC tumor and the present chronic liver disease such as viral hepatitis may affect DEC1 expression in adjacent non-tumor tissues.

Clinical significance of DEC1 nuclear expression in HCC

For the 36 cases with positive DEC1 nuclear staining, the proportion of DEC1 staining in well, moderately and poorly differentiated HCC tissues was 94.4% (17/18), 42.4% (14/33) and 41.7% (5/12), respectively. Negative DEC1 nuclear protein staining was associated with high histological HCC grade (Table 2). Well-differentiated tissue was significantly different from moderate and poorly differentiated tissues (*P* < 0.001). Factors such as sex (*P* = 0.886), age (*P* = 0.383), tumor size (*P* = 0.571), hepatitis B virus infection (*P* = 0.842) and cirrhosis (*P* = 0.616) had no significant association with DEC1 nuclear expression.

Nuclear DEC1 expression in HCC tissues is correlated with tumor differentiation

As a transcription factor, DEC1 plays its role in the nucleus. We found strong nuclear-positive staining and high nuclear DEC1 expression in well-differentiated HCC samples. In poorly differentiated tumors with large and pleomorphic cells, DEC1 nuclear expression was weak, even negative. We investigated the nuclear immunoreactivity of DEC1 related to differentiation status (Figure 2A-C). The proportion of high DEC1 nuclear expression in well, moderately and poorly differentiated HCC tissues was 83.3%, 27.3% and 16.7%, respectively (Figure 2D).

Table 2 Correlation between nuclear differentiated embryo chondrocyte 1 protein expression and clinicopathologic features of hepatocellular carcinoma

Clinicopathologic features	<i>n</i>	Nuclear DEC1 protein expression		<i>P</i> value
		Negative	Positive	
Sex				
Male	52	23	29	0.886 ¹
Female	11	4	7	
Age (yr)				
< 56	31	15	16	0.383
≥ 56	32	12	20	
Tumor size (cm)				
≤ 5	28	11	17	0.571
> 5	30	14	16	
Pathological grade				
Grade (I)	18	1	17	0.000
Grade (II-III)	45	26	19	
Venous infiltration				
Absent	9	2	7	0.070 ²
Present	10	7	3	
No. tumor nodules				
< 3	49	22	27	0.781 ¹
≥ 3	9	3	6	
Hepatitis B virus infection				
Yes	51	22	29	0.842 ¹
No	3	2	1	
Cirrhosis				
Yes	47	21	26	0.616
No	16	6	10	
α-fetoprotein level (ng/mL)				
> 20	40	18	22	0.713
≤ 20	20	8	12	

¹Corrected χ^2 analysis; ²Fisher's exact test.

We found a correlation between nuclear DEC1 protein expression and histological differentiation in HCC (*r* = 0.376, *P* = 0.024) (Table 3).

DISCUSSION

HCC is a complex and heterogeneous cancer, and the key drivers are not well known^[3,4]. Human DEC1 and the homologs rat SHARP-2 and mouse Stra13 belong to the bHLH family^[8]. In many tumor-derived cell lines and tumor tissues, DEC1 mRNA levels are upregulated^[9,18]. Its protein is also widely expressed with restricted patterns in many tissues^[9,10]. Nevertheless, the distribution and role of DEC1 in carcinogenesis and in the differentiation of human HCC are unknown. A previous study showed 5 of 6 cases of HCC with positive cytoplasmic and nuclear staining^[9]. However, the number of samples in this study was too small. In the current research, we studied the expression of DEC1 in 176 samples of HCC and normal liver tissues by immunohistochemistry.

We found that DEC1 protein was persistently expressed in the cytoplasm of both normal and malignant hepatocytes, with varying degrees of nucleic immunoreactivity and two staining patterns, granular and diffusive, in the cytoplasm of hepatocytes. Compared with the adjacent non-tumor tissues, HCC tissue showed predominant

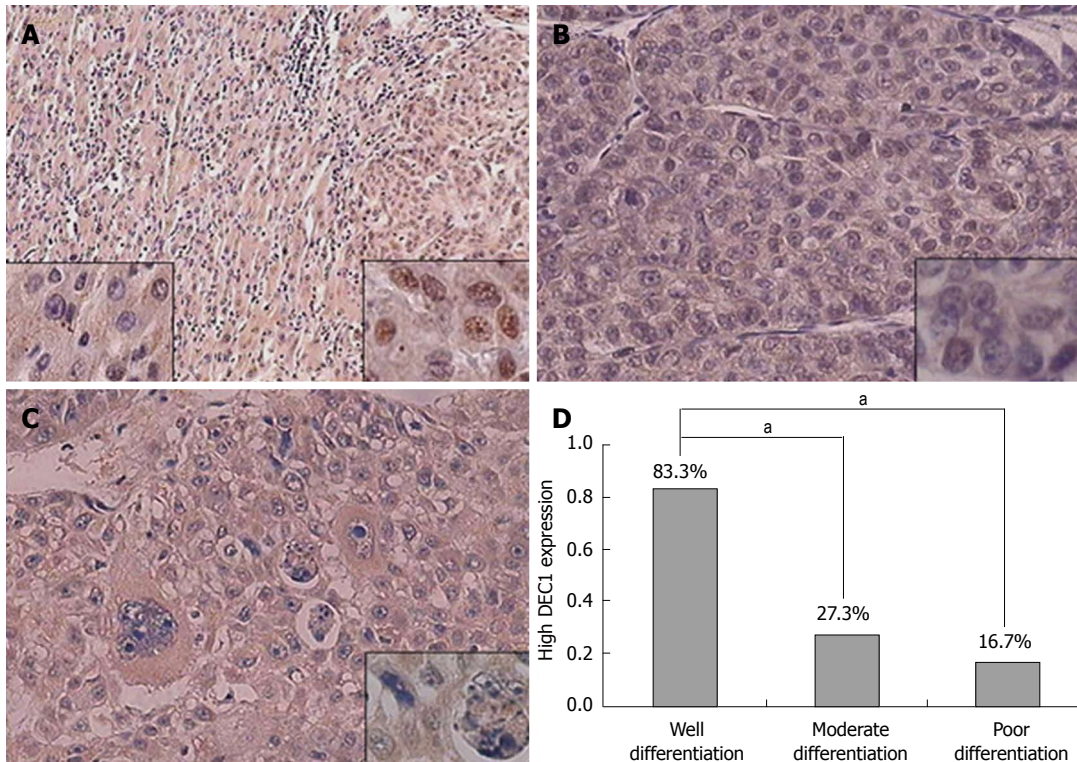


Figure 2 Differentiated embryo chondrocyte 1 expression in hepatocellular carcinoma by immunohistochemical staining. A: Well-differentiated hepatocellular carcinoma (HCC), $\times 100$. Intense nuclear staining in HCC (right inset), and weak nuclear staining in the corresponding adjacent non-tumor tissues (left inset), with no significant difference in cytoplasmic staining; B: Moderately-differentiated HCC, $\times 200$; C: Poorly-differentiated HCC, $\times 200$. Inserted figure (at high magnification) showing differentiated embryo chondrocyte 1 (DEC1) staining in the cytoplasm and nucleus, $\times 400$; D: Quantitation of DEC1 nuclear expression in HCC by different differentiation status. $^aP < 0.05$.

Table 3 Correlation between differentiated embryo chondrocyte 1 nuclear expression and hepatocellular carcinoma differentiation

Tumor differentiation (cases)	DEC1 nuclear expression ¹		<i>r</i> value	<i>P</i> value
	Low	High		
Well (<i>n</i> = 17)	2	15	0.376	0.024
Moderate (<i>n</i> = 14)	5	9		
Poor (<i>n</i> = 5)	3	2		

¹Number of cases. DEC1: Differentiated embryo chondrocyte 1.

DEC1 nuclear protein expression, but the nuclear expression of DEC1 was decreased from well to moderately and poorly differentiated HCC. It seems that low DEC1 expression is associated with poor histological differentiation and malignancy progression in HCC. However, the mechanism of this distribution of DEC1 expression in HCC needs further investigations.

The intracellular distribution of DEC1 protein shows cell specificity: in Hela cells, DEC1 is equally present in both the nucleus and cytoplasm; in HepG2 cells, DEC1 is located mostly in the cytoplasm; and in the 786-0 cell line, the nuclear expression of DEC1 is predominant^[9]. In the present study, DEC1 protein was persistently expressed in almost all cytoplasm of hepatocytes, with a varying degree of nuclear immunoreactivity. This expression pattern implies that DEC1 may take part in the normal vital

functions of the liver such as metabolism and detoxification. DEC1-positive granular staining gradually weakened outward from the central vein in the cytoplasm of hepatocytes. The mechanism underlying this phenomenon might be the hypoxic environment around the central vein. Hypoxia-induced factor-1 α (HIF-1 α), the most important hypoxia effector, can upregulate the expression of DEC1 by binding hypoxia response element (HRE) on *DEC1* gene promoter^[19]. We also found that the DEC1 expression level in HCC was higher than in normal liver tissues. The oxygen tension in HCC was similar to that in normal liver tissues, so the overexpressed HIF-1 α in HCC was independent of hypoxia^[20]. Thus, the diffuse staining pattern of DEC1 in normal liver tissues and HCC was independent of hypoxia, and the granular staining pattern of DEC1 may represent a low oxygen concentration. Whether the different patterns of staining imply a different function of DEC1 in the cytoplasm of hepatocytes awaits further investigations. Additional studies are required to unravel the mechanism by which DEC1 performs its function in cytoplasm. Furthermore, the subcellular localization of DEC1 may be helpful in identifying the primary origin of certain tissues and cells.

DEC1 usually acts as a transcription repressor, but it also can function as a transcriptional activator under particular circumstances. It has been shown that signal transducers and activators of transcription 3 (STAT3), which

are essential for the carcinogenesis of HCC, are a protein partner of DEC1^[21,22]. DEC1 can bind with phosphorylated STAT3 β and/or STAT3 α isoforms, thus activating the downstream transcription from STAT-dependent cis-elements^[22]. For example, co-expression of STAT3 α or STAT3 β with DEC1 can modify the transcription status of Fas, which can regulate cell survival and apoptosis^[22]. DEC1, together with STAT3, is involved in complex regulation of STAT downstream transcriptional targets. Thus, inappropriate co-activation of DEC1 and STAT3 may lead to oncogenic transformation in hepatocytes. Through this network, DEC1 may contribute to the regulation of critical processes of cell survival and growth in hepatocellular carcinogenesis.

Unlike other bHLH proteins, such as c-Myc and ID, which exhibit intrinsically growth-promoting activity, DEC1 can cause proliferation inhibition and differentiation promotion. In NIH 3T3 cells, induced Stra13 strongly represses the expression of the cell proliferation-associated gene c-Myc by interacting with the basal transcription factor TFIIB^[18]. The proximal promoter region of ID1 gene contains several potential DEC1 responsive elements by which DEC1 can inhibit ID1 expression, thus promoting cell differentiation^[23]. DEC1 has been involved in various differentiation processes, such as neurogenesis^[6,7], chondrogenesis^[5] and myogenesis^[24]. The differentiation inducer hydroxyurea can markedly induce the expression of DEC1^[13]. The promotion or inhibition function of DEC1 in cell differentiation is cell-type specific and differs in different original tissues. Overexpression of Stra13 inhibits mesodermal but promotes neuronal differentiation in P19 cells^[6]. In rats, the expression of sharp-2 mRNA was slightly higher in the well-differentiated than in the poorly differentiated malignant hepatoma cell lines^[25]. Similarly, in our study, high DEC1 nuclear expression was more frequently detected in well-differentiated HCC than in poorly differentiated HCC. Thereby, DEC1 may serve as a marker for the degree of HCC differentiation. As a transcription repressor, DEC1 can regulate expression of many other transcription factors and maintain the homeostasis of the tissues and cells^[8]. The reduced expression of DEC1 in poorly differentiated HCC might lead to exacerbation through impairing the homeostasis of the liver.

Epidemiological studies indicated that metabolic disturbance, especially abnormal lipid and glucose metabolism, is a major characteristic of the cause of HCC^[4]. Liver X receptor (LXR), together with sterol-regulatory-element-binding protein 1c (SREBP1c) and fatty acid synthase (FAS), enhances the development of HCC caused by chronic viral infection^[26]. And insulin resistance increases the risk of HCC^[27]. Recent reports have proved that DEC1 was closely associated with LXR, SREBP1c, FAS and insulin^[28-31]. However, further researches are needed about the relationship between the function of DEC1 interacted with these genes in liver metabolism and the differentiation grade of HCC.

In summary, we demonstrated DEC1 protein expression patterns in the liver and characterized the relationship

between nuclear DEC1 protein expression and histological differentiation of HCC. DEC1 might be a marker of HCC differentiation. However, DEC1 expression can be regulated through several pathways to contribute to its specific functions in different tissues. In the liver, DEC1 may be situated at the crossroads of a complex transcriptional network that is able to modulate metabolism, energy, physiological function and tumor genesis. Dysregulation of *DEC1* gene causes alteration of homeostasis in tissues and cells, leading to abnormal cell proliferation, differentiation and death as well as subsequent development of cancer in a cell type-dependent manner.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the common fatal cancers worldwide. The aggressive phenotype and resistance to therapy makes HCC particularly dangerous. Recent reports indicate that the transcription factor differentiated embryo chondrocyte 1 (DEC1) contributes to tumorigenesis. The expression of DEC1 and its role in human HCC are unknown. DEC1 may take part in the vital metabolism function of liver and in the procession of hepatocarcinogenesis.

Research frontiers

DEC1 is a new transcriptional factor with helix-loop-helix (HLH) domains. It plays an important role in the maintenance of the homeostasis of metabolism and energy, oncogenesis, cell growth and apoptosis, immune balance and circadian rhythm.

Innovations and breakthroughs

This is the first study to describe the expression of DEC1 in HCC and normal liver tissues. In this study, an inverse relationship was found between the nuclear DEC1 protein expression and the histological grade of HCC. DEC1 might be a marker for HCC differentiation. Based on all these results, DEC1 may be situated at the crossroads of a complex transcriptional network that is able to modulate metabolism, energy, physiological function and tumor genesis in the liver.

Applications

By understanding the distribution of DEC1 protein expression and the function of this bHLH molecule, this study may represent a future strategy for therapeutic intervention in patients with HCC.

Terminology

Dec1 and Hif are transcriptional factors with HLH domains. They are important in oncogenesis. Sterol-regulatory-element-binding protein 1c (SREBP1c), fatty acid synthase (FAS) and liver X receptor (LXR) play vital functions in metabolism and tumor genesis in the liver. All these proteins take part in the maintenance of the homeostasis of metabolism and energy, cell growth and apoptosis, oncogenesis.

Peer review

The morbidity and mortality of HCC are high in China. In the procession of hepatocarcinogenesis, there are already pre-existing chronic liver diseases. In this paper, the authors demonstrated for the first time that DEC1 protein expression patterns in normal liver tissue and HCC systematically. It is important for further studying the development of HCC and the function of DEC1.

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Apoptotic bone marrow CD34+ cells in cirrhotic patients

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CD34+ cells was $15.00\% \pm 15.81\%$ and $5.73\% \pm 1.57\%$ ($t = 2.367$, $P < 0.05$) in cirrhosis and control groups, respectively. The percentage of apoptotic marrow CD34+ cells was $6.25\% \pm 3.30\%$ and $20.92 \pm 18.5\%$ ($t = 2.409$, $P < 0.05$) in Child-Pugh A and Child-Pugh B + C cirrhotic patients, respectively. The percentage of late apoptotic marrow CD34+ cells was positively correlated with the total bilirubin and aspartate aminotransferase serum levels in patients with cirrhosis.

CONCLUSION: The status of CD34+ marrow cells in cirrhotic patients may suggest that the ability of hematopoietic progenitor cells to transform into mature blood cells is impaired.

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Key words: Cirrhosis; CD34; Hematopoietic stem cells; Hematopoietic progenitor cells; Apoptosis

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Abstract

AIM: To access the frequency and level of apoptotic CD34+ cells isolated from the marrow fluid of patients with post-hepatitis cirrhosis.

METHODS: The frequency of bone marrow CD34+ cells and apoptotic bone marrow CD34+ cells in 31 in-patients with post-hepatitis cirrhosis (cirrhosis group), and 15 out-patients without liver or blood disorders (control group) was calculated by flow cytometry. Parameters were collected to evaluate liver functions of patients in cirrhosis group.

RESULTS: The percentage of normal bone marrow CD34+ cells was $6.30\% \pm 2.48\%$ and $1.87\% \pm 0.53\%$ ($t = 3.906$, $P < 0.01$) while that of apoptotic marrow

INTRODUCTION

Cirrhosis represents the final stage for a wide variety of chronic liver diseases. One of its main clinical manifestations in its decompensatory stage is cytopenia. The causes have been generally recognized as hypersplenism and abnormal bone marrow^[1]. However, the more detailed mechanism underlying cytopenia in cirrhotic patients is not clear. An understanding of its mechanism underlying loss of peripheral blood cells would shade further insight

Table 1 Characteristics of patients in cirrhosis and control groups

Parameters	Control	Child-Pugh A	Child-Pugh B	Child-Pugh C	P value (cirrhosis vs control)
Demographic data					
Number	15	12	11	8	
Male/female	9/6	6/6	6/5	5/3	
Age	42 (32-53)	39 (35-50)	46 (32-60)	48 (29-60)	NS
Lab tests					
WBC ($\times 10^9$)	7.05 \pm 2.21	4.34 \pm 2.01	2.93 \pm 0.50	3.62 \pm 1.78	< 0.01
RBC ($\times 10^{12}$)	3.53 \pm 0.60	3.67 \pm 0.61	3.93 \pm 0.75	3.34 \pm 0.57	NS
PLT ($\times 10^9$)	210 \pm 105	141 \pm 114	58 \pm 42	54 \pm 27	< 0.01
ALT(IU/L)	16 \pm 14	55 \pm 49	87 \pm 48	61 \pm 31	< 0.01
AST(IU/L)	20 \pm 11	50 \pm 33	73 \pm 24	106 \pm 63	< 0.01
Albumin (g/L)	43 \pm 5	40 \pm 6	35 \pm 3	30 \pm 6	< 0.01
TBIL (μ mol/L)	10.3 \pm 3.5	15.8 \pm 7.6	40.7 \pm 23.0	78.4 \pm 57.8	< 0.01
γ -GT(IU/L)	15 \pm 12	64 \pm 63	143 \pm 171	100 \pm 108	< 0.01

TBIL: Total bilirubin; NS: No significance.

into the progress of cirrhosis and its treatment.

Peripheral blood cells are derived from hematopoietic stem cells (HSC). HSC are derived from mesenchymal cells located in wall of yolk sac during the embryonic stage. After establishment of embryonic blood circulation, HSC spread to the liver and hematopoiesis is initiated in the 6th week of embryonic stage and reoccurs in the spleen when hematopoiesis in the liver is decelerated. After birth, the production of blood cells in the liver and spleen dwindles, and almost halts altogether^[2]. HSC settle mainly in the bone marrow and produce blood cells for life. As yet, very few studies are available on the phenotypic change of HSC located in bone marrow of cirrhotic patients and its impact on the production of peripheral blood.

CD34, a well known marker for HSC, is a type of phosphoglycoprotein belonging to type 1 trans-membrane protein, helps HSC/hematopoietic progenitor cells (HPC) to adhere to marrow stroma, inhibits differentiation of hematopoietic cells, stimulates formation of HPC, and is involved in intracellular signal transduction, *etc.* The number of CD34+ cells including hematopoietic cells at several stages is heterogeneous and can differentiate to all blood cell lineages. The CD34 molecule gradually disappears when HSC/HPC differentiate into mature blood cells^[3].

In this study, the percentage of apoptotic CD34+ bone marrow cells in patients with post-hepatitis cirrhosis at different stages was calculated by flow cytometry.

MATERIALS AND METHODS

Study subjects

Thirty-one patients with post-hepatitis cirrhosis, admitted to Department of Infectious Diseases, Second Affiliated Hospital of Xi'an Jiaotong University from November 2008 to April 2009, were divided into three groups according to the Child-Pugh classification. The patients did not receive treatment with interferon and only 6 patients were treated with lamivudine or adefovir intermittently before they were enrolled in this study. The control group consisted of 15 patients attending the Outpatient Department of Hematology in our hospital. Bone marrow smears showed that they had no liver and hematological

disorders. Laboratory tests were performed to evaluate their liver functions. The clinical details of cirrhosis and control groups are shown in Table 1. Bone marrow was aspirated from each patient. Mononuclear cells (MNC) were isolated from 4 mL freshly-heparinized marrow fluid by Isopaque-Ficoll (Bioer, Hangzhou, China) gradient centrifugation. The study was approved by the local ethical committee, and informed consent was obtained from the patients.

Flow cytometry evaluation

After Ficoll gradient separation, the MNC were washed with 100 mL of phosphate-buffered saline (PBS), and 10^6 cells were stained with anti-CD34-FITC MAb (Biosynthesis, Beijing, China) for 30 min at 4°C. Percentage of CD34+ cells was assayed with a Keygen annexin V apoptosis detection kit containing annexin V-PE, propidium iodide (PI; Sigma, US) solution and annexin V binding buffer. CD34+ cells were analyzed to determine the early annexin V+/PI- or late annexin V+/PI+ apoptotic phase.

The MNC were stained again with anti-CD34-FITC MAb, annexin V-PE (Keygen, Nanjing, China) and PI, washed again with PBS and re-suspended in 100 mL of annexin V-PE for 15 min at room temperature. Another 200 mL of binding buffer and 5 mL of PI solution were added and 800 000 cells were obtained by flow cytometry (BD, USA)^[4].

Statistical analysis

Statistical analysis was conducted using the SPSS 13.0 statistical package. Difference in independent variables of apoptotic CD34+ cells was detected by *t*-test and one way ANOVA. *P* < 0.05 was considered statistically significant.

RESULTS

Percentage of bone marrow CD34+ cells in cirrhotic patients

Marrow CD34+ cells were gated from the side scatter height (SSC-H)/CD34 FITC dot plot according to the Milan protocol (Figure 1).

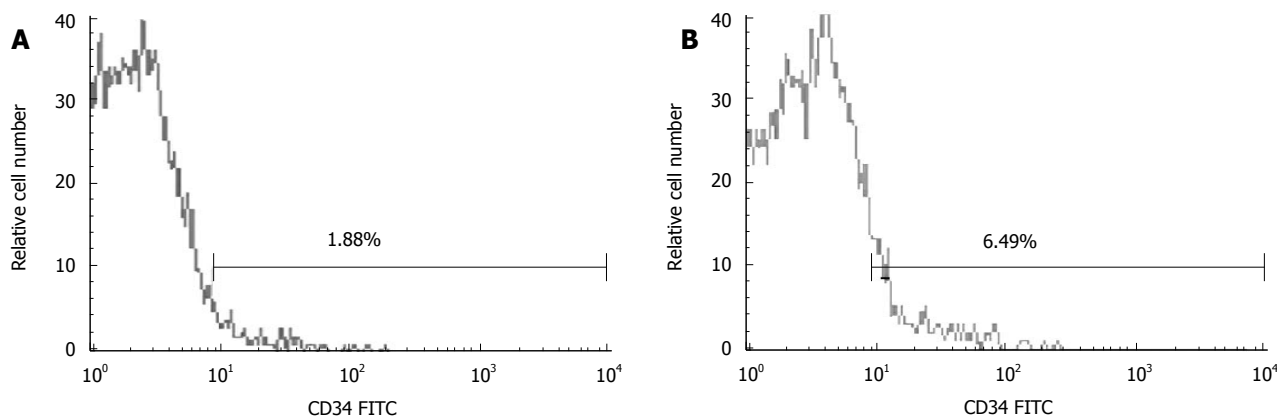


Figure 1 Flow cytometry showing the percentage of CD34+ cells in controls (A) and cirrhotic patients (B).

Table 2 Correlation between early, late and total CD34+ cells and laboratory parameters						
Parameters	Early apoptotic cells		Late apoptotic cells		Total CD34+ cells	
	r	P	r	P	r	P
Age	-0.124	0.625	0.225	0.385	0.048	0.839
RBC	0.024	0.924	-0.181	0.487	-0.107	0.653
WBC	-0.152	0.548	0.244	0.345	0.322	0.167
PLT	-0.216	0.389	0.195	0.452	0.218	0.355
TBIL	-0.186	0.490	0.717	0.003 ^b	0.314	0.205
ALT	-0.015	0.957	0.256	0.358	0.206	0.412
AST	-0.043	0.874	0.663	0.007 ^b	0.146	0.564
γ-GT	-0.117	0.667	0.136	0.630	0.471	0.048 ^a
Albumin	-0.283	0.270	-0.033	0.905	0.465	0.045 ^a

^aP < 0.05, ^bP < 0.01 vs early apoptotic cells.

The percentage of normal bone marrow CD34+ cells (out of mononuclear cells -CD34+/MNC) was 6.30% ± 2.48% and 1.87% ± 0.53% (*t* = 3.906, *P* < 0.01), respectively, in cirrhosis and control groups, while that of CD34+/MNC was 7.01% ± 2.1%, 4.58% ± 2.56%, and 7.72% ± 1.49% (*F* = 3.586), respectively, in Child-Pugh A-C cirrhosis patients (Figure 2).

Percentage of apoptotic bone marrow CD34+ cells in cirrhotic patients

The percentage of bone marrow CD34+ cells was determined by FACS analysis with annexin V/PI staining (Figure 3). The percentage of early apoptotic bone marrow CD34+ cells (out of total marrow CD34+ cells) was 6.60% ± 5.83% and 3.88% ± 1.22% (*t* = 0.912), respectively, while that of late apoptotic bone marrow CD34+ cells was 9.24% ± 13.67% and 1.86% ± 0.86% (*t* = 2.207, *P* < 0.05), respectively, in cirrhosis and control groups. The total percentage of apoptotic bone marrow CD34+ (out of total marrow CD34+ cells) cells was 15.00% ± 15.81% and 5.73% ± 1.57% (*t* = 2.367, *P* < 0.05), respectively, in cirrhosis and control groups.

The total percentage of apoptotic bone marrow CD34+ cells (out of total marrow CD34+ cells) was 6.25% ± 3.30% and 20.92% ± 18.5% (*t* = -2.409, *P* < 0.05), re-

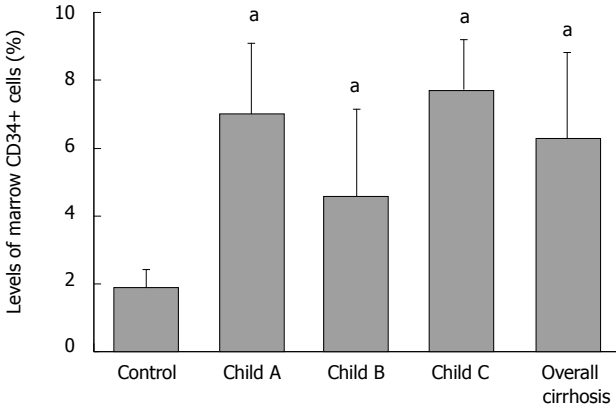


Figure 2 Percentage of bone marrow CD34+ cells in two groups. ^aP < 0.01 vs control control.

spectively, in early (Child-Pugh A) and late stage (Child-Pugh B+C) cirrhotic patients (Figure 4).

Correlation between apoptotic CD34+ bone marrow cells and reduced bilitubin and aminotransferase serum levels

The correlation between apoptotic CD34+ bone marrow cells and reduced bilitubin and aminotransferase serum levels was assessed (Table 2), which showed that the early apoptotic CD34+ bone marrow cells were positively correlated with the serum levels of γ-GT and albumin, while the late apoptotic CD34+ bone marrow cells were negatively correlated with the serum levels of total bilirubin and aspartate aminotransferase (AST) in cirrhotic patients.

DISCUSSION

HSC, the ancestors of different blood cells, are multipotent and self-renewable with a great ability to proliferate, and can differentiate into HPC which then differentiate into red blood cells, white blood cells and platelets. HPC, derived from HSC, are not able to renew. The number of HPC is retained by proliferation which expands different mature blood cells^[5].

In this study, the expression of CD34 antigen in bone

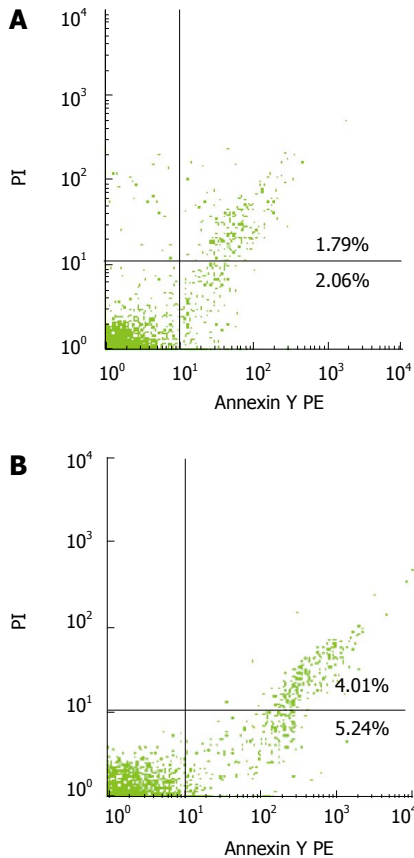


Figure 3 Scatter plot showing percentages of early and late apoptotic bone marrow CD34+ cells in control group (A) and cirrhosis group (B).

marrows of 31 patients with post-hepatitis cirrhosis was detected by flow cytometry. The percentage of bone marrow CD34+ cells was significantly higher in cirrhosis group than in control group, indicating that bone marrow CD34+ cells are activated in patients with post-hepatitis cirrhosis. HSC/HPC have a compensatory proliferation capacity for cytopenia in cirrhotic patients and enhance their ability to differentiate into mature blood cells, in order to make up the loss of mature blood cells because of hypersplenism, which is significantly different from that for aplastic anemia, in which cytopenia is caused by abnormal HSC/HPC^[6].

In this study, the percentage of apoptotic bone marrow CD34+ cells was significantly different in cirrhosis and control groups, and between early and late stage cirrhosis. The percentage of apoptotic CD34+ cells was significantly higher in cirrhosis group than in control group. The percentage of apoptotic bone marrow CD34+ cells ($20.92\% \pm 18.5\%$) was very high in late stage cirrhotic patients, and almost comparable between early stage cirrhotic patients and controls ($6.25\% \pm 3.30\%$ vs $5.73\% \pm 1.57\%$), suggesting that the compensatory ability of HSC/HPC to proliferate is impaired, thus limiting their ability to differentiate into mature blood cells, which may be the cause of cytopenia in late stage cirrhotic patients. This could also explain why the peripheral blood cell count in early stage cirrhotic patients remains normal and the peripheral blood cell count is decreased in late stage cirrhotic patients, although the number of bone marrow CD34+ HPC is still

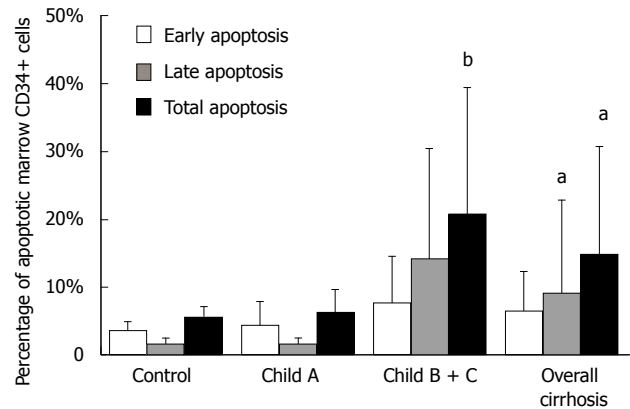


Figure 4 Percentage of apoptotic bone marrow CD34+ cells in controls and Child A, Child B + C cirrhotic patients. ^a $P < 0.05$ vs controls, ^b $P < 0.05$ vs Child A patients.

high^[7]. Our previous study showed that the Fas expression level is significantly higher in rats with carbon tetrachloride-induced cirrhosis than in normal rats^[8]. It is also known that the serum TNF- α and IFN- γ levels are higher in cirrhotic patients, thus up-regulating the expression of Fas, leading to cell apoptosis. We therefore speculate that the TNF- α and IFN- γ /Fas pathways may contribute to the apoptosis of bone marrow HSC/HPC^[9-13].

Finally, the correlation between parameters used to evaluate liver functions and apoptotic HSC/HPC was analyzed. The late apoptotic CD34+ bone marrow cells were positively correlated with the serum levels of total bilirubin and AST, indicating that the impaired function of bone marrow CD34+ cells is correlated with late stage cirrhosis. The establishment of such a correlation can be used in evaluating hematopoiesis in bone marrow of cirrhotic patients.

It has been shown that hypersplenism is the main cause of cytopenia in cirrhotic patients. Decreased production of thrombopoietin in liver may also contribute to thrombocytopenia^[14,15]. In this study, the number of HSC/HPC was increased and become more active in cirrhotic patients leading to compensatory effects of peripheral cytopenia. However, as cirrhosis progresses and apoptosis of HSC/HPC increases, hematopoiesis in bone marrow is impaired, eventually leading to disordered homeostasis and poor prognosis, in addition to liver failure and hypersplenism, in end-stage cirrhotic patients. The subjects involved in this study had hepatitis B or hepatitis C-associated cirrhosis. The virological impact on activation of bone marrow CD34 cells and functional impairment therefore merits further investigation.

In conclusion, the percentage of CD34+ bone marrow cells is high in cirrhotic patients, and increased apoptotic CD34+ HSC/HPC are correlated with late stage cirrhosis and total bilirubin and AST serum level.

COMMENTS

Background

Peripheral cytopenia is quite common in cirrhotic patients and the percentage of apoptotic CD34+ cells in bone marrow of cirrhotic patients was calculated.

Research frontiers

The relation between bone marrow CD34+ cells and liver cells has been reported. Bone marrow CD34+ cells can transform into hepatocytes *in vitro* and *in vivo*. Meanwhile, autologous or umbilical stem cell transplantation seems to be a promising new therapy for cirrhosis.

Innovations and breakthroughs

Bone marrow CD34+ cells were studied in cirrhotic patients. In this study, the percentage of bone marrow CD34+ cells was high in cirrhotic patients and that the increased apoptotic CD34+ HSC/HPC are correlated with late stage cirrhosis and the total bilirubin and AST serum level.

Applications

The findings in the study can partly explain why peripheral cytopenia occurs in patients with post-hepatitis cirrhosis.

Terminology

HSC: Multipotent stem cells that increase different blood cells including myeloid and lymphoid lineages and are defined by their ability to replenish different blood cells and their ability to self-renew.

Peer review

This is an interesting manuscript describing the role of CD 34+ cells, a well known marker for hematopoietic stem cells, in cirrhotic patients. The flow cytometry evaluation is adequate. The statistical analysis has well been conducted.

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A case-control study on the relationship between salt intake and salty taste and risk of gastric cancer

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Abstract

AIM: To investigate the relationship between salt intake and salty taste and risk of gastric cancer.

METHODS: A 1:2 matched hospital based case-control study including 300 patients with gastric cancer and 600 cancer-free subjects as controls. Subjects were interviewed with a structured questionnaire containing 80 items, which elicited information on dietary, lifestyle habits, smoking and drinking histories. Subjects were tested for salt taste sensitivity threshold (STST) using

concentrated saline solutions (0.22-58.4 g/L). Conditional logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (95% CI).

RESULTS: Alcohol and tobacco consumption increased the risk of gastric cancer [OR (95% CI) was 2.27 (1.27-4.04) for alcohol and 2.41 (1.51-3.87) for tobacco]. A protective effect was observed in frequent consumption of fresh vegetable and fruit [OR (95% CI) was 0.92 (0.58-0.98) for fresh vegetable and 0.87 (0.67-0.93) for fruit]. Strong association was found between STST ≥ 5 and gastric cancer [OR = 5.71 (3.18-6.72)]. Increased STST score was significantly associated with salted food intake and salty taste preference ($P < 0.05$).

CONCLUSION: A high STST score is strongly associated with gastric cancer risk. STST can be used to evaluate an inherited characteristic of salt preference, and it is a simple index to verify the salt intake in clinic.

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Key words: Gastric cancer; Salt taste sensitivity threshold; Salt taste preference

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Yang WG, Chen CB, Wang ZX, Liu YP, Wen XY, Zhang SF, Sun TW. A case-control study on the relationship between salt intake and salty taste and risk of gastric cancer. *World J Gastroenterol* 2011; 17(15): 2049-2053 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i15/2049.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i15.2049>

INTRODUCTION

Gastric cancer is the fourth most common cancer in the world, and is the second most common cause of death

from cancer^[1]. Its incidence shows wide geographical variation, but almost two-thirds of the cases are from developing countries, including 42% from China. Although gastric cancer is decreasing in most populations, the absolute number of cases is predicted to increase up to the year 2050 due to the aging of the population^[2]. In China, gastric cancer is the third most common cancer, with an age-standardized incidence of 37.1 and 17.4 cases per 100 000 person-years in men and women, respectively^[3]. Therefore, prevention of gastric cancer is one of the most important cancer control strategies both in China and around the world.

High intake of salt is hypothesized to be a cause of cancer and an important cause of gastric cancer^[4,5]. Evidence has proved that a high salt intake damages the gastric mucosa producing atrophy and intestinal metaplasia^[6,7]. In addition, a high salt diet has been shown to have a synergistic interaction with gastric carcinogens^[8,9]. In animal experiments, the co-administration of a high dietary salt intake enhances both the initiation and promotion of gastric cancer induced by carcinogenic N-nitroso compounds^[10]. It has been shown that a high intake of salted food is associated with increased risk for gastric cancer. But a recent meta-analysis showed a weak association between salt and gastric cancer^[4], and the relationship between quantity of salt intake and gastric cancer is hard to estimate. Most studies evaluated the salt preference only by the subjective feeling of subjects, and salty taste sensitivity test has the capacity to identify the flavor of salt, and its threshold can influence salt appetite or salt food preference^[11]. This method can be used to assess the association between salt preference and gastric cancer. The purpose of this study was to analyze the relationship between salt intake, salty taste and risk of gastric cancer.

MATERIALS AND METHODS

A hospital-based case-control study was carried out in the Shanyin People's Hospital in Shanyin of Shanxi. The study included 300 patients aged 40-75 years who had histologically confirmed diagnosis of gastric cancer from January 2006 to July 2010. Hospital-based controls were individually matched to cases by gender and age (± 5 years). Controls were patients selected from the Surgical Department, Plastic Surgery Department, ENT Department and Department of Gynecology. Ratio of cases to controls was 1:2. Totally, there were 600 controls who were non-cancer or cancer-free subjects.

A self-administered structured questionnaire consisting of 80 items was used in the study. It included questions about demographic information, dietary and lifestyle habits, smoking and drinking history and so on. Face to face interview was made for all subjects by trained interviewers. A completed questionnaire was obtained from 900 subjects. Cancer patients were asked of habits a year before the disease diagnosed. After interviewing for questionnaires, the salt taste sensitivity test was performed in all the subjects. The salt taste sensitivity threshold (STST) was measured using NaCl solutions on the tip of the tongue with

Table 1 Concentration of sodium chloride *n* (%)

STST score	NaCl concentration		Cases (<i>n</i> = 300)	Controls (<i>n</i> = 600)
	g/L	mol/L		
1	0.22	0.004	5 (2)	12 (2)
2	0.45	0.008	7 (2)	48 (8)
3	0.90	0.015	24 (8)	108 (18)
4	1.80	0.030	43 (14)	174 (29)
5	3.60	0.060	57 (19)	132 (22)
6	7.30	0.120	76 (25)	60 (10)
7	14.60	0.150	54 (18)	42 (7)
8	29.20	0.500	31 (10)	18 (3)
9	58.40	1	3 (1)	6 (1)

STST: Salt taste sensitivity threshold.

a dropper. Five drops of the test solution were dripped on the tongue. Ten seconds after closing the mouth, the cases and controls who tasted the usual food were perceived. The solutions were offered in increasing concentrations. Between the tests, the subjects were asked to wash their mouths with distilled water at a 30-s interval during the successive tests. The concentrations of each test NaCl solution were classified into ten grades from 0.22 g/L to 58.4 g/L, and the STST value for salt recognition in normal individuals was 0.015 mol/L of NaCl (0.9 g/L) (Table 1).

Questions included the frequency of intake of various food. For diet preference, the subjects chose one of the following frequencies: < 3 times/wk and ≥ 3 times/wk. Salty food preference was classified into not salty, medium, and salty. Cigarette smoking was measured in pack-years (number of cigarette smoking per day/20 \times smoking time in years) and divided into two categories: smokers who consumed < 40 packs/year and ≥ 40 packs/year or more; alcohol consumption was calculated according to the amount of alcohol consumed per day in grams. The subjects were classified into two categories: drinkers who consumed less than 22.8 g alcohol per day and ≥ 22.8 alcohol per day.

The ethics committee of each collaborating institution reviewed and approved the study, and informed consent was obtained from all the participants.

Statistical analysis

The conditional logistic regression was used to calculate odds ratios (ORs), and corresponding 95% confidence intervals (CI) for gastric cancer in relation to exposure of interest. Two models were examined: (1) none-adjusted; (2) age, sex, smoking, drinking, fresh fruit and fresh vegetables adjusted. Tests for trend were computed by fitting conditional logistic regression model to ordinal values representing levels of exposure. All reported trend test significance levels (*P* values) were two-sided^[12]. The relationship between STST score and lifestyle and dietary factors was evaluated by Chi-square test. The coherence of STST score with salty taste preference was detected by Anova test. All the calculations were performed by statistical package version 9, STATA 9, College Station, TX.

Table 2 Odds ratio and 95% CIs for lifestyle- and diet-related factors and gastric cancer *n* (%)

Characteristics	Cases (<i>n</i> = 300)	Controls (<i>n</i> = 600)	OR (95% CI)	<i>P</i> value
Age (yr)	52.1 ± 5.4	52.4 ± 4.9		> 0.05
Male	214 (23.7)	428 (71.3)		
Education				0.12
Literate	96 (32.0)	162 (27.0)	0.79 (0.59-1.06)	
Smoking	156 (52.0)	276 (46.0)	2.27 (1.27-4.04)	< 0.001
Drinking	72 (24.0)	134 (22.3)	2.41 (1.51-3.87)	< 0.001
Fresh vegetable				
< 3 times/wk	99 (33.0)	282 (47.0)	-	< 0.001
≥ 3 times/wk	201 (67.0)	318 (53.0)	0.92 (0.58-0.98)	
Fresh fruit				
< 3 times/wk	144 (48.0)	219 (36.5)	-	< 0.001
≥ 3 times/wk	156 (52.0)	381 (63.5)	0.87 (0.67-0.93)	
Salted food(meat and fishes, pickled vegetable)				
< 3 times/wk	120 (40.0)	298 (49.7)	-	
≥ 3 times/wk	180 (60.0)	302 (50.3)	1.54 (1.15-2.93)	< 0.05
STST score	5.52 ± 1.26	4.4 ± 0.91	1.66 (1.48-1.85)	< 0.001

STST: Salt taste sensitivity threshold. OR: Odds ratio.

Table 3 Relationship between salt taste sensitivity threshold score and lifestyle factors

	<i>n</i> (%)	STST score (mean ± SD)	<i>P</i>
Age (yr)			
< 50	224 (24.9)	4.79 ± 1.25	0.76
50-64	543 (60.3)	5.10 ± 0.83	
≥ 65	133 (14.8)	5.08 ± 1.56	
Smoking (packs/yr)			
< 40	660 (73.3)	4.88 ± 1.02	0.93
≥ 40	240 (26.7)	5.24 ± 1.51	
Drinking (g/d)			
< 22.8	517 (57.4)	5.10 ± 1.02	0.10
≥ 22.8	383 (42.6)	5.19 ± 1.12	
Fresh vegetable(times/wk)			
< 3	241 (26.8)	5.14 ± 1.08	0.18
≥ 3	659 (73.2)	5.07 ± 0.97	
Fresh fruit (times/wk)			
< 3	363 (40.3)	5.18 ± 1.41	0.11
≥ 3	537 (59.7)	5.07 ± 1.15	
Salted food intake (times/wk)			
< 3	418 (46.4)	4.68 ± 1.32	< 0.001
≥ 3	482 (53.6)	5.47 ± 0.98	

STST: Salt taste sensitivity threshold.

RESULTS

The characteristics of the subjects are listed in Table 2. Among the 300 cases and 600 controls, 71% were males, and their mean age was 52.1 and 52.4 years, respectively. There was a significant difference in educational level between cases and controls. Smokers and drinkers showed an increased risk of developing gastric cancer with OR (95% CI) = 2.27 (1.27-4.04) and 2.41 (1.51-3.87), respectively. In contrast, consumed protective effect was found in those who took 3 times/wk of fresh vegetable and fruit, OR (95% CI) = 0.92 (0.58-0.98) and 0.87 (0.67-0.93), respectively. The mean STST score of cases was significantly higher than that of controls.

We analyzed the association between STST score and other risk factors. The results showed that STST score was increased with age and duration of smoking and drinking, but no significant association was found (Table 3). STST score was significantly increased with a higher salted food intake.

The mean STST score of all subjects was 4.8 ± 1.1 , and the median NaCl concentration was 3.6 g/L (3.6-7.3) or 0.06 (0.06-0.12) mol/L corresponding to a score of 5 (Table 2). There were more patients with STST ≥ 5 than the controls (Table 4). We defined the STST cut-point as 5 (3.6 g/L or 0.06 mol/L). Subjects with STST ≥ 5 had 5.71 times greater risk of gastric cancer than those with STST < 5 (Table 4).

The relationship between the salty taste preference and STST score indicated that the salty taste preference was significantly associated with STST score ($P < 0.001$) (Table 5), which means that the subjects with a higher STST score was more likely to prefer a salty taste.

DISCUSSION

The present hospital based case-control study indicated that high consumption of smoking, drinking and salty taste preference elevated the risk of gastric cancer, and that the gastric cancer was associated with a higher STST score. The STST score of 3.6 g/L (0.03 mol/L) was independently associated with a high risk of gastric cancer with the OR (95% CI) of 5.71 (3.18-6.72).

STST is a personal characteristic of an individual and is a useful index to evaluate the salty preference^[13]. STST test was used for predicting hypertension in previous studies^[14,15], and it indicated that hypertensive individuals were more salt sensitive than the normal individuals^[16]. Our study proved that subjects with a higher STST score were more likely to have salty taste preference and a high intake of salty food (Table 4), and it further indicated that STST is a helpful test to evaluate the salty taste preference and

Table 4 Odds ratio and 95% CIs for salt-related factors and gastric cancer *n* (%)

Salt factors	Cases	Controls	OR1 (95% CI)	<i>P</i>	OR2 (95% CI)	<i>P</i> value
Salted taste preference						
Not salty	118 (39.3)	301 (50.2)	-	-	-	-
Medium	162 (54)	265 (44.2)	1.12 (0.79-1.89)	<i>P</i> = 0.45	1.34 (0.92-2.67)	<i>P</i> = 0.08
Salty	20 (6.6)	34 (5.7)	1.33 (1.02-1.75)	< 0.05	1.94 (1.37-4.76)	< 0.05
STST ≥ 5	221 (73.7)	258 (43)	4.03 (2.87-5.65)	< 0.001	5.71 (3.18-6.72)	< 0.001

OR1 for salt related factors and gastric cancer was none-adjusted; OR2 was adjusted for age, sex, smoking, drinking, fresh fruit and fresh vegetables; STST: Salt taste sensitivity threshold. OR: Odds ratio.

Table 5 Relationship between salt taste sensitivity threshold score and salt intake *n* (%)

Salty taste preference	STST score						<i>P</i>
	1-2	3-4	5-6	7-8	9	Total	
Dislike	60 (6.7)	233 (25.9)	98 (10.9)	26 (2.9)	2 (0.2)	419 (46.6)	$\chi^2 = 220.1$ <i>P</i> < 0.001
Not prefer	11 (1.2)	104 (11.6)	206 (22.9)	104 (11.6)	2 (0.2)	427 (47.4)	
Like	1 (0.1)	12 (1.3)	21 (2.3)	15 (1.7)	5 (0.6)	54 (6.0)	
Total	72 (8.0)	349 (38.8)	325 (36.1)	145 (16.1)	9 (1.0)	900 (100)	

STST: Salt taste sensitivity threshold.

salt intake. The 24 h urinary excretion of salt was used previously as an objective method for salt intake measurement^[17,18], and epidemiologic studies indicated that gastric cancer mortality is weakly or non-significantly correlated with dietary salt measured by the 24 h urinary salt excretion. This method is impracticable for a large-scale population study and case-control study because it can only reflect the situation of 24 h salt intake. However, STST test is simpler, cheaper and more acceptable than the 24 h urinary salt excretion, and patients can identify the taste before diagnosis.

In our study, significantly increased risk for gastric cancer was observed among those with a high STST score. Age, histories of smoking and drinking, and consumption of fruit and vegetables did not show any significant interactions with STST for gastric cancer. STST ≥ 5 showed a higher risk for gastric cancer in our study, and the possible explanation may be that the high STST is associated with a high intake of salty food such as salted meat or fish (Table 3). Previous studies indicated that ingestion of salt could induce gastritis and co-administration with N-methyl-N-nitro-N-nitrosoguanidine could enhance the effect of gastric carcinogens^[9,19], and high intragastric salt concentration could destroy the mucosal barrier, leading to inflammation and damage such as diffuse erosion and degeneration. The induced proliferous change might enhance the effect of food-derived carcinogens.

There are various methods for measuring salt intake and salt preference. Most studies only use the frequency of salt consumption and self-reported salty taste preference, but these methods could not objectively reflect the real situation of the subjects. The mean salt intake varies among different populations, and salt consumption levels which were considered high in one study might be considered low in another one^[19]. Salt can be derived from different food species, so it is difficult to calculate the total salt consumption from all food. Self-reported salty taste preference is a method often biased

by subject's feelings. Therefore, the methods of measuring salt intake from food consumption and self-reported salty taste preference could induce measurement bias and confounding bias. In contrast, STST test is a simple method which is related to salt intake and consumption, and it could indirectly reflect the objective salty preference and avoid the measurement bias.

Several limitations of this study should be considered. Firstly, the STST test was not conducted before the cancer occurred, and the patients may change their salt preference after clinical symptom appearance, so there is recall bias in our study. But as the recall bias could not be avoided in every case-control study, we used the method of STST and the habits were defined to a year before the disease was diagnosed. Secondly, the cases and controls were selected from a hospital, which may have selection bias. We selected controls from the Surgical Department, Plastic Surgery Department, ENT Department and Department of Gynecology. Thirdly, we did not detect the *Helicobacter pylori* (*H. pylori*) infection in both the cases and controls, and *H. pylori* could bias the effect of STST score in gastric cancer, but the *H. pylori* could not reverse the result of STST score in gastric cancer due to the high OR. We will detect the *H. pylori* infection in the future studies. Fourthly, the objective method of measuring salt intake is the 24 h urinary salt excretion, but we did not use it to verify STST in our study. Because 24 h urinary salt excretion could not reflect the previous salt habit, we could only used salt preference questionnaire to verify it. Further cohort studies on the relationship between STST and 24 h urinary salt excretion are needed. Finally, there may be variability in the taste of each subject, and it may induce measurement bias. In order to avoid the bias, we offered the same concentration of NaCl solution to subjects after they chose the usual taste concentration.

To summarize, this study suggests that a high STST score is strongly associated with gastric cancer risk. STST

may be used as a test to evaluate an inherited characteristic of salt preference, and a useful index to verify the salt intake in clinic. However, the role of STST has to be further studied to answer the questions raised from the present study.

COMMENTS

Background

A high intake of salt is hypothesized to be a cause of cancer and an important cause of gastric cancer. But the salt intake and salt preference are hard to measure. salt taste sensitivity threshold (STST) test was once used to predict hypertension, and this study used it to measure the salt intake in an attempt to explore its association with gastric cancer.

Research frontiers

Salt taste sensitivity is independently associated with gastric cancer, and it is proved to be a better index to reflect the salt preference in this study. This method could help identify the risk population of gastric cancer.

Innovations and breakthroughs

This study for the first time explored the relationship between salt taste sensitivity and risk of gastric cancer. A high STST score was found to be strongly associated with gastric cancer risk, and STST score could also reflect the salt preference and salt intake of the subjects, and it may be used for predicting the risk population of gastric cancer.

Applications

STST test is a cheap and fast examination to evaluate an inherited characteristic of salt preference, and it is a simple method to verify the salt intake in clinic.

Peer review

This is an interesting study, and the data suggest that STST may be a potentially useful tool to screen patients at risk of developing gastric cancer.

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Laparoscopic repair of hiatal hernia with mesenterioaxial volvulus of the stomach

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nia, volvulus, and gastroesophageal reflux.

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Abstract

Although mesenterioaxial gastric volvulus is an uncommon entity characterized by rotation at the transverse axis of the stomach, laparoscopic repair procedures have still been controversial. We reported a case of mesenterioaxial intrathoracic gastric volvulus, which was successfully treated with laparoscopic repair of the diaphragmatic hiatal defect using a polytetrafluoroethylene mesh associated with Toupet fundoplication. A 70-year-old Japanese woman was admitted to our hospital because of sudden onset of upper abdominal pain. An upper gastrointestinal series revealed an incarcerated intrathoracic mesenterioaxial volvulus of the distal portion of the stomach and the duodenum. The complete laparoscopic approach was used to repair the volvulus. The laparoscopic procedures involved the repair of the hiatal hernia using polytetrafluoroethylene mesh and Toupet fundoplication. This case highlights the feasibility and effectiveness of the laparoscopic procedure, and laparoscopic repair of the hiatal defect using a polytetrafluoroethylene mesh associated with Toupet fundoplication may be useful for preventing postoperative recurrence of hiatal her-

INTRODUCTION

Intrathoracic gastric volvulus is an uncommon disease entity characterized by an enlarged esophageal hiatus and weakened gastrosplenic and gastrocolic ligaments^[1]. Although gastric volvulus possibly occurs in all ages, it often occurs over the fourth decade of life. If undiagnosed, it can lead to ulceration, perforation, hemorrhage or ischemia of the incarcerated gastrointestinal tract^[2]. Two types of gastric volvulus have been recognized: an organoaxial form and a mesenterioaxial form^[3,4]. The mesenterioaxial form is characterized by rotation at the transverse axis, extending from the middle of the greater curvature to the porta hepatis.

We recently experienced a case of intrathoracic mesenterioaxial volvulus of the distal portion of the stomach and duodenum, which was successfully treated with laparoscopic repair of the hiatal defect using a polytetrafluoroethylene mesh associated with Toupet fundoplication. The laparoscopic technique employed is described and its therapeutic implications, mesh-related complications, and long-term clinical outcome are discussed.

CASE REPORT

A 70-year-old Japanese woman had been diagnosed with hiatal hernia for several years by her home doctor. Because the patient had complained of no specific symptoms related to the hiatal hernia, no treatment had been undertaken during that period. She had no other past history of any diseases except for a significant kyphosis. She was admitted to our hospital because of the sudden onset of epigastralgia and nausea. Physical examination at admission revealed no abdominal tenderness suggesting diffuse peritonitis. A chest X-ray film showed a large-sized air pocket associated with air-fluid level in the chest, which was likely to be a dilated stomach incarcerated into the intrathoracic cavity (Figure 1). She was dehydrated and vomited during the placement of a nasogastric tube which resulted in aspiration pneumonia. She then presented with acute prerenal failure as a complication, and further treatment was required. Total parenteral nutrition was performed for the management of the intermittent abdominal pain and nausea. The patient responded to intravenous fluid management and the administration of antibiotics. The patient soon recovered from the critically-ill condition and further examinations were performed for precise diagnosis. An upper gastrointestinal series revealed an incarceration of the distal portion of the stomach and the duodenum into the thoracic cavity (Figure 2). An abdominal computed tomography scan also showed an incarcerated upper gastrointestinal tract (Figure 3). These findings were compatible with a mesenterioaxial volvulus of the stomach and duodenum incarcerated into the thoracic cavity through an esophageal hiatus. A laparoscopic approach for the repair of hiatal hernia was selected for complete repair.

During surgery, pneumoperitoneum was established at an intraabdominal pressure of 12 mmHg. A total of 4 ports were placed, 3 ports of 5 mm diameter in the right and left upper abdomen and left upper abdomen and 1 port of 12 mm diameter in the right middle abdomen. The endoscopic port was placed in the umbilicus. The laparoscopic view during the operation showed a hiatal hernia of the sliding type and also showed that the distal portion of the stomach and the proximal portion of the duodenum were rotated at its transverse axis of the upper gastrointestinal tract, and were incarcerated through the porta of the left crus of the diaphragm. The incarcerated distal stomach and proximal duodenum through the esophageal hiatus was covered with the hernia sac. Because there was no adhesion of the herniated content with the surrounding tissues, the incarcerated stomach and duodenum were deliberately pulled out from the thoracic cavity through a relatively large-sized paraesophageal hiatal defect (Figure 4A). The hernia sac and crus of the diaphragm were then dissected. The lower esophagus was also mobilized. The hiatal defect was closed and was reinforced by using a polytetrafluoroethylene (PTFE) mesh (Figure 4B). Toupet fundoplication was then added to prevent gastroesophageal reflux. The fundic wrap was sutured with the right crus and left-sided diaphragm with interrupted sutures. No intra- and/or postoperative complications were observed. Postoperative upper gastrointestinal

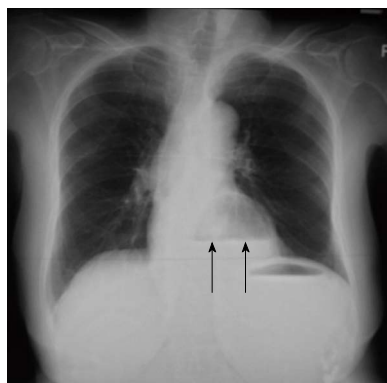


Figure 1 A chest X-ray film at admission, revealing air in the dilated stomach associated with air-fluid level (arrows), which was dislocated into the chest.

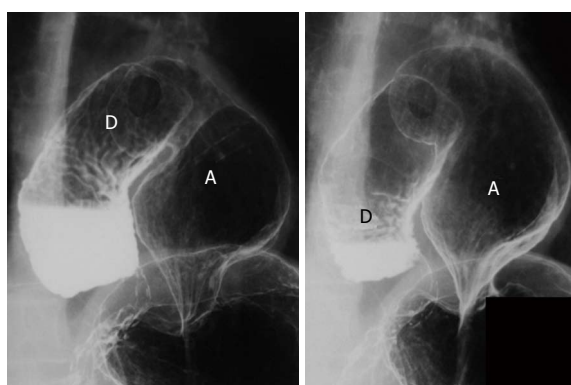


Figure 2 An upper gastrointestinal series performed preoperatively, revealing dislocated distal portion of the stomach (A) and duodenum (D) into the thoracic cavity rotated in the mesenteric axis, which corresponded to a mesenterioaxial volvulus of the upper gastrointestinal tract.

series performed 5 d after surgery revealed appropriate arrangement of the upper gastrointestinal tract and no sign of recurrence of the hiatal hernia (Figure 5).

The postoperative course was uneventful and the patient was discharged from the hospital 15 d after surgery. The patient was in good condition, and complained of no symptoms suggesting the recurrence of hiatal hernia and/or intrathoracic gastric volvulus for 4 years after surgery. The patient has undergone annual check-ups using upper gastrointestinal endoscopic examinations for 4 years after surgery and no recurrence has been found to date.

DISCUSSION

Although hiatal hernia is commonly encountered, hiatal hernia with mesenterioaxial intrathoracic gastric volvulus is extremely uncommon. Two types of gastric volvulus have been recognized: an organoaxial form and a mesenterioaxial form^[3,4]. The organoaxial type is a common type of gastric volvulus, in which the stomach rotates on a vertical axis. This type of gastric volvulus is usually caused by eventuation, diaphragmatic hernia, pyloric obstruction, adhesions, or enlarged esophageal hiatus. This is also described as an intrathoracic stomach or upside down stomach. Another type is the mesenterioaxial form. In this form, the stomach

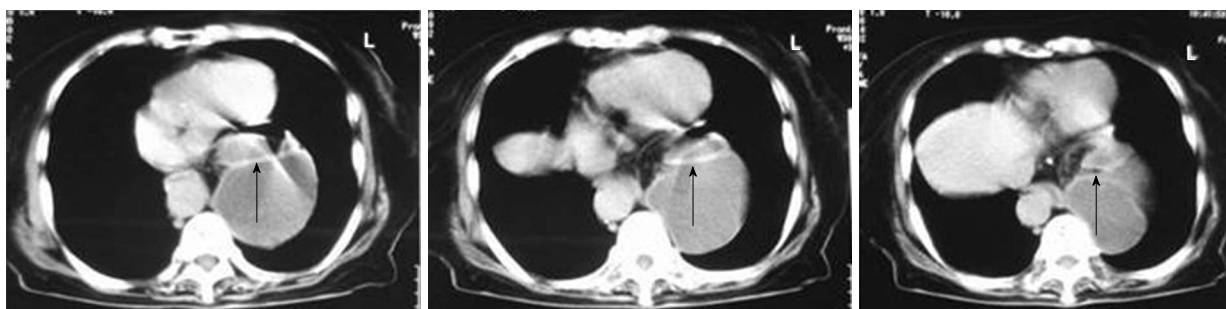


Figure 3 Abdominal computed tomography scan. An incarcerated upper gastrointestinal tract in the thoracic cavity (arrows). The gastrointestinal tract dislocated in the thoracic cavity was significantly dilated, where fluid had accumulated.

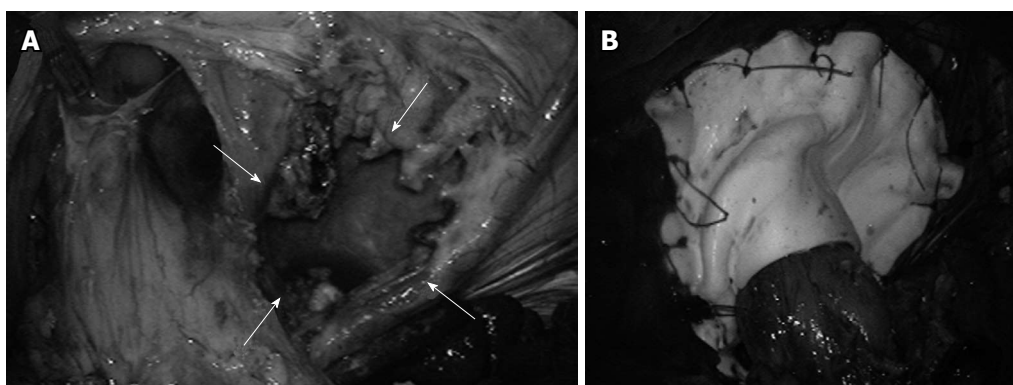


Figure 4 Laparoscopic view after initial reduction of the stomach from the chest into the abdomen. Sliding hernia and parahiatal hernia. A: A paraesophageal aperture (arrows) was large-sized and was located at the left side of the left crus of the esophageal hiatus; B: Hernia orifice was closed using the mesh reinforcement.



Figure 5 A postoperative upper gastrointestinal series performed at 5 d after surgery, showing the normal intraperitoneal location of the stomach and duodenum.

rotates on a horizontal axis, which extends from the middle of the greater curvature to the porta hepatis. The rotated stomach is located in the chest from the hiatal defect. Mesenterioaxial volvulus is even more uncommon and represents about 29% of all torsions occur in the stomach. Of mesenterioaxial volvulus, the idiopathic pattern was 37%^[3].

In the present case, the patient had gastrointestinal obstruction, dehydration and acute prerenal failure caused by the volvulus of the stomach. Elective surgery was performed after recovering from these complications. In fact, emergency surgery is occasionally required for cases with

acute gastric obstruction associated with strangulation due to gastric volvulus^[5]. If there is no gastrointestinal ischemia and/or necrosis requiring emergency surgery, placement of a nasogastric tube may be useful for the effective decompression of the stomach, which may allow a reduction of the volvulus and thereby enabling elective surgery.

Treatment of gastric volvulus has classically involved reduction of the stomach, gastropexies in its normal intra-abdominal position, and correction of the associated diaphragmatic defect^[4]. The laparoscopic repair of intrathoracic gastric volvulus and large paraesophageal hernias has been proved to be feasible and safe^[4,6-9]. Previously published reports have confirmed the significant benefits of the minimally-invasive approach in these often elderly and/or debilitated patients^[7]. Therefore, laparoscopic repair of gastric volvulus is best suited as a minimally-invasive approach as long as the precise repair of hiatal aperture and reinforcement are added.

In the present case, Toupet fundoplication was added to prevent gastroesophageal reflux because a simple reduction of the volvulus associated with gastropexy has been shown to be unsatisfactory^[10]. A concomitant antireflux procedure has been recommended^[8]. The fundoplication is mandatory as it prevents reflux because of the extensive dissection at the hiatus, and provides a good anchor for the repair. This approach is considered to be safe and effective, and it also provides for rapid recovery from the operation.

In the present case, neither mesh-related complications

nor recurrence of hiatal hernia was found for 4 years after surgery. It has been reported that primary laparoscopic hiatal hernia for paraesophageal hernia is associated with up to a 42% recurrence rate^[11,12]. The most important reason is the absence of strong fascia at the hiatal aperture leading to suture pullout^[13]. We decided to use synthetic mesh reinforcement for the crural repair, because direct suturing of the aperture of paraesophageal defect was difficult due to the size and the absence of strong fascia. Although mesh repair improved the recurrence rate^[14], the use of mesh for the repair of hiatal hernia has still been controversial because mesh-related complications have frequently reported. Several clinical studies with significant patient numbers have demonstrated the safety and effectiveness of synthetic mesh reinforcement for the repair of hiatal hernia^[14,15,16]. An improved recurrence rate has been reported in cases with the use of mesh for the tension free crural repair^[17]. However, some surgeons have pointed out the risk of erosion into the esophageal or gastric lumen caused by placing the mesh^[15,11,18]. Because several types of non-absorbable mesh have recently been used for the repair of hiatal hernia^[9,19], further evaluation of clinical outcome may be required. Stadlhuber *et al.*^[17] analyzed 28 cases of mesh-related complications in patients undergoing laparoscopic or open hiatal closure. They demonstrated that 23 cases required reoperation, and surprisingly 7 patients required esophagectomy. Among the patients who required reoperation, PTFE was used in 12 cases for the hiatal repair. A total of 10 patients had mesh intraluminal erosion, 2 patients had dense hiatal fibrosis. Although there is no apparent relationship between mesh type and configuration with the complications, they suggested that it may be due both to the technical aspects of mesh placement and to the type of mesh material used^[17]. Nonetheless, they concluded that complications related to synthetic mesh placement at the esophageal hiatus are more common than previously reported and that further studies are needed to determine the best method and type of mesh for implantation.

In summary, we experienced a case with mesenterioaxial volvulus of the stomach and duodenum associated with hiatal hernia, which was successfully treated with complete laparoscopic repair of the large-sized hiatal defect using a PTPE mesh associated with Toupet fundoplication. These laparoscopic procedures are safe and useful to obtain short-term as well as long-term clinical outcomes of patients with a large-sized hiatal hernia. Further accumulation of cases is required to precisely determine the best method and type of mesh for reinforcement and long-term clinical outcome.

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Treatment of advanced rectal cancer after renal transplantation

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Abstract

Renal transplantation is a standard procedure for end-stage renal disease today. Due to immunosuppressive drugs and increasing survival time after renal transplantation, patients with transplanted kidneys carry an increased risk of developing malignant tumors. In this case report, 3 patients with advanced rectal cancer after renal transplantation for renal failure were treated with anterior resection or abdominoperineal resection plus total mesorectal excision, followed by adjuvant chemotherapy. One patient eventually died of metastasized cancer 31 mo after therapy, although his organ grafts functioned well until his death. The other 2 patients were well during the 8 and 21 mo follow-up periods after rectal resection. We therefore strongly argue that patients with advanced rectal cancer should receive standard oncology treatment, including operation and adjuvant treatment after renal transplantation. Colorectal cancer screening in such patients appears justified.

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Key words: Rectal cancer; Renal transplantation; End-stage renal disease; Treatment; Screening

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INTRODUCTION

Renal transplantation, commonly performed for end-stage renal disease (ESRD), is an alternative to dialysis. An increased incidence of malignancy in transplant recipients is well recognized, which may be related to impaired immunosurveillance, direct neoplastic action of immunosuppressive agents, oncogenic viruses such as Epstein-Bar virus or cytomegalovirus, and chronic antigenic stimulation, uremia, or genetic predisposition^[1].

There is evidence that renal transplant recipients are approximately three times more likely to develop cancer than the general population^[2,3]. Their risks vary in different tumors. The risk of Kaposi's sarcoma is the highest (200 times increased risk) followed by that of non-melanocytic and melanocytic skin cancer (9-20 times increased risk)^[2,3]. The risk other solid-organ cancers, such as colorectal cancer, is increased by approximately 2-3 times higher in renal transplant recipients than in general population^[3,4]. Cancers occurring in transplanted patients are generally de novo and mainly diagnosed after the third year with an increase after 10 years^[5]. The mean onset time of colorectal malignancies is 10.4 years^[1].

The prognosis of renal transplant recipients with advanced-stage cancer is extremely poor. Currently, posttransplant malignancies are an important cause of mortality and the leading reason of death within the next 20 years^[6]. Immunosuppression and late diagnosis have been implicated^[7]. We report 3 cases of advanced rectal cancer after renal transplantation for renal failure.

CASE REPORT

In the past 5 years, 3 male patients at a mean age of 55.3 years who developed rectal cancer (RC) after renal transplantation were diagnosed and treated in Shanxi Cancer Hospital (Taiyuan, China). The mean elapsed time from renal transplantation to development of RC was 6.5 years. The 3 patients who underwent anterior resection (AR) or abdominoperineal resection (APR) plus total mesorectal excision (TME) had an uneventful postoperative course. The clinical data about each patient are listed in Table 1.

Case 1

A 68-year-old man who underwent renal transplantation for ESRD due to hydropigenous nephritis in 1993 at the age of 54 years. He received a second left renal transplantation in 2002 for graft failure followed by immunosuppressive therapy. In 2007, he underwent colonoscopy for rectal bleeding and dyschezia, which revealed a 5.0 cm mass at the upper rectum with 80% luminal occlusion. Biopsy showed a well-differentiated adenocarcinoma. Laboratory tests showed that his preoperative CEA level was 26.20 µg/L and his cellular immune function was low. The patient underwent AR with TME. Pathologic examination revealed a 5.0 cm moderately-differentiated adenocarcinoma with invasion through the serous membrane. Of the removed 7 lymph nodes, 1 was positive (pT₄N₁M₀). The postoperative course was uneventful. Following the operation, the patient did not receive any chemotherapy and radiotherapy due to his refusal. In November 2009, he received 3 cycles of Xeloda after liver and lung metastasis was discovered. The patient died in March, 2010.

Case 2

A 44-year-old man who received immunosuppressive therapy for ESRD in 2004 after renal transplantation. In 2009, he underwent colonoscopy for heme-positive stools, which revealed a 4 cm mass at the rectum with 60% luminal occlusion. Biopsy showed an adenocarcinoma of the rectum. Laboratory tests showed that his preoperative CEA level was 0.04 µg/L and his cellular immune function was low. The patient underwent APR with TME. Pathologic examination revealed a 4.0 cm poorly-differentiated adenocarcinoma with invasion of the adjacent perirectal fatty tissues. Eight lymph nodes were found with no malignant lymph node involved (pT₃N₀M₀). The patient was recovered uneventfully. He received a course of Xeloda and was well during the 21-mo follow-up period.

Case 3

A 54-year-old man with chronic pyelonephritis who underwent renal transplantation for ESRD in 2009. After the operation, he received immunosuppressive therapy. In 2010, 6 mo after renal transplantation, the patient presented with diarrhea and rectal bleeding. Colonoscopy showed a rectal mass with 70% luminal occlusion and a pedunculated polyp in sigmoid colon which was excised by electrocautery. Histology of the rectal mass showed a well-differentiated adenocarcinoma of the rectum while histology

of the polyp suggested a tubular adenoma. Laboratory tests showed that his preoperative CEA level was 1.05 µg/L and his cellular immune function was low. The patient underwent APR with TME. Pathologic examination revealed a 5 cm moderately-differentiated adenocarcinoma and partial mucinous adenocarcinoma with invasion of the pericolic adipose tissue. Thirteen lymph nodes were found with no lymph node metastasis (pT₃N₀M₀). Cancer emboli were identified in vessels of the mass. He received a course of Xeloda and recovered uneventfully during the 8-mo follow-up period.

DISCUSSION

It has been shown that the incidence of cancer is significantly higher in patients who underwent renal transplantation than in those who did not undergo renal transplantation^[2,3]. For example, the risk of colorectal cancer is increased by approximately 2-3 times higher in patients than in general population^[3,4]. However, some of the tumors may arise in renal recipients without any relation with renal transplantation, because they might have already presented at the time of renal transplantation but not detected. As a general rule, tumors detected within the first 12 mo after renal transplantation are considered pre-existed. Such patients should be excluded from the “*de novo*” group.

In our study, the 3 RC patients who underwent renal transplantation were males, and the onset interval from renal transplantation was 14 years, 5 years and 0.5 year, respectively. One patient was diagnosed with RC within the first 12 mo after renal transplantation, and pathological stage was pT₃N₀M₀ (advanced cancer). The other 2 patients were diagnosed with post-transplantation RC with a mean onset interval of 9.5 years. The 3 patients had radical AR and APR with TME.

It is well known that immunosuppressive treatment increases the incidence of cancers, which is supported by the fact that the incidence of tumors is higher in patients treated with immunosuppressants following renal transplantation due to chronic renal failure than in normal population. The causes for this difference might be explained by the immunological abnormalities induced by immunosuppressants^[8-10]. Therefore, screening and early diagnosis of tumors are essential both before and after renal transplantation, which means that tumors, if existed, should be detected, thus unnecessary renal transplantation can be avoided. Furthermore, annual tumor screening after renal transplantation should be conducted so that treatment can be commenced at an early stage of malignancy. In our study, the patient who was diagnosed with advanced RC within 6 mo after renal transplantation had no tumor screening before renal transplantation. It is likely that he developed RC while he was on dialysis and waiting for renal transplantation.

In general, the prognosis of transplant recipients who develop malignancy following immunotherapy are poor due to delayed diagnosis^[6,8,11-14]. Most cancers are at advanced stages when they are diagnosed, and usually progress rapidly with more than 50% of such patients died within the first year of diagnosis^[15]. The average survival time is proximally 25.8 mo^[15]. Evidence from National Cancer Institute Surveillance Epidemiology and End Results Database suggests

Table 1 Parameters of patients with rectal cancer after renal transplantation

Age (yr)	Elapsed time (yr)	Location	Grade	pTNM	Operation	Screening	Outcome
68	14	Rectum	Moderate	pT4N1M0	AR + TME	No	Died after 31 mo
44	5	Rectum	Poor	pT3N0M0	APR+TME	No	Alive after 21 mo
54	0.5	Rectum	Moderate to poor	pT3N0M0	APR + TME	No	Alive after 8 mo

that transplant patients develop colorectal cancer at a younger age (58 *vs* 70 years, $P < 0.001$) and have a worse 5-year survival rate than the general population (overall, 44% *vs* 62%, $P < 0.001$; Dukes A and B, 74% *vs* 90%, $P < 0.001$; Dukes C, 20% *vs* 66%, $P < 0.001$; and Dukes D, 0% *vs* 9%, $P = 0.08$) mainly due to chronic immunosuppression which results in a more aggressive tumor biology^[16]. RC patients who underwent renal transplantation usually develop more advanced (AJCC stage $> \text{II}$) colon cancer with a worse disease-specific survival rate (all stages) than those who did not undergo renal transplantation. Multivariate analyses showed that renal transplantation is a negative risk factor for survival, and cancer stage at diagnosis is the most profound negative survival predictor^[17], indicating that colorectal cancers in transplant recipients are biologically more aggressive, thus resulting to a worse prognosis in such patients than in general population. Moreover, Ho *et al*^[7] also highlighted that immunosuppression and late diagnosis should be blamed for the poor prognosis of colorectal cancer patients after renal transplantation. In the present study, of the 3 patients with advanced rectal cancer, 1 died of multiple liver and lung metastases 31 mo after operation, indicating that frequent colorectal cancer screening should be warranted after renal transplantation.

Zittel *et al*^[18] reported a case of a 48-year-old patient who developed advanced RC 6.5 years after pancreas-kidney-transplantation for type I diabetes. The patient received neo-adjuvant radio and chemotherapy followed by low anterior rectal resection with total mesorectal excision. Within the next thirteen months, he underwent consecutive resections for a solitary hepatic metastasis, a solitary pulmonary metastasis and a chest wall metastasis. The patient eventually died of metastasis 32 mo after the initial therapy although the organ grafts functioned well until his death, suggesting that although a higher degree of morbidity might be encountered, transplantation patients should receive standard oncology treatment, including neo-adjuvant therapy, if their general condition is good and the organ graft functions well.

In conclusion, early prevention, detection and treatment of malignancies after renal transplantation are the important management strategies for improving the survival time and quality of life of cancer patients because malignancies develop more frequently in cancer patients after renal transplantation than in general population. Surgical resection is still the first choice of treatment which is a safe procedure when indicated. However, if the patients have an advanced disease (local or metastatic), the standard oncology treatment, including neo-adjuvant treatment can be used.

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Pancreatic hyperechogenicity on endoscopic ultrasound examination

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Abstract

There is an ongoing discussion on how to diagnose a hyperechogenic pancreas and what is the clinical significance of diffusely hyperechogenic pancreas. Computerized tomography and magnetic resonance imaging are the more appropriate methods to diagnose pancreatic hyperechogenicity when compared with transcutaneous or endoscopic ultrasound examination. More importantly, pancreatic hyperechogenicity may not be a certain indicator of pancreatic fat infiltration. Even if it is true, we do not know the clinical significances of pancreatic fat accumulation. Some suggested that excess fat in the pancreas is associated with chronic pancreatitis. However, several histological studies on human alcoholic chronic pancreatitis did not prove the presence of fatty pancreas in such cases. Thus, except for aging, it is very rare to have truly steatotic pancreas in the absence of certain human diseases.

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Key words: Hyperechogenic pancreas; Fatty pancreas; Endoscopic ultrasound; Aging; Chronic pancreatitis

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TO THE EDITOR

We read with interest the article by Choi *et al*^[1] entitled "Associated factors for a hyperechogenic pancreas (HP) on endoscopic ultrasound examination". The authors investigated the risk factors for hyperechogenic pancreas on endoscopic ultrasound (EUS). Their study group included 53 cases of HP and the control group consisted of 79 cases having various indications for endosonographic examination with normal pancreas echogenicity on EUS. They noted that HP was significantly associated with fatty liver, male gender, age older than 60 years, hypertension and visceral adipose tissue area (cm²).

Pancreatic fat, readily observed on EUS, is only suspected when an overt HP is noted. However, as the authors in their study noted, mild hyperechogenic pancreas with respect to liver is a normal finding on ultrasound examination. Since the quantitative analysis of pancreatic parenchymal echogenicity was not conducted in their study, how could the authors be sure that pancreatic echogenicity they saw can be "hyper"? The authors also indicated the limitations of their study as the absence of direct determination of the pancreatic fat and visceral fat in pancreatic tissue. It would be unethical to get pancreatic biopsy samples. However, they could estimate the pres-

ence of pancreatic steatosis with the help of computerized tomography (CT) imaging which was already done to estimate the visceral adipose tissue area in all cases in their study. CT can be very helpful for the diagnosis and quantification of the existence of pancreatic steatosis^[2].

We also do not know the clinical consequences of pancreatic steatosis as yet. Some epidemiologic data suggest that obesity is a risk factor for pancreatic cancer development^[3] and that obese patients develop more severe pancreatitis than lean individuals^[4]. Furthermore, postoperative fistula develops more commonly in obese subjects than in lean individuals^[5]. However, in the absence of regular alcohol consumption, the obese patients with increased visceral adiposity are not accepted as having increased risk for chronic pancreatitis. We know that diffusely increased parenchymal echogenicity has not been suggested to be a EUS finding associated either with early or with late stage chronic pancreatic inflammation. Unlike the current evidence for the association between fatty liver and steatohepatitis, there is no similar evidence as yet to suggest that steatotic pancreas progresses to pancreatohepatitis and then to chronic pancreatitis. Indeed, pancreatic hyperechogenicity may not be a certain indicator of pancreatic fat infiltration. The belief that hyperechogenicity of the pancreas indicates the presence of fat in this organ has now been largely abandoned^[6]. Moreover, several histological studies on human alcoholic chronic pancreatitis did not support the presence of fatty pancreas^[7-9].

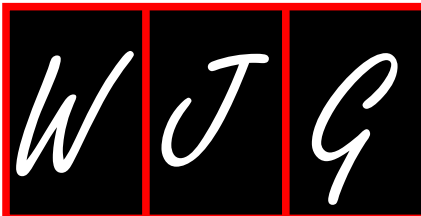
Thus, it would not be appropriate to diagnose diffuse HP solely on EUS, but CT or Magnetic resonance imaging would be more reliable for such a diagnosis. More importantly, we need to clarify what is the clinical importance of HP on EUS. We are even not sure that HP

represents pancreatic steatosis. Even if it does, we do not know the clinical consequences of pancreatic steatosis.

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MEETINGS

Events Calendar 2011

January 14-15, 2011

AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011

Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011

Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011

9. Gastro Forum München, Munich,
Germany

February 4-5, 2011

13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011

Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011

APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011

Inflammatory Bowel Diseases
2011-6th Congress of the European
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Dublin, Ireland

February 24-26, 2011

2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011

International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011

Canadian Digestive Diseases Week,
Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011

Childhood & Adolescent Obesity:

A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011

42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011

Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011

British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011

41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011

Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011

UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011

MedicReS IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011

26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011

IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011

International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011

Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011

Pediatric Emergency Medicine:
Detection, Diagnosis and Developing

Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011

9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011

The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011

Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011

4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011

Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011

2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011

1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011

22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011

4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011

The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011

Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011

International Scientific Conference
on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011

ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011

XI Congreso Interamericano
de Pediatría "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium

178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne,
Martinstr. 29-37, 50667 Cologne,
Germany

September 10-11, 2011

New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011

ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011

Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011

Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise,
Papeete, French Polynesia

October 22-26, 2011

19th United European
Gastroenterology Week,
Stockholm, Sweden

October 28-November 2, 2011

ACG Annual Scientific Meeting &
Postgraduate Course,
Washington, DC 20001,
United States

November 11-12, 2011

Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku,
Tokyo 107-0052, Japan

December 1-4, 2011

2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



GENERAL INFORMATION

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

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

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h; blood glucose concentration, *c* (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 ± 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, *etc.*

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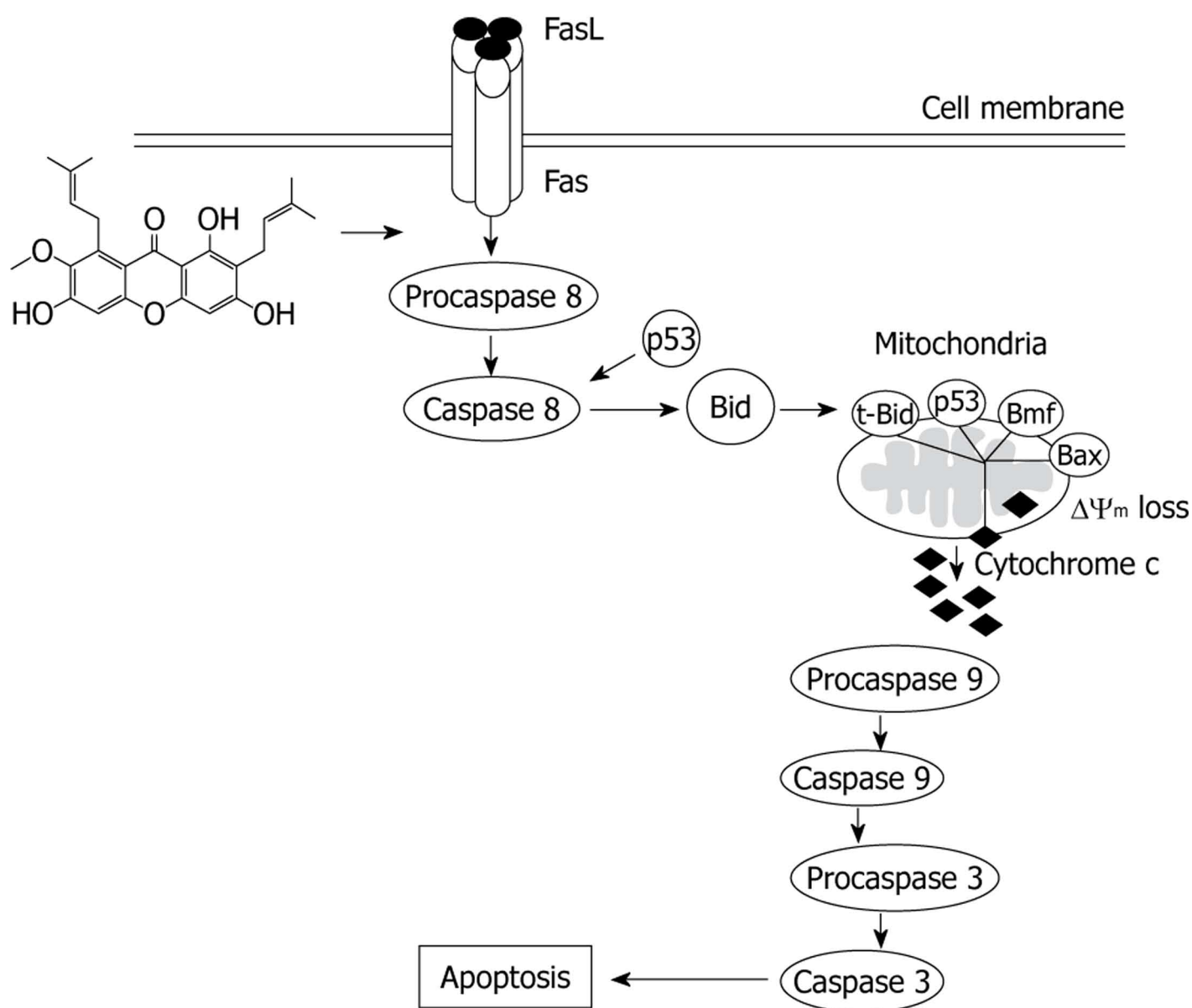
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**EDITORIAL**

- 2063** Targeting the cell cycle in esophageal adenocarcinoma: An adjunct to anticancer treatment
Dibb M, Ang YS
- 2070** Optimizing management in autoimmune hepatitis with liver failure at initial presentation
Potts JR, Verma S

TOPIC HIGHLIGHT

- 2076** A practical approach to the diagnosis of autoimmune pancreatitis
Frulloni L, Amodio A, Katsotourchi AM, Vantini I
- 2080** Endoscopic ultrasonography findings in autoimmune pancreatitis
Buscarini E, De Lisi S, Arcidiacono PG, Petrone MC, Fuini A, Conigliaro R, Manfredi G, Manta R, Reggio D, De Angelis C

ORIGINAL ARTICLE

- 2086** Effects of α -mangostin on apoptosis induction of human colon cancer
Watanapokasin R, Jarinthan F, Nakamura Y, Sawasjirakij N, Jaratrungtawee A, Suksamrarn S
- 2096** Chemometrics of differentially expressed proteins from colorectal cancer patients
Yeoh LC, Dharmaraj S, Gooi BH, Singh M, Gam LH

BRIEF ARTICLE

- 2104** Dietary treatment of colic caused by excess gas in infants: Biochemical evidence
Infante D, Segarra O, Luyer BL
- 2109** Levels of matrix metalloproteinase-1 and tissue inhibitors of metalloproteinase-1 in gastric cancer
Kemik O, Kemik AS, Sümer A, Dulger AC, Adas M, Begenik H, Hasirci I, Yilmaz O, Purisa S, Kisli E, Tuzun S, Kotan C

BRIEF ARTICLE

- 2113 Sunitinib for Taiwanese patients with gastrointestinal stromal tumor after imatinib treatment failure or intolerance
Chen YY, Yeh CN, Cheng CT, Chen TW, Rau KM, Jan YY, Chen MF
- 2120 MELD score can predict early mortality in patients with rebleeding after band ligation for variceal bleeding
Chen WT, Lin CY, Sheen IS, Huang CW, Lin TN, Lin CJ, Jeng WJ, Huang CH, Ho YP, Chiu CT
- 2126 Study on chronic pancreatitis and pancreatic cancer using MRS and pancreatic juice samples
Wang J, Ma C, Liao Z, Tian B, Lu JP
- 2131 *Ku80* gene G-1401T promoter polymorphism and risk of gastric cancer
Li JQ, Chen J, Liu NN, Yang L, Zeng Y, Wang B, Wang XR
- 2137 Effects of penethyclidine hydrochloride on rat intestinal barrier function during cardiopulmonary bypass
Sun YJ, Cao HJ, Jin Q, Diao YG, Zhang TZ
- 2143 *p53* gene therapy in combination with transcatheter arterial chemoembolization for HCC: One-year follow-up
Guan YS, Liu Y, He Q, Li X, Yang L, Hu Y, La Z

CASE REPORT

- 2150 Celiac disease and microscopic colitis: A report of 4 cases
Barta Z, Zold E, Nagy A, Zeher M, Csipo I
- 2155 Pure red cell aplasia caused by pegylated interferon- α -2a plus ribavirin in the treatment of chronic hepatitis C
Chang CS, Yan SL, Lin HY, Yu FL, Tsai CY

LETTERS TO THE EDITOR

- 2159 Enucleation for gastrointestinal stromal tumors at the esophagogastric junction: Is this an adequate solution?
Peparini N, Carbotta G, Chirletti P

ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

APPENDIX I Meetings
I-VI Instructions to authors

ABOUT COVER Watanapokasin R, Jarinthanan F, Nakamura Y, Sawasjirakij N, Jaratrungtaewee A, Suksamrarn S. Effects of α -mangostin on apoptosis induction of human colon cancer.
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Targeting the cell cycle in esophageal adenocarcinoma: An adjunct to anticancer treatment

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Abstract

Esophageal adenocarcinoma is a major cause of cancer death in men in the developed world. Continuing poor outcomes with conventional therapies that predominantly target apoptosis pathways have led to increasing interest in treatments that target the cell cycle. A large international effort has led to the development of a large number of inhibitors, which target cell cycle kinases, including cyclin-dependent kinases, Aurora kinases and polo-like kinase. Initial phase I/II trials in solid tumors have often demonstrated only modest clinical benefits of monotherapy. This may relate in part to a failure to identify the patient populations that will gain the most clinical benefit. Newer compounds lacking the side effect profile of first-generation compounds may show utility as adjunctive treatments targeted to an individual's predicted response to treatment.

Key words: Esophageal adenocarcinoma; Cell cycle; Cyclin-dependent kinase; Aurora kinases; Polo-like kinase

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INTRODUCTION

Esophageal cancer is a major cause of cancer death worldwide^[1]. It was the fourth most common cause of death from cancer in men in the United Kingdom between 2004 and 2006^[2]. Although in the developed world the incidence and mortality of cancer in general has decreased with advances in diagnosis and treatment, the incidence and mortality of esophageal carcinoma have increased^[1].

Esophageal cancer carries a poor prognosis with a 5-year survival rate of < 10%^[3]. This probably reflects the fact that the majority of esophageal cancers present late with symptoms after invasion of the muscularis propria and lymph node metastasis have occurred^[4]. Extensive disease means that few patients are suitable for definitive surgical therapy^[4,5]. Poor outcomes from conventional therapies including surgery and radiochemotherapy have led to increasing interest in understanding the molecular mechanisms that underpin the development of esophageal cancer. This may assist in developing new diagnostic techniques and identifying potential therapeutic targets.

The mechanism by which cells reproduce has fascinated biologists since Virchow's 1855 observation that cells could only arise from pre-existing cells. By the

early 20th century, pathologists had recorded extensive descriptions of the cytological events of cell division, including division of the nucleus and partitioning of the cytoplasm to the formation of two daughter cells^[6]. It has become increasingly clear since those early descriptions of the normal cell cycle that disorders in this process can lead to disease. It was not however until the 1970s, that molecular biology allowed a deeper understanding of the cell cycle and its role in health, disease and cancer development. The past three decades, in particular, have seen major advances in our understanding of the genetic and molecular mechanisms by which cells reproduce and how this process is regulated and controlled. It has also been aptly described that cell cycle deregulation, in the form of growth self-sufficiency and insensitivity to growth inhibitory signals, have become fundamental hallmarks of cancer development^[7-9]. Targeting these pathways in cancer development for diagnostic and therapeutic use has become increasingly important. We assess in this review the potential for targeting the cell cycle to treat esophageal adenocarcinoma.

HALLMARKS OF CANCER

It is clear that cellular reproduction is carefully controlled and regulated to prevent uncontrolled proliferation of cells^[10]. A number of alterations in cell physiology are required to lead to carcinogenesis^[7]. First, a cell must become able to move from its dormant inactive state (known as quiescence) to enter the cell cycle without stimulation from external growth factors. Second, the cell must lose response to growth-inhibitory signals. Cells must evade senescence and programmed cell death to gain limitless replicative potential. Finally, it must be able to develop and maintain an adequate blood supply (angiogenesis), which allows the cancer cell to invade and metastasize throughout the organism^[11].

Many genes responsible for the carcinogenesis have been identified. Broadly, they fall into two categories: oncogenes and tumor suppressor genes. Oncogenes are created by mutations in genes that cause them to become constitutively active, whereas in tumor suppressor genes, mutations reduce or inactive the gene product^[12]. Oncogenes and tumor suppressor genes increase tumor cell number by stimulation of cell division or prevention of cell death.

CELL CYCLE

Embryonic cells can undergo DNA replication and nuclear division at rapid rates. A full cycle of embryonic cell division can last just 30 min^[13]. Division of adult stem cells requires more complex control (Figure 1). Gaps or pauses are inserted between the phases of nuclear division (M phase) and DNA synthesis (S phase). These gaps are known as G1 (between M and S phases) and G2 (between S and M phases).

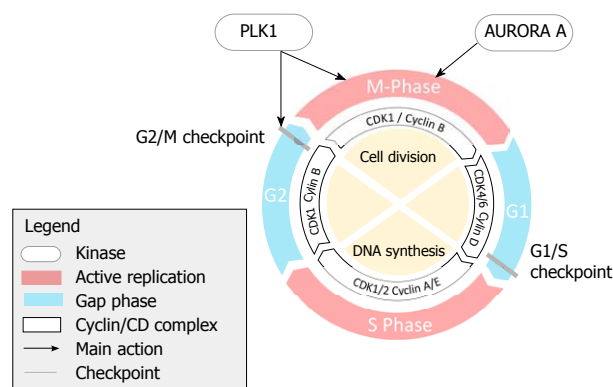


Figure 1 Cell cycle.

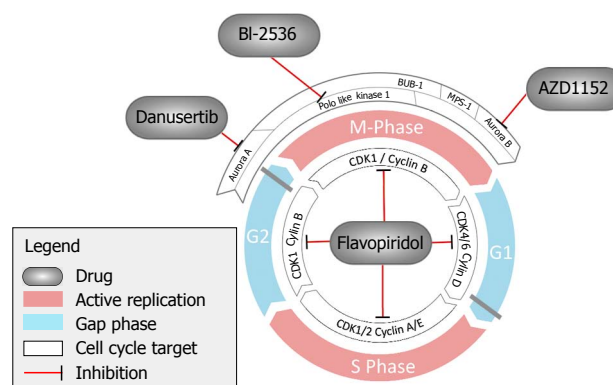


Figure 2 Compounds targeting the cell cycle.

Events in the cell cycle happen in a temporally organized sequence, with later events depending on successful completion of earlier events^[14].

Control of the cell cycle is driven by the cyclin-dependent kinases (CDKs), a family of serine/threonine kinases. Cells cannot enter S phase, without CDK activation. In order to become catalytically active, CDKs need to bind to a cyclin subunit that acts as an activator. CDKs can also be modulated by inhibitors such as CDK inhibitor 1A (p21^{CIP1}), CDK inhibitor 1B (p27^{KIP1}) or CDK inhibitor 2B (p15^{INK4B})^[10]. It has previously been thought that mammalian cells require the sequential activation of a number of the CDKs to complete the cell cycle successfully^[15]. Recent evidence from mouse models has suggested that CDK1 alone is sufficient to complete the cell cycle, although other CDKs are required for normal development and cell type specialization^[16]. Cell cycle defects can contribute to esophageal cancer development in a number of different ways (Figure 2).

Mitosis itself contains a series of phases that lead to chromosome separation and cell division. Mitosis is a vital step in the cell cycle, which involves carefully regulated interactions between multiple proteins. Abnormalities throughout the cell cycle can lead to genomic instability through unrestrained proliferation or defects in the transmission of genetic information to daughter cells. A number of established chemotherapy agents, including

the vinca alkaloids and the taxanes work by targeting the mitotic phase of the cell cycle.

CELL CYCLE CHECKPOINTS

Cells need mechanisms that prevent progression of the cell cycle if there is significant genomic damage, until the damage is repaired or the cell undergoes apoptosis. These have become known as cell cycle checkpoints. There are two major checkpoints: the G1/S checkpoint and the G2/M checkpoint. Checkpoint kinases ATM and ATR mediate these checkpoints, through effector kinases such as CHK1 and CHK2, by preventing activation of CDKs and progression through the cell cycle^[17]. Double-stranded DNA breaks activate preferentially ATM, whereas UV light activates ATR kinase. Defects in this DNA damage response can contribute to cancer formation by allowing tumor cell survival despite genome instability and enhanced mutation rates^[18,19]. The DNA damage response is commonly activated in early neoplastic lesions^[20,21].

G1/S checkpoint

The G1/S checkpoint occurs towards the end of the G1 phase, prior to entry into G2. During G1, the cell remains responsive to external mitogenic and anti-mitogenic stimuli. These can either cause the cell to become quiescent (entering the G0 phase) or allow re-entry to the cell cycle. This decision is controlled by the pocket protein RB. Immediately after mitosis, RB is dephosphorylated by protein phosphatase type 1. Whilst in this dephosphorylated state, RB binds to a group of transcription factors called E2Fs and inhibits their activity. During G1, RB is hypophosphorylated by the complex of CDK4 and cyclin D. CDK2 and cyclin E complexes then act to hyperphosphorylate RB, which causes dissociation from E2Fs. Free E2Fs trigger increased transcription of CDK2 and cyclin E, which creates a positive feedback loop that drives the cell into DNA synthesis (S phase). CDC25 phosphatases act to regulate CDK and cyclin complexes by removing inhibitory phosphate groups thereby promoting cell cycle progression^[13]. In genomic damage, CHK2 activates the p53 pathway, which stimulates production of p21^{CIP1} as well as phosphorylation of CDC25A. This prevents activation of the CDK/cyclin complexes^[13].

G2/M Checkpoint

The G2/M checkpoint acts as a final check to prevent mitosis occurring if the genome is damaged. A complex of cyclin B and CDK1 regulates this transition. Throughout G2, the inhibitory kinases CHK1, WEE1 and MYT1 phosphorylate CDK1, which prevents its activation and progression to mitosis. Polo-like kinase 1 (PLK1) protein levels begin to accumulate during S phase and G2/M phases, having been relatively low during G1^[22,23]. PLK1 transcription is most abundant in cells that are in G2/M phase^[24]. In the absence of DNA damage, PLK1 is phosphorylated by Aurora A at its phosphorylation site

at T210^[25]. Phosphorylated PLK1 then activates CDC25 phosphatases that remove inhibitory phosphates from the ATP-binding site located at Thr14 and Tyr15 in human CDK1. This causes the activation of the CDK1/cyclin B complex and drives the cell into mitosis^[26]. PLK1 also increases phosphorylation-dependent cyclin B import to the nucleus^[27]. PLK1 phosphorylates WEE1 and MYT1, which leads to ubiquitination and degradation of WEE1 and inhibition of MYT1^[28,29]. PLK1 is then inactivated and degraded during anaphase by ubiquitin-dependent degradation mediated by the anaphase promoting complex^[30]. Cell cultures show severely impaired growth when PLK1 is either overexpressed or functionally depleted^[31,32].

CELL CYCLE AS A TARGET FOR CANCER THERAPEUTICS

Many oncogenes and tumor suppressors have downstream effects on cellular functions involving cell cycle entry and exit. Healthy or normal cells have the ability to stop at pre-determined checkpoints in the cell cycle in the presence of damage or unfavorable conditions. Cancer cells develop mechanisms that eliminate these checkpoints, which leads to uncontrolled proliferation. One example of this is the INK4 family member *p16*. This occurs as a result of epigenetic silencing by DNA hypermethylation at the *p16* promoter, which leads to reduced transcription and loss of gene expression. *p16* is a CDK inhibitor and loss of *p16* function leads to unrestrained cellular proliferation. This has been demonstrated to occur with a number of different tumors^[33]. Abnormalities of *p16* function have been described in Barrett's esophagus and esophageal adenocarcinoma^[34]. DNA hypermethylation of the *p16* promoter has also been shown to be a strong predictor of the progression to high-grade dysplasia and esophageal adenocarcinoma^[35].

CDK inhibitors

Abnormal expression of CDKs and their partner cyclins has been noted in esophageal cancer^[36-39]. Polymorphisms of *CCND1*, which encodes cyclin D1 has been shown to be associated with an increased risk of esophageal adenocarcinoma^[40]. *CCND1* amplification and nuclear staining of cyclin D1 have been shown to correlate negatively with survival^[41,42]. Abnormal activity of the CDK/cyclin complexes in esophageal adenocarcinoma has been shown to be a marker of acquired chemoradioresistance^[42,43]. The observation that inhibition of CDKs leads to cell cycle arrest and apoptosis has led to the development of CDK inhibitors as antitumor drugs. There are a number of drugs that target these pathways. The pioneer compound for this group is flavopiridol, a semi-synthetic inhibitory flavonoid of CDKs. Flavopiridol prevents the phosphorylation and activation of CDK1, CDK2, CDK4 and CDK6, which leads to reduced expression of cyclin D1, cell cycle arrest, and induction of apoptosis^[44].

In vitro, it has been demonstrated that even at nanomolar doses, flavopiridol can enhance the antitumor activity

of cytotoxic drugs by increasing apoptosis^[45]. Phase I and II studies have been undertaken with various combinations of chemotherapeutic agents with variable results. Most promising is the combination with irinotecan and cisplatin. A phase I trial of relapsed gastric and esophageal cancer patients showed that eight out of 14 patients achieved a partial response^[46]. Further clinical studies are awaited.

Aurora kinases inhibitors

The Aurora kinase family is an important family of serine/threonine kinases that are evolutionarily conserved and act as mitotic regulators throughout the cell cycle. There are three mammalian aurora kinases, Aurora A, Aurora B, and Aurora C, which have differing roles throughout mitosis^[47]. Aurora A is required for centrosome maturation and spindle formation, in addition to its role at the G2/M checkpoint described above. Aurora B is required for chromosome segregation and cytokinesis. Small molecule inhibitors of Aurora B lead to premature mitotic exit without successful chromosome separation. Continued inhibition of Aurora B results in large multiploid cells that eventually undergo apoptosis^[48]. This potentially has the advantage that Aurora B inhibitors could be combined with other agents that act during other phases of the cell cycle. Aurora C is abundant in the testes. Its global functions are unclear, however, it has recently been shown to have some overlap with the functions of Aurora B during mitosis^[49]. Aurora kinases have been shown to be overexpressed in a number of different tumors. Aurora A has been shown to be overexpressed in Barrett's esophagus and esophageal adenocarcinoma^[50,51]. Cell line models suggest that Aurora A overexpression protects developing esophageal adenocarcinoma cells against drug-induced apoptosis^[51]. In other forms of cancer, Aurora A expression has been shown to correlate with chromosomal instability^[52]. A number of Aurora kinase inhibitors are undergoing phase I and II evaluation. Danusertib, a pan-Aurora kinase inhibitor has undergone phase I testing in patients with advanced solid tumors. Forty-six percent of patients treated with danusertib had stable disease following treatment and a number of prolonged objective responses were noted^[53,54]. The major dose limiting effect of these drugs is neutropenia.

PLK1 inhibitors

PLKs form a group of prominent mitotic kinases. They were first described in mutants that failed to undergo a normal mitosis in *Drosophila melanogaster* (polo)^[55,56]. They are highly conserved from yeast to humans. There are four members of the polo family in mammals (PLK1-4)^[57,58]. They are involved in multiple functions throughout the cell cycle in mitosis and meiosis. PLK1 is the best characterized of the four known PLKs^[58].

PLK1 is a candidate for development as a therapeutic target because it contains two functionally relevant sites: a C-terminal regulatory region containing two polo box domains (PBDs) and an N-terminal catalytic kinase domain^[59]. The highly conserved PBD has been identi-

fied as a phosphopeptide-binding motif^[60]. The polo box motif is only observed in the PLK family and contains a characteristic sequence. Drugs that target the PBD are specific to the human family of PLKs.

PLK1 is overexpressed in a broad range of primary gastrointestinal tumors, including gastric, colorectal and pancreatic carcinoma^[61-63]. In contrast, one study has noted downregulation of PLK1 within tumor cells^[64]. There is now increasing evidence that PLK1 expression levels have prognostic significance within different cancers, including esophageal cancer^[63]. Two separate reports of PLK1 overexpression in esophageal carcinoma primarily relate to squamous cell carcinoma (SCC) in the far east^[63,65]. Given the high impact of environmental factors (e.g. aflatoxin) on SCC development in these populations, it is unclear whether the findings can be directly applied to western populations. There are no data on PLK1 expression in adenocarcinoma patients. Some reports of other cancers have suggested that PLK1 expression is a reliable marker of metastasis^[66]. PLK1 has also been used in the context of larger arrays of genes as a prognostic marker to predict metastasis in breast cancers^[67]. Current cancer staging systems and histological assessments often fail to predict individual outcomes reliably but correlation of PLK1 protein and mRNA expression levels with clinical stage has the potential to improve clinical decision making in a number of different tumors^[68].

The unique PLD of PLK1 also makes it a good candidate for the development of alternative cancer therapies. Initial efforts have focused on specific phosphorothioate antisense oligonucleotides that are able to block protein translation^[69]. Use of siRNAs, which cause depletion of PLK1, has also been considered. Whilst there are drawbacks of siRNAs, including off-target effects and nuclease sensitivity, these hold promise in cancers such as bladder cancer in which they can act locally^[70]. There are now a number of small molecule inhibitors of PLK1, which act either in an ATP-competitive or non-ATP-competitive manner^[68]. The multiple actions of PLK1 throughout the cell cycle mean that these new agents need to be carefully assessed for specificity and side effects. In particular, it is possible that anti-PLK1 agents have similar toxicity to other microtubule inhibitors. PLK1 inhibitors are now in early clinical testing (phase I and II). Early clinical experience suggests that neutropenia and thrombocytopenia are dose-related effects, although neuropathy has not been seen^[71].

MPS1 inhibitors

Cell cycle translational research has focused on the development of inhibitors of the major kinases discussed above. There are additional mitotic kinases that may have relevance for inhibiting tumor growth. Inhibitors of MPS1, a kinetochore-associated kinase that is involved in the spindle assembly checkpoint, have been shown to arrest tumor cell proliferation *in vitro*^[72,73]. This appears to be mediated at least in part by impaired Aurora B func-

Table 1 Compounds targeting the cell cycle under active development

Inhibitor	Main target	Sponsor	Clinical trials
BI2536	PLK1 (partial inhibition of PLK2/3)	Boehringer Ingelheim	Phase II pancreatic cancer
Danusertib (Formerly PHA-739358)	Pan-aurora kinase inhibitor	Pfizer Italia	Phase II advanced solid tumors
MLN8237	Aurora a inhibitor	Millennium	Phase I / II advanced solid tumours
BI6267	PLK1 inhibitor	Boehringer Ingelheim	Phase II ovarian cancer/phase I advanced solid tumors
P276-00	Small molecule cyclin inhibitor	Piramal Life Sciences	Phase I advanced malignancy/phase II head and neck malignancy
NMS-1286937	PLK1 selective inhibitor	Nerviano Medical Sciences	Phase I advanced solid tumours
P1446A-05	CDK selective inhibitor	Piramal Life Sciences	Phase I advanced malignancy
SCH727965	CDK inhibitor	Schering-Plough	Phase I advanced malignancy
Seliciclib (Roscovitine)	CDK inhibitor	Cyclacel Pharmaceuticals	Phase I advanced malignancy

CDK: Cyclin-dependent kinase; PLK1: Polo-like kinase 1.

tion at centromeres, which leads to impaired alignment of chromosomes^[74]. Detailed information on MPS1 in esophageal cancer is lacking, however, MPS1 inhibition has been demonstrated as a chemotherapy sensitization strategy *in vitro*^[75].

CONCLUSION

Established esophageal carcinoma chemotherapy regimes are relatively blunt tools that predominantly target apoptosis pathways and are often associated with significant side effects. This has led to a large international effort to develop targeted therapy.

Current therapies that target the cell cycle have largely disappointed with relatively modest effects seen in phase I / II trials (Table 1). This may be in part related to failure to identify the patient populations that will gain the most clinical benefit. Few treatments are targeted towards specific pathways or personalized to the individual tumor proteome or genomic signature.

Efforts are now being made to assess gene expression profiles from histological specimens from solid tumors such as breast cancer in an attempt to predict response to chemotherapy^[76]. Initial steps in this direction have been taken by the UK Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) Study Group, which has demonstrated a four-gene signature associated with poor prognosis in esophageal adenocarcinoma, as well as a larger group of genes associated with lymph node metastasis^[77]. Efforts have also been made to identify Barrett's esophagus patients who are likely to progress to adenocarcinoma, however, little work has been undertaken on response to chemotherapy in the esophagus^[78]. Careful studies are needed in esophageal adenocarcinoma to define patient populations that are likely to respond well to treatment with both established and novel chemotherapy regimes. Optimizing individual chemotherapy regimens for patients will assume greater significance as health economics demand most clinical benefit from limited resources. In this setting of personalized targeted therapy, new cell cycle treatments may hold promise as carefully selected adjuncts to existing chemotherapy regimes.

Patients with esophageal adenocarcinoma unfortunately often still present late with a large burden of dis-

ease. Given the large number of cells involved it is likely that some tumor cells will abrogate the inhibited pathways and escape from chemotherapy-induced apoptosis. Targeted cell cycle therapy in esophageal cancer presents an alternate strategy as cell cycle inhibitors affect multiple essential pathways involved in replication and DNA damage repair. They may provide a useful adjunct in patients with late presenting esophageal tumors who have failed standard chemotherapy regimens.

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Optimizing management in autoimmune hepatitis with liver failure at initial presentation

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understatement that multicenter prospective studies are urgently needed to address this important clinical issue.

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Abstract

Autoimmune hepatitis (AIH) is a disease of unknown etiology, its hallmark being ongoing hepatic inflammation. By its very nature, it is a chronic condition, although increasingly, we are becoming aware of patients with acute presentations, some of whom may have liver failure. There are very limited published data on patients with AIH with liver failure at initial diagnosis, which consist mostly of small retrospective studies. As a consequence, the clinical features and optimal management of this cohort remain poorly defined. A subset of patients with AIH who present with liver failure do respond to corticosteroids, but for the vast majority, an urgent liver transplantation may offer the only hope of long-term survival. At present, there is uncertainty on how best to stratify such a cohort into responders and non-responders to corticosteroids as soon as possible after hospitalization, thus optimizing their management. This editorial attempts to answer some of the unresolved issues relating to management of patients with AIH with liver failure at initial presentation. However, it must be emphasized that, at present, this editorial is based mostly on small retrospective studies, and it is an

INTRODUCTION

Autoimmune hepatitis (AIH) is a disease that is characterized by chronic hepatic inflammation, presence of autoantibodies [antinuclear antibody (ANA), anti-smooth muscle antibody (SMA), and liver kidney microsomal (LKM) antibody], female preponderance and elevated serum gamma-globulins, especially IgG^[1]. Earlier studies have established the beneficial effects of corticosteroids in AIH and up to 80% of patients can now achieve remission with immunosuppressants^[2,3]. At accession, 10%-20% of patients with AIH can be negative for the conventional autoantibodies^[4], although their outcomes, especially response to immunosuppression, are no different from those that are autoantibody-positive^[5].

AIH can have protean manifestations, with the majority of patients presenting with subclinical or chronic disease. However, in > 25%, the disease may present acutely with jaundice, a subset of whom may have fulminant or sub-acute liver failure (LF)^[6-8]. Fulminant hepatic failure (FHF) is a devastating clinical condition that occurs in patients

with no prior history of liver disease, and is characterized by development of hepatic encephalopathy and coagulopathy within 8 wk after onset of jaundice^[9]. In contrast, those with subacute LF present with encephalopathy at 8–26 wk after onset of symptoms^[10]. In a survey in the United States carried out between 1998 and 2008, the major etiologies of FHF in 1147 patients were acetaminophen overdose (46%), followed by indeterminate causes (14%), drug-induced (11%), hepatitis B virus (7%), other causes (7%), AIH (5%), ischemic hepatitis (4%), hepatitis A virus (3%) and Wilson's disease (2%)^[11]. Similar data were reported from Europe where 2%–5% of patients with FHF have AIH as the underlying etiology^[12,13]. Unfortunately, neither the International Autoimmune Hepatitis Group (IAIHG) criteria^[14] nor the simplified diagnostic criteria for diagnosis of AIH^[15] have been extensively validated in patients with LF; largely because of the small number of cases encountered. Thus, diagnosis of AIH and LF remains clinical and is supported by positive autoantibodies, negative viral serology, absence of alcohol excess and culprit drugs, and compatible liver biopsy. This has been corroborated by an earlier study in which 28 patients with FHF were clinically diagnosed with AIH, but after application of the IAIHG criteria and simplified scoring systems only 50% and 46%, respectively, fulfilled the criteria, with the concordance of the two scoring systems being only 46%^[16].

Immunoparesis is commonly seen in critically ill patients with LF in whom both autoantibodies and/or elevated IgG concentrations may be absent^[17]. In addition, because of the severity of the hepatic insult (massive/submassive necrosis), histological evaluation may be difficult or impossible^[16]. Although challenging, AIH can still be diagnosed in such a scenario by excluding other liver diseases, and by testing for other autoantibodies [perinuclear antineutrophil cytoplasmic antibodies (pANCA), and antibodies to soluble liver antigen (SLA)]^[18,19]. Furthermore, if the patient is HLA B8, DR3 or DR4 positive, has a concurrent immunological disorder, and responds to corticosteroid therapy, this further lends credence to the diagnosis of AIH^[4]. Nonetheless, the decision to initiate corticosteroids in patients who do not fulfill conventional diagnostic criteria for AIH must be made on an individual basis, and remains the prerogative of the treating hepatologist.

AIH AND LF

There is a paucity of published data on patients with AIH with LF at initial diagnosis; consisting mostly of anecdotal case reports or small case series^[20,21]. Thus the clinical characteristics, response to immunosuppression, and outcomes with/without liver transplantation (LT) of this cohort remain poorly described. Much of the controversy hinges on a critical management issue, namely should such patients be given a trial of corticosteroids, be priority listed for LT, or both. If corticosteroids are indeed initiated, how and at what time point do we define failure of medical treatment? This editorial attempts to address some of these controversies with the aim to develop strategies that could optimize

management of patients with AIH that present with LF.

We therefore searched the medical literature (PubMed) to collect published data on AIH with initial presentation with LF. Only studies providing data on type and duration of immunosuppressive therapy and outcomes were included. Case reports/small case series, and studies in which authors reported acute AIH in the absence of LF were excluded. We identified five studies that met our inclusion criteria and these included a total of 85 patients with AIH and LF^[7,22–25] (Table 1). In three of the five studies^[7,23,24], patients were diagnosed with AIH according to IAIHG criteria, although information regarding probable or definite AIH was only available in two^[7,24]. In the remaining two studies^[22,25], the diagnosis of AIH was based on the presence of autoantibodies, elevated IgG levels, exclusion of Wilson's disease, negative viral serology, absence of culprit drugs, and compatible liver histology (1). The patients were very heterogeneous as regards ethnicity, presence/absence of cirrhosis, and inclusion of acute and subacute LF. It is well known that these factors have a prognostic value in patients with AIH and in those with LF^[7,26–28]. In addition, all the studies were retrospective, and one has only been published in an abstract form^[22]. Nonetheless, these five studies do provide valuable information about the natural history of AIH with LF at initial presentation.

In these five studies, the prevalence of LF at initial presentation in patients with AIH varied from 8.7% to 19.8%^[7,23]. In all but one patient this was the first presentation of their disease. The majority (> 75%) were women in the third to the sixth decade with type 1 AIH. Almost all patients had either encephalopathy at admission and/or had significant coagulopathy (Table 1). IgG levels were available in two studies^[24,25], and 74% had levels in excess of 1800 mg/dL.

OUTCOMES IN PATIENTS WITH AIH AND LF

Table 2 shows treatment data and outcomes in these five above studies. Of the total of 85 patients, 69 (89.2%) received immunosuppression, mostly corticosteroids (Table 2). For the majority of the patients, there was no rationale provided for initiation or withholding corticosteroids, and the decision appeared to have been made on an *ad hoc* basis. The remission rates with immunosuppression varied from 8.3% to 50% (average: 33.3%, 23/69) (Table 2). Overall, 43.5% (37/85) either underwent or were listed for LT and 32.9% (28/85) died. These outcomes are certainly poorer than those reported in patients with chronic AIH (remission with corticosteroids ~80%^[2,3], need for LT 1.4%–8.4% and mortality 1.8%–4.9%^[27,29]), and makes for dismal reading.

The variability in remission rates with corticosteroid therapy in these five studies is most certainly a reflection of the heterogeneous patient population. Unsurprisingly, the lowest remission rates were seen in the study of Ichai *et al.*^[25], which had the sickest patients, as reflected by their high admission MELD scores. However, those patients with AIH and LF that did respond to corticosteroid

Table 1 Clinical characteristics of patients with autoimmune hepatitis with liver failure at initial presentation

	Villamil <i>et al</i> ^[22] (<i>n</i> = 28)	Kessler <i>et al</i> ^[23] (<i>n</i> = 10)	Miyake <i>et al</i> ^[24] (<i>n</i> = 11)	Ichai <i>et al</i> ^[25] (<i>n</i> = 16)	Verma <i>et al</i> ^[7] (<i>n</i> = 20)
Study design	Retrospective	Retrospective	Retrospective	Retrospective	Retrospective
Age (yr) ²	41	40 ± 15.9	53 (16-75)	36 ± 13.1	41.3 ± 14.2
Definition of LF	NA	NA	PT < 40% and HE ≥ grade 2	HE within 12 wk of jaundice	Any grade HE and/or INR > 2
Symptoms duration ²	NA	3.2 wk	24 (16-52) d	NA	2.1 ± 2.5 mo ³
Female	NA	8 (80%)	11 (100%)	14/16 (87.5%)	15 (75%)
Ethnicity or country of origin	South American	80% White	Japanese	French	70% black
Definite/probable AIH (IAIHG ⁴ criteria)	NA	NA ⁵	3(36%)/8 (64%)	NA	9(45%)/11(55%)
LC/LKM ⁶ positive	6 (21.4)	1 (10%)		3 (18.7%)	NA
ANA/SMA ⁷ positive	22 (78.5%)	7 (70%)	NA	11 (68.7%)	20 (100%)
Bilirubin ² (mg/dL)	398 ⁸	16.97 ± 9.83	20.6 (5.9-31)	425 (278-850) ⁸	19.3 ± 10.3
AST or ALT ²	NA	1179 ± 1127.17	220 (59-1094)	678 (60-2867)	1147.1 ± 711.4
INR ² or PT	30%	49.3 ± 66.9	29% (6%-38%)	5.36 (1.7-12.2)	2.7 ± 1.4
HE ⁹ at onset	28 (100%)	8 (80%)	11 (100%)	10 (62.5%)	19 (95%)
Cirrhosis	None	2/10 (20%)	NA	None	8/20 (40%)
MELD ²	NA	NA	NA	37 (24-47)	28 ± 7.41
Sub-massive or massive necrosis (SMN, MN)	19/23 (82.6%) 17 needed LT and/or died	5/10 (50%)	NA	16/16 (100%) 15 needed LT and/or died	12/19 (63.1%), 10 needed LT and or died
Immunosuppressant regimen used	Prednisone 60 mg/d	Corticosteroids (Dose NA) and other ¹⁰	Prednisolone 40-60 mg/d and steroid pulse	Prednisone 1 mg/kg per day and other ¹⁰	Corticosteroids ¹¹ 20-1250 mg/d
Poor prognostic criteria	1: PT < 20%; 2: Grade 4 HE; 3: SMN at diagno- sis; 4: 20% increase in PT at day 3 of steroids	NA	1: High bilirubin at onset; 2: Worsening bilirubin during days 8-15 of steroid therapy	NA	1: Absence of cirrhosis; 2: MELD > 28; 3: Worsening trend in bilirubin and INR after 3.7 ± 0.6 d of steroid therapy
Septic events	NA	NA	NA	7 (43.7%), of whom 6 had received steroids	2 (10%), of whom 1 received steroids

¹Published only in abstract form; ²Data presented as mean ± SD or median (range); ³Duration from first symptom (and not necessarily jaundice/hepatic encephalopathy) to hospitalization; ⁴IAIHG: International Autoimmune Hepatitis Group; ⁵Met IAIHG criteria, data on probable or definite disease unavailable; ⁶LKM/LC: Liver kidney microsomal antibody/liver cytosol antibody; ⁷ANA/SMA: antinuclear antibody/anti-smooth muscle antibody; ⁸Values in μmol/L; ⁹HE: Hepatic encephalopathy; ¹⁰Additional immunosuppression was used in nine patients in the study of Kessler *et al* (azathioprine, tacrolimus, mycophenolate mofetil, 6-mercaptopurine, cyclosporine) and in one patient in the study of Ichai *et al* (azathioprine and cyclosporine); ¹¹Included prednisone, hydrocortisone and methylprednisone, (converted to equivalent doses of prednisone); LT: Liver transplantation; PT: Prothrombin time; AIH: Autoimmune hepatitis.

therapy survived, obviating the need for a subsequent LT. Unfortunately, among the non-responders to corticosteroids in these five studies (*n* = 46), death was the inevitable outcome in the absence of LT (Table 2). The duration of steroid therapy prior to death was highly variable (3-95 d). Clearly, in some, the illness was so fulminant that death occurred rapidly after hospitalization, thereby precluding LT, and in others, there were active contraindications to transplantation, such as sepsis (Table 2). Nevertheless, in these five studies, there were a subset of patients with AIH and LF in whom death may have been preventable had LT been more aggressively pursued. It is conceivable that initiation of steroids provided a false sense of security, thereby delaying transplant evaluation.

One could argue that the low remission rates to corticosteroids in this cohort were partly related to delay in initiating therapy. However, where available, the data do not support this conclusion, as corticosteroids were initiated promptly, especially in the sicker patients. In our study, subsequent non-responders to corticosteroids were commenced on therapy within 2.6 ± 1.8 d of admission, compared to 6.4 ± 5.5 d in those who eventually responded to

corticosteroids^[7]. It is more likely that non-responders to corticosteroids had aggressive disease at the time of diagnosis with a critical degree of liver cell death already having occurred prior to the introduction of medical treatment^[24]. This hypothesis is supported by the study of Ichai *et al*^[25], in which all patients had massive/sub-massive liver necrosis (median MELD score at admission: 37), with only 8.3% responding to corticosteroids and > 80% needing LT.

OPTIMIZING MANAGEMENT IN PATIENTS WITH AIH AND LF

Assessing patients with LF for LT is a complex process. The most widely used criteria for prioritizing patients for LT are the King's College criteria^[30]. However, neither the King's College criteria^[29] nor the more recently developed MELD score^[31] have been validated in patients with AIH and LF. This is most likely due to the fact that the prevalence of AIH in patients with LF being evaluated for LT is low (0%-5%)^[12,13,32]. As is evident from the published data^[7,22-25], there certainly are a subset of patients with AIH and LF who will respond to corticosteroids. Inappropri-

Table 2 Outcomes of patients with autoimmune hepatitis and initial presentation with liver failure

Study	Villamil <i>et al.</i> ^[22] (<i>n</i> = 28)	Kessler <i>et al.</i> ^[23] (<i>n</i> = 10)	Miyake <i>et al.</i> ^[24] (<i>n</i> = 11)	Ichai <i>et al.</i> ^[25] (<i>n</i> = 16)	Verma <i>et al.</i> ^[7] (<i>n</i> = 20)
Treated with IS ¹	25	10	8	12	14
Responders to steroids	9 (36%) (alive)	4 (40%) (alive)	2 (25%) (alive)	1 (8.3%) (alive)	7 (50%) (alive)
Non responders	16	6	6	11	7
LT	11 (2 Died)	3	1	10 (1 Died)	1 (Died)
Listed for LT	-	1	-	-	1 (Died)
Died without LT	5	2	5	1 ⁴	5 ²
Not treated with IS ¹	3	-	3 ³	4	6
Spontaneous survival	-	-	3	-	-
LT	1	-	-	3	5 (1 Died)
Listed for LT	-	-	-	-	-
Died	2	-	-	1	1
Overall underwent LT or listed for LT	12/28 (42.8%)	4/10 (40%)	1/11 (9%)	13/16 (81.2%)	7/20 (35%)
Overall mortality	9/28 (32.1%)	2/10 (20%)	5/11 (45.4%)	3/16 (18.7%)	9/20 (45%)

¹IS: Immunosuppression; ²Four died while being evaluated for liver transplantation, in 1 sepsis precluded liver transplantation evaluation; ³Treated with plasmapheresis and or stronger neo-minophagen; ⁴Not evaluated for LT due to sepsis; LT: Liver transplantation. Additional outcome data obtained by personal communication with authors.

ate transplantation in such patients would mean subjecting them to unnecessary surgery (and its attendant complications) and lifelong immunosuppression. In addition, it would deprive another more suitable recipient from receiving the graft^[33]. On the other hand, denying LT to a patient with AIH and LF who is unlikely to respond to corticosteroids means condemning them to a certain death, which is unacceptable, especially since post-transplant survival for AIH is excellent [estimated 5-year survival probability after first LT is 0.73 (95% CI: 0.67-0.77)]^[34].

The contentious issue thus is how best to stratify patients with AIH and LF into likely responders and non-responders to corticosteroids as soon as possible after hospitalization; hence optimizing their management. In our study^[7], all responders to corticosteroid therapy had a MELD score ≤ 28 at admission. This is also supported by Ichai *et al.*^[25], who showed that the only patient to respond to corticosteroids had a MELD score of 24, and none with an initial MELD score > 28 responded to corticosteroids. Furthermore, in our study, responders to corticosteroids were more likely to have either an improvement or stabilization in bilirubin and INR within 3.7 ± 0.6 d of initiation of corticosteroid therapy, whereas non-responders tended to have a trend for higher bilirubin and INR^[7]. Villamil *et al.*^[22] also observed that a 20% increase in prothrombin time (PT) at day 3 of corticosteroid therapy to be a predictor of poor outcome, along with PT $< 20\%$, grade 4 encephalopathy, and LKM antibody/liver cytosol (LC) antibody positivity at diagnosis. Histological evidence of sub-massive/massive necrosis is also invariably associated with need for LT and/or death (Table 1). Surprisingly, in our study, the presence of cirrhosis was more likely was associated with response to corticosteroids^[7]. Although the impact of cirrhosis on the natural history of AIH remains controversial^[27,28,35,36], it is likely that this group has long-standing indolent disease that progresses to cirrhosis, with LF representing an acute relapse of AIH^[37]. This is in contrast with the study of Ichai *et al.*^[25], in which absence of significant hepatic fibrosis in all the patients indicated a *de novo* fulminant disease process.

CORTICOSTEROIDS AND INFECTIONS

Whether steroids increase the risk of septic complications in patients with severe liver disease is subject to an ongoing debate. The issue becomes even more contentious in the presence of LF because in itself that has been associated with an increased risk of bacterial and fungal infections^[25,38,39]. In fact, earlier studies have shown that up to 35% of patients with LF can develop bacteremia in the pre-transplant period^[39]. This increased propensity for sepsis is further aggravated in the post-transplant setting due to use of immunosuppression. Therefore, not surprisingly, sepsis with or without multiorgan failure, accounts for almost one-third of all deaths in patients undergoing LT for LF and is the most common cause of mortality in this cohort^[40]. In the study of Ichai *et al.*^[25] (which had the sickest cohort of patients with a median MELD score of 37 at admission), 42.3% developed a septic event, and this prevalence is not higher than that reported previously^[39]. It is however noteworthy that in Ichai *et al.*'s study septic events were more likely to occur in those initiated (6/12) versus those not initiated (1/4) on corticosteroids^[25]. It is unclear whether patients received prophylactic antibiotics in this study. Reich *et al.*^[41] also have reported an increased trend for wound infection in corticosteroid-treated patients with AIH undergoing LT (30.7% *vs* 5.2%). In a recent publication that analyzed data from the European Transplant Registry, in comparison with transplantation for primary biliary cirrhosis and alcoholic cirrhosis, the probability of infectious complications limiting patient survival was significantly increased after transplantation for AIH. This was especially relevant to patients aged > 50 years and within the first 3 mo of transplantation^[34]. Unfortunately, data on disease severity and use of pre-transplant immunosuppression and prophylactic antibiotics were not available in that study. On the other hand, others have reported corticosteroids not to be associated with increased risk of infections in patients with severe AIH^[42]. These discordant results most likely reflect the heterogeneous patient groups (in-

cluding the whole spectrum from chronic disease to FHF), use of varying immunosuppressive regimens, and inconsistent use of prophylactic antibiotics. Nonetheless, Ichai *et al*^[25] caution against injudicious use of corticosteroids in patients with AIH and LF, and on the contrary, emphasize the need for expedited LT evaluation in such a cohort. Furthermore, it lends credence to the argument for the use of prophylactic antibiotics and antifungal agents, because such a strategy has been shown to reduce the risk of infections in the pre-transplant setting^[43].

THE FUTURE

Prospective multicenter studies are clearly needed to address this complex and important clinical issue. In future, testing for additional autoantibodies and HLA typing might also help risk-stratify patients. For example, presence of antibodies to SLA have been associated with DRB1 *0301, and such patients have aggressive disease and are more likely to require LT and/or die^[44,45].

CONCLUSION

The diagnosis and management of patients with AIH with AF at initial diagnosis can be challenging. Although there are only limited published data available, mostly in the form of small retrospective studies, up to 8.7%-19.8% of patients with AIH may have this form of presentation. On the whole, about one-third can respond to corticosteroids and have a good outcome, although for the vast majority, LT may offer the only hope of long-term survival. A MELD score at admission of ≤ 28 , more severe hepatic fibrosis, absence of sub-massive/massive necrosis, and early (within 4 d) improvement or stabilization in bilirubin and INR, identify those who are likely to respond to corticosteroid therapy, and thus survive without the need for LT. If clinical and biochemical improvement does not occur within the first few days, then continuation of corticosteroids may be a futile exercise, as it would be unlikely to change the clinical outcome, and on the contrary, may result in adverse events, especially sepsis. Nonetheless, if a decision is made to continue therapy with corticosteroids it is imperative that LT be actively pursued concomitantly. Furthermore, it may not be unreasonable to consider prophylactic antimicrobial and antifungal agents in such high-risk patients. It must however be emphasized that, at present, these recommendations are based on small retrospective studies. This underlines the urgent need for prospective multicenter studies to address this important clinical issue.

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A practical approach to the diagnosis of autoimmune pancreatitis

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Abstract

Autoimmune pancreatitis is a disease characterized by specific pathological features, different from those of other forms of pancreatitis, that responds dramatically to steroid therapy. The pancreatic parenchyma may be diffusely or focally involved with the possibility of a low-density mass being present at imaging, mimicking pancreatic cancer. Clinically, the most relevant problems lie in the diagnosis of autoimmune pancreatitis and in distinguishing autoimmune pancreatitis from pancreatic cancer. Since in the presence of a pancreatic mass the probability of tumour is much higher than that of pancreatitis, the physician should be aware that in focal autoimmune pancreatitis the first step before using steroids is to exclude pancreatic adenocarcinoma. In this review, we briefly analyse the strategies to be followed for a correct diagnosis of autoimmune pancreatitis.

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Key words: Autoimmune diseases; Pancreatitis; Therapy; Diagnosis

INTRODUCTION

Autoimmune pancreatitis (AIP) is now a well defined entity among the inflammatory diseases of the pancreas^[1-3]. The number of studies in literature has constantly increased since the first one published in 1995 by Yoshida *et al*^[4] (Figure 1). Despite the fact that in the first paper from Japan the disease was described as diffusely involving the pancreatic gland^[5-8], later publications pointed out that the pancreas may also be focally involved by the autoimmune process^[3,9-12]. Therefore, some authors have classified AIP as focal or diffuse^[3]. Focal AIP is characterized by a segmental involvement of the parenchyma with the possibility of a low-density mass being present at imaging.

Clinically, the focal form, particularly in the presence of a low-density pancreatic mass, requires a more careful patient evaluation, since it may be easily confused with pancreatic cancer. Several series indicate that in 5%-21% of resected pancreatic masses suspected of being cancer, the final diagnosis excluded malignancy (Table 1)^[13-20]. Since AIP responds dramatically to steroid treatment^[1], a correct diagnosis of the disease is important to avoid surgery. On the other hand, in the presence of a resectable pancreatic mass, the probability of cancer is very high (> 90%). A

misdiagnosis of AIP implies 2-3 week's steroid treatment and a one month delay in surgery, with the consequent risk of not operating because of the progression of the malignancy with the onset of metastasis or of vascular involvement. A correct and quick diagnosis of AIP is therefore an important goal in clinical practice, particularly in focal AIP.

AIP diagnosis may be attained through well established diagnostic criteria. There is agreement on the use of four main criteria based on histological findings, radiological features, other organ involvement and clinical and instrumental response to steroid therapy. HISORt criteria introduced by Chari *et al*^[21] in 2006 and based on surgical specimens of operated AIP patients can be considered standard criteria for the diagnosis of AIP. Serum IgG4^[22-24] and positive IgG4+ plasma cells in pancreatic surgical specimens or pancreatic biopsies may also support the diagnosis of AIP^[25-29].

There is agreement on the use of these diagnostic criteria (pathology, imaging, presence of other organ involvement, response to steroids), but not on the strategy to be followed in making the diagnosis.

THE STRATEGIES IN THE DIAGNOSIS OF AIP

Three main strategies, from Japan, the USA and Italy, have been suggested. The clinical approach to the disease by these strategies is different.

In the USA distinguishing the different pathological subtypes of AIP^[21,30,31] is considered prominent for the diagnosis. In the USA and in Europe, AIP may be classified as type 1 (or Lympho-Plasmacytic Sclerosing Pancreatitis-LPSP) and type 2 (or Idiopathic Duct-centric Chronic Pancreatitis-IDCP)^[31-34]. Since the clinical evolution of these forms seems to be different, some authors have suggested obtaining the diagnosis of AIP subtypes from EUS-guided biopsy^[31,35].

The main pathological and serological features in type 1 AIP are^[31,36]: (1) Prevalence of storiform fibrosis, with obstructive phlebitis; (2) high levels of serum IgG4; (3) presence of IgG4+ plasma cells in the involved pancreatic tissue; and (4) absence of granulocytic epithelial lesions (GEL), that are the expression of an aggression against epithelial ductal cells, with rupture and destruction of ductal structures. The pathological characteristics in type 2 AIP are on the contrary^[31,36]: (1) prevalence of inflammation; (2) presence of GEL; and (3) absence of serum IgG4 and of IgG4+ plasmavcells in the inflamed pancreatic tissue.

The clinical aspects and the evolution are different in type 1 and 2 AIP^[31,36,37]. In type 1 AIP (LPSP), there is a prevalence of males, patients are older, other organs may be involved (more commonly salivary glands, biliary tract, kidney, lung, retroperitoneum) and the relapse of the disease is more frequent after steroid treatment. In type 2 (IDCP), male/female ratio is about 1, patients are younger, the colon only may be involved (ulcerative colitis) and relapse after steroids is infrequent. Both forms respond quickly to steroid treatment^[31,36-38].

The diagnostic approach is therefore aimed at diagnosing

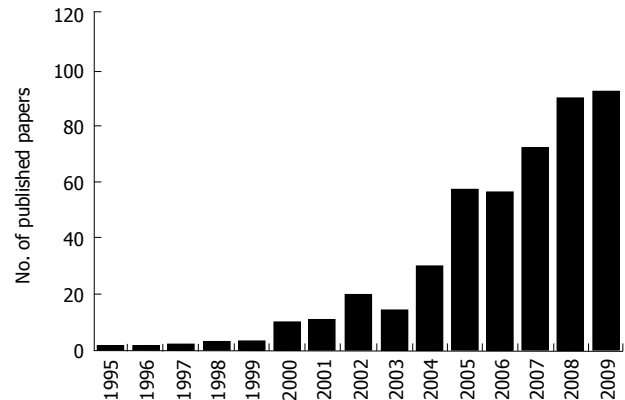


Figure 1 Increased number of published papers on autoimmune pancreatitis obtained by searching in Pubmed up to 2009 (search terms: Autoimmune pancreatitis, limit: Field title).

Table 1 Frequency of benign lesions in patients who undergo pancreatoduodenectomy in the presence of a pancreatic mass suspected of being pancreatic adenocarcinoma

Authors	Yr	No. of pts	Frequency of benign lesions	
			n	%
Smith <i>et al</i> ^[13]	1994	603	29	5
Barens <i>et al</i> ^[14]	1996	510	108	21
van Gulik <i>et al</i> ^[15]	1997	220	14	6
Abraham <i>et al</i> ^[16]	2003	442	47	10.4
Weber <i>et al</i> ^[17]	2003	1287	159	12
Kennedy <i>et al</i> ^[18]	2006	162	21	12.9
De La Fuente <i>et al</i> ^[19]	2010	494	37	7.4
Hurtuk <i>et al</i> ^[20]	2010	461	35	8
All studies	-	4179	450	10.8

AIP subtypes, mainly through pancreatic core biopsy, and this appears to have a good sensitivity and specificity^[28,29,39].

In Japan, only type 1 AIP (LPSP) is considered an autoimmune disorder and an IgG4-mediated systemic disorder associated with pancreatic lesions^[40]. Only in a few cases has type 2 AIP (IDCP) been described in Japan and it is not considered an autoimmune disease, despite its quick response to steroids just as type 1 AIP. Instrumentally, in the majority of cases the disease diffusely involves the pancreas. Several diagnostic algorithms have been suggested in Japan and Korea^[8,41-44]. A comprehensive diagnosis should be based on pancreatic imaging (including ERCP), serological tests (IgG4, total IgG, non organ specific autoantibodies, antibodies to carbonic anhydrase type I and II, antibodies to lactoferrin) and pathological findings. The presence of extrapancreatic lesions may suggest the possibility of AIP.

THE ITALIAN STRATEGY: A CLINICAL APPROACH TO THE DISEASE

The Italian proposal for the diagnosis of AIP, which is different from that suggested in Japan and the USA, is based on the instrumental distinction between focal and diffuse forms of the disease^[2,3].

A wide range of symptoms are reported by patients at the clinical onset of the disease. Jaundice, abdominal pain, usually mild, symptoms secondary to pancreatic exocrine and endocrine insufficiency (weight loss, diabetes), and persistent elevation of serum levels of pancreatic enzymes may be observed in AIP patients. In a few cases AIP is discovered incidentally by US or other imaging techniques performed without an indication for a pancreatic disorder.

On the basis of imaging, these patients can be divided in those with focal involvement of the pancreas and those with diffuse enlargement of the pancreatic gland^[12]. In the case of focal AIP, particularly in the presence of a low-density pancreatic mass, the clinical challenge is to exclude pancreatic cancer and correctly diagnose AIP. Therefore, focal and diffuse types AIP should be strictly separated, since the problem of differential diagnosis with pancreatic cancer involves only focal AIP.

Diffuse AIP may be confused with acute pancreatitis. The clinical picture of diffuse AIP, however, differs from those observed in acute pancreatitis. In AIP, pain, if present, is mild, no risk factors for pancreatitis (biliary lithiasis, alcohol) are present, a persistent increase in serum pancreatic enzymes may be observed, jaundice is caused by enlargement of the pancreas without the presence of a mass, with a stricture on the intrapancreatic tract of the common bile duct. Since pancreatic necrosis has never been described in AIP, the differential diagnosis should be with oedematous pancreatitis. This can be achieved through imaging, since the radiologic features of AIP are different from those observed in acute oedematous pancreatitis. Hypodensity of the pancreas in arterial phase and the absence of a peripancreatic strand appear to differentiate AIP from acute oedematous pancreatitis, where the pancreatic gland shows normal perfusion and the peripancreatic strand is a common radiological picture (personal unpublished data). We do not suggest pancreatic biopsy in diffuse AIP. The diagnosis may be definitely made after treatment with steroids, which produces complete disappearance of the pancreatic changes.

In the diffuse form associated with jaundice secondary to a common bile duct stricture, a diagnosis of cholangiocarcinoma should be considered and, if necessary, ruled out before steroid therapy through ERCP with biliary biopsies and/or intraductal biliary ultrasonography.

In the focal form, particularly in the presence of a low-density pancreatic mass at imaging, the first diagnostic goal is to exclude pancreatic cancer, even if the presence of clinical (young age, other organ involvement), radiological (perfusion of the pancreatic mass suggestive of inflammation, no or mild dilation of the main pancreatic duct) and serological (high level of IgG4, presence of autoantibodies, low serum levels of Ca 19-9) findings are suggestive of AIP. Therefore, pancreatic biopsy is mandatory, preferably EUS-guided, first of all to exclude neoplasia and possibly to confirm the diagnosis of AIP.

If pancreatic biopsy confirms the diagnosis of AIP, a 3 wk steroid treatment is indicated. The diagnosis of AIP is final in the presence of a significant clinical and radiological response. Since significant improvement/resolution

of jaundice is an indication of response to steroid therapy, biliary stenting is not recommended, unless serum bilirubin levels are very high.

If pancreatic biopsy is only suggestive of AIP or non diagnostic, a careful evaluation of HISORt criteria is necessary to decide whether the patient should be treated with steroids or undergo resective surgery. The decision is actually a challenge and should be made in experienced centres only, because it requires expert clinicians, radiologists, pathologists and surgeons. After a complete or significant response to steroid therapy, a definitive diagnosis of AIP may be made.

CONCLUSION

The diagnosis of AIP still remains difficult. The diagnostic algorithm is different in the diffuse and focal forms of the disease, particularly in the presence of a low-density pancreatic mass at imaging. Biopsy or fine needle aspiration cytology is mandatory in the presence of a low-density pancreatic mass. In some cases, only a full or significant response to steroids allows a final diagnosis of AIP to be made. Agreement among experienced clinicians, radiologists, pathologists and surgeons is needed to adopt the response to steroid therapy as a diagnostic criterion in patients where the diagnosis cannot be made through pancreatic biopsy.

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Endoscopic ultrasonography findings in autoimmune pancreatitis

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INTRODUCTION

Endoscopic ultrasonography (EUS) is superior to standard imaging techniques in detecting pancreatic cancer or masses and in the assessment of early parenchymal changes in chronic pancreatitis^[1,2]; however, its role in the diagnosis of autoimmune pancreatitis (AIP) has yet to be standardized, even though its high accuracy together with its safety make it a promising tool in the management of this disease.

To date, no consensus about the diagnosis of AIP has been reached^[3]. Several criteria have recently been proposed, reflecting the different clinical entities that AIP can adopt worldwide^[4-9]. The diffuse form of AIP has typically been included in the first set of criteria^[4], and in 2008 only, a focal pancreatic enlargement evident upon imaging was classified as a form of AIP.

Obstructive jaundice is the most common presentation of AIP and, together with biochemical and imaging features, can mimic neoplastic conditions^[10,11]. Because

Abstract

Endoscopic ultrasonography is an established diagnostic tool for pancreatic masses and chronic pancreatitis. In recent years there has been a growing interest in the worldwide medical community in autoimmune pancreatitis (AIP), a form of chronic pancreatitis caused by an autoimmune process. This paper reviews the current available literature about the endoscopic ultrasonographic findings of AIP and the role of this imaging technique in the management of this protean disease.

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Table 1 Endoscopic ultrasonography features of autoimmune pancreatitis

	Pancreas		Extrahepatic bile duct	Gallbladder	Lymph nodes ¹	Peripancreatic vessels
	Diffuse AIP	Focal AIP				
EUS	Gland volume: increased Echotexture: echopoor, with echogenic interlobular septa Gland border: thickened Wirsung: narrowed Wirsung wall: thickened ¹	Gland volume: focal enlargement/s Echotexture: echopoor, with echogenic interlobular septa Wirsung: narrowed within the lesion, dilated upstream to the lesion Wirsung wall: thickened ¹	Caliber: dilated Wall: diffuse and uniform thickening, “sandwich-pattern” ¹	Wall: diffuse and uniform thickening, “sandwich-pattern” ¹	Volume: enlarged (also substantially) Echotexture: echopoor Sites: liver hylum, peripancreatic, celiac	Loss of interface between pancreas and portal or mesenteric veins ¹
IDUS			Wall: diffuse and uniform thickening, “sandwich-pattern”; differential diagnosis with cholangiocarcinoma ¹			

¹Indicates the features which are detected only or substantially better by endoscopic ultrasonography (EUS) and not seen in conventional cross-sectional imaging. AIP: Autoimmune pancreatitis; IDUS: Intraductal ultrasonography.

AIP is a benign disease, a definitive diagnosis without the need for surgery is desirable.

AIP can present with extrapancreatic lesions, the most frequent being IgG4-related sclerosing cholangitis (IgG4-SC), followed by hilar lymphadenopathy^[12].

EUS can display both of these conditions in addition to parenchymal, ductal and vascular lesions. Moreover, this technique offers the advantage over other diagnostic tools of allowing clinicians to perform biopsies to achieve a definitive diagnosis^[13-16].

In this article, we describe the EUS findings of AIP (Table 1) by reviewing the currently available literature in this field.

PANCREATIC FINDINGS

The predominant finding in the diffuse form of AIP is a diffuse pancreatic enlargement with altered echotexture (Figure 1)^[14,15]. A recent retrospective study proposed to differentiate early from advanced stage AIP according to EUS findings^[15]. In 19 patients with AIP, the presence of parenchymal lobularity and a hyperechoic pancreatic duct margin were significantly correlated with early stage AIP^[17]. Other EUS findings that are indicative of AIP, such as reduced echogenicity, hyperechoic foci and hyperechoic strands (Figure 1), are found in both AIP stages^[17]. Should these results be confirmed in prospective studies, EUS would acquire an essential role in the identification of early stage AIP, which is characterized by a prompt response to steroid therapy. Stones and cysts similar to those described in chronic alcoholic pancreatitis can occur in the late stage of AIP^[18].

The Sahai criteria for chronic pancreatitis were found

to be inadequate to evaluate parenchymal and ductal changes in AIP^[19]. According to the scoring system, a series of 25 patients with AIP were classified as normal or displaying mild disease^[16].

In the focal form of AIP a solitary (Figure 2), irregular hypoechoic mass, generally located in the head of the pancreas, is observed^[13-15]. In addition, upstream dilatation of the main pancreatic duct could be observed^[13,17]. In this setting, the overlap with EUS findings of pancreatic cancer is remarkable, and EUS-elastography (Figure 1) can provide further information about pancreatic lesions. In a case-control study of five patients with AIP, EUS-elastography showed a typical and homogeneous stiffness pattern of the focal lesions and of the surrounding parenchyma that is different from that observed in ductal adenocarcinoma^[20].

COMMON BILE DUCT FINDINGS

The common bile duct is the most frequent extrapancreatic organ involved in AIP and was found to affect 58% of patients in a Japanese survey^[12]. Biliary strictures can mimic both sclerosing cholangitis and biliary cancer. EUS allows visualization of the entire common bile duct and enables identification of the cause of a biliary stricture. In patients with either diffuse or focal AIP, EUS can show dilatation of the common bile duct and thickening of its wall better than other diagnostic techniques^[3,13-16,21]. The typical EUS feature of the common bile duct is a homogeneous, regular thickening of the bile duct wall, called “sandwich-pattern”, which is characterized by an echopoor intermediate layer and hyperechoic outer and inner layers, has been described as a EUS feature of the

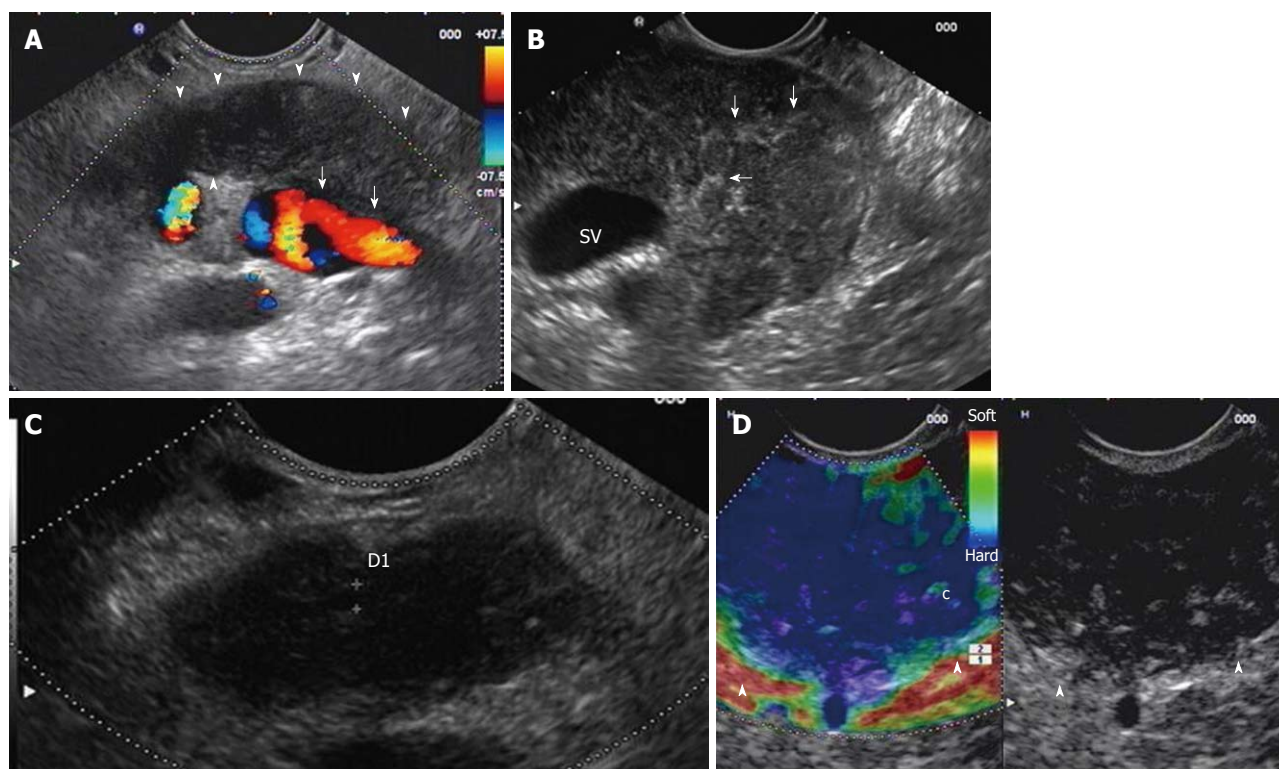


Figure 1 Diffuse form of autoimmune pancreatitis. A: Endoscopic ultrasonography (EUS) linear scanning shows a diffuse pancreatic enlargement (arrowheads) with echopoor echotexture, and with loss of interface with splenic vein (arrows); B: Parenchymal lobularity and hyperechoic strands (arrows) are visible in the enlarged gland; C: Pancreatic duct calliper is 1.8 mm; D: EUS-elastography demonstrates the diffuse pancreatic stiffness (arrowheads).

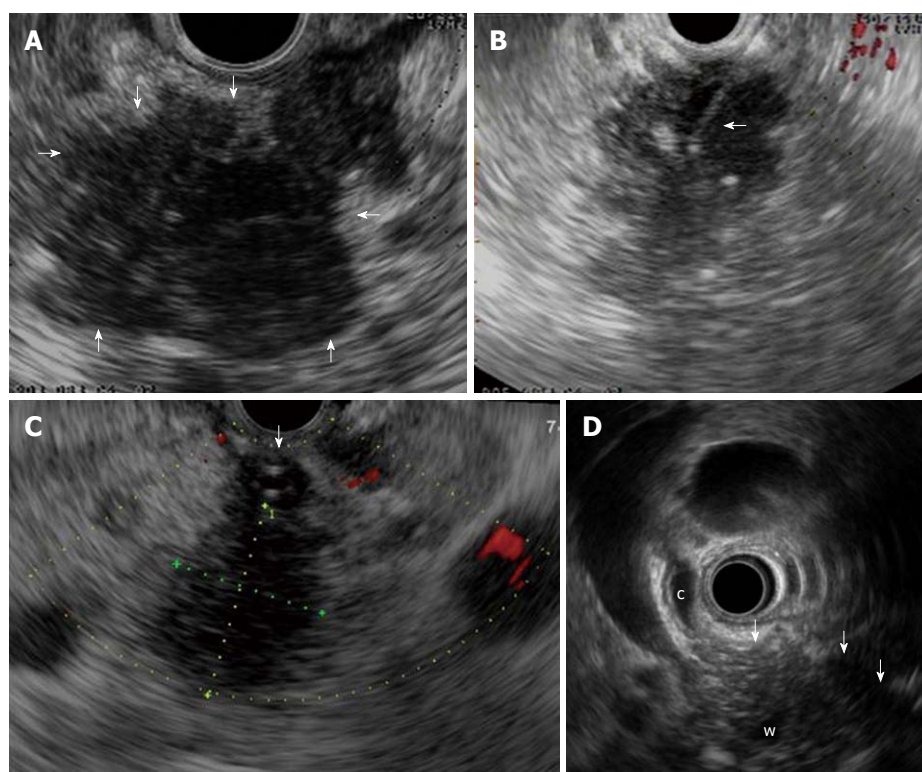


Figure 2 Focal form of autoimmune pancreatitis. A: Endoscopic ultrasonography (EUS) shows a focal lesion (arrows) of pancreatic head which is echopoor with hyperechoic strands; B: A EUS-guided fine needle aspiration is performed (arrow) for tissue characterization; C: Another case of focal autoimmune pancreatitis (AIP) with echopoor lesion of pancreatic head (between callipers) and marked echopoor thickening of the choledochal wall (arrow); D: In this case of focal AIP EUS shows a echopoor lesion (arrows) of pancreatic head, with upstream dilatation of both common bile duct (c) and pancreatic duct (w); notice the thickened choledochal wall.

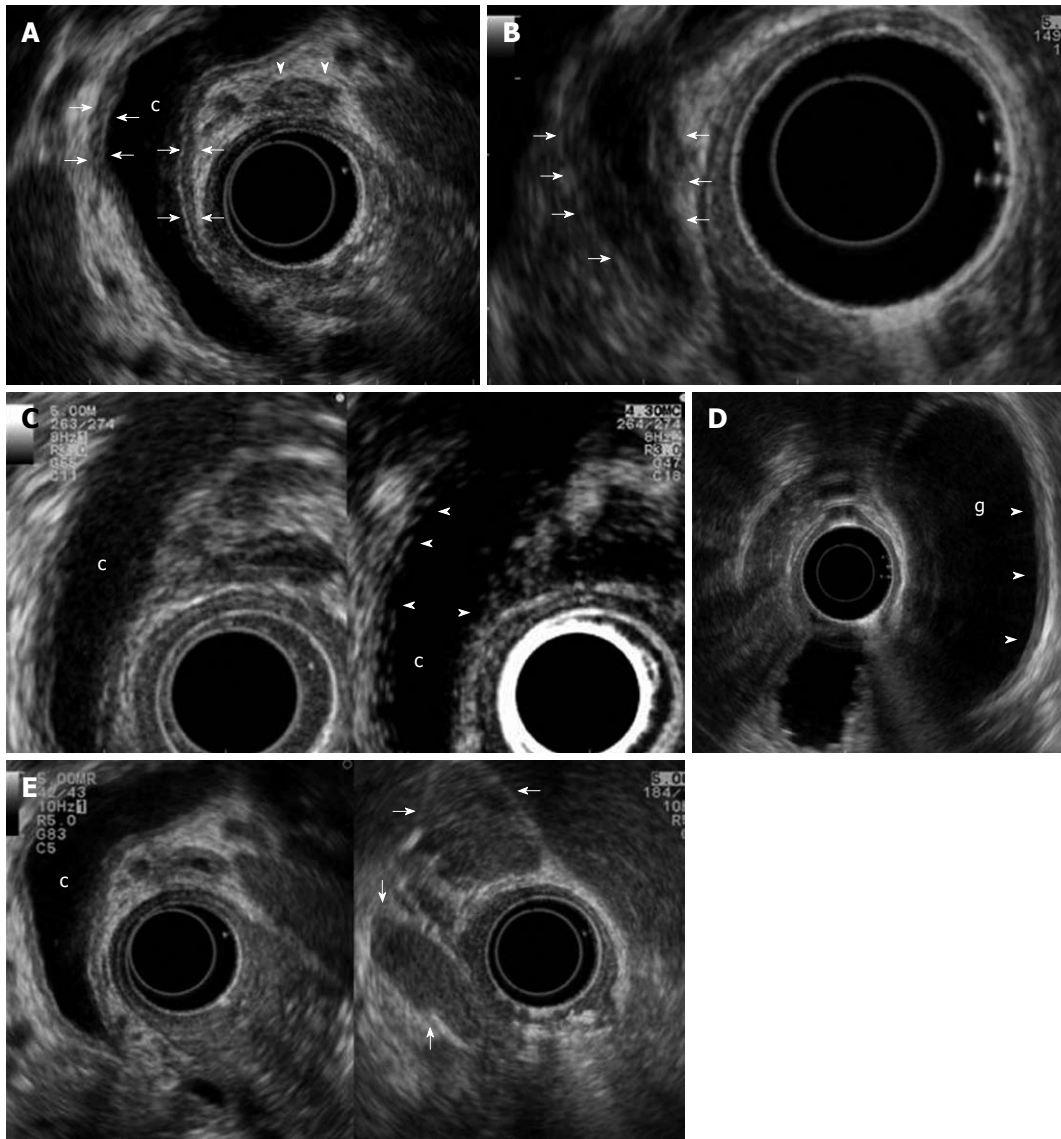


Figure 3 Biliary and peripancreatic findings in autoimmune pancreatitis. Autoimmune pancreatitis presenting with jaundice: A: Endoscopic ultrasonography (EUS) shows a dilated common bile duct (c) upstream to a distal funnel-shaped stenosis; EUS demonstrates the diffuse thickening of the biliary wall (between arrows) with "sandwich-pattern", either of common bile duct or of cystic duct (arrowheads). This thickening is equally visible both in the dilated region of the common bile duct; B: In the distal strictured tract (arrows); C: After contrast administration (Sonovue, Bracco) the biliary wall shows an early and persistent enhancement (arrowheads); D: EUS shows the same thickening of the gallbladder (g) wall (arrowheads); E: Enlarged lymph nodes to the hepatic hilum (arrows).

common bile duct; the cause of the biliary stricture is the thickened wall itself rather than extrinsic pancreatic compression (Figure 3)^[3,13].

A further application of EUS is intraductal ultrasonography (IDUS), which can be performed during endoscopic retrograde cholangiography for the characterization of biliary stenosis. Naitoh *et al*^[22] recently evaluated IDUS findings in 23 patients with IgG4-SC. They found that a circular, symmetric wall thickness, smooth inner and outer margins and a homogeneous intermediate layer in the stricture were significantly more common in AIP than in cholangiocarcinoma. The wall thickness in IgG4-SC in regions of non-stricture on the cholangiogram was significantly greater than that in cholangiocarcinoma and therefore a bile duct wall thickness exceeding 0.8 mm in regions of non-stricture on the cholangiogram was

highly suggestive of IgG4-SC. Contrast enhancement of conventional EUS and IDUS showed an inflammatory pattern of the bile duct wall, with a long-lasting enhancement starting in the early phase instead of the poor enhancement found in bile duct cancer (Figure 3)^[21].

PERIPANCREATIC FINDINGS

Hilar lymphadenopathy is one of the most frequently described extrapancreatic lesions^[12]. Other sites of enlarged lymph nodes are the peripancreatic and celiac regions. EUS nodal features that accurately predict nodal metastasis have been previously identified in patients with esophageal cancer^[23]; they include size (> 1 cm in diameter on the short axis), hypoechoic appearance, round shape, and smooth border. However, these conventional EUS cri-

teria have proven inaccurate for staging non-esophageal cancers, including those that are biliopancreatic^[24,25]. EUS can detect single or multiple enlarged lymph nodes in patients with AIP (Figure 3), reflecting the underlying inflammatory process, which can involve extra-pancreatic organs^[14,15].

Hoki *et al.*^[16] reported a significant difference in detection of lymphadenopathy by EUS imaging over CT (72% *vs* 8%) in patients with AIP. Moreover, in the same series, a trend toward a higher prevalence of lymphadenopathy in AIP compared to pancreatic cancer was reported.

In the absence of specific nodal features indicating malignancy, the differential diagnosis with biliopancreatic neoplasms is arduous but can be achieved by evaluating the broad spectrum of clinical and imaging data of AIP patients.

EUS criteria for vascular invasion of pancreatic cancer have been established^[26,27].

In a series of 14 patients with AIP, EUS suspected invasion of the portal or mesenteric veins in 21% of patients compared to 14% on CT. No pancreatic cancer developed during the follow-up of these patients. Such EUS features, easily mistaken for malignancy, are due to the inflammatory process of AIP, which can involve medium and large-sized vessels (Figure 1)^[15].

Peripancreatic fluid collections are less common and not specific for AIP.

INTERVENTIONAL EUS IN AIP

In recent years, the possibility of guiding tissue sampling with either fine needle aspiration (EUS-FNA) (Figure 2) or Tru-cut biopsy (EUS-TCB) has increased the diagnostic potential of EUS by the acquisition of cytological and histological specimens from gastrointestinal lesions^[28-30].

In the setting of AIP, EUS-FNA can be employed to yield specimens of pancreatic lesions, the common bile duct wall or lymph nodes^[13,15]. Although a cytologic pattern specific for AIP has not been identified, high cellularity of stromal fragments with lymphoplasmacytic infiltrate has emerged as a discriminating feature in a retrospective series of 16 patients with an AIP diagnosis confirmed by histology of the respective specimens. Indeed, 56% of AIP patients presented such a feature *vs* 19% of patients with pancreatic carcinoma, and none of the chronic pancreatitis controls exhibited this feature^[31]. Immunohistochemical staining can show IgG4-positive plasma cells which are a useful marker for the tissue diagnosis of AIP.

EUS-FNA can fail in diagnosis because of the small size of the specimens that do not have preserved tissue architecture. Moreover, sampling error due to the patchy distribution of AIP can occur. EUS-TCB can overcome these limitations by acquiring large samples fit for histological examination^[30-32].

A recent study compared EUS-FNA and EUS-TCB performed in 14 patients for the diagnosis of AIP. EUS-TCB showed higher sensitivity (100%) and specificity (100%) compared to EUS-FNA (36% and 33%, respec-

tively). Both procedures were found to be safe, with no complications^[33].

However, the diagnostic accuracy of EUS-FNA for pancreatic cancer has been reported to range between 60% and 90%^[34-36], and the shortcomings of EUS-TCB due to technical difficulties of the sampling of lesions in the pancreatic head should also be considered.

Hence, when AIP is suspected, a sequential sampling strategy has been proposed based on using EUS-FNA first, which is followed by EUS-TCB when cytologic examination is inconclusive^[33].

In cases of inconclusive cytology, an additional aid for AIP diagnosis could come from molecular analysis of EUS-FNA samples, which has shown high accuracy in the differential diagnosis between AIP and pancreatic cancer^[37].

CONCLUSION

AIP represents 20%-25% of benign diagnoses undergoing resection for presumed malignancy^[38,39]. Thus, a definitive diagnosis based on safe and reliable methods should be obtained. In this setting, EUS could play an important role in diagnosis, identifying typical features of AIP and distinguishing it from biliopancreatic neoplasms. The higher sensitivity over standard imaging for pancreatic, biliary and nodal lesions should make it a cornerstone in the process of diagnosing AIP. If validated in large prospective series, newly available techniques such as EUS-elastography and CE-EUS could add useful information about focal lesions without resorting to invasive procedures. Finally, EUS-FNA or EUS-TCB can provide pathological specimens, as required by some diagnostic criteria.

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Effects of α -mangostin on apoptosis induction of human colon cancer

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mitochondrial membrane potential. The molecular mechanisms of α -mangostin mediated apoptosis were further investigated by Western blotting analysis including activation of caspase cascade, cytochrome c release, Bax, Bid, p53 and Bcl-2 modifying factor.

RESULTS: The highest inhibitory effect of α -mangostin on cell proliferation of COLO 205, MIP-101 and SW 620 were $9.74 \pm 0.85 \mu\text{g/mL}$, $11.35 \pm 1.12 \mu\text{g/mL}$ and $19.6 \pm 1.53 \mu\text{g/mL}$, respectively. Further study showed that α -mangostin induced apoptotic cell death in COLO 205 cells as indicated by membrane blebbing, chromatin condensation, DNA fragmentation, cell cycle analysis, sub-G1 peak ($P < 0.05$) and phosphatidylserine exposure. The executioner caspase, caspase-3, the initiator caspase, caspase-8, and caspase-9 were expressed upon treatment with α -mangostin. Further studies of apoptotic proteins were determined by Western blotting analysis showing increased mitochondrial cytochrome c release, Bax, p53 and Bcl-2 as well as reduced mitochondrial membrane potential ($P < 0.05$). In addition, up-regulation of tBid and Fas were evident upon treatment with α -mangostin ($P < 0.01$).

CONCLUSION: α -Mangostin may be effective as an anti-cancer agent that induced apoptotic cell death in COLO 205 *via* a link between extrinsic and intrinsic pathways.

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Key words: α -mangostin; Apoptosis; Caspases; Colon cancer; Mitochondria

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Abstract

AIM: To investigate the effect of α -mangostin on the growth and apoptosis induction of human colon cancer cells.

METHODS: The three colorectal adenocarcinoma cell lines tested (COLO 205, MIP-101 and SW 620) were treated with α -mangostin to determine the effect on cell proliferation by MTT assay, cell morphology, chromatin condensation, cell cycle analysis, DNA fragmentation, phosphatidylserine exposure and changing of

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INTRODUCTION

Searching for new biologically active compounds, novel chemotherapeutic agents derived from active phytochemicals, could be used to improve the anti-carcinogenicity of standard drug treatment. A variety of tropical plants have useful biological activities and some offer potential therapeutic applications. Mangosteen (*Garcinia mangostana* L.) in the Clusiaceae family has been used in Southeast Asia as traditional medicine for treatment of wounds, skin infection, diarrhea and chronic ulcer^[1]. Phytochemical studies showed that the fruit hull of mangosteen is rich in a variety of oxygenated and prenylated xanthenes^[2,3] which possess different biological properties, such as anti-mycobacterial^[4], anti-fungal^[5], anti-oxidant^[6-8], cytotoxicity^[9-12] and anti-inflammatory activities^[13]. However, the underlying molecular mechanisms of α -mangostin in COLO 205 cells are not yet reported.

Apoptosis plays a vital role in controlling cell number in many physiological and developmental stages, tissue homeostasis, and regulation of immune system^[14], while insufficient apoptosis is an integral part of cancer development^[15]. Mammalian cells have two major apoptotic pathways. One pathway (extrinsic pathway) is triggered when ligands [Fas/CD95, tumor necrosis factor (TNF)- α] bind to receptors on cell surface leading to the activation of caspase-8 and -3^[16], respectively. The other involves mitochondrial (intrinsic) pathway induced by anti-cancer drugs, prostaglandin, *etc.* resulting in disruption of mitochondrial membrane and release of various pro-apoptotic factors^[17-19]. The pro-apoptotic and anti-apoptotic members of B-cell CLL/lymphoma 2 (Bcl-2) family regulate the release of cytochrome c, a mitochondrial protein that can activate caspases.

Fas/CD95 belongs to the TNF superfamily and is the prototype of death receptor that initiates an apoptotic cascade^[20]. FasL, the tumor necrosis factor-related cytokine, is a ligand of Fas and synthesized as a type II membrane protein^[21]. Upon FasL binding, activated death receptors engage the Fas associated death domain (FADD)^[22], which in turn recruits caspase-8 and forming the death inducing signaling complex (DISC). The DISC then activates caspase-8 through a proximity-inducing dimerization mechanism. In type I cells, caspase-8 directly activates caspase-3, -6 and -7, leading to cell death. In contrast, in type II cells the small amount of active caspase-8 generated at the DISC is not sufficient to induce cell death, therefore the mitochondria-dependent apoptosis pathway is needed^[23]. As such, the pro-apoptotic signal has to be amplified *via* cleavage of the BH3-only protein Bid, Bax/Bak-assisted release of cytochrome c from the mitochondria, an activation of caspase-9 and subsequently caspase-3^[24].

The objective of the present study was to purify the α -mangostin from the fruit hull of *Garcinia mangostana* L. and explore its effect on apoptosis induction and mechanisms involved in COLO 205 cells.

MATERIALS AND METHODS

α -mangostin preparation

Mangosteen fruit (*G. mangostana*) was collected from Kambang District, Chantaburi Province, Thailand in April, 2007. A voucher specimen (Ms Porntip Wongnapa No. 002) was deposited at the Faculty of Science, Ramkhamhaeng University. The dried and pulverized fruit hull of *G. mangostana* (0.5 kg) was thoroughly extracted with ethyl acetate (EtOAc) at 50°C. The combined extract after filtration was concentrated under reduced pressure to yield the extract as a yellowish solid (285 g). A portion of the extract was subjected to repeated column chromatography over silica gel using a gradient of hexane/acetone which yielded the pure major compound, α -mangostin, including other minor xanthenes. Purity of α -mangostin exceeded 98% as determined by LC analysis and its spectroscopic data (NMR and MS) was consistent with the reported values^[12].

Cell lines and culture conditions

Three human colorectal cancer cell lines were used: COLO 205 (colorectal adenocarcinoma), MIP-101 (colorectal carcinoma) and SW620 (colorectal adenocarcinoma). COLO 205 and SW620 were obtained from the American Type Culture Collection (Manassas, VA). MIP-101 was a generous gift from Peter Thomas, Boston University School of Medicine, Boston, MA. COLO 205 and MIP-101 were maintained in the RPMI 1640 medium (Invitrogen), supplemented with 10% fetal calf serum (Invitrogen). SW620 were cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal calf serum. All cell lines were maintained in culture at 37°C in an atmosphere of 5% CO₂.

Cell proliferation and cell viability assays

The cytotoxic activity of α -mangostin was determined by cell proliferation analysis using MTT assays as previously described^[25]. Briefly, cells were cultured in 96-well plates at a density of 1×10^4 /well in complete medium. Then the cells were treated with varying concentrations of α -mangostin and incubated at 37°C for 24 h. The final DMSO concentration in each well was 0.05%, at which concentration no appreciable effect on cell proliferation was seen. Then, 100 μ L of 5.0 mg/mL MTT in culture media was added to each well and incubated at 37°C for 2 h. The metabolic product of MTT, formazan, in each well was dissolved in DMSO, and the absorbance was determined at 595 nm. Effect of α -mangostin on the viability of COLO 205 cells was analyzed by using a trypan blue exclusion method. Briefly, cells were cultured in 96-well plates at cell density of 1×10^4 /well at 37°C for 24 h. α -mangostin was then added to culture wells at 0, 10, 20,

30 and 40 $\mu\text{g/mL}$ or vehicle (in DMSO), incubated at 37°C then collected periodically (0, 3, 6, 9 and 12 h). The number of viable cells was determined with hemocytometers under a light microscope. Cell viability was expressed as a percentage of the number of viable cells to that of the control, to which no α -mangostin was applied.

Apoptosis assay

Characterization of the anticancer activity of α -mangostin in COLO 205 cells was further conducted. Based on the preliminary experiments, 20 $\mu\text{g/mL}$ of α -mangostin was used to study cell and nuclear morphology, cytochrome c release, mitochondrial transmembrane potential, expression of pro-apoptotic proteins, Fas and truncated-Bid (*t*-Bid). However, a selective range of test concentrations, ranging from 10 to 30 $\mu\text{g/mL}$ was used to study DNA fragmentation and for cell cycle analysis and annexin V-FITC assays.

Microscopic analysis of cell and nuclear morphology

COLO 205 cells were cultured in 24-well culture plates at the initial number of 2×10^4 /well in the presence of 20 $\mu\text{g/mL}$ α -mangostin for 3, 6, 9 and 12 h. As controls, cells were cultured in the same fashion in the absence of α -mangostin. The cells were examined under a phase-contrast inverted microscope (model CKX31/CKX41, Olympus) for cell morphology.

The nuclear morphology was analyzed by treatment of COLO 205 cells with 20 $\mu\text{g/mL}$ α -mangostin for 3, 6, 9 and 12 h. Control cells were grown in the same manner in the absence of α -mangostin. Cells were trypsinized and fixed with methanol. Then, cell nuclei were stained by treatment with 1 $\mu\text{g/mL}$ Hoechst 33342 (Sigma) at 37°C for 15 min in the dark. Stained cells were examined under a fluorescence inverted microscope (model BX50, Olympus).

Analysis of DNA fragmentation

COLO 205 cells were treated with varying concentrations, 10, 20 and 30 $\mu\text{g/mL}$, of α -mangostin for 12 h and then lysed in 500 μL of lysis solution, consisting of 5 mmol/L Tris-Cl (pH 8.0), 0.5% Triton X-100, and 20 mmol/L ethylenediaminetetraacetic acid (EDTA). The cells were then treated with RNase A (0.5 mg/mL) for 1 h at 37°C. DNA fractions were prepared using phenol-chloroform-isoamyl alcohol (25:24:1) and electrophoresed on 1.8% agarose gels. Approximately 20 μg of DNA was loaded in each well and the agarose gels were run at 50 V for 2 h in Tris-borate/EDTA electrophoresis buffer. DNA was stained with ethidium bromide and visualized under a UV light trans-illuminator and photographed.

Flow cytometer analysis

The cell cycle analysis, annexin V binding and mitochondrial transmembrane potential were investigated by flow cytometric analysis (FACScan, Becton Dickinson). The proper filters and optimal setting of the instrument were chosen, the histograms generated by FACS were analyzed by Cell Quest™ software (Becton Dickinson).

Cell cycle analysis using flow cytometer

COLO 205 cells were cultured in 6-well culture plates at 4×10^6 cells/well and treated with 0, 10, 20 and 30 $\mu\text{g/mL}$ of α -mangostin for 3 h. The cells were then harvested, washed with PBS and resuspended in 200 μL PBS and fixed in 800 μL of ice-cold 70% ethanol at -20°C, overnight. The cells were stained with 1 mL of 50 $\mu\text{g/mL}$ propidium iodide solution (containing 0.1% Triton X-100, and 0.1% sodium citrate) for 30 min at 37°C. The samples were then analyzed by a flow cytometer (FACScan, Becton Dickinson). Excitation was done at 488 nm, and emission filter at 600 nm. Histograms generated by FACS were analyzed by Cell Quest™ software (Becton Dickinson) to determine the percentage of cells in each phase.

Annexin V-FITC assay

Percentage of α -mangostin-treated cells undergoing apoptosis was determined using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD) Bioscience, San Jose, CA). COLO 205 cells at 1×10^5 cells/mL were treated with 0, 10, 20 and 30 $\mu\text{g/mL}$ of α -mangostin for 3 h and resuspended in 100 μL of annexin V binding buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl_2 , 1.8 mmol/L CaCl_2), then incubated with 5 μL of 1 $\mu\text{g/mL}$ FITC-conjugated annexin V and 1 $\mu\text{g/mL}$ propidium iodide for 15 min at room temperature prior to analysis on a FACScan, Becton Dickinson.

Analysis of mitochondrial transmembrane potential

COLO 205 cells at 1×10^6 cells/mL were treated with 20 $\mu\text{g/mL}$ of α -mangostin for 3 h, and then incubated with 10 $\mu\text{g/mL}$ JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) at 37°C for 10 min in darkness. Stained cells were washed with PBS, followed by FACS analysis. The mitochondrial function was assessed as JC-1 green (uncoupled mitochondria) or red (contact mitochondria).

Western blotting analysis of cytochrome c

COLO 205 cells were cultured in 6-well culture plates at 4×10^6 cells/well and then incubated in the absence or presence of 20 $\mu\text{g/mL}$ α -mangostin for 3, 6, and 9 h. Cell lysates was prepared as previously described^[25]. Briefly, cell suspensions were sonicated for 10 s and the cell lysates was centrifuged at 4°C at $10000 \times g$ for 30 min. The supernatants (cytosol fractions) were subjected to SDS-PAGE using 12% polyacrylamide gels, and transferred onto Immobilon P membrane and subjected to immuno-detection of cytochrome c using a mouse monoclonal antibody against human cytochrome c (7H8, mouse monoclonal Ig G 2b, Santa Cruz, Biotechnology) with goat anti-mouse IgG conjugates to horse radish peroxidase (Cell Signaling Technology) and detected using an ECL Plus Western Blotting Detection System (Amersham Biosciences).

Western blotting analysis of caspases, Bid, p53, Bax, Bmf and Fas

COLO 205 cells were treated with 20 $\mu\text{g/mL}$ α -mangostin

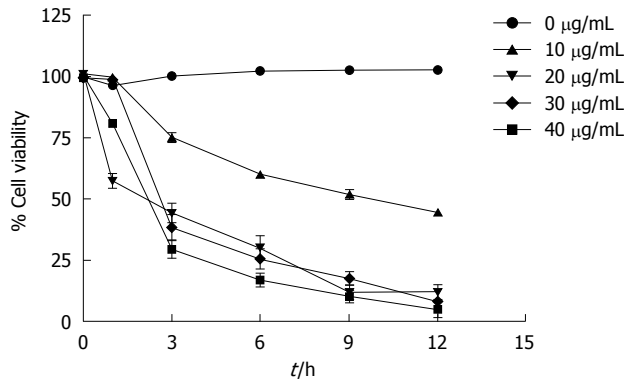


Figure 1 Effect of α -mangostin on the viability of COLO 205 cells. Different concentrations of α -mangostin (0–40 $\mu\text{g/mL}$) at different incubation times were studied. Cell viability is expressed as a percentage of the number of viable cells to that of the control, to which no α -mangostin was applied. Each data point shown is the mean \pm SD from three independent experiments.

for 3 h, lysed in lysis buffer. Cell lysates were subjected to SDS-PAGE using 12% Tris/HCl ready gels (BioRad). The transferred proteins were incubated with appropriate antibodies at 4°C overnight: rabbit polyclonal anti-caspase-3 (8G10); mouse polyclonal anti-caspase-8 (1C12); mouse monoclonal anti-caspase-9 (C9); rabbit polyclonal anti-Bid; mouse monoclonal anti-p53; rabbit polyclonal anti-Bax, anti-Bmf (Cell Signaling Technology) and mouse monoclonal anti-Fas (CD 95) (CH 11, MBL international). After the removal of unbound primary antibodies, the blots were incubated with a secondary antibody (goat anti-rabbit IgG and goat anti-mouse IgG, each of which was conjugated with horse radish peroxidase; (Cell Signaling Technology) as described for Western blotting analysis of cytochrome c above.

Statistical analysis

Data were expressed as mean \pm SD. Statistical comparisons were performed by using one-way analysis of variance. A *P* value less than 0.05 was considered statistically significance.

RESULTS

Cell growth inhibition by α -mangostin

The IC_{50} value of the three human colonic cancer cell lines, after 24 h incubation with serial dilutions of α -mangostin, COLO 205, MIP-101 and SW 620 were $9.74 \pm 0.85 \mu\text{g/mL}$, $11.35 \pm 1.12 \mu\text{g/mL}$ and $19.6 \pm 1.53 \mu\text{g/mL}$, respectively. Among the three cell lines tested, the highest inhibitory effect of α -mangostin on cell proliferation was detected with COLO 205. Thus, COLO 205 cells were used as the primary target in subsequent experiments.

The viability of COLO 205 cells decreased by the treatment with α -mangostin in both concentration- and time-dependent fashions (Figure 1). Treatment of COLO 205 cells with α -mangostin at 20 $\mu\text{g/mL}$ or higher for 12 h reduced the number of viable cells to approximately 5%–10% of the control cells, to which no α -mangostin was applied.

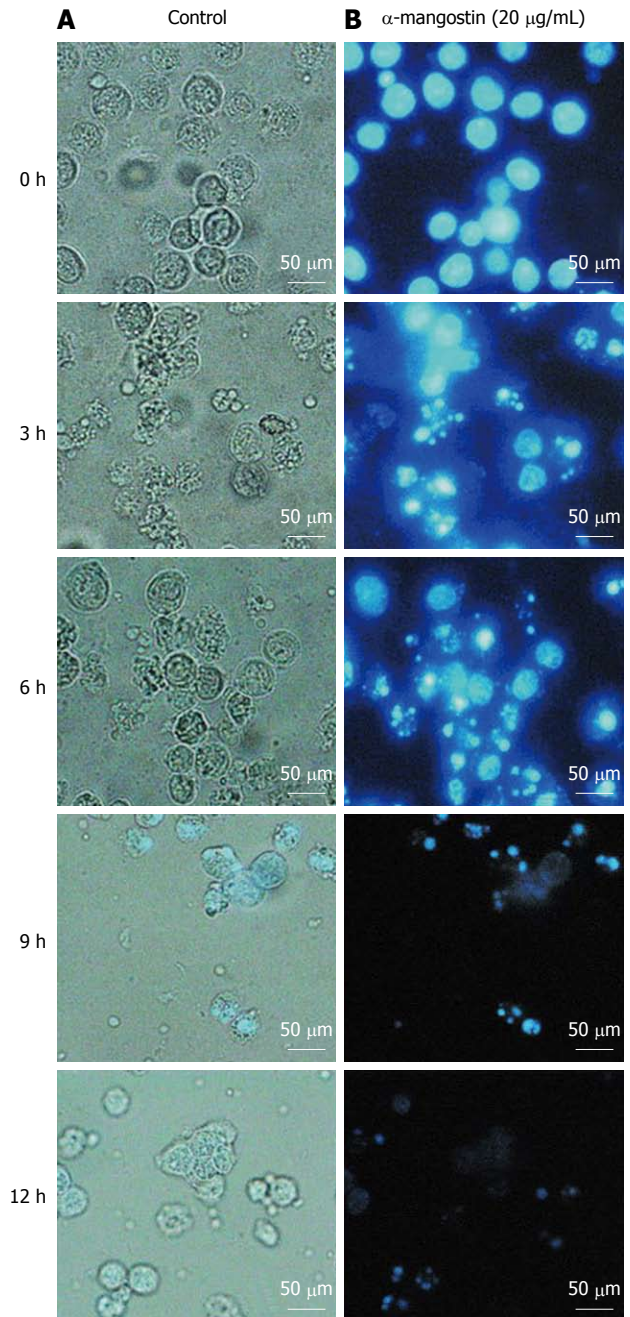


Figure 2 Effect of α -mangostin on the cell morphology and nuclear condensation of COLO 205 cells. A: Untreated control cells were examined for cell morphology and Hoechst 33342 stained cells were examined for nuclei morphology; B: Cells were cultured for 0, 3, 6, 9 and 12 h in the presence of 20 $\mu\text{g/mL}$ α -mangostin.

Morphological and nuclei changes

Cells treated with 20 $\mu\text{g/mL}$ α -mangostin for 3, 6, 9 and 12 h showed evident morphological changes including rounding and blebbing as well as the presence of apoptotic bodies (Figure 2A, 3, 6, 9 and 12 h). Such morphological changes were not seen with control cells (without the α -mangostin treatment) (Figure 2A, 0 h). For nuclei staining with Hoechst 33342, chromatin condensation and destructive fragmentation of the nucleus with intact cell membrane was seen with COLO 205 cells which had been

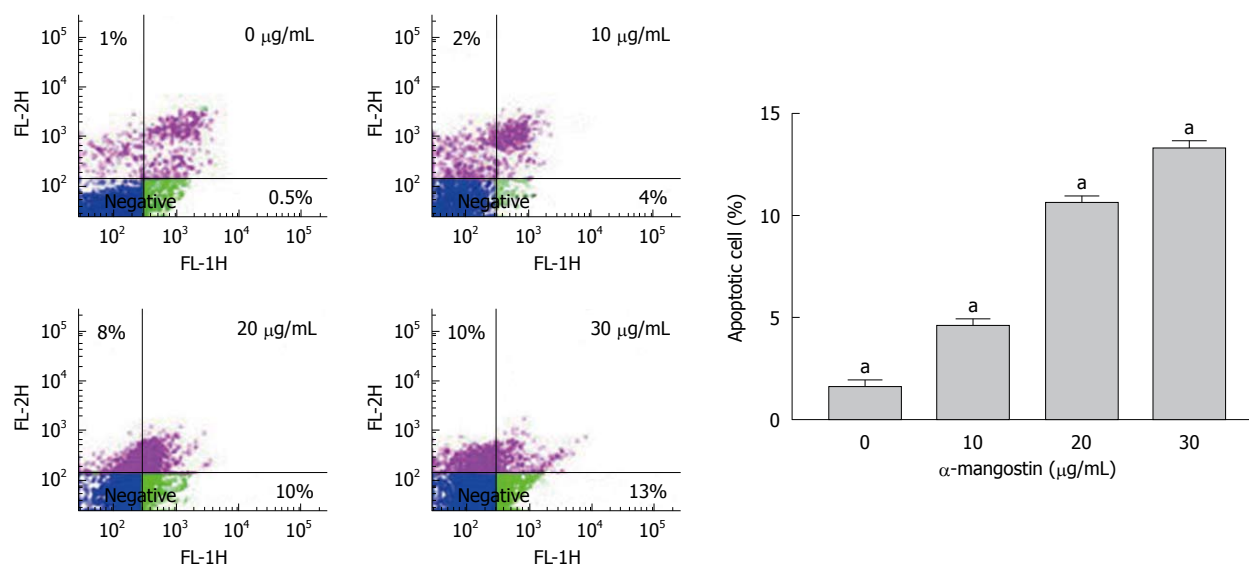


Figure 3 Fluorescent-activated cell sorter analysis of COLO 205 cells stained with Annexin V-FITC. Cells were treated with 10, 20 and 30 µg/mL α-mangostin for 3 h. α-mangostin induced early apoptosis in a concentration dependent manner. The values are expressed as mean ± SD; ^a $P < 0.05$.

treated with 20 µg/mL of α-mangostin for 3, 6, 9 and 12 h (Figure 2B, 3, 6, 9 and 12 h), indicated early apoptosis while the nuclei of control cells without α-mangostin treatment showed normal morphology (Figure 2B, 0 h).

α-mangostin mediated apoptotic cell death

The early apoptosis was detected upon treatment of COLO 205 cells with 0, 10, 20 and 30 µg/mL of α-mangostin for 3 h using Annexin V-FITC assay. The results indicated significant increases in apoptotic populations in COLO 205 cells approximately $0.50\% \pm 0.02\%$, $4.00\% \pm 0.25\%$, $10.00\% \pm 0.50\%$ and $13.00\% \pm 0.30\%$, ($P < 0.05$) respectively (Figure 3). Exposure of COLO 205 cells to increasing concentrations (0, 10, 20 and 30 µg/mL) of α-mangostin for 3 h resulted in increased percentage of cells arrested in sub G-1 phase (apoptotic cell death) of $1.03\% \pm 0.15\%$, $7.00\% \pm 0.20\%$, $51.00\% \pm 1.53\%$ and $80.00\% \pm 2.08\%$ ($P < 0.05$), respectively (Figure 4). It is evident that the formation of apoptotic cells at sub-G1 phase was directly proportional to the increased concentration of α-mangostin.

Fragmentation of chromosomal DNA

Fragmentation of genomic DNA was apparent by the presence of DNA ladders when COLO 205 cells were treated with 10, 20 and 30 µg/mL α-mangostin for 12 h (Figure 5). The characteristic ladder pattern of discontinuous DNA fragments was observed only in the treated cells, whereas the untreated control showed no DNA fragmentation.

Activation of caspases upon α-mangostin treatment

The activation of caspases-3, -8 and -9 was detected (Figure 6). The amount of the pro-enzyme form of caspase-3 (pro-caspase-3, 35 kDa), pro-caspase-8 (57 kDa) and pro-caspase-9 (47 kDa) decreased with increasing concentration of α-mangostin (10, 20, 30 and 40 µg/mL).

Accordingly, cleaved activated forms of caspase-3 (19 and 20 kDa), caspase-8 (41 and 43 kDa) and caspase-9 (35 and 37 kDa) ($P < 0.01$) became apparent upon treatment of α-mangostin at 20 µg/mL or higher. Activated caspase-3 was apparent upon treatment with 20 µg/mL α-mangostin but not at higher concentrations (30 and 40 µg/mL α-mangostin) due to cell death at higher concentrations, as the effector caspase-3 is the late event of the pathways. On the other hand, caspase-8 and -9 were also detected implying the consecutive activation of caspases of the intrinsic pathway. In addition, induction of apoptosis by α-mangostin was accompanied by increased phospho-p53, pro-apoptotic Bax and Bmf ($P < 0.01$) (Figure 7). The release of cytochrome c from mitochondria to cytosol was evident upon treatment of COLO 205 cells with 20 µg/mL α-mangostin for 3 h and 6 h (Figure 8). The non-cytosolic fraction (pellet) of the treated cells showed no cytochrome c expression (data not shown). At 9 h of α-mangostin treatment, cytochrome c was reduced due to increasing cell death. No appreciable amount of cytochrome c was detected in the cytosol fraction of control COLO 205 cells, to which no α-mangostin was applied (Figure 8). This implied that α-mangostin mediated apoptosis is accompanied by mitochondrial dysfunction, which could be further strengthened by mitochondrial membrane depolarization detected by a carbocyanine fluorescence dye, JC-1, upon treatment with α-mangostin. The results indicated the increased percentage of cells with depolarized mitochondrial membrane potential (red to green) approximately 82% after α-mangostin treatment for 3 h (Figure 9). These results further confirmed that α-mangostin is an efficient inducer of apoptosis that both extrinsic and intrinsic pathway may be involved. Thus, we further conducted the Western blotting analysis of Bid, t-Bid, the linker between extrinsic and intrinsic pathway, and Fas receptor and found that they were up-regulated upon α-mangostin treatment ($P < 0.01$) (Figure 7).

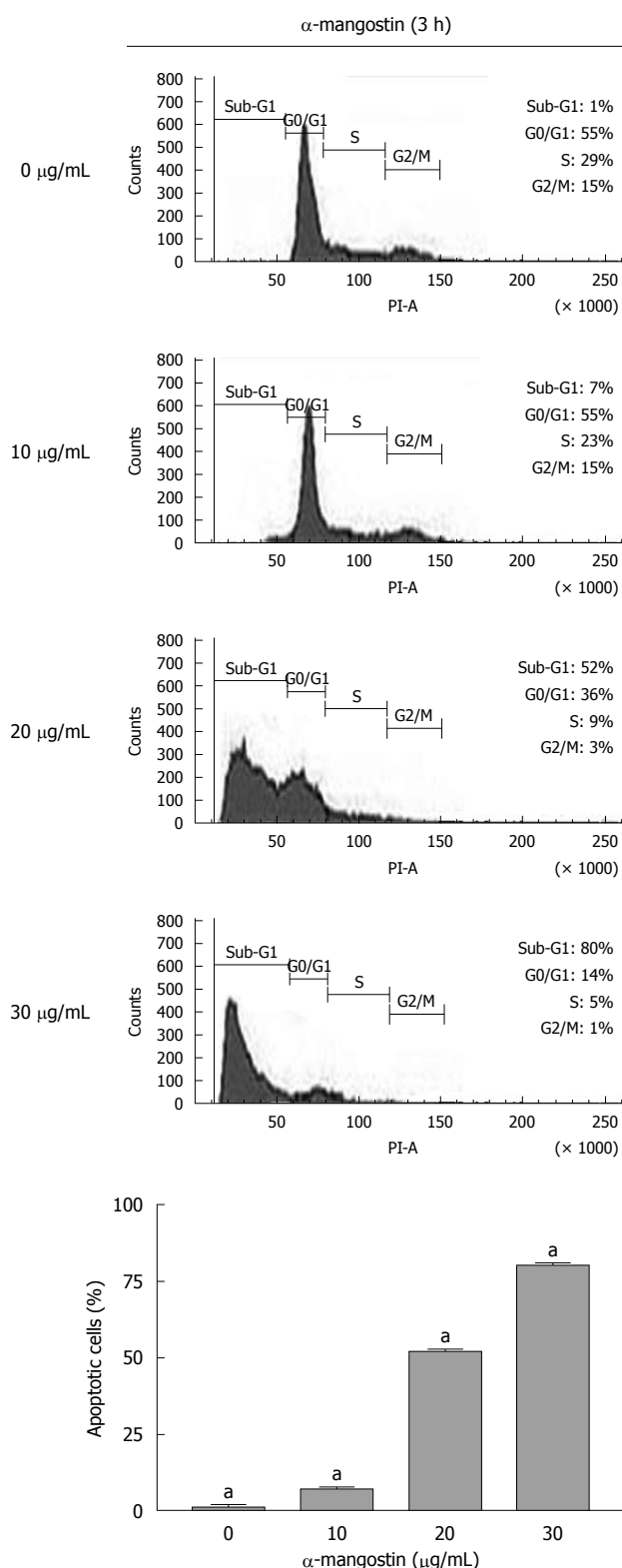


Figure 4 Flow analysis of cell cycle. Representative plots of PI staining of COLO 205 cells that were treated with 0 (control), 10, 20 and 30 µg/mL α-mangostin for 3 h. The values are expressed as mean ± SD; **P* < 0.05.

DISCUSSION

The evident goal of medical research is to be able to manipulate the machinery of cell death. Regulation of apoptosis might also lead to new possibilities for cancer ther-

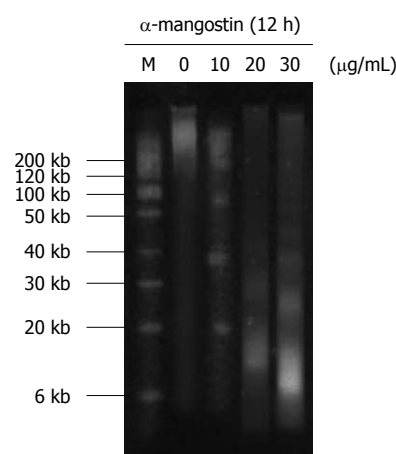


Figure 5 Analysis of DNA integrity in COLO 205 cells upon treatment with α-mangostin. COLO 205 cells were treated with varying concentrations of α-mangostin (10, 20 and 30 µg/mL) for 12 h. Cells were lysed, followed by phenol-chloroform extraction. DNA fractions were then electrophoresed on 1.8% agarose gels. DNA was stained with ethidium bromide and visualized under a UV light trans-illuminator.

apy^[26]. In the present study, we showed that α-mangostin treatment of human colon COLO 205 induced cytotoxic effects in a dose and time dependent fashion. We then investigated the apoptotic effects of α-mangostin in COLO 205 cells to advance our knowledge of its biological functions and also health advantages. The membrane shrinkage, chromatin condensation and fragmentation were detected. As cancer growth is associated with the loss of cell cycle checkpoints, which regulate the DNA integrity and ensure that the genes are co-ordinately expressed^[27]. The sub-G1 fraction and phosphatidylserine translocation is an indication of apoptosis cell death that naturally occurs in cells and is beneficial for cancer therapy^[28]. Therefore, to characterize apoptotic cells upon treatment of COLO 205 cells with α-mangostin, a bi-parametric cytofluorimetric analysis was performed using PI and annexin V-FITC, which stained DNA and phosphatidylserine residues, respectively. In the early apoptotic process, a phosphatidylserine residue became exposed on the cell surface by flipping from the inner to outer leaflet of the cytoplasmic membrane^[29,30]. Our results demonstrated the increased sub-G1 population and numbers of early apoptotic cells upon treatment of COLO 205 cells with 20 µg/mL α-mangostin for 3 h as compared to untreated control. The Bcl-2 family of proteins regulates apoptosis and it has been shown that the gene products of Bcl-2 and Bax play important roles in apoptotic cell death^[14]. The Bcl-2 family comprises of both pro-apoptotic and anti-apoptotic proteins that elicit opposite effects on mitochondria. Anti-apoptotic members include Bcl-2, Bcl-xL, Bcl-W, Mcl-1, whereas pro-apoptotic members are Bid, Bax, Bakm, Bmf and others. Several pathways involve p53-mediated apoptosis, and one of these is the Bcl-2 and Bax proteins. The Bax protein is a p53 target and known to promote cytochrome c release from mitochondria which in turn activates caspase-3. Regulation of Bax/Bcl-2 and caspases activity becomes impor-

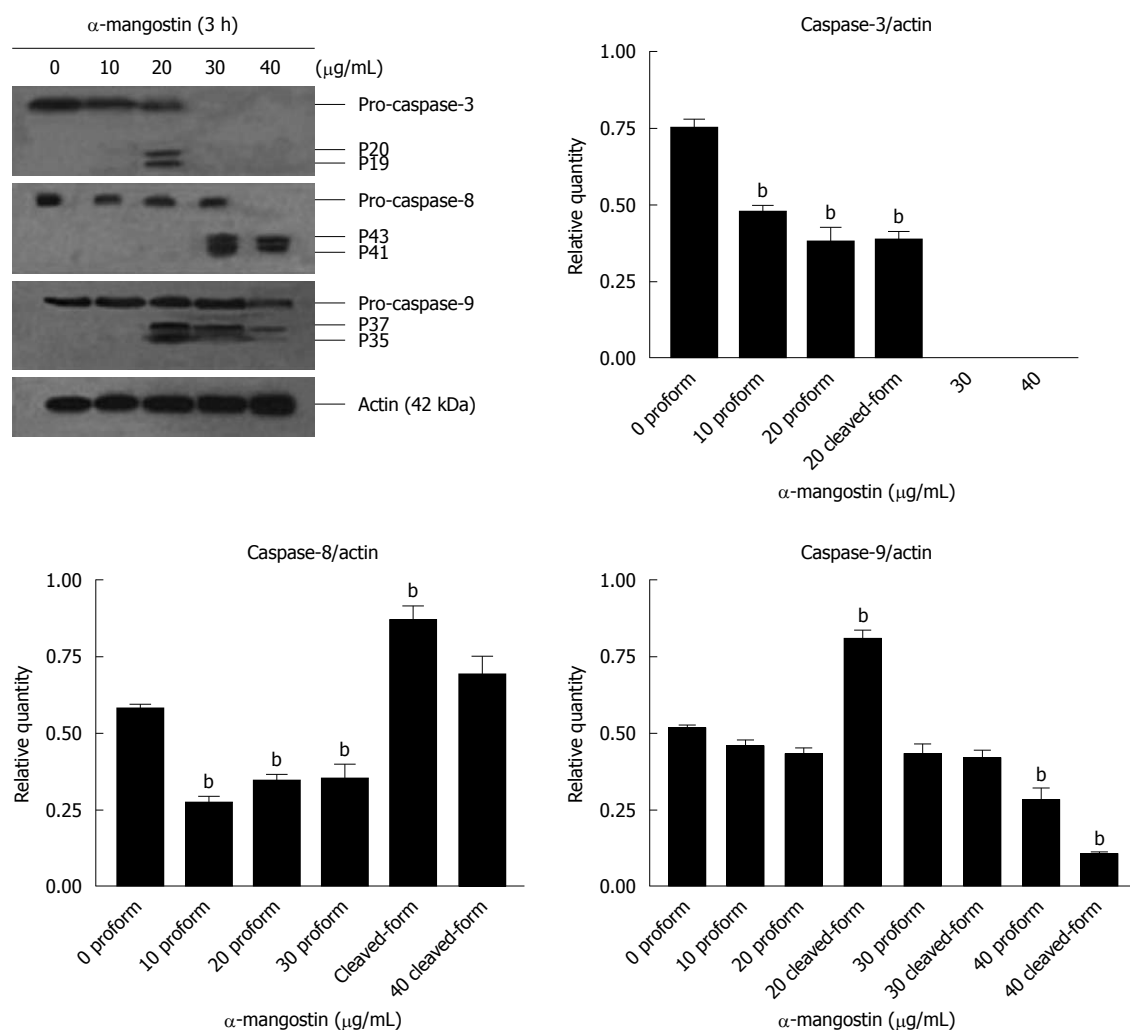


Figure 6 Effects of α -mangostin on the activation of caspase-3, -8 and -9 in COLO 205 cells. Cells were treated with 10, 20, 30 and 40 μ g/mL α -mangostin for 3 h. Cell lysates were separated by SDS-PAGE using 12% polyacrylamide gels. Proteins were subjected to immuno-detection of caspases-3, -8, and -9 using appropriate anti-caspase antibodies at 4°C. The expression of cleaved-caspase-3, -8 and -9 were detected. The density of each band was determined, equal protein loading was verified by β -actin staining. The values are expressed as mean \pm SD; ^b $P < 0.01$.

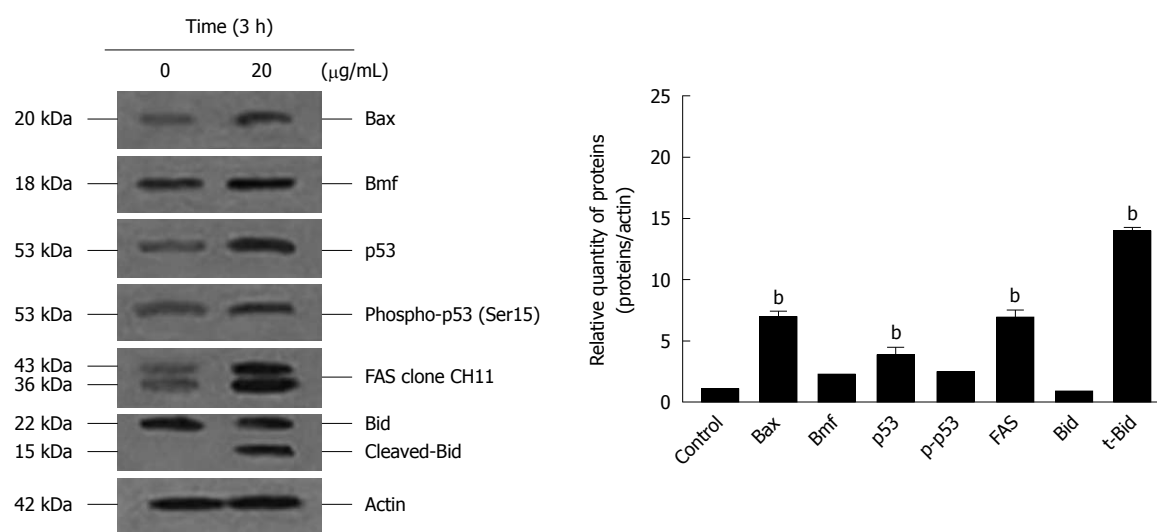


Figure 7 Effects of α -mangostin on the activation of Bax, Bmf, p53, Fas and Bid. Cells were treated with 20 μ g/mL α -mangostin for 3 h. Cell lysates were separated by SDS-PAGE using 12% polyacrylamide gels. Proteins were subjected to immuno-detection of Bax, Bmf, p53, p-p53, Fas, Bid and t-Bid using appropriate antibodies. The density of each band was determined, equal protein loading was verified by β -actin staining. The values are expressed as mean \pm SD; ^b $P < 0.01$.

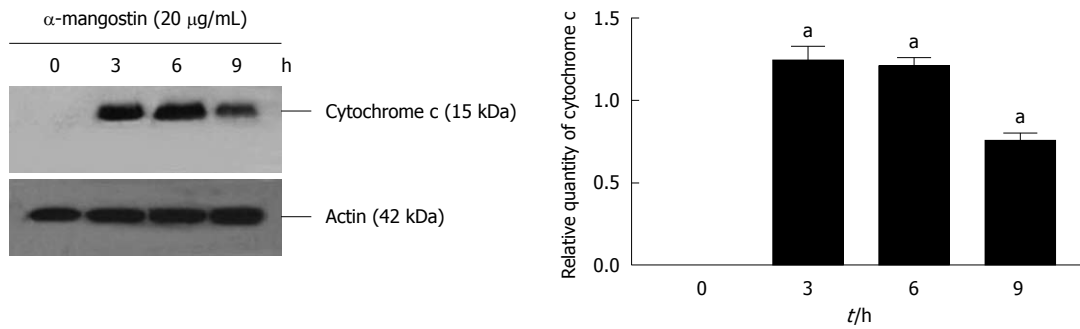


Figure 8 Release of cytochrome c from the mitochondria in COLO 205 cells. Upon treatment with 20 µg/mL α-mangostin for 3, 6 and 9 h, cytosol fractions were prepared from these cells and separated by SDS-PAGE using 12% polyacrylamide gels. Proteins were subjected to immuno-detection of cytochrome c using a mouse monoclonal antibody against human cytochrome c. The density of each band was determined, equal protein loading was verified by β-actin staining. The values are expressed as mean ± SD; ^a*P* < 0.05.

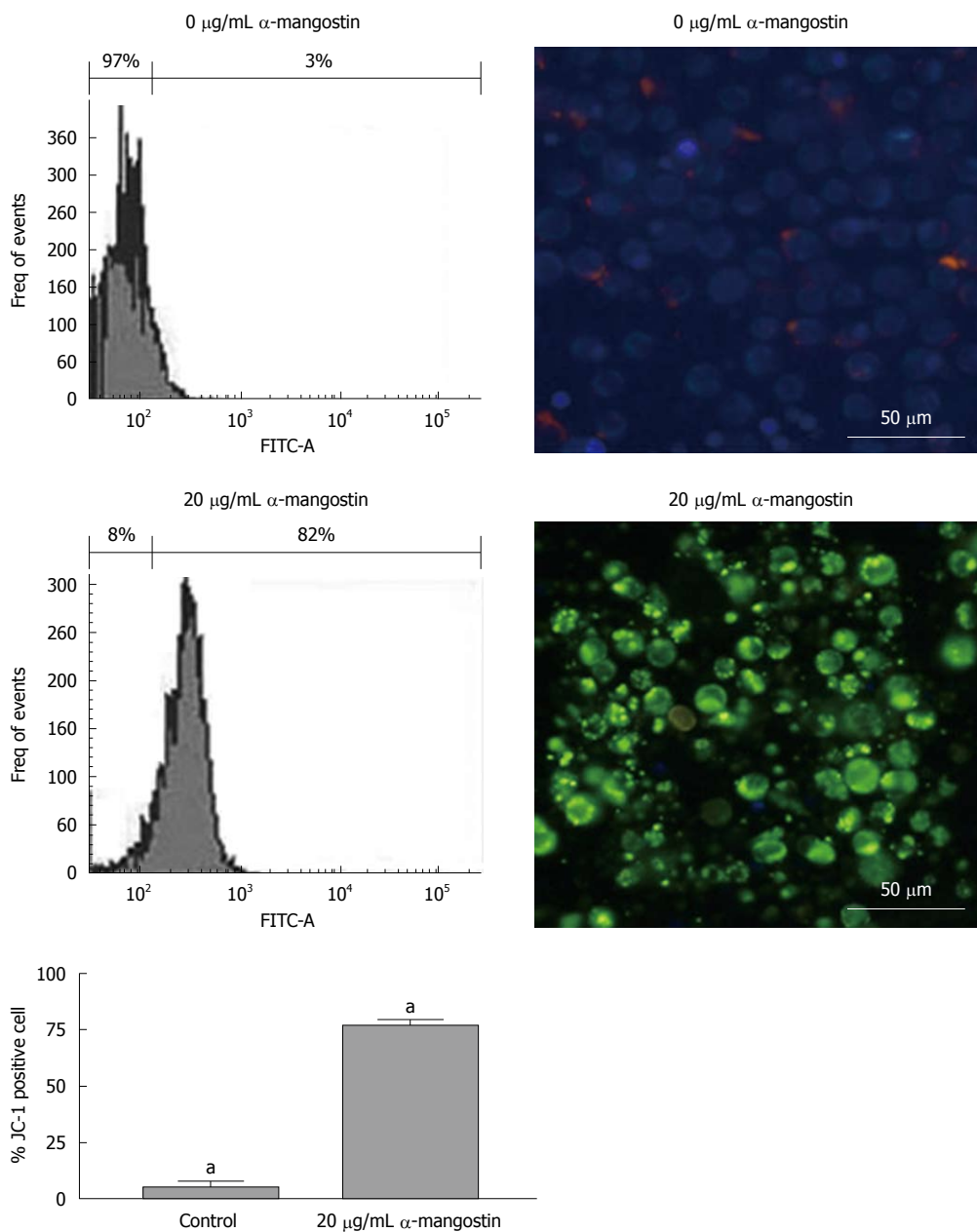


Figure 9 Measurements of mitochondrial membrane depolarization in COLO 205 cells. Cells were treated with 0 (control) and 20 µg/mL α-mangostin for 3h and then incubated with JC-1 (10 µg/mL in PBS) at 37°C for 10 min. Stained cells were subjected to FACS analysis. The mitochondrial function was assessed as JC-1 green. The values are expressed as mean ± SD; ^a*P* < 0.05.

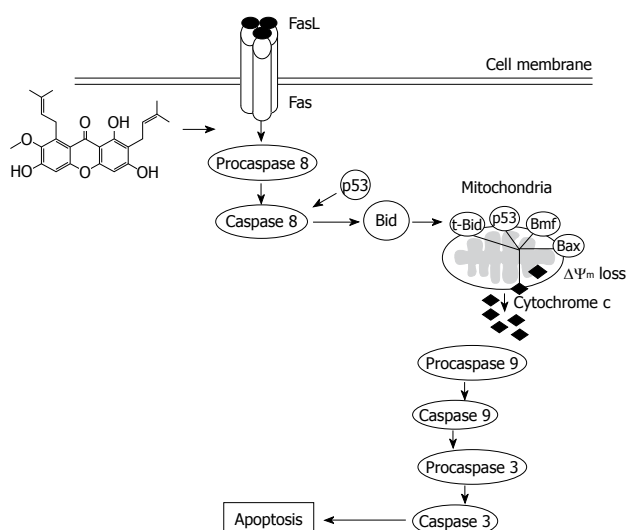


Figure 10 A proposed diagram for α -mangostin-induced apoptosis in COLO 205 cells. Upon α -mangostin treatment, extrinsic pathway was activated, procaspase-8 was cleaved to caspase-8 which then further activated the cleavage of Bid to t-Bid. The t-Bid then translocates to mitochondria resulting in the activation of mitochondrial apoptotic pathway.

tant targets for cancer intervention. Caspase-3 being the major executioner caspase^[31], thus we examined whether activated caspase-3, -8 and -9 is involved in apoptotic induction and found evident expression of activated caspase-3, -8 and -9.

Under the influence of α -mangostin treatment in COLO 205 cells, a cell death pathway both *via* death receptor pathway and mitochondrial pathway may be involved, as caspase-8 and -9 were expressed. We further demonstrated the loss of mitochondrial membrane potential, release of cytochrome c into the cytosol and DNA fragmentation. Our results are in agreement with a study showing that apoptosis was associated with a loss of mitochondrial membrane potential, which may correspond to the opening of an outer membrane pore, leading to cytochrome c release from mitochondria into the cytosol. The released cytochrome c later triggered the cleavage and activation of caspases and onset of apoptosis^[32]. The expression of Fas and caspase-8 implies the death receptor pathway, while regulation of mitochondrial membrane permeability by upregulation of Bax and p-Bad triggering the release of cytochrome c from mitochondria to cytosol. Our further investigation showed the expression of Fas, Bid, t-Bid, p-53, phospho-p53 and the proteins in Bcl-2 family (Bax and Bmf). Expression of t-Bid is the important linkage between death receptor and mitochondrial pathway. When Bid is cleaved into t-Bid by the activated caspase-8 and translocated towards the mitochondria to activate Bax and Bak, then changing the mitochondrial membrane permeability and the release of cytochrome c^[33] which form a complex with apoptotic enzyme activators and caspase-9. It activates and starts a caspase cascade reaction, which further activates the downstream caspase-3 and other caspase family members for apoptosis induction. We also noticed that α -mangostin

up-regulated the expression of Bax in COLO 205 cells at protein level, suggesting that Bcl-2 family protein regulate α -mangostin mediated apoptotic cell death.

Taken together our results evaluating the molecular mechanism that α -mangostin induced apoptosis cell death in COLO 205 cells may occur *via* caspase-8 dependent cleavage of Bid to tBid providing a link between extrinsic and intrinsic pathways (Figure 10). This could be a promising chemotherapeutic agent and may also serve as a model to develop and design new derivatives which may be more potent.

COMMENTS

Background

Cancer causes significant morbidity and mortality and is a major public health problem worldwide. Globally, colorectal cancer is one of the most common types of cancer in both men and women. Phytochemical studies showed that a variety of tropical plants have useful biological activities and some offer potential therapeutic applications. However, the molecular mechanisms underlying α -mangostin induced apoptosis in human colorectal adenocarcinoma have not yet been fully understood.

Research frontiers

Apoptosis plays a vital role in controlling cell number in many physiological and developmental stages, tissue homeostasis, and regulation of the immune system, while insufficient apoptosis is an integral part of cancer development. The main function of apoptosis is to dispose of a cell without causing damage or stress to neighbouring cells. Thus, the anti-cancer drug that induces apoptotic cell death would be more suitable for use in patients and should be further developed. Therefore, the effect of α -mangostin on the growth and apoptosis induction of human colon cancer cells was investigated in this study.

Innovations and breakthroughs

The results showed that α -mangostin induced apoptotic cell death in COLO 205 cells indicating by membrane blebbing, chromatin condensation, DNA fragmentation, cell cycle analysis, sub-G1 peak, and phosphatidylserine exposure. The expression of caspase-3, caspase-8 and caspase-9, cytochrome c release, Bax, p53 and Bcl-2 modifying factor (Bmf) as well as reduced mitochondrial membrane potential were demonstrated. In addition, upon treatment with α -mangostin, up-regulation of tBid and Fas were evident. Therefore, α -mangostin may be effective as an anti-cancer agent that induces apoptotic cell death in COLO 205 *via* a link between extrinsic and intrinsic pathways.

Applications

α -mangostin could be used as a future promising anti-cancer agent for the treatment of colorectal adenocarcinoma cells.

Terminology

The fruit hull of mangosteen (*Garcinia mangostana* L.) in the Clusiaceae family is rich in a variety of oxygenated and prenylated xanthenes which possess different biological properties, such as anti-mycobacterial, anti-fungal, anti-oxidant, cytotoxicity and anti-inflammatory activities.

Peer review

In this study the authors examined the effect of α -mangostin on the growth and apoptosis induction of human colon cancer cells. They showed that α -mangostin induced apoptotic cell death in COLO 205 cells, activated caspase-3, -8, and -9, increased the mitochondrial cytochrome c release, Bax, p53 and Bmf, reduced mitochondrial membrane potential, and up-regulated tBid and Fas. From these results, the authors suggested that α -mangostin may be effective as an anti-cancer agent *via* a link between extrinsic and intrinsic pathways. It is interesting that the authors clearly showed the mechanism through which α -mangostin induced apoptosis in colon cancer cells.

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Chemometrics of differentially expressed proteins from colorectal cancer patients

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Abstract

AIM: To evaluate the usefulness of differentially expressed proteins from colorectal cancer (CRC) tissues for differentiating cancer and normal tissues.

METHODS: A Proteomic approach was used to identify the differentially expressed proteins between CRC and normal tissues. The proteins were extracted using Tris buffer and thiourea lysis buffer (TLB) for extraction of aqueous soluble and membrane-associated proteins, respectively. Chemometrics, namely principal component analysis (PCA) and linear discriminant analysis (LDA), were used to assess the usefulness of these proteins for identifying the cancerous state of tissues.

RESULTS: Differentially expressed proteins identified were 37 aqueous soluble proteins in Tris extracts and 24 membrane-associated proteins in TLB extracts. Based on the protein spots intensity on 2D-gel images, PCA

by applying an eigenvalue > 1 was successfully used to reduce the number of principal components (PCs) into 12 and seven PCs for Tris and TLB extracts, respectively, and subsequently six PCs, respectively from both the extracts were used for LDA. The LDA classification for Tris extract showed 82.7% of original samples were correctly classified, whereas 82.7% were correctly classified for the cross-validated samples. The LDA for TLB extract showed that 78.8% of original samples and 71.2% of the cross-validated samples were correctly classified.

CONCLUSION: The classification of CRC tissues by PCA and LDA provided a promising distinction between normal and cancer types. These methods can possibly be used for identification of potential biomarkers among the differentially expressed proteins identified.

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Key words: Colorectal cancer; Proteomics; Marker protein; Principal component analysis; Linear discriminant analysis

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Yeoh LC, Dharmaraj S, Gooi BH, Singh M, Gam LH. Chemometrics of differentially expressed proteins from colorectal cancer patients. *World J Gastroenterol* 2011; 17(16): 2096-2103 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i16/2096.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i16.2096>

INTRODUCTION

Proteomic research has made great achievements in biomarker discovery, especially when incorporated with high-

throughput analytical tools and technology, for example 2D-PAGE and LC-MS/MS^[1]. Two-dimensional gel electrophoresis is a fundamental tool for protein analysis to detect alterations in protein expression between control and disease states of cells, which can lead to the discovery of various biomarkers that contribute to pathogenesis or carcinogenesis^[2]. Biomarkers can be used to discriminate variables for subsequent classification of normal and diseased groups^[3]. The complexity of variables generated by mass spectra, microarray and immunohistochemistry often requires advanced statistical techniques or chemometrics to evaluate their clinical value.

Multivariate analyses including the dimension reduction method known as principal component analysis (PCA), and classification methods such as linear discriminant analysis (LDA) are often employed in proteomic studies. PCA reduces the number of variables for further data analysis and interpretation while identifying the variables that retain most of the data variance^[4]. A principal component (PC) is defined as a new variable to explain the maximum amount of variance in the original data and corresponds to a linear combination of the original variables. PCs are presented orthogonally to each other, which provides a more effective representation of the data than the original variables^[2]. LDA is a multivariate technique to classify observations into groups or categories. LDA forms new variables from the original data and identifies the variables that provide the best discrimination between the groups^[5].

Djijda *et al.*^[6] have used a novel approach that combines matrix-assisted laser desorption ionization-ion mobility separation-mass spectrometry (MALDI-IMS-MS) and PCA-discriminant analysis (PCA-DA) to generate tumor classification models based on pancreatic cancer protein patterns. Furthermore, Kamath *et al.*^[7] have used PCA-based k-nearest neighbor analysis to classify normal and cancerous autofluorescence spectra of colonic mucosal tissues. Zwielly *et al.*^[8] have investigated the use of Fourier transform infrared microscopy for colon cancer diagnosis. Their model uses PCA to define spectral changes among normal and cancerous human biopsied colon tissues. Ragazzi *et al.*^[9] have reported the use of multivariate techniques on plasma proteins to diagnose colorectal cancer (CRC). The plasma protein profile generated by MALDI-MS is analyzed by PCA and LDA to discriminate ionic species from normal subjects and CRC patients.

In this study, we carried out the comparison of 2-D images of cancerous and normal colorectal tissues. The differentially expressed proteins from Tris and thiourea lysis buffer (TLB) extractions were respectively tested on a PCA-LDA model to find out the possibility of using protein expression to classify the disease and non-disease tissues of CRC.

MATERIALS AND METHODS

Tissue specimen collection

Matching pairs of normal colonic mucosa and cancerous colonic tissue (located 10 cm from each other) from 26

CRC patients were collected after surgery at the Penang General Hospital, Penang, Malaysia. The study was approved by the Human Ethical Committee of Universiti Sains Malaysia. Informed written consent was received from all patients before the study was conducted. Prior to surgery, the patients did not receive preoperative neoadjuvant chemotherapy and radiotherapy. The tissues were confirmed as cancerous and normal, respectively, by the hospital's pathologist. The cancerous tissues were classified using the TNM system. Surgically removed samples were stored at -80°C until use.

Protein analysis

The method of protein analysis was as described in Yeoh *et al.*^[10]. Frozen tissue (250 mg) was rinsed in distilled water to remove cell debris and excess blood. The tissues were homogenized in ice-cold Tris buffer (0.5 g tissue/mL buffer) [40 mmol/L Tris and 1 × Protease Inhibitor Cocktail (Sigma, St Louis, MO, USA)] and centrifuged at 12000 rpm for 15 min at 18°C. The supernatant was recovered and labeled as Tris extract. The pellet was subjected to further extraction using TLB (1 g tissue/1 mL buffer) [8 mol/L urea, 2 mol/L thiourea, 4% (w/v) CHAPS, 0.4% (w/v) carrier ampholytes and 50 mmol/L dithiothreitol] and centrifuged at 12000 rpm for 15 min at 18°C. The supernatant was recovered and labeled as TLB extract. The extracts were subjected to 2D gel separation on 11 cm ReadyStrip™ IPG strip (linear pH 4-7, Bio-Rad, USA) followed by separation on 10% (w/v) PAGE at a constant voltage of 200 V. The gels were stained with Coomassie Blue. The images obtained were analyzed by PDQuest version 7.3 (Bio-Rad). Comparison of the protein expression levels was carried out between cancerous and normal tissues. Differentially expressed proteins were defined as proteins with a spot intensity that was 1.5-fold higher or lower in cancerous tissues when compared to that in the corresponding normal tissues. A differentially expressed protein was defined as upregulated when it was found at greater intensity in cancerous tissue than in the corresponding normal tissue. The downregulated proteins were detected at greater intensity in normal tissues than in the corresponding CRC cancerous tissues.

Protein identification

The differentially expressed proteins were excised from the gel and subjected to in-gel digestion using trypsin and the tryptic peptides were analyzed by LC/MS/MS using an electrospray ionization ion trap mass analyzer (Agilent Technologies, Santa Clara, CA, USA). The MS/MS data were subjected to the MASCOT protein database search engine for protein identification. The identities of a few proteins (dependent on the availability of antibodies) were further confirmed using western blotting.

Statistical analysis

The differential expression of the proteins was tested by the paired Student's *t* test that is included in PDQuest, to determine their statistical significance (*P* < 0.05). For

Table 1 Clinicopathological features of 26 colorectal cancer patients involved in study

Patient No.	Age (yr)	Race	Sex	pTNM	Stage	Degree of differentiation	Tumor location
1	62	Malay	Male	pT3N1Mx	III B	MD	Sigmoid colon
2	79	Malay	Male	pT2NoM0	I	MD	Descending colon
3	74	Malay	Male	pT3N0M0	II A	MD	Ascending colon
4	-	Malay	Male	pT3N2Mx	III C	MD	Rectum
5	37	Malay	Male	pT3N0M0	II A	MD	Transverse colon
6	58	Malay	Female	pT3N0Mx	II A	MD	Recto-sigmoid
7	59	Malay	Female	pT4N2Mx	III C	MD	Ileocecal
8	69	Malay	Male	pT3N0Mx	II A	MD	Sigmoid colon
9	63	Malay	Female	pT3N0Mx	II A	MD	Recto-sigmoid
10	84	Chinese	Female	pT4N0M0	II B	MD	Rectum
11	58	Chinese	Male	pT3N0Mx	II A	MD	Recto-sigmoid

MD: Moderately differentiated adenocarcinoma.

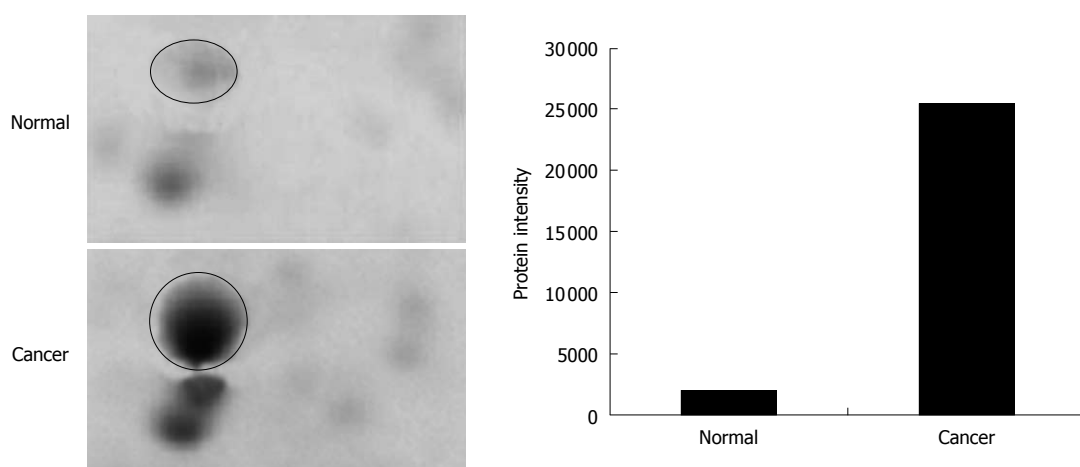


Figure 1 Comparison of protein spot intensity between normal and colorectal cancer tissues for glutathione S-transferase P.

PCA and LDA, the protein spot intensities were exported out from PDQuest and imported into SPSS version 15.0 (Chicago, IL, USA) to perform multivariate analyses. Protein spot intensities were used as variables.

RESULTS

The tissues specimens from each patient were collected in pairs of cancerous and normal tissues. Table 1 shows the details of the tissues used in the analysis. The tissues were subjected to a sequential extraction method to extract aqueous soluble proteins and membrane-associated proteins in two different fractions using Tris and TLB, respectively. Tables 2 and 3 show the 37 and 24 differentially expressed proteins identified in Tris and TLB extracts, respectively. The average fold change indicates the degree of differentiation in expression levels of the protein in cancerous tissues compared to normal tissues in all the patients tested, where a positive sign indicates a greater expression level in cancerous tissues, whereas a negative sign indicates a greater expression level in normal tissues. The MOWSE score refers to the score values given by the MASCOT search. Tables 4 and 5 show the mean intensity of spots and SD, and percentage coefficient of variation (%CV) of spot intensity of differentially ex-

pressed proteins in all patients for Tris and TLB extracts, respectively. An example of the differentially expressed protein, as represented by different intensities of protein spots between normal and cancerous tissues for glutathione S-transferase P (GST-P), is shown in Figure 1; the bar chart was plotted according to the intensity of the respective protein spots. GST-P was detected as upregulated in cancerous tissues.

Data analysis

The significance of the expression levels of the differentially expressed proteins in both Tris and TLB extracts was analyzed by Student's *t* test. After univariate analysis was performed, the normalized intensities of 37 differentially expressed protein spots in Tris extracts were subjected to PCA. The PCA reduced the original data to 12 PCs based on an eigenvalue of > 1 , and these 12 PCs contributed 76.43% of the total data variance of the Tris extract data. Figure 2 shows the 3D PC plot with the x-, y- and z-axes representing the first, second and third PC number. The variables that had the highest loadings were those that contributed most to the differentiation of the disease state. Figure 3 shows the scree plot of Tris extracts. Six PCs were chosen and these components contributed 53.97% of the total variance of the Tris extract

Table 2 List of proteins found in 2D gel of Tris extracts

Spot No.	Protein name	Swissprot No. ¹	MOWSE score ²	MW (Da)	pI	Sequence coverage (%)	GRAVY	Average fold change ³
1	Proteasome subunit β type 6	P28072	134	25573	4.80	16	0.034	-2.967
2	14-3-3 protein ζ	P63104	336	35567	6.97	40	-0.744	11.659
3	Tropomyosin α -3C-like protein	A6NL28	127	27407	4.71	31	-0.992	44.183
4	Rho GDP-dissociation inhibitor 1	P52565	167	23120	5.03	29	-0.700	-7.607
5	14-3-3 protein ζ	P63104	282	27919	4.73	16	-0.621	4.127
6	Tubulin β -2C chain	P68371	524	50304	4.83	40	-0.362	-52.184
7	Cathepsin B	P07858	74	22981	5.20	18	-0.433	33.149
8	Rho GDP-dissociation inhibitor 2	P52566	48	22901	5.10	18	-0.799	-10.625
9	SEC13 homolog	P55735	78	36040	5.22	9	-0.372	6.873
10	Hsc70-interacting protein	P50502	164	28464	8.92	21	-0.653	20.959
11	Apolipoprotein A-I	P02647	143	30777	5.56	26	-0.717	-4.478
12	Proteasome subunit α type 3	P25788	201	15958	6.82	41	0.008	4.249
13	Actin, cytoplasmic 2	P63261	105	26169	5.65	14	-0.156	28.601
14	60 kDa heat shock protein	P10809	151	61348	5.70	14	-0.074	131.219
15	Peroxiredoxin-2	P32119	283	21935	5.67	42	-0.210	1.250
16	Guanine nucleotide binding protein subunit β 2	P62879	112	37954	5.60	11	-0.183	-14.442
17	F-actin-capping protein subunit β	P47756	259	34187	6.02	37	-0.574	33.554
18	GST-P	P09211	730	23442	5.44	60	-0.131	4.834
19	Haptoglobin-related protein	P00739	49	39529	6.42	3	-0.308	56.209
20	Cathepsin Z	Q9UBR2	100	27787	5.48	15	-0.545	-60.766
21	F-actin-capping protein subunit β	P47756	245	21280	7.93	34	-0.540	13.278
22	Actin-related protein 3	P61158	148	47704	5.61	27	-0.271	15.881
23	Abhydrolase domain-containing protein 14B	Q96IU4	200	25429	6.82	26	-0.023	0.765
24	Nucleoside diphosphate kinase A	P15531	87	19873	5.42	36	-0.075	73.120
25	L-lactate dehydrogenase B chain	P07195	228	36928	5.71	14	0.056	3.513
26	Fibrinogen β chain	P02675	151	56624	8.54	22	-0.758	41.329
27	Leukocyte elastase inhibitor	P30740	170	42857	5.90	11	-0.249	10.458
28	PDI A3	P30101	674	57202	5.98	35	-0.506	7.579
29	Gelsolin	P06396	238	86103	5.90	20	-0.415	-11.917
30	Heat shock 27 kDa protein	P04792	256	22840	5.98	47	-0.567	-1.508
31	DJ-1 protein	Q99497	122	20079	6.33	54	0.004	4.981
32	Fibrinogen β chain	P02675	75	56624	8.54	22	-0.758	-72.722
33	Selenium-binding protein 1	Q13228	502	52971	5.93	21	-0.254	-26.544
34	Selenium-binding protein 1	Q13228	592	52938	5.93	30	-0.254	27.403
35	Selenium-binding protein 1	Q13228	979	52938	5.93	37	-0.254	-1.887
36	Leukotriene A-4 hydrolase	P09960	215	69792	5.80	22	-0.259	29.759
37	Proteasome subunit α type 6	P60900	71	20988	8.57	39	-0.247	0.768

¹Protein accession number at SwissProt at <http://www.expasy.org/uniprot/>; ²MOWSE score from MASCOT protein database search at <http://www.matrixscience.com>, where score > 41 is statistically significant ($P < 0.05$); ³Average ratio of the spot intensity in normal mucosa over tumor tissue (negative variation or decrease) or tumor tissue over normal tissue (positive variation or increase). GST-P: Glutathione S-transferase P; PDI: Protein disulfide isomerase.

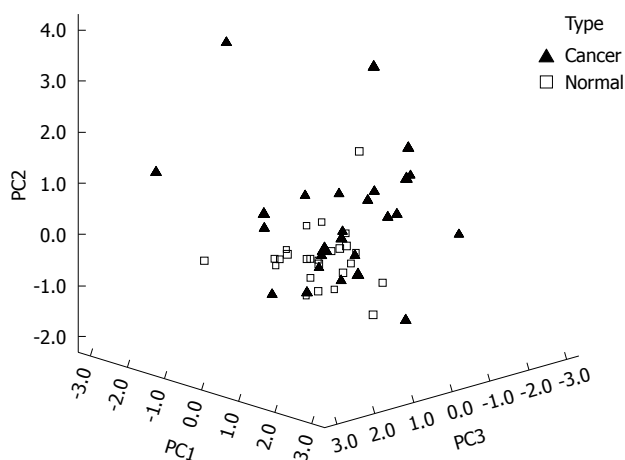


Figure 2 Principal component plot of Tris proteins.

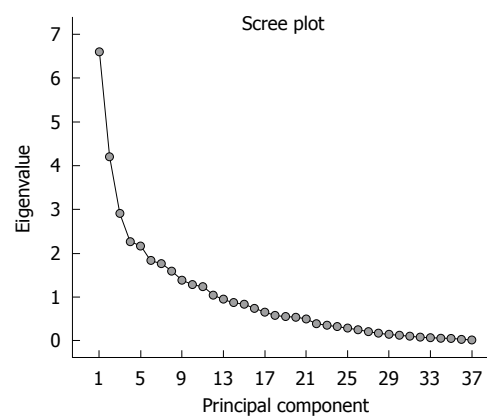


Figure 3 Scree plot showing principal components and their eigenvalues in Tris extracts.

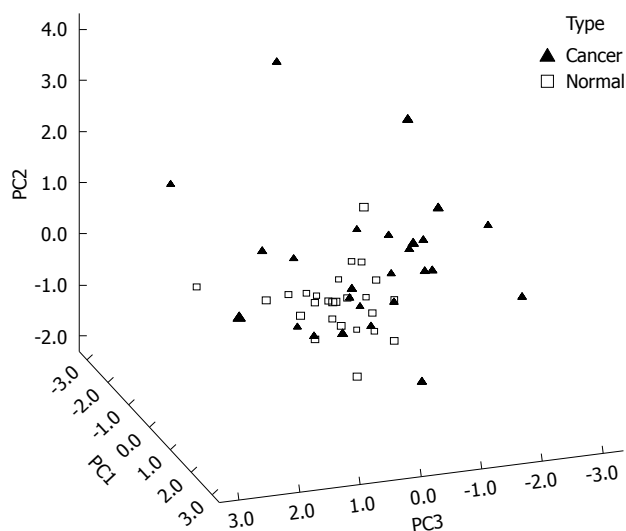
data. Table 6 shows the LDA results for Tris extract proteins, where 22 out of 26 original normal tissues, and 21

out of 26 original cancer tissues were correctly classified. In cross-validated samples, 22 out of 26 normal tissues and 21 out of 26 cancer tissues were correctly classified.

Table 3 List of proteins found in 2D gel in thiourea lysis buffer extracts

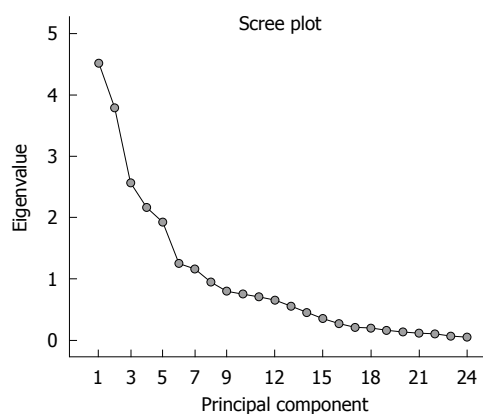
Spot No.	Protein name	SwissProt No. ¹	MOWSE score ²	MW (Da)	pI	Sequence coverage (%)	GRAVY	Average fold change ³
1	Tropomyosin α -4 chain	P67936	139	28506	4.67	33	-1.033	-51.151
2	Putative tropomyosin α -3-chain-like protein	A6NL28	53	27407	4.71	25	-0.992	4.922
3	GC1q-R, mitochondrial	Q07021	123	31768	4.74	20	-0.461	-3.333
4	Calreticulin	P27797	73	47092	4.30	11	-1.191	1.394
5	Prohibitin	P35232	421	29890	5.57	41	0.024	0.032
6	Heat shock 70 kDa protein	P11021	775	72488	5.07	42	-0.487	-32.940
7	Tubulin β -2C chain	P68371	299	48142	4.70	25	-0.347	-9.060
8	PDI	P07237	266	57510	4.82	42	-0.450	-1.515
9	ATP synthase subunit β , mitochondrial	P06576	1096	56559	5.26	43	0.018	-15.661
10	ATP synthase D chain	O75947	117	18406	5.22	32	-0.569	-5.129
11	Chloride intracellular channel protein 1	O00299	299	27123	5.09	30	-0.293	20.288
12	Tubulin α -1 chain	Q71U36	61	50800	4.94	6	-0.229	-30.291
13	Apolipoprotein A-I	P02647	129	28078	5.27	37	-0.840	78.135
14	Actin, cytoplasmic 2	P63261	52	42009	5.31	4	-0.205	-26.716
15	Actin, aortic smooth muscle	P62736	261	42154	5.23	21	-0.233	46.181
16	Stomatin-like protein 2	Q9UJZ1	151	38644	6.88	28	-0.161	-29.709
17	60 kDa heat shock protein, mitochondrial	P10809	451	61386	5.70	28	-0.074	14.023
18	Triosephosphate isomerase	P60174	167	26828	6.51	24	-0.126	16.757
19	Annexin A5	P08758	195	35994	4.94	39	-0.330	-2.019
20	Cytochrome b-c1 complex subunit 1, mitochondrial	P31930	96	53342	5.94	18	-0.141	13.151
21	Annexin A3	P12429	140	36396	5.63	22	-0.430	31.244
22	Annexin A4	P09525	165	35983	5.85	33	-0.447	11.890
23	α -enolase	P06733	143	47385	6.99	12	-0.226	85.960
24	Lamin-A/C	P02545	198	65192	6.40	25	-0.947	-3.378

¹Protein accession number as SwissProt at <http://www.expasy.org/uniprot/>; ²MOWSE score from MASCOT protein database search at <http://www.matrixscience.com>, where score > 41 is statistically significant ($P < 0.05$); ³Average ratio of the spot intensity in normal mucosa over tumor tissue (negative variation or decrease) or tumor tissue over normal tissue (positive variation or increase). PDI: Protein disulfide isomerase.

**Figure 4** Principal component plot of thiourea lysis buffer proteins.

Both original and cross-validation samples had an average 82.7% correct classification.

Figure 4 shows the 3D view of the PCs plot for the TLB extract. PCA reduced the original data of the TLB extract to seven PCs based on an eigenvalue one of > 1, and the seven PCs accounted for 72.46% of the total data variance. The 3D view indicates that tissues can be grouped according to CRC disease state. Figure 5 shows the scree plot of the TLB extracts. Six PCs were chosen based on the slope of scree plot, which contributed

**Figure 5** Scree plot showing principal components and their eigenvalues in thiourea lysis buffer extracts.

67.61% of the total data variance of TLB extracts. Table 7 shows the LDA results of TLB extracts, where 22 out of 26 original normal tissues, and 19 out of 26 original cancerous tissues were correctly classified. In cross-validated samples, 21 out of 26 normal tissues and 16 out of 26 cancerous tissues were correctly classified. The average percentages of correct classification for original and cross-validation samples were 78.8% and 71.2%, respectively.

DISCUSSION

The expression levels of the differentially expressed protein between colorectal cancerous and normal tissues were

Table 4 mean \pm SD and percentage coefficient of variation of spot intensities of Tris proteins

Protein spot No.	Intensity of spots (mean \pm SD)	% CV of spot intensity
1	2565.84 \pm 2247.86	87.60
2	3865.47 \pm 3766.11	97.42
3	2424.01 \pm 1847.71	76.23
4	4957.17 \pm 2923.49	58.97
5	3901.55 \pm 3900.52	99.97
6	2105.64 \pm 2444.14	116.08
7	2572.91 \pm 1765.28	68.61
8	2959.95 \pm 2177.86	73.58
9	2478.29 \pm 1697.98	68.51
10	1253.48 \pm 1472.88	117.50
11	3373.93 \pm 2451.35	72.66
12	3247.26 \pm 2519.26	77.58
13	9413.58 \pm 10685.11	113.51
14	2735.49 \pm 2665.85	97.45
15	8354.35 \pm 4824.59	57.75
16	7370.39 \pm 7935.67	100.34
17	14200.72 \pm 16194.91	114.04
18	6254.81 \pm 5105.54	81.63
19	14364.73 \pm 10849.77	75.53
20	10753.33 \pm 14509.06	134.93
21	5171.49 \pm 3304.12	63.89
22	3230.12 \pm 1905.24	58.98
23	2114.69 \pm 1164.19	55.05
24	2331.41 \pm 2122.56	91.04
25	9254.07 \pm 4830.01	52.19
26	9118.41 \pm 9336.23	102.39
27	3750.45 \pm 3869.35	103.17
28	8098.16 \pm 5450.79	67.31
29	3984.55 \pm 2658.12	66.71
30	4236.70 \pm 4229.74	99.84
31	3932.80 \pm 2507.88	63.77
32	1681.49 \pm 2019.10	120.08
33	6600.04 \pm 4860.85	73.65
34	3121.51 \pm 2694.58	86.32
35	8587.77 \pm 5871.40	68.37
36	939.46 \pm 1682.25	179.07
37	3780.67 \pm 1967.05	52.03

CV: Coefficient of variation.

analyzed using PCA based on a multivariate analysis approach, to assess their usefulness in classifying colorectal tissues as cancerous or normal. The differentially expressed proteins identified showed good consistency in their expression levels in cancerous and normal tissues. The proteins were extracted in two fractions according to their polarities. In the PCA-LDA model, the selected proteins from the first few PCs were able to discriminate colorectal tissues with and without CRC.

A scree plot was derived by plotting the eigenvalues against the PC number. The shape of the plot was used to evaluate the number of PCs to be retained. In general, the point at which the scree plot straightens out indicates the number of PCs to be extracted^[11]. Cross-validation is a method to estimate the accuracy of a predicted classification model if performed using new future data sets (samples); this is because a classification model is considered incomplete until the prediction error is estimated^[12]. One method of cross validation is leave-one-out cross-validation, where one sample from the data set of N

Table 5 mean \pm SD and percentage coefficient of variation of spot intensities of thiourea lysis buffer proteins

Protein spot No.	Intensity of spots (mean \pm SD)	% CV of spot intensity
1	10918.80 \pm 8005.09	73.31
2	8516.42 \pm 7898.33	92.74
3	3986.45 \pm 3471.51	87.08
4	36146.18 \pm 24859.84	68.78
5	13329.50 \pm 7123.20	53.44
6	4091.51 \pm 4636.51	113.32
7	6512.40 \pm 6048.73	92.88
8	13401.28 \pm 8031.43	59.93
9	24196.99 \pm 14907.64	61.61
10	4861.29 \pm 4327.71	89.02
11	4128.52 \pm 3764.18	91.18
12	3522.46 \pm 2821.84	80.11
13	9624.81 \pm 8295.52	86.19
14	5407.19 \pm 5270.17	97.47
15	4683.89 \pm 6994.94	149.34
16	2633.26 \pm 2593.91	98.51
17	10104.77 \pm 10369.91	102.62
18	16086.82 \pm 19928.39	123.88
19	6791.99 \pm 5063.21	74.55
20	7596.19 \pm 4759.49	62.66
21	2685.37 \pm 3298.54	122.84
22	5022.01 \pm 3735.74	74.39
23	5957.62 \pm 7526.42	124.65
24	2323.67 \pm 2269.62	97.67

CV: Coefficient of variation.

Table 6 Percentage of correct classification of normal and colorectal cancer tissues in Tris extracts using linear discriminant analysis

Type	Predicted group membership		% correct classification
	Cancer	Normal	
Original count			
Cancer (26)	21	5	82.7
Normal (26)	4	22	
Cross-validated count			
Cancer (26)	21	5	82.7
Normal (26)	4	22	

Table 7 Percentage of correct classification of normal and colorectal cancer tissues in thiourea lysis buffer extracts using linear discriminant analysis

Type	Predicted group membership		% correct classification
	Cancer	Normal	
Original count			
Cancer (26)	19	7	78.8
Normal (26)	4	22	
Cross-validated count			
Cancer (26)	16	10	71.2
Normal (26)	5	21	

samples is removed, the discriminant rule is recalibrated, and a classification model is built based on the remaining $N - 1$ data. The one sample that is left out is classified in this model and the process repeated N times^[12].

PCA and LDA results from Tris extract indicated that six out of 37 proteins were reliable to determine the tissues with CRC. The proteins comprised five upregulated proteins, namely GST-P, tropomyosin α -3C-like protein, F-actin capping protein subunit β , selenium binding protein 1 and DJ-1 protein, and one downregulated protein, namely, proteasome subunit β type 6. DJ-1 protein and GST-P contributed the most to the first PC based on the weight of their loadings. This was followed by the tropomyosin α -3C-like protein and proteasome subunit β type 6 that contributed to the second PC, while F-actin capping protein subunit β and selenium binding protein 1 contributed to the third PC. The initial PCA reduced the original data and therefore enabled LDA to be carried out because LDA is sensitive to the number of variables. In LDA, the six PCs chosen were shown to be capable of predicting whether the tissues were with or without CRC. Two-way validation by using original and cross-validation analyses was applied to validate the state of the tissues, where the cancerous and normal tissues were classified correctly at 82.7% for both original and cross-validation samples.

Two proteins that contributed most to PC1 in Tris extract were DJ-1 and GST. DJ-1 is a putative oncoprotein that is able to transform cells with H-Ras^[13]. Overexpression of DJ-1 activates protein kinase B, which subsequently increases cell survival. Furthermore, increased DJ-1 expression also activates Nrf2 (nuclear factor erythroid 2-related factor), which in turn increases expression of antioxidant enzymes that confer a survival advantage to tumor cells^[14]. Upregulation of DJ-1 protein in esophageal squamous cell carcinoma is correlated with lymph node metastasis^[15]. Although there is no reported role of DJ-1 in CRC, its upregulation in CRC is undeniable, and we have shown that its expression can be used to discriminate between CRC cancerous and normal tissues.

GST catalyzes the conjugation of reduced glutathione to electrophiles^[16]. GST functions to remove peroxides from endogenous compounds such as lipids and DNA^[17]. Overexpression of GST-P1 in CRC may be involved in cell proliferation, differentiation and apoptosis^[18]. GST-P1 is overexpressed in liver cancer cells^[19].

In TLB extract, six of the 24 differentially expressed proteins identified were found to be useful in discriminating CRC cancerous from normal tissues. These proteins were protein disulfide isomerase (PDI), complement component 1 Q subcomponent-binding protein (GC1q-R), chloride intracellular channel protein 1, triosephosphate isomerase, annexin A5 and actin cytoplasmic 2. All the proteins were downregulated in TLB extracts, except chloride intracellular channel protein 1 and triosephosphate isomerase. PDI and GC1q-R contributed the most to the first PC based on the weight of their loadings. This was followed by the chloride intracellular channel protein 1 and triosephosphate isomerase that contributed the most to the second PC, while annexin A5 and actin cytoplasmic 2 contributed most to the third PC. In LDA, the six PCs that explained 67.61% of the total variance were able to distinguish CRC cancerous from

normal tissues. The leave-one-out cross-validation obtained 71.2% correct classification of normal and cancerous tissues. The value for original grouped samples was higher with 78.8% correct classification.

Two proteins that contributed most to PC1 in TLB extracts were PDI and GC1q-R. PDI catalyzes the formation and breakage of disulfide bonds between two cysteine residues^[20]. PDI regulates cell transformation and intracellular and extracellular redox activities *via* its reductase activity^[21]. PDI regulates STAT3 signaling and proliferation, which is thought to induce malignancy^[22]. PDI is upregulated in CRC cell lines and its upregulation is correlated with cancer cell differentiation^[23,24].

GC1q-R is a cell surface glycoprotein, which binds to the globular heads of C1q molecules^[25]. C1q molecules bind to a variety of cells such as B cells, monocytes, macrophages, endothelial and smooth muscle cells^[26]. C1q elicits responses such as phagocytosis in monocytes and activation of tumor cytotoxicity of macrophages^[27,28]. GC1q-R is overexpressed in colon cancer cells and may be involved in tumor metastasis. However, PDI and GC1q-R were downregulated when using average fold change to determine their expression levels.

Proteins are the expression components that regulate cell activity. Differential expression of proteins is expected upon transformation of normal cells to cancerous cells. These differentially expressed proteins are useful in diagnosis and prognosis of the disease. In the present study, the specimens used in the analysis comprised tissues from female and male patients who were diagnosed with various stages, grades and locations of CRC. Regardless of the sex of the patients and pathological specification of the tissues, we showed that the differentially expressed protein identified from 2D protein profiles of cancerous and normal tissues could be used to separate and classify normal and cancerous tissues by combining PCA and LDA. The data reduction technique of PCA was sufficient to provide a classification of tissues according to CRC disease state. These statistical models simplify the data management through the reduced dimensionality of protein spots from the 2D gel images. Therefore, multivariate analysis of differentially expressed proteins identified from cancerous and normal tissues may be used as a tool for diagnosis and prognosis of CRC disease state.

ACKNOWLEDGMENTS

We want to express our appreciation to Universiti Sains Malaysia for provided the grant under RU funding to conduct this project. We would also like to thank National Institute of Pharmaceutical and Nutraceutical for their generosity in allowing us to conduct the LC/MS/MS experiments.

COMMENTS

Background

Colorectal cancer (CRC) is one of the leading causes of death worldwide. Dif-

ferentially expressed proteins between cancerous and normal colonic tissues were identified using 2D gel separation followed by LC/MS/MS analysis. The protein spot intensities of the 2D gel images were analyzed using principal component analysis (PCA) and linear discriminant analysis (LDA) for their possible use in classification of disease state.

Research frontiers

Multivariate analyses, including the dimension reduction method known as PCA and classification methods such as LDA, are used in cancer proteomic studies to identify the protein variables that provide the best discrimination between the cancerous and normal tissues.

Innovations and breakthroughs

The authors used sequential protein extraction to extract aqueous soluble and membrane-associated proteins from colorectal tissues. Differentially expressed proteins were analyzed using a combination of PCA and LDA to determine their usability in differentiating normal and cancerous colonic tissues. Using this method, the authors successfully classified the tissues according to their respective types. DJ-1 protein and glutathione S transferase P1 of the aqueous soluble proteins, protein disulfide isomerase and complement component 1 Q subcomponent-binding protein of the membrane-associated proteins gave the best classification of the tissues.

Applications

The identified biomarkers may be used for the diagnosis and prognosis of CRC.

Terminology

Chemometrics is defined as the information aspects of complex biological and chemical systems. Chemometrics utilize mathematical, statistical or formal logic-based methods to extract chemical information, which in this case, is for biomarker discovery.

Peer review

This study investigated the use of PCA and LDA of differential protein expression between normal and cancerous tissues for classification of disease state. The method gave good classification of cancerous and normal colonic tissues.

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Dietary treatment of colic caused by excess gas in infants: Biochemical evidence

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breath test decreased significantly (10 ± 2.5 ppm, $P < 0.01$).

CONCLUSION: This study showed an association between clinical improvement and evidence of decreased levels of hydrogen when the infants were fed with a specially designed, low-lactose formula.

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Key words: Infants; Colic; Lactose; Hydrogen breath test

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Abstract

AIM: To evaluate the impact of feeding colicky infants with an adapted formula on the hydrogen breath test and clinical symptoms.

METHODS: Hydrogen expiration was measured by SC MicroLyzer gas chromatography at inclusion and 15 d after treatment with an adapted low-lactose formula in 20 colicky infants.

RESULTS: All babies were symptomatic: 85% with excess gas, 75% with abnormal feeding pattern, and 85% with excessive crying. The hydrogen breath test at inclusion was abnormal: 35 ± 3.1 ppm. After 15 d feeding with an adapted low-lactose formula, crying and flatulence decreased in 85% of patients ($P < 0.001$). For infants in whom no decrease of gas was reported, crying was still reduced ($P < 0.01$). Moreover, the feeding pattern was improved in 50% of infants when it was initially considered as abnormal. Finally, the hydrogen

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INTRODUCTION

Infant colic continues to be one of the most disconcerting issues in pediatric medicine. Wessel in 1954 established the famous "rule of three" criteria: "a symptomatic disorder characterized by paroxysms of fussing, agitation or crying, lasting more than 3 h a day and occurring more than 3 d a week for at least 3 wk"^[1]. These criteria are now outdated and as there is no clear definition for the condition, studies on its causes, prevalence and treatment inevitably include a heterogeneous group of infants with different problems^[2-4]. The definition of "excessive infant crying syndrome"^[5] is preferred, although the word

“colic” is still used and can be defined as an acronym standing for “Cause Obscure Lengthy Infant Crying”. It is characterized by paroxysms of excessive and inconsolable crying. The infant might present with a tense abdomen, flex the leg to the abdomen, and appear flushed. Symptoms typically start around the second week of life, peak around 3–6 wk and resolve by 3 mo^[4,6]. This term now includes digestive disorders such as constipation, gastroesophageal reflux, allergy to cow milk proteins, and excess intestinal gas due to malabsorption of lactose, and its prevalence has been established^[6,7].

These disorders, although not serious from a medical point of view, can be very distressing for the baby and his/her family, and can be associated with symptoms of depression in the mother in the first months after birth^[8]. For most of these disorders, some dietary solutions have been developed in compliance with the international expert group coordinated by ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition)^[9].

The objective of this study was to provide clinical and biochemical evidence of the efficacy of an adapted formula in colic caused by excessive gas, due to physiological hypolactasia, which led to excessive infant crying syndrome.

MATERIALS AND METHODS

We included formula-fed infants who were referred to their pediatrician and/or the Unit of Gastroenterology, Hepatology and Nutrition, Children's Hospital, Vall d'Hebron, Barcelona, Spain because of excessive crying reported by their parents. Infants with vomiting/regurgitation, constipation or cutaneous rash were excluded. When the parents reported “rumbling tummies” excessive flatus, and frothy stools, we considered it as suggestive of carbohydrate malabsorption. Other symptoms were taken into account such as feeding difficulties (e.g. crying during meals) and excessive crying time per 24 h. The hydrogen breath test was performed in case of suspected excess intestinal gas, or infant crying for > 3 h/d to diagnose possible reduced lactose absorption.

Twenty consecutive infants with positive hydrogen breath test were included in this study. All infants were fed with an adapted formula, from various brands having a lactose content of 7 g/100 mL equivalent to 10.4 g of lactose/100 kcal (caloric density of formulas 67 kcal/100 mL).

All of them were eutrophic, Caucasian, healthy full term infants whose growth and development had been normal since birth. Infants were 3.7 wk old on average (range: 1.7–6 wk). They were switched to an adapted formula, Novalac AC (United Pharmaceuticals SA, Paris, France) during the intervention period (Table 1).

Duration of crying, intestinal bloating and behavior during feeding (such as interruption of the meal due to crying) were evaluated through a questionnaire at baseline and during a second consultation 15 d later. A second hydrogen breath test was also performed during the second visit in these 20 infants.

Table 1 Composition of Novalac AC®

Average composition for 100 mL	
Energy (kcal)	65.7
Proteins (g)	1.4
Carbohydrates (g)	7.5
Lactose (g)	2.3
Maltodextrin (g)	5.2
Fat (g)	3.3
C18:2 (mg)	610.0
C18:3 (mg)	59.8
Calcium (mg)	50.7
Phosphorus (mg)	31.2

The persons legally responsible for the children were invited to give their informed consent for participation in the study. The study was approved by the local ethics committee.

Method of hydrogen breath test

Breath samples were collected before the start of feeding, as well as at 90, 120 and 180 min after the beginning of the meal. The feeding schedule was not modified. Samples were taken using face masks with a two-way valve and a pot system. Breath samples were collected in duplicate. The samples were injected in an SC MicroLyzer gas chromatograph (Quinton Instrument Company, USA) for simultaneous detection of hydrogen, CO₂ and methane^[10]. This model had an internal gas chromatographic column through which the sample was flushed. Material in the column retarded components which might have interfered with the measurement, and hydrogen thus appeared by itself at the detector and was accurately measured. The gas was inserted in a SvRite-10 cartridge before being analyzed. Prior calibration was performed with a standard gas that contained 102 ppm hydrogen, 23 ppm methane, and 5% CO₂. The results were expressed as parts per million (ppm). The result was considered to be positive when there was an increase > 20 ppm; the normal level for methane being < 10 ppm applying a correction factor for CO₂. The hydrogen breath data were analyzed as a nested factorial design by analysis of variance. For each infant, the maximum hydrogen value was defined as being the highest mean of the hydrogen breath test, because the actual time from the beginning of the feeding did not have a consistent impact on the value.

Statistical analysis

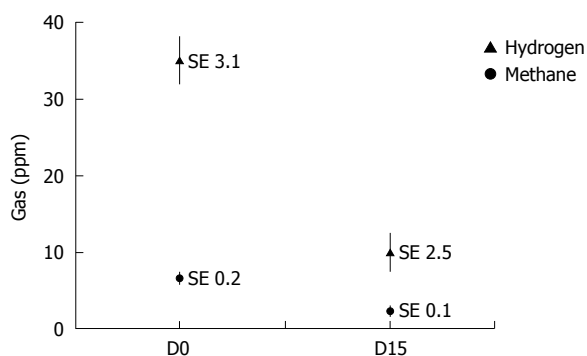
Hydrogen values were expressed as mean ± SE. The values of expired hydrogen were compared using Student's *t* test. A χ^2 test was used for categorical variables when both groups were compared. *P* < 0.05 was considered significant. The statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Table 2 shows the clinical evolution (data reported by

Table 2 Clinical evaluation of associated symptoms in infants with crying secondary to excess gas *n* (%)

	Inclusion	After 15 d	χ^2	<i>P</i>
Excess gas	17 (85)	5 (25)	14.56	< 0.001
Abnormal feeding	15 (75)	6 (30)	8.22	< 0.01
Duration of crying				
< 1 h/d		85%		
1-3 h/d	13 (65)	3 (15)	10.41	< 0.001
> 3 h/d	7 (35)	0 (0)	8.48	< 0.01


Figure 1 Evolution of breath hydrogen and methane before (D0) and after 15 d (D15) of consumption of low-lactose formula. Values are expressed as mean \pm SE. Only the change in expired hydrogen was significant ($P < 0.01$).

relatives) of infants who were included because of crying secondary to excess gas. The duration of crying was reduced in all infants regardless of the initial duration, and 85% cried for < 1 h/d. Moreover, of the 85% of infants reported with excessive gas at inclusion, only 25% still had excessive gas at the end of the study period ($P < 0.001$). Four out of the five infants who were described by their parents as having excessive gas were crying for < 1 h/d. The proportion of infants for whom feeding was described as abnormal decreased from 75% at inclusion to 30% after 2 wk feeding with Novalac AC ($P < 0.01$). The level of hydrogen expired (biochemical evidence) decreased from 35 ± 3.1 ppm at inclusion to 10 ± 2.5 ppm ($P < 0.01$) after 2 wk feeding with Novalac AC. The methane excretion was unchanged (Figure 1).

DISCUSSION

Many authors tend to include digestive disorders in the etiology of “excessive infant crying syndrome” (COLIC), and recommend dietary treatment of this functional disorder^[6,7]. Therefore, this study focused on transient lactose intolerance in infants with colic^[11,12] and we do not discuss other etiologies such as food hypersensitivity, which has been reviewed by Hill *et al*^[13].

As a result of the failure to break down all the lactose ingested, part of it enters the large bowel where it becomes a substrate for lactobacilli and bifidobacteria. This reaction (fermentation) produces hydrogen and other substances. Therefore, subsequent increases in breath hydrogen are accepted as an indirect sign of hypolactasia^[6,11,12,14].

Miller *et al*^[12] have shown that the expired hydrogen in 65 infants with colic was significantly higher than in control subjects (29 ppm *vs* 11 ppm, $P < 0.001$). Sixty-two percent of children with colic had expired hydrogen > 20 ppm, but 38% of control subjects also had abnormal levels of expired hydrogen. Similar results have been shown by Moore *et al*^[14], with 80% of colicky infants having positive expired hydrogen *vs* 36% in normal infants. The assignment of infants to the colicky or control groups according to the mothers’ perception of the duration of crying might not distinguish clearly colicky or non-colicky infants. This might explain the dissociation between clinical and hydrogen values reported by some studies^[12-14]. Moreover, among infants with positive expired hydrogen, individual response to abdominal distension might vary, and this is why some infants cry more than others. Therefore, the issue of personal susceptibility to stimuli and a lengthy crying response varies considerably between infants.

Our baseline data are close to those reported by Miller *et al*^[12]. In this pilot study, we assessed the efficacy of the studied formula only in children younger than 6 wk who had a positive expired hydrogen test and clearly defined symptoms.

Mature breast milk contains 7 g lactose per 100 mL (10.2 g/100 kcal), as do several standard infants formulas. In the first few weeks, infants present a physiological or functional lactase deficiency that limits the amount of lactose that they can digest^[15]. Twenty-seven point five percent of neonates present a positive hydrogen breath test (i.e. > 20 ppm) after lactose ingestion, regardless of sex or gestational age, with only weight playing a significant role: hydrogen expired is more important in infants with a birth weight < 2.5 kg than in those > 2.5 kg^[16]. One study^[17] has found that infants who weigh < 1.8 kg, fed with a low-lactose formula (< 5% lactose) ingested more calories, finished their bottles sooner, presented less milk residue in the stomach, and required feeding more often and with less interruptions than those fed with a formula with normal lactose content (7 g/100 mL). The study of Douwes *et al*^[18] has indicated that abnormal expired hydrogen is more frequent in breast-fed infants or those fed with a 7.5% lactose formula, than in infants fed with a 1% lactose formula.

Furthermore, the concentration of hydrogen in the breath of healthy infants increases from birth to reach its highest level in the second month of life, and declines to low concentrations by 3-4 mo of age. This pattern is parallel to the evolution of crying in infants with COLIC^[4]. Children selected in our study were almost as old as those in the clinical studies of Barr *et al*^[4] or Moore *et al*^[14] (3.7 wk *vs* 28.4 d and 2.6 wk, respectively) and younger than those studied by Miller *et al*^[12] (median age: 8 wk).

Fermentation of the disaccharide generates osmotic active substances such as lactic acid, short chain fatty acids, and hydrogen and/or methane. Methane production in lactose malabsorbers is normal, and without significance^[10].

Colic can result directly from the hyper-peristaltic

stimulus of the fluid load imposed by the osmotic action of unabsorbed lactose in the small intestine, and gas or pharmacologically active metabolites might be responsible for the symptomatic signs. In many susceptible infants, this excess of gas is responsible for triggering colic.

Several studies have investigated lengthy crying and have related it to excess intestinal gas^[14,19,20]. The rapid production of hydrogen in the lower bowel distends the colon, which causes different symptoms. This model of colic implies that symptoms could be relieved by reducing the lactose content of the infant feed. Four clinical trials that have used artificial lactase have been published^[19,21-23]. When lactase is added to a formula 30 min before it is fed, 30% of the lactose is not hydrolyzed. In a double-blind, placebo-controlled crossover study, 10 colicky infants were fed breast milk and cows' milk formulas, untreated and treated with lactase^[19]. This study showed no evidence that low-lactose milk reduced the severity and amount of crying^[19]. In another double-blind, placebo-controlled crossover trial in 12 infants, no effects on duration of crying and fussing were demonstrated^[21]. In a third study with the same methodology, the lactase-treated formula reduced crying time by 1.14 h/d^[22]. Yet another double-blind placebo-controlled crossover study was performed on 53 infants with colic who were treated with placebo or lactase added to their formula 4 h before they were fed. Data on 46 infants were available for crying time analysis and hydrogen breath tests were available in 34. Only 32 infants complied with treatment. In these infants, crying time and median expired hydrogen were significantly lower in the active group than in the placebo group^[23]. The results of our study were in agreement with Kanabar's study that also included an expired hydrogen measurement.

Differences in hydrogen breath excretion between colicky and control infants might also be associated with factors other than the amount or rate of delivery of lactose to the colon. The microbiota, the colonic bacterial metabolic pathways, the partial pressure of hydrogen in the colon, the buffering capacity of the colon, gut perfusion, and incomplete monosaccharide absorption might all play a part. Therefore, the volume of gas released by a fecal sample reflects the end result of a complex interaction of several factors.

This pathophysiological mechanism explains the clinical and biochemical response of these infants to an adapted, low-lactose diet^[22,23]. However, when calcium absorption is enhanced by the presence of lactose^[24], the formula has a lactose content (3 g/100 mL) that provides a daily amount close to absorption capacity in young infants of 4.5 g/kg per day^[23].

Even in the absence of a placebo control group, we believe that the clinical improvement observed in our study was related to dietary management. This clinical improvement appeared earlier (after 15 d feeding with the test formula only) than the usual resolution of colic. Moreover this improvement was endorsed by a decrease in expired hydrogen.

In conclusion this non-randomized, non-placebo-con-

trolled pilot study demonstrates that the use of an adapted infant formula with a low lactose concentration leads to clinical improvement and a decrease in expired hydrogen in colicky infants. Thus, infants with colic might benefit from a switch from standard formula to this specific adapted formula. Larger randomized clinical trials on the efficacy of this formula are needed.

COMMENTS

Background

Infant colic is still one of the most disconcerting issues in pediatric medicine. This term now includes digestive disorders such as constipation, gastroesophageal reflux, allergy to cow milk proteins, and excess intestinal gas due to lactose malabsorption.

Research frontiers

In infants with colic, the possibility of functional lactase deficiency has led to clinical trials with lactase supplementation of infant formulas. These studies have given conflicting results.

Innovations and breakthroughs

This article reports the clinical and biological efficiency of an adapted low lactose formula in infants with colic and positive hydrogen expiration.

Applications

In colicky infants with excess abdominal gas, a diet with an adapted low lactose formula can be tried for several days. If the results are positive, the diet can be continued for several months.

Peer review

The experiments were well designed and the paper is well written. However, there is one major concern about the data analysis.

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Levels of matrix metalloproteinase-1 and tissue inhibitors of metalloproteinase-1 in gastric cancer

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using an enzyme-linked immunosorbent assay.

RESULTS: Higher serum MMP-1 and TIMP-1 levels were observed in patients than in controls ($P < 0.001$). Serum MMP-1 and TIMP-1 levels were positively associated with morphological appearance, tumor size, depth of wall invasion, lymph node metastasis, liver metastasis, perineural invasion, and pathological stage. They were not significantly associated with age, gender, tumor location, or histological type.

CONCLUSION: Increased MMP-1 and TIMP-1 were associated with gastric cancer. Although these markers are not good markers for diagnosis, these markers show in advanced gastric cancer.

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Key words: Gastric cancer; Matrix metalloproteinase-1; Tissue matrix metalloproteinase-1

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Abstract

AIM: To evaluate the levels of preoperative serum matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in gastric cancer.

METHODS: One hundred gastric cancer patients who underwent gastrectomy were enrolled in this study. The serum concentrations of MMP-1 and TIMP-1 in these patients and in fifty healthy controls were determined

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases that play a significant role in the degradation of all matrix partitions, which

are crucial for malignant tumor growth, invasion, and metastasis^[1,2]. MMPs are inhibited by tissue inhibitors of metalloproteinase (TIMPs), which are secreted proteins. TIMPs bind to enzymatically active MMPs at a 1:1 molar stoichiometry, thus inhibiting proteolysis^[3]. The role of TIMPs in the imbalance of the extracellular matrix is significant and may inhibit or stimulate tumorigenesis^[4].

MMP-1 is also known as collagenase (EC 3.4.23.7)^[5]. Saffarian *et al*^[6] showed that activated MMP-1 acts by processing on the collagen fibril. The biological implications of MMP-1 acting as a molecular retainer, tied to the cell surface, prompted recent mechanisms for its status in tissue remodeling and cell-matrix interaction to be proposed. MMP-1 in the stromal tumor microenvironment can change the behavior of cancer cells to promote cell migration and invasion^[7].

TIMP-1 is a 28.5 kDa glycoprotein that has been studied in many human malignancies, including gastric cancer^[8]. TIMP-1 mRNA expression is increased in gastric, esophageal, and pancreatic cancer^[9-11]. TIMP-1 is present in human peripheral blood and body fluids^[12]. MMP-1 and TIMP-1 levels have been studied in plasma or serum of patients with cumulative malignancies^[13,14].

Our study was carried out to analyze serum MMP-1 and TIMP-1 levels in gastric cancer patients and to investigate their clinicopathological correlations.

MATERIALS AND METHODS

A total of 100 patients who underwent gastrectomy with gastric cancer between December 2007 and April 2010 were enrolled. Their median age was 58.5 years (range, 34-78 years), and the ratio of men/women was 47/53. There were 50 healthy volunteer controls without family history of cancer, whose average age was 56 years (range, 48-65 years) (22 men, 28 women). Peripheral venous blood of patients and controls was taken before gastrectomy and stored at 4°C. Blood from controls was taken on the day of a physical examination. The blood samples were centrifuged 1000 rpm, in 15 min, at 20°C to separate the serum, which was stored at -70°C until analysis. The mean storage time of all samples was 2 mo (45-80 d).

Resected tumor specimens were studied pathologically according to the criteria of the UICC's pTNM classification^[15]. Information recorded included age, gender, tumor location, tumor size, wall invasion, resection margin, histological type, lymph node metastasis, vascular invasion, lymphatic invasion, and perineural invasion. The histological features were classified into two types: (1) intestinal or differentiated type, consisting of papillary and/or tubular adenocarcinomas; and (2) diffuse or undifferentiated type, consisting of poorly differentiated, signet-ring cells, and/or mucinous adenocarcinomas.

Enzyme-linked immunosorbent assay (ELISA) for serum MMP-1 and TIMP-1 was performed using an ELISA kit (R&D System, USA) following the manufacturer's instructions.

As appropriate, the Mann-Whitney *U* test or Fisher's exact test was used for group comparisons. Correlations

Table 1 Serum matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 levels in patients and controls

Variables	Controls (<i>n</i> = 50)	Patients (<i>n</i> = 100)	<i>P</i>
Age (yr)	56 (48-65)	58 (47-64)	
Gender female (%)	37	40	
MMP-1 (ng/mL)	256 (109-342)	785 (457-900)	< 0.0001
TIMP-1 (ng/mL)	220 (198-267)	725 (417-1134)	< 0.0001

MMP-1: Matrix metalloproteinase-1; TIMP-1: Tissue inhibitor of metalloproteinase-1.

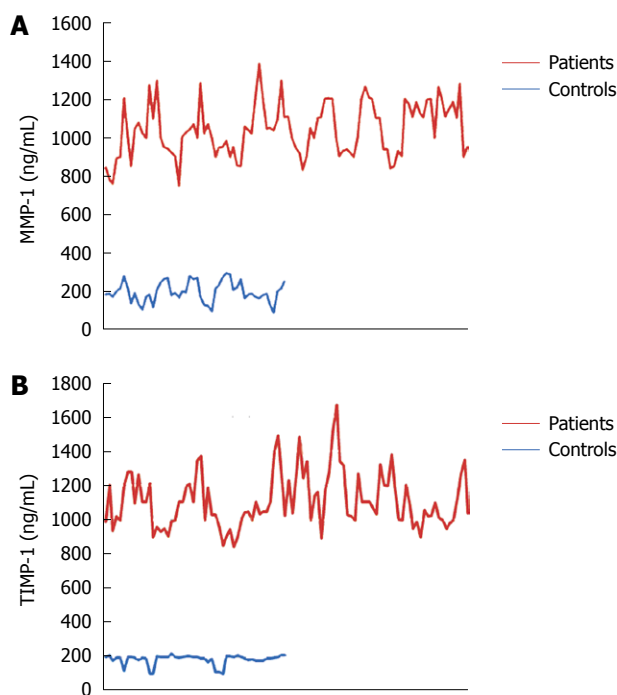


Figure 1 Serum matrix metalloproteinase-1 (A) and tissue inhibitor of metalloproteinase-1 (B) levels of controls and patients. MMP-1: Matrix metalloproteinase-1; TIMP-1: Tissue inhibitor of metalloproteinase-1.

between parameters were tested by Spearman's correlation coefficient. A *P* < 0.05 was considered statistically significant.

RESULTS

Serum MMP-1 and TIMP-1 levels in gastric cancer patients and controls are shown in Table 1 and Figure 1A and B. The serum levels of MMP-1 and TIMP-1 in gastric cancer patients were significantly higher than in the control group (*P* < 0.0001). Clinicopathological variables are shown in Table 2. Serum MMP-1 and TIMP-1 levels were positively associated with the depth of wall invasion (*P* < 0.01), lymph node metastasis (*P* < 0.001), and lymphatic invasion (*P* < 0.001). The serum levels of MMP-1 and TIMP-1 were closely associated with distant metastasis (*P* < 0.001). In particular, higher MMP-1 and TIMP-1 levels were significantly associated with positive lymphovascular invasion (*P* < 0.001), tumor size ≥ 4 cm (*P* < 0.001), positive lymph node metastasis (*P* < 0.001), T stage

Table 2 Clinicopathological variables of serum matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in patients

Variables	MMP-1	TIMP-1	P
Lymphovascular invasion			
Negative	543 (500-678)	489 (450-573)	< 0.001
Positive	801 (768-845)	642 (567-703)	
Tumor size (cm)			
< 4	478 (460-501)	429 (425-479)	< 0.001
≥ 4	675 (509-725)	671 (532-690)	
Lymph node metastasis			
Negative	563 (503-650)	642 (598-709)	< 0.001
Positive	742 (657-799)	756 (570-876)	
T stage			
T0-2	521 (498-599)	598 (564-783)	< 0.001
T3-4	674 (578-783)	749 (570-794)	
TNM stage			
I	469 (458-502)	476 (423-512)	< 0.001
II	534 (467-563)	521 (478-589)	
III	714 (546-857)	753 (512-699)	
IV	765 (699-900)	975 (812-1134)	

MMP-1: Matrix metalloproteinase-1; TIMP-1: Tissue inhibitor of metalloproteinase-1.

(T3-T4) ($P < 0.001$), or TNM stage (III and IV) ($P < 0.001$). MMP-1 and TIMP-1 levels were not significantly associated with negative lymphovascular invasion, tumor size < 4 cm, negative lymph node metastasis, T stage (T0-T2), and TNM stage (I and II). Overall, they were associated with pathological stage ($P < 0.001$). Serum MMP-1 and TIMP-1 levels were not associated with age ($P = 0.237$), gender ($P = 0.281$), tumor location ($P < 0.142$), histological type ($P = 0.103$), vascular invasion ($P = 0.247$), or peritoneal seeding ($P = 0.271$).

Higher serum MMP-1 and TIMP-1 levels were correlated with gastric cancer ($P < 0.001$, $r = 0.77$). Figure 1A shows that MMP-1 levels in patients with gastric cancer were significantly higher than in control groups. Figure 1B shows that TIMP-1 levels in patients with gastric cancer were significantly higher than in control groups.

DISCUSSION

In our study, we investigated MMP-1 and TIMP-1 levels in gastric cancer patients and compared them with a control group. We also investigated their associations with clinicopathological features.

Matrix metalloproteinases are involved in many normal biological processes (e.g. embryonic development, blastocyst implantation, organ morphogenesis, nerve growth, ovulation, cervical dilatation, postpartum uterine involution, endometrial cycling, hair follicle cycling, bone remodeling, wound healing, angiogenesis, and apoptosis) and pathological processes (e.g. arthritis, cancer, cardiovascular disease, nephritis, neurological disease, breakdown of the blood brain barrier, periodontal disease, skin ulceration, corneal ulceration, liver fibrosis, emphysema, and fibrotic lung disease). Although the main function of matrix metalloproteinases is elevation of ECM during tissue resorption and progression of many diseases, it is

obvious that matrix metalloproteinases also alter the biological functions of ECM molecules by definite proteolysis. MMP-1 and TIMP-1 are thought out to be involved in dissemination of cancer cells by dissolving the ECM, but they are also important in creating an environment that supports the initiation and growth of primary and metastatic tumors. These effects may be associated with proteolytic release of growth factors and/or modification of cellular environments^[16].

The most important finding in our study was the association between high MMP-1 and TIMP-1 levels in gastric cancer patients. In addition, high MMP-1 and TIMP-1 levels were significantly associated with certain clinicopathological variables. High MMP-1 expression has been associated with hematogenous metastasis^[17,18], rising depth of invasion, and metastasis in colorectal cancer^[18,19]. Our study also suggested that MMP-1 levels are associated with depth of invasion and metastasis.

Patients with colorectal cancer, ovary, lung, and liver diseases have increased TIMP-1 levels compared to control groups^[14,20-22]. Wang *et al.*^[23] suggested that serum TIMP-1 levels were higher in gastric cancer patients than control groups and were associated with clinicopathological variables. However, they suggested that serum TIMP-1 levels were associated with depth of wall invasion, distant metastasis, peritoneal seeding, lymphatic invasion, lymph node metastasis, and perineural invasion. However, we did not find that serum TIMP-1 levels were associated with peritoneal seeding and perineural invasion.

MMP-1 is associated with the primal pace of invasion and angiogenesis in gastric cancer, which may make it a useful marker for prognosis. TIMP-1 is more simply released into the blood^[24]; therefore, the sensitivity of the assay is higher than that for MMP-1.

High blood levels of MMP-1 and TIMP-1 are associated with poor prognosis of malignancies. Thus, they might useful as markers for malignant potential (i.e. tumor growth and/or differentiation) for cancer. Notably, serum TIMP-1 levels have been established as an independent factor in gastric cancer^[23].

Some metalloproteinases have been shown to degrade over time when measured in stored blood samples. However, we do not think that such protein decay is a significant factor when proteins are stored for 2 mo. This assumption is supported by the work of Papazoglou *et al.*^[25], Kardeşler *et al.*^[26] and Karapanagiotidis *et al.*^[27].

MMP-1 and TIMP-1 can be considered as 'traditional' and conventional serum biomarkers; many studies have measured both of these proteins as serum biomarkers^[28].

This study demonstrated that high serum MMP-1 and TIMP-1 levels in gastric cancer patients are significantly associated with disease progression. Their levels are important markers of tumor progression or advanced tumor stages.

COMMENTS

Background

The incidence of gastric cancer is rising worldwide. Collagenases may play a role

in degradation of the cell matrix, possibly leading to growth of malignant tumors, lymph node metastasis, increased depth of invasion and other metastases.

Research frontiers

Matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) change the environment of cancer cells to promote cell migration and invasion. Changes caused by these endopeptidases have a role in the progression of the gastric cancer.

Innovations and breakthroughs

High blood levels of MMP-1 and TIMP-1 are associated with poor prognosis of malignancies, making them potentially useful biomarkers for the malignant potential (i.e. tumor growth and/or differentiation) of cancer. These effects may be associated with proteolytic release of growth factors and/or modification of tumor cells.

Applications

The data generated in this paper might be used to explain the development of gastric cancer, to prevent metastasis, and to aid early diagnosis.

Terminology

MMP-1 and TIMP-1 zinc-dependent neutral endopeptidases. The role of MMP-1 and TIMP-1 in the imbalance of the extracellular matrix is significant and may inhibit or stimulate tumorigenesis. These effects have been demonstrated, and these molecules may represent useful markers of tumorigenesis.

Peer review

It is a nice study, with interesting results.

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Sunitinib for Taiwanese patients with gastrointestinal stromal tumor after imatinib treatment failure or intolerance

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(PR), and 9 stationary disease (SD); 15/23]. In 12 patients harboring mutations of the kit gene at exon 11, the clinical benefit rate (CR, PR, and SD) was 75.0% and 6 patients with tumors containing kit exon 9 mutations had a clinical benefit of 50.0% (not significant, $P = 0.344$). The progression free survival (PFS) and overall survival (OS) did not differ between patients whose GISTs had wild type, KIT exon 9, or KIT exon 11 mutations. Hand-foot syndrome was the most common cause of grade III adverse effect (26.1%), followed by anemia (17.4%), and neutropenia (13.0%). During the median 7.5-mo follow-up after sunitinib use, the median PFS and OS of these 23 GIST patients after sunitinib treatment were 8.4 and 14.1 mo, respectively.

CONCLUSION: Sunitinib appears to be an effective treatment for Taiwanese with IM-resistant/intolerant GISTs and induced a sustained clinical benefit in more than 50% of Taiwanese advanced GIST patients.

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Key words: Sunitinib; Gastrointestinal stromal tumors; Imatinib; Failure or intolerance

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Chen YY, Yeh CN, Cheng CT, Chen TW, Rau KM, Jan YY, Chen MF. Sunitinib for Taiwanese patients with gastrointestinal stromal tumor after imatinib treatment failure or intolerance. *World J Gastroenterol* 2011; 17(16): 2113-2119 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i16/2113.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i16.2113>

Abstract

AIM: To report preliminary results of the efficacy and safety of sunitinib in the management of Taiwanese gastrointestinal stromal tumors (GIST) patients facing imatinib mesylate (IM) intolerance or failure.

METHODS: Between 2001 and May 2010, 199 Taiwanese patients with metastatic GIST were treated at Chang Gung Memorial Hospital. Among them, 23 (11.6%) patients receiving sunitinib were investigated.

RESULTS: Sixteen male and 7 female patients with a median age of 59 years (range: 24-83 years) received sunitinib. Twenty-two GIST patients changed to sunitinib because of IM failure and 1 because of intolerance. The median duration of sunitinib administration was 6.0 mo (range: 2-29 mo). The clinical benefit was 65.2% [2 complete response (CR), 4 partial response

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) primarily arise from mesenchymal tissue in the gastrointestinal (GI) tract

and abdomen. Although GISTs are rare, representing only an estimated 0.1% to 3% of all GI tract tumors^[1] GISTs account for the most common mesenchymal malignancy of the GI tract with unknown incidence^[2]. GISTs appear to be related to the interstitial cells of Cajal^[3] and express the cell surface transmembrane receptor KIT, which has tyrosine kinase activity. Gain-of-function mutations of KIT are frequent in GISTs and result in constitutive activation of KIT signaling and lead to uncontrolled cell proliferation and resistance to apoptosis^[4,5]. The KIT tyrosine kinase inhibitor imatinib mesylate (IM) has shown a promising clinical result for an advanced GIST patient^[6], and several trials have shown a promising effect of this target therapy^[6,7]. Our previous study showed that IM significantly affected survival in GIST patients^[8,9].

Surgical resection remains the mainstay therapy for GIST, but recurrence is common. The 5-year survival rates for GIST after complete resection range from 40% to 65%^[6,10-13]. Unresectable or metastatic GIST is a fatal disease that resists conventional chemotherapy. IM selectively inhibits certain protein tyrosine kinases: intracellular ABL kinase, chimeric BCR-ABL fusion oncoprotein of chronic myeloid leukemia, the transmembrane receptor KIT, and platelet-derived growth factor receptors (PDGFR)^[14-17]. IM induced a sustained objective response in more than 50% of patients with advanced GISTs in the West and in Taiwan^[8,9]. However, progression of GIST eventually develops and emerges as a challenge.

Sunitinib is an oral multi-targeted tyrosine kinase inhibitor with activity against KIT and PDGFRs, as well as vascular endothelial growth factor receptors (VEGFRs), glial cell line-derived neurotrophic factor receptor (rearranged during transfection; RET), colony-stimulating factor 1 receptor (CSF-1R), and FMS-like tyrosine kinase-3 receptor (FLT3)^[18-23]. Sunitinib received multi-national approval for the treatment of GIST after failure of IM because of resistance or intolerance based on the results of an international, randomized, double-blind, placebo-controlled phase III trial^[24]. The clinical safety and efficacy of both IM and sunitinib in GIST have primarily been established in Western patients living in the USA or Europe and have not been thoroughly studied in Asian patients. Fifty-six centers in 11 countries participated in the phase III trial of sunitinib in GIST, but only 15 of the 312 patients were of Asian descent (10 and 5 in the sunitinib and placebo groups, respectively)^[25]. Therefore, we report our preliminary results to clarify the efficacy and safety of sunitinib in management of Taiwanese GIST patients facing IM intolerance or failure.

MATERIALS AND METHODS

Between 2001 and May 2010, 199 patients who had histologically confirmed, recurrent, unresectable, or metastatic GIST that expressed CD117 or CD34 and were treated at the Department of Medical Oncology, and Surgery, Chang Gung Memorial Hospital were retro-

spectively reviewed. Failure of prior IM therapy, as demonstrated by disease progression [based on Response Evaluation Criteria in Solid Tumors (RECIST)]^[26] or discontinuation of IM due to toxicity was the inclusion criteria in this study. Additional eligibility criteria included an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and adequate cardiac, hepatic, renal, coagulation, and hematologic function. Key exclusion criteria included lack of recovery from the acute toxic effects of previous anticancer therapy or imatinib treatment, discontinuation of imatinib therapy within 2 wk or of any other approved or investigational drug for GIST within 4 wk before starting sunitinib treatment, clinically significant cardiovascular events or disease in the previous 12 mo, diabetes mellitus with clinical evidence of peripheral vascular disease or diabetic ulcers, or a diagnosis of any second malignancy within the previous 5 years. Patients could have previously received chemotherapeutic regimens (the last chemotherapy treatment must have been at least 4 wk before study entry) and undergone radiotherapy, or surgery, or both. The study was approved by the local institutional review board of Chang Gung Memorial Hospital, and written informed consent for drug administration and the analysis of tumor-associated genetic alteration was obtained independently from each patient.

Study design and follow-up study

A retrospective study was conducted to evaluate the effect of sunitinib in inducing objective response in Taiwanese GIST patients. Patients were administered 50 mg (4 wk on and 2 wk off; for clinical trial) or 37.5 mg continuously of sunitinib in 12.5 mg capsules taken orally daily with food. Patients had regular physical examinations and evaluations of performance status, body weight, complete blood count, and serum chemistry. The administration of each dose and any adverse events were recorded for each patient. Standard computed tomography (CT) was performed on each patient every 3 mo for the first 3 years and every 6 mo for the following 2 years to assess patient response. Measurement of efficacy was based on objective tumor assessments made using RECIST with a minor modification to allow use of standard radiographic protocols for spiral CT. Time to response (TTR) was defined as the interval for better drug response during sunitinib treatment. Time to progression (TTP) was defined as the interval for worse drug response during sunitinib treatment. Progression free survival (PFS) was defined as no progression after sunitinib use. Overall survival (OS) was defined as survival after administration of sunitinib and death was the endpoint of the study. Response rate, PFS, OS, TTR, duration of response, and TTP were recorded. Safety and tolerability were assessed by analysis of adverse events, physical examinations, vital signs, ECOG performance status, and laboratory abnormality assessments (for example, complete blood count with differential count, serum electrolyte measurements, and electrocardiogram). Cardiac function was assessed at screening, at day 28 of

all treatment cycles, and treatment end with 12-lead electrocardiogram and multigated acquisition scans. Toxic effects were recorded in accordance with the National Cancer Institute Common Toxicity Criteria^[27].

Analysis of KIT and PDGFRA mutations

Sections were prepared from formalin-fixed, paraffin-embedded pretreatment specimens trimmed to enrich tumor cells. Polymerase chain reaction amplification of genomic DNA for KIT and PDGFRA was performed and amplification was analyzed for mutations as previously described^[28].

Statistical analysis

All data are presented as percentages of patients or means with standard deviation. Pearson χ^2 test and Fisher exact test were used for nominal variables. Survival rate was calculated and plots constructed by the Kaplan-Meier method and compared between groups with a log-rank test. All statistical analyses were performed using the SPSS computer software package (Version 10.0, Chicago, IL, USA). A *P*-value < 0.05 was considered statistically significant.

RESULTS

Clinical features

Table 1 summarizes the demographic features of 23 GIST patients receiving sunitinib. There were 16 male and 7 female patients with a median age of 59 years (range from 24 to 83 years). The stomach was the most common site for GISTs treated with sunitinib (8/23; 35%), followed by the jejunum (5/23; 22%), the ileum (5/23; 22%), and the rectum (3/23; 13%) (Table 1).

Treatment and outcomes before and after use of sunitinib

In Taiwan, sunitinib has been approved for treatment of metastatic GIST patients facing IM intolerance or failure since February 2009. Before 2009, sunitinib was administered to selected patients with unresectable or metastatic (advanced) GISTs facing IM failure or intolerance because they were enrolled in clinical trials. Sunitinib (12.5-50 mg/d) was given to 23 patients and all 23 patients were followed after administration of sunitinib at regular intervals until death or until the time of this manuscript writing. The median follow-up time after sunitinib was 7.5 mo, range: 1.2-58.0 mo. Overall, 2 patients (8.7%) had a complete response (CR), 4 (17.4%) had a partial response (PR), 9 had stationary disease (SD) (39.1 %), and 8 had progressive disease (PD) (34.8%). A clinical benefit was observed in 65.2% of GIST patients. Among the 23 patients, the median TTR for 2 patients with CR was 3.73 mo and was 3.67 mo for 4 PR patients. The median TTP was 2.37 mo and the median survival is still unknown in the 8 PD patients (Table 2). During the median 7.5 mo follow-up after sunitinib use, the median PFS and OS of these 23 GIST patients after sunitinib treatment was 8.4 and 14.1 mo, respectively (Figures 1 and 2).

Table 1 Demographic and genetic data of 23 Taiwanese gastrointestinal stromal tumor patients with imatinib failure or intolerance treated with sunitinib *n* (%)

	Sunitinib (<i>n</i> = 23)
Age (median/range, yr)	59.0/24-83
Gender (male:female)	16:7
Location	
Stomach	8 (26.6)
Duodenum	1 (12.5)
Jejunum	5 (23.4)
Ileum	5 (14.1)
Mesentery	1 (18.8)
Rectum	3 (4.7)
Tumor recurrence	
Liver	15
Peritoneum	6
Local recurrence	2
Genetic spectrum	21 (84.4)
Exon 11	12
Deletion mutation	
Deletion and insertion mutation	
Missense mutation	
Exon 9 (insertion mutation)	6
Exon 13	1
No mutation (wild type)	1
PDGFRA (exon 18)	1
Median duration of sunitinib use (mo)	6

PDGFRA: Platelet derived growth factor α .

Table 2 Antitumor response of 23 Taiwanese with advanced gastrointestinal stromal tumor treated with sunitinib

	<i>n</i> (%)	Sunitinib duration (median, mo)	TTR/TTP (median, mo)	OS (median, mo)
CR	2 (8.7)	9.85	3.73/NA	NA
PR	4 (17.4)	12.3	3.67/12.71	NA
SD	9 (39.1)	11.9	1.87/13.53	14.03
PD	8 (34.8)	3.63	2.37	NA

CR: Complete response; PR: Partial response; SD: Stationary disease; PD: Progression of disease; TTR: Time to response; TTP: Time to progression; OS: Overall survival.

Spectrum of mutations in 23 advanced GIST patients

Tumor specimens suitable for genetic analysis were available from 21 (84.4%) of the 23 GIST patients with IM failure or intolerance. Overall, 18 (85.7%) of the 21 examined GISTs had activated mutations of KIT exon 9 and 11. Six of 21 (28.6%) GISTs had exon 9 mutation, 12 (57.1%) had exon 11 mutation, and 1 (4.8%) had no mutation of KIT. One PDGFRA exon 18 mutation was found. One patient had a concurrent deletion mutation in exon 11 and a missense mutation in exon 13; however, the exon 13 mutation was followed by the deletion mutation in exon 11. This patient developed acquired resistance and expired from disease progression. All 6 GISTs had KIT exon 9 mutation and displayed in-frame duplication of nucleotides, resulting in insertion of alanine (A) and tyrosine (Y) at codons 502 and 503. The KIT exon 11 mutations in the 12 GIST patients included insertion and deletion mutations, deletion mutations, and missense mutations.

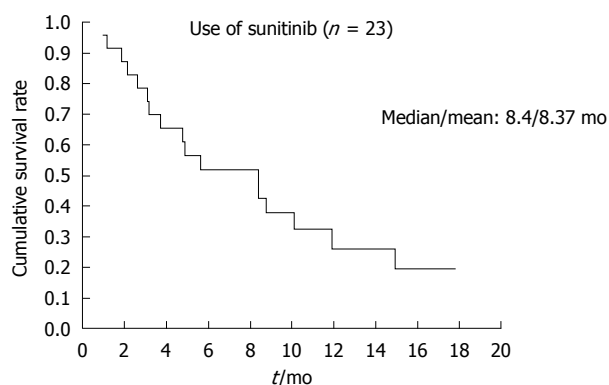


Figure 1 Progression free survival of 23 Taiwanese with metastatic gastrointestinal stromal tumor treated with sunitinib after imatinib failure or intolerance.

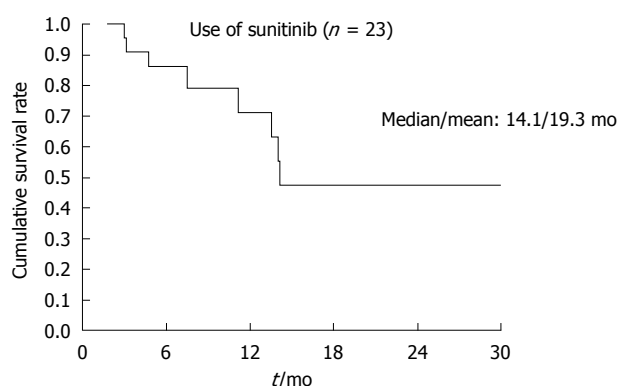


Figure 2 Overall survival of 23 Taiwanese with metastatic gastrointestinal stromal tumor treated with sunitinib after imatinib failure or intolerance.

Table 3 Correlation between antitumor response and mutation status of 21 Taiwanese with advanced gastrointestinal stromal tumor treated with sunitinib

	CR	PR	SD	PD	P	CR + PR + SD	PD	P
Exon 9 (n = 6)	0	1	2	3	0.610 ¹	3	3	0.344 ¹
Exon 11 (n = 12)	2	2	5	3		9	3	
Exon 13 (n = 1)	0	0	0	1				
No mutation (n = 1)	0	0	1	1				
PDGFRA (n = 1)	0	1	0	0				

¹Exon 9 vs exon 11. CR: Complete response; PR: Partial response; SD: Stationary disease; PD: Progression of disease; PDGFRA: Platelet derived growth factor α .

Treatment and outcomes after use of sunitinib in terms of mutation status

In 12 patients with GISTs harboring KIT exon 11 mutations, the clinical benefit rate was 75% (2 CR, 2PR, and 5 PR) and 3 of 6 patients with tumors containing a KIT exon 9 mutation had a clinical benefit of 50% (1 PR and 2 SD) (not significant, $P = 0.344$) (Table 3). The median PFS and OS for the 12 GIST patients who had KIT exon 11 mutations after sunitinib use was 8.8 mo and still not reached, respectively. The median PFS and OS for the 6

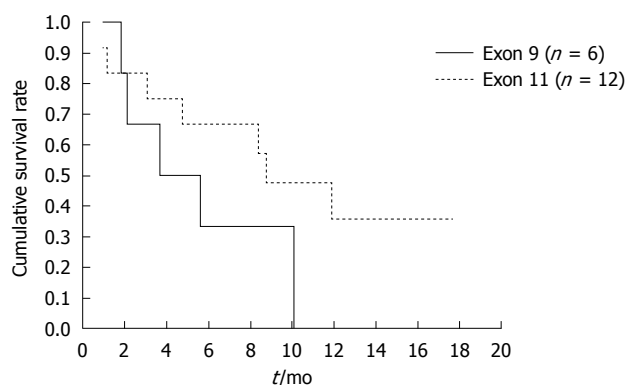


Figure 3 Progression free survival of 18 Taiwanese with metastatic gastrointestinal stromal tumor treated with sunitinib after imatinib failure or intolerance (exon 9 vs exon 11). Median/mean (mo): 3.7/5.6 (exon 9), 8.8/10.2 (exon 11); 95% CI: 0-7.9/2.5-8.6 (exon 9), 2.24-14.4/6.5-14 (exon 11). Log-rank test, $P = 0.221$.

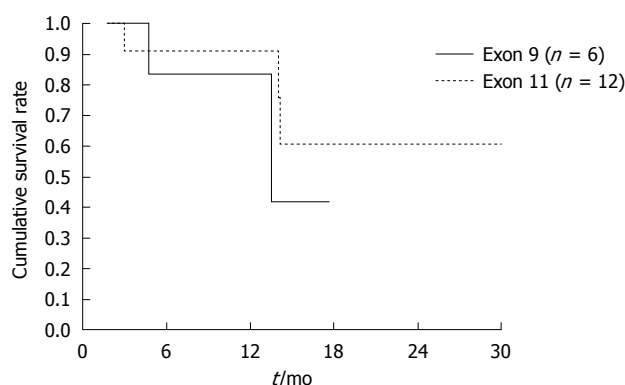


Figure 4 Overall survival of 18 Taiwanese with metastatic gastrointestinal stromal tumor treated with sunitinib after imatinib failure or intolerance (exon 9 vs exon 11). Median/mean (mo): 13.5/13.7 (exon 9), Not achieved/22.6 (exon 11); 95% CI: 0.9-26.1/9.8-17.7 (exon 9), NA/16.1-29.1 (exon 11). Log-rank test, $P = 0.473$.

patients with tumors containing a KIT exon 9 mutation were 3.7 and 13.5 mo, respectively. The twelve GIST patients who had KIT exon 11 mutations had similar PFS and OS to that of 6 patients with tumors containing a KIT exon 9 mutation (Figures 3 and 4).

Adverse events in 23 advanced GIST patients receiving sunitinib

Hand-foot syndrome was the most common cause of grade III adverse effects (26.1%), followed by anemia (17.4%), and neutropenia (13.0%). None of 11 patients had hypothyroidism after use of sunitinib (Table 4).

DISCUSSION

We had shown that IM significantly prolongs the post-recurrence and OS of Taiwanese patients with advanced GISTs^[8,9]. However, approximately 50% of GIST patients eventually develop progression in 24 mo after IM treatment and emerge as a challenge^[7]. This study confirmed the positive effect of sunitinib on improving PFS

Table 4 Adverse events and selected laboratory abnormalities

Variable	Sunitinib (n = 23)			
	Grade 1	Grade 2	Grade 3	Grade 4
Adverse event				
Anorexia	5	1	0	0
Diarrhea	6	8	0	0
Constipation	1	2	0	0
Fatigue	2	2	0	0
Nausea	0	0	0	0
Mucositis/stomatitis	4	0	0	0
Vomiting	1	0	0	0
Hypertension	4	4	1	0
Hand-foot syndrome	1	2	6	0
Rash	1	4	0	0
Skin discoloration	5	0	0	0
Fever	2	0	1	0
Laboratory abnormalities				
Leukopenia	4	6	1	0
Neutropenia	2	4	3	0
Febrile neutropenia	0	0	1	0
Anemia	8	6	4	0
Elevated creatinine	4	4	0	0
Thrombocytopenia	6	7	0	0
AST	7	1	0	0
ALT	4	0	0	0
Total bilirubin	3	1	1	0
GFR	3	2	0	0
Hypothyroidism	0	0	0	0

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GFR: Glomerular filtration rate.

and OS of advanced GIST patients facing IM failure or intolerance. This study reported a median PFS and OS for 23 advanced GIST patients of 8.4 and 14.1 mo, respectively, after sunitinib administration for a median period of 6.0 mo.

Sunitinib induced a sustained clinical benefit in more than 50% of Taiwanese patients with advanced GISTs (15/23; 65.2%)^[29] in our study, which was better than Heinrich's report. A CR induced by tyrosine kinase inhibitors on GIST patients has been sporadically reported. The US S0033 phase III study revealed that the CR rate was 3% for 751 metastatic or unresectable GIST patients receiving 400 or 800 mg IM daily^[30]. In the EORTC 62005 phase III study, the CR rate was 4.76% for 923 metastatic or unresectable patients receiving 400 or 800 mg imatinib daily^[31]. In contrast to the 2 previous studies, the CR rate in this study was 8.7% (2/23) and the median TTR for 2 patients that had a CR was 3.73 mo. The high incidence of CR in this study, even for patients using the second line tyrosine kinase, is because 1 of these 2 patients underwent surgery to achieve complete tumor removal. The limited experience on CR after sunitinib treatment for advanced or metastatic GIST patients facing IM failure or intolerance may still not justify the use of surgery as an adjunct method for target therapy in selected patients.

Regarding the relationship between response rate and kinase mutation, KIT exon 11 and exon 9 mutations predict a favorable response to IM^[32]. Heinrich reported that the clinical activity of sunitinib after IM failure is

significantly impacted by both primary and secondary mutations in the predominant pathogenic kinases, which has implications on optimal treatment of patients with GIST. Heinrich reported that both the clinical benefit and the objective response rates with sunitinib were higher in patients with primary KIT exon 9 mutations than with exon 11 mutations. Similarly, PFS and OS were significantly longer in patients with primary KIT exon 9 mutations or a wild-type genotype than in those with KIT exon 11 mutations^[29]. A possible explanation is that the potency of sunitinib against wild-type and exon 9 mutant KIT was superior to that of imatinib *in vitro*, whereas both drugs exhibited similar potency against KIT exon 11 mutant kinases. These results suggest that the greater clinical benefit seen in sunitinib-treated patients with exon 9 mutant or wild-type imatinib-resistant GISTs may be related to the greater potency of sunitinib against these kinases^[29]. In contrast to Heinrich's study, the clinical benefit, PFS, and OS did not differ between the groups of patients whose GISTs had KIT exon 9 or exon 11 mutation. Although the KIT oncoproteins encoded by exon 9 and exon 11 mutants were unequally sensitive to sunitinib *in vitro*^[29], the limited case number and racial difference might partly explain the similar clinical response rate of sunitinib in terms of KIT exon mutations in Taiwanese GIST patients.

Sunitinib was reasonably well tolerated in our study and the most common treatment-related adverse events were fatigue, diarrhea, skin discoloration, and nausea. Treatment-related adverse events of any severity grade were reported in 83% of sunitinib-treated patients, and serious treatment-related adverse events were reported in 20% of patients^[24]. In contrast to western GIST patients, hand-foot syndrome was the most common cause of grade III adverse events in our study. The reason for this discrepant incidence of hand-foot syndrome is still unknown and needs to be fully clarified. Racial differences in drug metabolism or pharmacokinetics are possible reasons for this observation^[33]. However, Lee *et al*^[34] reported a higher frequency of hand-foot syndrome in Asian patients at Asian sites compared to Asian patients at non-Asian sites and in non-Asian patients in more than 4000 renal cell carcinoma patients receiving sunitinib. A lower frequency of some GI-related adverse events (AEs) in Asian patients at non-Asian sites compared to frequencies in Asian patients at Asian sites and in non-Asian patients has been observed. Recent evidence suggest that heterogeneity in toxicity and efficacy among patients receiving anti-VEGF therapy can be partially explained by genomic variability, including single-nucleotide polymorphisms, providing a possible explanation for the differences in AE frequencies between Asians and non-Asians in this analysis^[34].

Sunitinib-induced hypothyroidism was reported as a side effect in 12% of GIST patients. No hypothyroidism was noted in our series and primary hypothyroidism is not a common complication of therapeutic drugs. Drugs known to affect thyroid function are lithium, thioamides,

amiodarone, and cytokines such as interferon and interleukin-2. The molecular mechanisms of sunitinib-induced hypothyroidism are currently unknown but one possible mechanism by which sunitinib directly affects the thyroid is through the inhibition of VEGFR and/or PDGFR. Recent studies in a mouse model have shown that VEGFR inhibition can induce capillary regression in various organs, including the thyroid. Moreover, the vasculature of the thyroid showed the greatest regression of all organs^[35,36].

In conclusion, sunitinib appears to be a safe and effective treatment for Taiwanese patients with imatinib-resistant/intolerant GIST. Sunitinib induced a sustained clinical benefit in more than 50% of Taiwanese advanced GIST patients, even those facing imatinib failure or intolerance, with a median 8.4 mo PFS. ORR, PFS, and OS did not differ between patients whose GISTs had wild type KIT, KIT exon 9 mutation, or KIT exon 11 mutation. However, hand-foot syndrome accounted for the most common cause of grade III adverse event.

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COMMENTS

Background

The clinical safety and efficacy of both imatinib mesylate (IM) and sunitinib in gastrointestinal stromal tumors (GIST) have primarily been established in Western patients living in the USA or Europe and have not been thoroughly studied in Asian patients. Fifty-six centers in 11 countries participated in the phase III trial of sunitinib in GIST, but only 15 of the 312 patients were of Asian descent (10 and 5 in the sunitinib and placebo groups, respectively).

Research frontiers

To clarify the efficacy and safety of sunitinib in management of Taiwanese GIST patients facing IM intolerance or failure. The response of this second line target therapy also correlates with genetic status of the tumor.

Innovations and breakthroughs

Sunitinib appears to be a safe and effective treatment for Taiwanese patients with imatinib-resistant/intolerant GIST. Sunitinib induced a sustained clinical benefit in more than 50% of Taiwanese advanced GIST patients, even those facing imatinib failure or intolerance, with a median 8.4 mo progression free survival (PFS). ORR, PFS, and overall survival did not differ between patients whose GISTs had wild type KIT, KIT exon 9 mutation, or KIT exon 11 mutation. However, hand-foot syndrome accounted for the most common cause of grade III adverse event.

Applications

The preliminary report helps to clarify the efficacy and safety of sunitinib in management of Taiwanese GIST patients facing IM intolerance or failure.

Peer review

This is a review of therapeutic effects of sunitinib on 22 Taiwanese patients with metastatic GISTs after IM failure. Their data showed that sunitinib was helpful in 15 of the 23 patients, and the clinical benefits of sunitinib did not differ in patients with either primary KIT exon 9 or exon 11 mutation. Although the finding of this study is not new for sunitinib has been shown effective for IM failure, it is an interesting report showing authors' experience in using sunitinib in Taiwanese patients.

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MELD score can predict early mortality in patients with rebleeding after band ligation for variceal bleeding

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Abstract

AIM: To investigate the outcomes, as well as risk factors for 6-wk mortality, in patients with early rebleeding after endoscopic variceal band ligation (EVL) for esophageal variceal hemorrhage (EVH).

METHODS: Among 817 EVL procedures performed for EVH between January 2007 and December 2008, 128 patients with early rebleeding, defined as rebleeding within 6 wk after EVL, were enrolled for analysis.

RESULT: The rate of early rebleeding after EVL for acute EVH was 15.6% (128/817). The 5-d, 6-wk, 3-mo, and 6-mo mortality rates were 7.8%, 38.3%, 55.5%, and 58.6%, respectively, in these early rebleeding patients. The use of beta-blockers, occurrence of hypovolemic

shock, and higher model for end-stage liver disease (MELD) score at the time of rebleeding were independent predictors for 6-wk mortality. A cut-off value of 21.5 for the MELD score was found with an area under ROC curve of 0.862 ($P < 0.001$). The sensitivity, specificity, positive predictive value, and negative predictive value were 77.6%, 81%, 71.7%, and 85.3%, respectively. As for the 6-mo survival rate, patients with a MELD score ≥ 21.5 had a significantly lower survival rate than patients with a MELD score < 21.5 ($P < 0.001$).

CONCLUSION: This study demonstrated that the MELD score is an easy and powerful predictor for 6-wk mortality and outcomes of patients with early rebleeding after EVL for EVH.

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Key words: Model for end-stage liver disease score; Esophageal variceal hemorrhage; Rebleeding; Cirrhosis; Mortality

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INTRODUCTION

Esophageal variceal hemorrhage (EVH) is a serious com-

plication of liver cirrhosis and causes 70% of all upper gastrointestinal bleeding episodes in patients with portal hypertension^[1]. According to the Baveno Consensus Workshop in portal hypertension, endoscopic variceal band ligation (EVL) therapy is recommended for acute EVH, although endoscopic sclerotherapy may be used if ligation is technically difficult^[2]. According to the natural course of EVH, the risk of a recurrent episode of EVH increases after the first EVH but becomes similar to non-bleeding esophageal varices (EV) after 6 wk^[3]. Therefore, rebleeding within 6 wk after the first EVH is coined as early rebleeding. Secondary prophylaxis could reduce the early rebleeding rate to 20%^[11]. Several factors have been identified as predictors of mortality after EVH, including early rebleeding, bacterial infection^[4], hepatic venous pressure gradient (HVPG) > 20 mmHg measured shortly after admission^[5], active bleeding at initial endoscopy, severity of initial bleeding, hematocrit level, AST levels, presence of portal vein thrombosis or of hepatocellular carcinoma (HCC), alcoholic liver disease, serum bilirubin and albumin levels, Child-Turcotte-Pugh (CTP) score^[11], and Model for End-stage Liver Disease (MELD) score^[6-8]. Among these predictors, early rebleeding is the most important one^[3,9]. However, little information is known about the risk factors for mortality in the group of patients with early rebleeding. Thus, the goal of this retrospective study was to investigate the predictive factors for mortality in patients with early rebleeding.

MATERIALS AND METHODS

A total of 817 consecutive EVL procedures for esophageal variceal bleeding were recorded and evaluated in a 3500-bed tertiary referral medical center between January 2007 and December 2008. All of the patients with early rebleeding, defined as rebleeding between one day and 6 wk after ligation, were enrolled. The patients without endoscopic confirmation of rebleeding focus were excluded. The appropriately convened Institutional Review Board approved this study. Finally, 128 cirrhotic patients (15.6%) with early rebleeding were enrolled in our study. Among these patients, 49 patients who died within 6-wk after rebleeding were classified as the mortality group. The remaining 79 patients who survived more than 6 wk were classified as the survival group. The clinical characteristics and laboratory data of the patients in these 2 groups were collected for comparison. Vasoactive drug therapy (terlipressin, somatostatin, or octreotide) was routinely administered before diagnostic endoscopic examination and was continued for at least 3 d according to national insurance guidelines of Taiwan for variceal hemorrhage. Prophylactic antibiotic treatment with intravenous ceftriaxone and non-selective beta-blockers were prescribed for some, depending on the patients' clinical condition, contraindication, adverse effect with tolerability, and physician's preference. Diagnosis of liver cirrhosis was based on a previous liver biopsy or compatible clinical, laboratory, and imaging findings. Hepatocellular carcinoma (HCC) was diagnosed by liver biopsy, fine needle aspiration cytology, or com-

bined typical dynamic imaging appearance and elevated α -fetoprotein (AFP). According to the tumor size, patients with HCC were divided into early (one nodule \leq 5 cm or maximum three nodules, each < 3 cm) or advanced (one nodule > 5 cm or > 3 nodules). The diagnosis of infection was made by positive results of blood, sputum, urine, and ascites bacterial culture or elevated ascites fluid and absolute neutrophil count (ANC) \geq 250 cells/ μ L. In addition, 6-wk mortality was defined as death occurring within 6 wk after rebleeding. The first EVL procedure applied to esophageal variceal bleeding during our study period was considered as the index EVL and index bleeding. The following definitions were used on the basis of the recommendations of the Baveno Consensus Workshop: (1) esophageal variceal bleeding: (a) visible oozing or spurting of blood from a esophageal varix, (b) white nipple sign or blood clot adherent to a varix, (c) presence of medium or large esophageal varices with no other potential bleeding lesion; (2) EVL ulcer bleeding: bleeding from esophageal ulcers after endoscopic EVL with one of the following: (a) active bleeding from the ulcer site, (b) adherent clot at the ulcer site, or (c) absence of other potential bleeding lesions; (3) bleeding duration: the acute bleeding episode was considered finished at the beginning of the first 24-h interval with no hematemesis, stable hemoglobin concentration without blood transfusions, and stable hemodynamic condition; (4) early rebleeding: recurrence of clinically significant hemorrhage (hematemesis/melena, aspiration of greater than 100 mL of fresh blood from nasogastric tube or > 3 g/dL decrease of Hb if no transfusion is given) within 6 wk after index bleeding episode was considered finished; (5) rebleeding 5-d failure: uncontrolled bleeding, death, or recurrent hemorrhage within 5 d since rebleeding; and (6) portal hypertensive gastropathy (PHG) bleeding: a macroscopic finding of a characteristic mosaic-like pattern of gastric mucosa with red-point lesions, cherry red spots, and/or black-brown spots (severe PHG) and the absence of other potential bleeding lesions.

Statistical analysis

Statistical analysis was performed after proper tabulation of data. Continuous variables were expressed as mean with range, and categorical variables were expressed as count with percentage. Groups were compared using Student's software *t*-test for continuous variables and χ^2 test for categorical variables. Multivariate analysis was performed using logistic regression, and a receiver operating characteristic (ROC) curve was generated to assess the predictive accuracy of the variables. All of these values were considered statistically significant if the *P*-value was < 0.05. Cumulative survival estimates were calculated by using the Kaplan-Meier method. All statistical analyses were performed with SPSS statistical for Windows (Version 16; SPSS, Inc., Chicago, IL, USA).

RESULTS

The relevant characteristics of these 128 rebleeding pa-

Table 1 Characteristics of patients with rebleeding after endoscopic variceal band ligation for esophageal varices bleeding (mean \pm SD) *n* (%)

Variable	Overall (<i>n</i> = 128)
Sex (male/female)	107 (83.6)/21 (16.4)
Age (yr)	53.6 \pm 13.9
Etiology	
Virus	61 (47.7)
Alcohol	31 (24.2)
Virus + alcohol	28 (21.9)
Others	8 (6.2)
Index EGD finding	
Active EV bleeding	39 (30.5)
Blood in lumen	49 (38.3)
Clean	40 (31.2)
Duration between rebleeding and Index EVL	14.0 (10.8)
1-5 d	33 (25.8)
6-42 d	95 (74.2)
Index EVL	
CTP score	9.8 \pm 2.3
CTP classification	
A	12 (9.40)
B	44 (34.4)
C	72 (56.2)
MELD	19.8 \pm 8.9
Rebleeding	
CTP score	10.1 \pm 2.6
CTP classification	
A	14 (10.9)
B	36 (28.1)
C	78 (60.9)
MELD	21.4 \pm 9.8

EVL: Endoscopic variceal band ligation; EV: Esophageal varices; CTP: Child-turcotte-pugh; MELD: Model for end-stage liver disease; EGD: Esophago-gastroduodenoscopy.

tients are reported in Table 1. The mean age of the patients was 54 years old (range 14-82); 83.6% were male and 16.4% were female. The etiologies of liver cirrhosis were virus (47.7%), alcohol (24.2%), or combined virus and alcohol (21.9%). The endoscopic findings at index bleeding were active EV bleeding (30.5%), blood in the esophageal or gastric lumen (38.3%), and clean esophago-gastro-duodenal lumen (31.2%). The average interval between index EVL and rebleeding was 14 ± 10.8 d. The average CTP scores at the time of index bleeding and rebleeding were 9.8 ± 2.3 and 10.1 ± 2.6 , and the average MELD scores were 19.8 ± 8.9 and 21.4 ± 9.8 , respectively.

The surveillance of rebleeding sites was carried out by upper GI endoscopy within 1 day for all enrolled patients; the rebleeding site, therapeutic methods, and outcomes are shown in Table 2. The surveillance revealed 75 patients with residual EV bleeding (58.6%), 24 patients with EVL-related esophageal ulcer bleeding (18.8%), 7 patients with gastric variceal bleeding (5.5%), 11 patients with peptic ulcer bleeding (8.6%), and 11 patients with PHG-related bleeding (8.6%). The management methods for rebleeding included combined endoscopic and pharmacologic therapy (71.9%) and pharmacologic therapy only for EVL ulcers or PHG with mild oozing (27.3%). Only one patient was treated with surgical intervention (0.8%). In total, 24 pa-

Table 2 Rebleeding focus, therapy and outcome of patients with rebleeding after endoscopic variceal band ligation for esophageal varices bleeding *n* (%)

	Overall (<i>n</i> = 128)
Rebleeding focus	
EV bleeding	75 (58.6)
Post-EVL ulcer bleeding	24 (18.8)
GV bleeding	7 (5.5)
Peptic ulcer bleeding	11 (8.6)
PHG	11 (8.6)
Therapy for rebleeding	
Endoscopic and pharmacologic therapy	92 (71.9)
Pharmacologic therapy only	35 (27.3)
Surgery	1 (0.8)
Rebleeding 5-d failure	24 (18.8)
Uncontrolled bleeding and died within 5 d	8 (6.3)
Died of non-bleeding cause	2 (1.6)
Recurrent bleeding within 5 d	14 (10.9)
5-d mortality	10 (7.8)
6-wk mortality	49 (38.3)
6-wk mortality causes	
Sepsis-induced multi-organ failure	26 (53.1)
GI bleeding	18 (36.7)
Liver failure	5 (10.2)
3-mo mortality	71 (55.5)
6-mo mortality	75 (58.6)

EV: Esophageal varices; EVL: Endoscopic variceal band ligation; GV: Gastric varices; PHG: Portal hypertensive gastropathy; GI: Gastrointestinal.

tients (18.8%) were associated with 5-d failure at rebleeding of which 8 patients had uncontrolled bleeding and died within 5 d, 2 patients died of other causes within 5 d, and 14 patients had recurrent bleeding within 5 d. The rebleeding mortality rates at 5 d, 6 wk, 3 mo, and 6 mo were 7.8%, 38.3%, 55.5%, and 58.6%, respectively. The causes of death within 6 wk after rebleeding were sepsis-induced multiple organ failure (53.1%), upper gastrointestinal tract hemorrhage (36.7%), and liver failure (10.2%).

We then further divided the patients into two groups according to their mortality or survival during the 6-wk period after rebleeding. The clinical characteristics of patients in the 6-wk mortality group and the survival group are displayed and compared in Table 3. There were no significant differences between these two groups with regard to gender, age, etiology of cirrhosis, HCC, PVT, duration between rebleeding and index EVL, rebleeding focus and treatment methods, antibiotic use, serum platelet count, and sodium and potassium level at rebleeding. However, higher CTP score (11.9 vs 8.9), higher MELD score (28.9 vs 16.8), and hypovolemic shock during rebleeding ($P < 0.001$); higher serum total bilirubin, creatinine, and white cell count levels; lower serum albumin and hemoglobin levels; longer prothrombin time (INR) and active bleeding on endoscopy; and higher hepatic encephalopathy grade were markedly seen in the 6-wk mortality group. Beta-blocker use after rebleeding was also significantly associated with 6-wk mortality.

Furthermore, by multivariate logistic regression analysis, hypovolemic shock (OR = 9.25, 95% CI: 1.68-50.93, $P = 0.011$), beta-blocker use after rebleeding (OR = 0.18, 95%

Table 3 Variables associated with 6-wk mortality in patients with rebleeding after endoscopic variceal band ligation for esophageal varices bleeding (mean \pm SD) *n* (%)

Variable	6-wk mortality (<i>n</i> = 49, 38.3%)	6-wk survival (<i>n</i> = 79, 61.7%)	<i>P</i> -value
Sex (male/female)	40 (81.6)/9 (18.8)	67 (84.8)/12 (17.6)	0.637
Age (yr)	53.3 \pm 13.3	53.7 \pm 14.3	0.866
Etiology			0.683
Virus	23 (46.9)	38 (48.1)	
Alcohol	11 (22.4)	20 (25.3)	
Virus + alcohol	13 (26.5)	15 (19.0)	
Others	2 (4.1)	6 (7.6)	
HCC			0.291
No or small	29 (59.2)	54 (68.4)	
Advanced	20 (40.8)	25 (31.6)	
PVT			0.786
Main trunk	12 (24.5)	18 (22.8)	
Branch	5 (10.2)	9 (11.4)	
Duration between rebleeding and index EVL	12.8 \pm 9.0	14.7 \pm 11.7	0.528
1-5 d	13 (26.5)	20 (25.3)	0.879
6-42 d	36 (73.5)	59 (74.7)	
Rebleeding laboratory findings			
CTP score	11.9 \pm 1.9	8.9 \pm 2.3	0.000
CTP classification			
A	1 (2.0)	13 (16.5)	0.017
B	3 (6.1)	33 (41.8)	0.000
C	45 (91.8)	33 (41.8)	0.000
MELD score	28.9 \pm 9.0	16.8 \pm 7.0	0.000
HE (grade)	1.3 \pm 1.5	0.3 \pm 0.8	0.000
Cr (μ mol/L)	203.3 \pm 159.1	114.9 \pm 97.2	0.000
Na (mmol/L)	138.7 \pm 9.6	134.6 \pm 14.3	0.084
K (mmol/L)	4.2 \pm 1.0	3.9 \pm 0.9	0.171
Bil (μ mol/L)	263.3 \pm 236.0	90.6 \pm 135.1	0.000
Alb (g/L)	25 \pm 5	29 \pm 6	0.000
Hb (g/L)	83 \pm 18	90 \pm 17	0.041
WBC (10^9 /L)	12.5 \pm 6.4	8.2 \pm 4.7	0.000
Platelets (10^9 /L)	102.4 \pm 69.2	101.0 \pm 72.3	0.915
INR (PT)	2.2 \pm 0.9	1.5 (0.4)	0.000
Rebleeding 5-d failure	18 (36.7)	6 (7.6)	0.000
Died within 5 d	10	0	0.000
Recurrent bleeding within 5 d	8	6	0.150
Hypovolemic shock	21 (42.9)	3 (3.8)	0.000
Rebleeding focus			0.500
EV bleeding	28 (57.1)	47 (59.5)	
Post-EVL ulcer bleeding	9 (18.4)	15 (19.0)	
GV bleeding	3 (6.1)	4 (5.1)	
Peptic ulcer bleeding	6 (12.2)	5 (6.3)	
PHG	3 (6.1)	8 (10.1)	
Rebleeding EGD finding			0.019
Active bleeding	20 (40.8)	30 (38.0)	
Blood in lumen	25 (51.0)	27 (34.2)	
Clean	4 (8.2)	22 (27.8)	
EGD Tx			0.716
Endoscopic therapy + medical	36 (73.5)	56 (70.9)	
Medical therapy only	13 (26.5)	22 (27.8)	
Surgery	0 (0.0)	1 (1.3)	
Rebleeding infection	42 (85.7)	36 (45.6)	0.000
Rebleeding antibiotic use	35 (71.4)	46 (58.2)	0.132
Rebleeding lnderal use	8 (16.3)	37 (46.8)	0.000

The *P*-values were calculated by using Student's *t*-test for continuous variables and χ^2 test for categorical variables. EV: Esophageal varices; EVL: Endoscopic variceal band ligation; HCC: Hepatocellular carcinoma; PVT: Portal vein thrombosis; CTP: Child-turcotte-pugh; MELD: Model for end-stage liver disease; INR (PT): International normalised ratio of a patient's prothrombin time to a normal control sample; PHG: portal hypertensive gastropathy; EGD: Esophagogastroduodenoscopy; GV: Gastric varices.

CI: 0.05-0.63, *P* = 0.007), and higher MELD score (OR = 1.17, 95% CI: 1.10-1.25, *P* < 0.001) at rebleeding were found to be independent factors for 6-wk mortality in these patients and this is reported in Table 4. The ROC curve

was used for predicting 6-wk mortality in cirrhotic patients with early rebleeding, and the area under ROC curve (AU-ROC) of the MELD score for predicting 6-wk mortality was 0.862 (95% CI: 0.80-0.93, *P* < 0.001). An optimized

Table 4 Logistic regression models for variables associated with 6-wk mortality in patients with rebleeding after endoscopic variceal band ligation for esophageal varices bleeding

Variables	UV		MV			
	Estimate	P-value	Estimate	OR	95% CI	P-value
Rebleeding laboratory						
CTP score	0.61	< 0.001				
MELD score	0.17	< 0.001	0.16	1.17	1.10-1.25	< 0.001
HE (grade)	0.76	< 0.001				
Cr	0.61	0.001				
Bil	0.09	< 0.001				
Alb	-1.18	0.001				
Hb	-0.23	0.045				
WBC	0.15	< 0.001				
INR (PT)	1.85	< 0.001				
Rebleeding	1.96	< 0.001				
5-d failure						
Hypovolemic shock	2.94	< 0.001	2.23	9.25	1.68-50.93	0.011
Rebleeding	-1.47	0.011				
EGD finding						
Rebleeding	1.97	< 0.001				
infection						
Rebleeding	-1.51	0.001	-1.7	0.18	0.05-0.63	0.007
liveral use						

UV: Univariate analysis; MV: Multivariate analysis; CTP: Child-turcotte-pugh; MELD: Model for end-stage liver disease; HE: Hepatic encephalopathy; INR (PT): International normalised ratio of a patient's prothrombin time to a normal control sample; EGD: Esophagogastroduodenoscopy.

cut-off value of the MELD score is 21.5. As shown in Table 5, the MELD score had a good sensitivity of 78%, specificity of 81%, positive predictive value (PPV) of 72%, and negative predictive value (NPV) of 85% for predicting 6-wk mortality. By the above analyses, a MELD score of ≥ 21.5 was subsequently chosen as the value for identifying patients with a high risk of death at 6 wk after rebleeding.

The Kaplan-Meier survival curves in patients classified according to a MELD score of < 21.5 and ≥ 21.5 revealed a significant difference as shown in Figure 1. The mortality rate was 14.7% in patients with MELD < 21.5 and 71.7% in patients with MELD ≥ 21.5 at 6 wk ($P < 0.001$); 36% in patients with MELD < 21.5 and 83% in patients MELD ≥ 21.5 at 3 mo ($P < 0.001$); and 40% in patients with MELD < 21.5 and 84.9% in patients MELD ≥ 21.5 at 6 mo ($P < 0.001$), respectively.

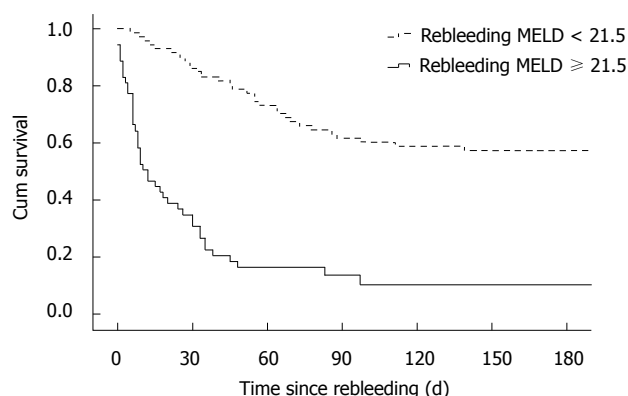
DISCUSSION

Our study has revealed that potential rebleeding sources in cirrhosis cases after index EVL for EVH were esophageal varices (58.6%), esophageal ulcer (18.8%), peptic ulcer (8.6%), PHG (8.6%), and gastric varices (5.5%). The results were consistent with a previous study^[1] reporting that residual esophageal varices were a major source of rebleeding. In addition, beta-blocker usage after rebleeding, hypovolemic shock, and higher MELD score at the time of rebleeding were independent predictors of 6-wk mortality. In addition, we found that the 6-wk mortality rate was 14.7% for patients with MELD scores less than 21.5 and

Table 5 Sensitivity, specificity, positive predictive value, and negative predictive value for predicting 6-wk mortality in patients with rebleeding after endoscopic variceal band ligation for esophageal varices bleeding *n* (%)

6-wk mortality	Sensitivity	Specificity	PPV	NPV
MELD score ≥ 21.5	77.55	81.01	71.7	85.33
Hypovolemic shock (+)	42.86	96.20	87.5	26.92
Beta-blocker use after rebleeding	83.67	46.84	49.4	82.22

PPV: Positive predictive value; NPV: Negative predictive value; MELD: Model for end-stage liver disease.

**Figure 1** Kaplan-Meier survival curves in patients classified according to model for end-stage liver disease score < 21.5 or ≥ 21.5 ($P < 0.001$). MELD: Model for end-stage liver disease.

71.7% for patients with MELD scores more than 21.5.

The mortality rate within 6 wk in our study was 38.3%, which is higher than the mortality rate of patients after acute variceal bleeding^[1,6]. This is probably because the patients with rebleeding after EVH were more advanced in disease severity than patients with initial acute EVH. This difference in severity could be reflected in the mean MELD score; the mean score was 21.4 in the rebleeding patients of our study group, higher than the group of acute variceal bleeding with a MELD score of 12 as previously reported^[6].

The presence of HCC could influence both early rebleeding and mortality in patients with EVH, as reported previously^[11,10]. However, in the present study, advanced HCC and portal vein thrombosis were not predictors of 6-wk mortality, which is consistent with previous reports that advanced HCC is not an independent risk factor but MELD score is a good predictor for early mortality after EVH^[11].

Another independent factor associated with 6-wk mortality in our study was rebleeding related to hypovolemic shock. This observation was similar to previous studies that found that the severity of the hemorrhage was predictive of 6-wk mortality in acute EVH of all cirrhotic patients^[1,6]. The third independent factor associated with 6-wk mortality was the use of beta-blockers, which reflected the general consensus that the use of beta-blockers for the secondary prevention of EVH could reduce mortality^[12]. Overall, 49 patients (38.3%) died within 6 wk after early rebleeding;

among of them, 10 died within 5 d, and 39 died from day 6 to day 42. In our study, the causes of death were sepsis-related multiple organ failure (53.1%), GI bleeding-related complications (36.7%), and liver failure-related complications (10.2%); our results were similar to a recent study reporting that early mortality after cessation of initial EV bleeding is significantly associated with bacterial infection and rebleeding^[13]. This finding provides evidence to support the AASLD guidelines for the treatment of acute variceal bleeding regarding the early use of pharmacological agents and emergent endoscopic procedure within 12 h^[14]. Additionally, for reducing sepsis-related multi-organ failure, prophylactic use of antibiotics for all patients with cirrhosis and GI hemorrhage should be encouraged^[2,15]. Patients with a CTP classification of A respond well to current therapies with minimal risk of death and represented only 2% of the patients in the 6-wk mortality group in our study. Whether current treatment recommendations should be applied to all patients should be further investigated^[16].

In conclusion, this study examined the focuses of rebleeding and treatment outcomes in cirrhotic patients with early rebleeding after EVL for acute EVH. Specifically, the study revealed that hypovolemic shock and MELD scores ≥ 21.5 at the time of rebleeding are predictors for 6-wk mortality in patients with early rebleeding after EVL for acute EVH. Also, beta-blocker use after rebleeding was associated with lower 6-wk mortality.

COMMENTS

Background

The management of variceal bleeding remains a clinical challenge with high mortality. At the present time, available treatments have reduced the 6-wk rebleeding rate to 20%. Early rebleeding is a strong predictor of death from variceal bleeding. Endoscopic therapy increases control of bleeding and decreases the risk of rebleeding and mortality. Despite the fact that endoscopic variceal ligation (EVL) is recommended for acute esophageal variceal bleeding in recent practice guidelines, there has been relatively little research investigating the situation of early rebleeding after EVL for esophageal variceal bleeding.

Research frontiers

Currently, treatment recommendations are applied to all patients with variceal bleeding. At present, only 40% of deaths are directly related to bleeding, while the majority are caused by infection-related multiple organ failure that is paralleled with the severity of liver cirrhosis. Patients with Child-Pugh classification A have good response to current therapy, with a minimal risk of mortality. However, treatment strategies might be different with different Child-Pugh classification.

Innovations and breakthroughs

This study provides evidence that there are independent predictors for 6-wk mortality and rebleeding origin in cirrhotic patients with early rebleeding after therapeutic endoscopic band ligation of initial esophageal varices bleeding.

Applications

This article shows significantly less beta blocker use in the mortality group and recognizes this as an independent poor predictor. To prevent rebleeding with associated mortality, secondary prophylaxis with beta blockers should start as soon as possible from the day after stopping usage of vasoactive drugs. Furthermore, the authors demonstrate that Model for End-Stage Liver Disease (MELD) score is an easy and accurate predictor of 6-wk mortality of patients with early rebleeding after EVL for esophageal variceal bleeding. Accurate predictive rules are provided for early recognition of high risk patients.

Terminology

The Model for End-Stage Liver Disease, or MELD, is a scoring system for assessing the severity of chronic liver disease. It was initially developed

to predict death within three months of surgery in patients who had undergone a transjugular intrahepatic portosystemic shunt procedure, and was subsequently found to be useful in determining prognosis and prioritizing for receipt of liver transplant instead of the older Child-Pugh score. MELD Score = $[0.957 \times \ln(\text{Serum Cr}) + 0.378 \times \ln(\text{Serum Bilirubin}) + 1.120 \times \ln(\text{INR}) + 0.643] \times 10$.

Peer review

The manuscript is a well designed retrospective study with the aim to investigate the predictive factors for mortality in patients with early rebleeding.

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Study on chronic pancreatitis and pancreatic cancer using MRS and pancreatic juice samples

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The parameters were as follows: spectral width, 15 KHz; time domain, 64 K; number of scans, 512; and acquisition time, 2.128 s.

RESULTS: The main component of pancreatic juice included leucine, iso-leucine, valine, lactate, alanine, acetate, aspartate, lysine, glycine, threonine, tyrosine, histidine, tryptophan, and phenylalanine. On performing 1D ¹H and 2D total correlation spectroscopy, we found a triplet peak at the chemical shift of 1.19 ppm, which only appeared in the spectra of pancreatic juice obtained from patients with alcoholic chronic pancreatitis. This triplet peak was considered the resonance of the methyl of ethoxy group, which may be associated with the metabolism of alcohol in the pancreas.

CONCLUSION: The triplet peak, at the chemical shift of 1.19 ppm is likely to be the characteristic metabolite of alcoholic chronic pancreatitis.

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Abstract

AIM: To investigate the markers of pancreatic diseases and provide basic data and experimental methods for the diagnosis of pancreatic diseases.

METHODS: There were 15 patients in the present study, among whom 10 had pancreatic cancer and 5, chronic pancreatitis. In all patients, pancreatic cancer or chronic pancreatitis was located on the head of the pancreas. Pathology data of all patients was confirmed by biopsy and surgery. Among the 10 patients with pancreatic cancer, 3 people had a medical history of long-term alcohol consumption. Of 5 patients with chronic pancreatitis, 4 men suffered from alcoholic chronic pancreatitis. Pancreatic juice samples were obtained from patients by endoscopic retrograde cholangiopancreatography. Magnetic resonance spectroscopy was performed on an 11.7-T scanner (Bruker DRX-500) using Call-Purcell-Meiboom-Gill pulse sequences.

Key words: Pancreatic juice; Pancreatic cancer; Chronic pancreatitis; Magnetic resonance spectroscopy; Magnetic resonance imaging

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INTRODUCTION

Pancreatic cancer accounts for about 2% of all cancer cases, but it has the worst prognosis of all cancers with a 5-year

survival rate of less than 3%^[1,2]. Because of the deep-seated location of the pancreas and no apparent symptoms at the initial stages of pancreatic cancer, it is difficult to diagnose this disease in the early stages. Chronic pancreatitis is a kind of localized or diffuse inflammation, and is caused by many factors. One of the medical dilemmas is to distinguish pancreatic cancer from chronic pancreatitis with a mass in the head of the pancreas; both these diseases have similar clinical behavior and imaging features^[3]. A puncture biopsy is usually preferred over an operation when diagnosing the disease, but this is an injurious procedure and may lead to some complications^[4].

Magnetic resonance imaging (MRI) is the most common procedure used in the diagnosis of pancreatic cancer. An MRI helps obtain images of the pancreas and its surrounding structures^[5]. The deep-seated location of the pancreas and similar clinical manifestations of chronic pancreatitis and pancreatic cancer are the main barriers in differentiating between these two diseases even with advanced MRI techniques. Magnetic resonance spectroscopy (MRS) has high sensitivity and resolution, allows in vitro testing of metabolites, and has been widely used in the field of metabolomics^[6-12]. MRS will be the most potent tool to help differentiate between pancreatic cancer and chronic pancreatitis, and it will be the most effective tool for the early diagnosis of a pancreatic tumor.

Usually, in the process of cancerization, gene and metabolite abnormalities appear before tissue structure transformation. Detection of abnormalities in metabolites facilitates early diagnoses of tumors. Clinically, the serum marker CA19-9^[13] and gene tumor markers such as the K-ras gene^[14,15], p53 anti-oncogene, and p53 protein^[16-19] are widely used as markers of pancreatic cancer; however, these markers are not sensitive, show low specificity, and are used as auxiliary tools^[20]. Beger *et al.*^[10] had successfully used MRS and mass spectrum (MS) to analyze blood constituents of patients with pancreatic cancer and of healthy volunteers; they were able to make a good distinction between pancreatic cancer and the control group by performing lipid profiling of the blood. Pancreatic juice is the exocrine of the pancreas, and is closely related with pancreatic tissues. We wanted to investigate whether it is possible to obtain some information to help differentiate between chronic pancreatitis and pancreatic cancer by analyzing pancreatic juice samples. In the present study, we used MRS technology to analyze the pancreatic juice of patients with pancreatic cancer or chronic pancreatitis with a mass in the head of the pancreas and tried to explore the markers of these diseases. We provided basic data and experimental methods for the study of pancreatopathy.

MATERIALS AND METHODS

The initial subject population comprised 35 patients with pancreatic cancer (24 men and 11 women; mean age, 67.2 years; age range, 47-85 years) recruited between January 2006 and June 2009. We selected 10 subjects (7 men and 3 women; mean age, 67.7 years; age range, 57-74 years) with surgically confirmed pancreatic cancer. The mean maxi-

mum lesion diameter was 26.1 mm (range, 11-51 mm), and all lesions were located in the head of the pancreas. We chose 5 patients (4 men and 1 woman; mean age, 58.3 years) with chronic pancreatitis, confirmed by in vivo biopsy, and lesions located at the head of the pancreas. The medical history of every patient was recorded in detail. All study protocols were approved by our Institutional Review Board, and informed consent was obtained from all patients before they were enrolled in this study.

Pancreatic juice samples were obtained from patients by endoscopic retrograde cholangio-pancreatography (ERCP) in frozen tubes and immediately placed in liquid nitrogen before storing the tubes in a -80°C refrigerator for MRS experiments. All patients were diagnosed by biopsy analyses of pathology data. Both the tumor marker CA19-9 and the cancer gene maker p53 were detected in patients with pancreatic cancer, whereas these markers were not detected in patients with chronic pancreatitis.

MRS experiments

MRS experiments were performed on a Bruker DRX-500 spectrometer (¹H frequency, 500.13 MHz; Bruker Biospin, Rheinstetten, Germany). Pancreatic juice samples were diluted with phosphate buffer in D₂O and placed in sample tubes (diameter, 5 mm), which are used in MRS experiments. Spectra were acquired at 300.0 K using Call-Purcell-Meiboom-Gill (CPMG) pulse sequence^[21,22] along with water presaturation during the relaxation delay of 2 s. The CPMG pulse sequence was applied as a T₂ filter to suppress signals from molecules with short T₂ values (such as macromolecules and lipids), using a total echo time (TE) time of 320 ms. The main parameters for the 1D ¹H-MRS spectra were as follows: spectral width (SW), 15 KHz; time domain (TD), 64 K; number of scans (NS), 512; and acquisition time (AQ), 2.128 s. Spectral assignments were confirmed by 2D ¹H-¹H TOCSY^[23] and J-resolved (JRES) along with the values obtained from the literature^[24]. The main parameters used for TOCSY were as follows: TD (F1-dimensional), 512; TD, (F2-dimensional), 1 K; SW (F1 and F2-dimensional), 5 KHz; and NS, 32. The main parameters used for JRES were as follows: TD (F1-dimensional), 256; TD (F2-dimensional), 8 K; SW (F1-dimensional), 78 Hz; SW (F2-dimensional), 8 KHz; and NS, 32. In both the 2D MRS experiments, the delay time was 2 s. The stability of the pancreatic juice samples was evaluated by repeating a 1D MRS experiment after overall acquisition. No biochemical degradation of any of the pancreatic juice samples was observed.

RESULTS

On combining 2D MRS experimental results (Figure 1) obtained in the present study with results from related literature^[25-27] we identified the main resonances in ¹H MRS spectra of pancreatic juice. TOCSY is a useful 2D MRS technology, it can be used to distinguish the frequencies in the total spin system and improve the sensitivity of detecting small J couplings^[28]. On the basis of TOCSY data, the resonances in 1D ¹H MRS spectra (Figure 2) of some

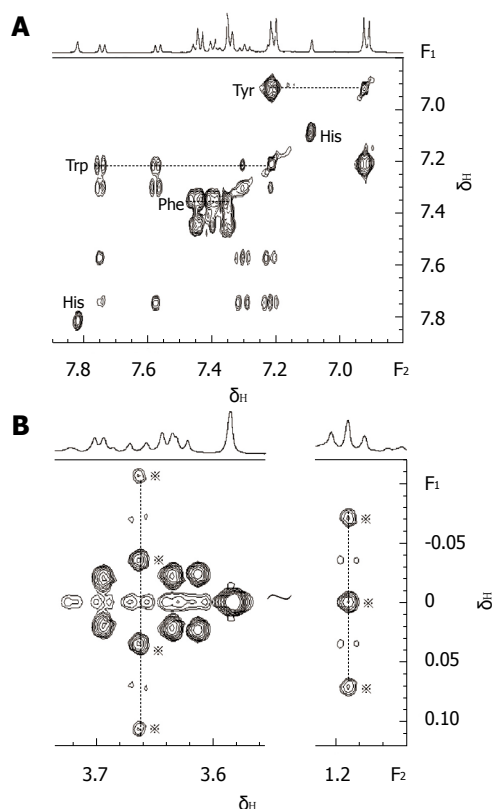


Figure 1 Assignments of partial resonances in ^1H spectra of pancreatic juice. A: 2D Total correlation spectroscopy (TOCSY) of human pancreatic juice; B: JRES spectra of human pancreatic juice. Tyr: Tyrosine; His: Histidine; Trp: Tryptophan; Phe: Phenylalanine.

amino acids were identified. The components, including leucine (Leu), iso-leucine (Ileu), valine (Val), lactate (Lac), alanine (Ala), acetate (Ace), aspartate (Asp), lysine (Lys), glycine (Gly), threonine (Thr), tyrosine (Tyr), histidine (His), tryptophan (Trp) and phenylalanine (Phe), and the locations were also identified.

From the analysis of 1D ^1H MRS spectra of all pancreatic juice samples, it was easy to find a triplet peak at the chemical shift of 1.19 ppm (Figure 3), which only appeared in some spectra. Four of the pancreatic juice samples of patients with chronic pancreatitis showed a triplet peak at the chemical shift of 1.19 ppm on ^1H MRS, whereas the spectra of the pancreatic juice of patients with pancreatic cancer did not show a peak at the chemical shift of 1.19 ppm. When subjected to 2D TOCSY, the spectra of pancreatic juice samples of patients with chronic pancreatitis only showed one correlation peak at the chemical shift of 1.19 ppm and 3.36 ppm (Figure 4). By chemical shift and J coupling constant, we found that the peak at the chemical shift of 1.19 ppm was the ^1H peak of the methyl of ethoxy group ($\text{CH}_3\text{CH}_2\text{O}-$). The 1D ^1H spectra of the 4 pancreatic juice samples of patients with chronic pancreatitis showed a triplet peak at the chemical shift of 1.19 ppm; further, these patients had a history of drinking, which was found from the analysis of pathology data. The 1D ^1H spectra of pancreatic juice obtained from the female patient with chronic pancreatitis did not show a triplet peak at the chemical shift of 1.19 ppm.

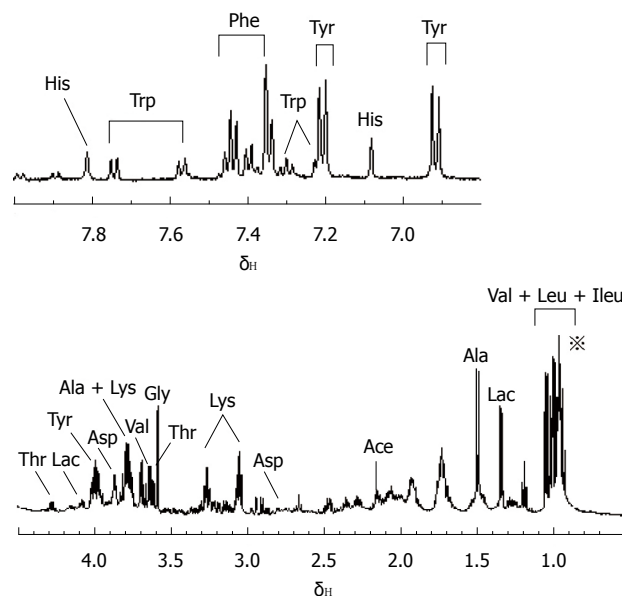


Figure 2 The assignment of proton magnetic resonance spectroscopy spectra of pancreatic juice with chronic pancreatitis. Ace: Acetate; Ala: Alanine; Asp: Aspartate; Gly: Glycine; His: Histidine; Ileu: Iso-leucine; Lac: Lactate; Leu: Leucine; Lys: Lysine; Phe: Phenylalanine; Thr: Threonine; Tyr: Tyrosine; Trp: Tryptophan; Val: Valine.

Among the 10 patients with pancreatic cancer, diagnosed by postoperative analyses of pathology data, 3 people had a medical history of long-term alcohol consumption. Of 5 patients with chronic pancreatitis, 4 men had a medical history of long-term alcohol consumption, and they were typical patients with alcoholic chronic pancreatitis. The female patient suffered from auto-immune chronic pancreatitis by analyses of the pathology data.

DISCUSSION

In recent years, there has been much debate on the relation between chronic pancreatitis and pancreatic cancer. While some scholars^[29] believe that both diseases have a close connection, others^[30] disagree. The results of our study show that there is no apparent difference between the components of pancreatic juice obtained from patients with chronic pancreatitis and pancreatic cancer, except that 1D ^1H spectra of pancreatic juice obtained from the former group of patients who suffered from alcoholic chronic pancreatitis shows a triplet peak of the methyl of ethoxy group ($\text{CH}_3\text{CH}_2\text{O}-$) at the chemical shift of 1.19 ppm. This finding may be used to differentiate pancreatic cancer from alcoholic chronic pancreatitis with a mass in the head of the pancreas.

In the present study of the 10 patients with pancreatic cancer, 3 had a medical history of long-term alcohol consumption, but the 1D ^1H spectra of their pancreatic juice did not show the triplet peak of the ethoxy group ($\text{CH}_3\text{CH}_2\text{O}-$); further, the pathology data of these patients did not show symptoms related to chronic pancreatitis. Hence, we can conclude that alcohol is not the main factor that causes pancreatic cancer. It is controversial whether

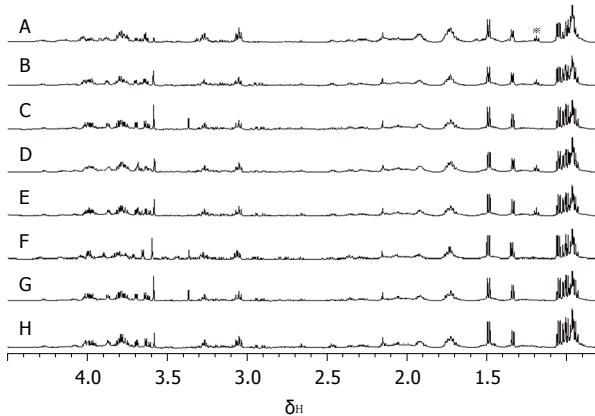


Figure 3 ^1H magnetic resonance spectroscopy spectra of human pancreatic juice with chronic pancreatitis and pancreatic cancer. A-E: 1D ^1H MRS spectra of pancreatic juice of patients with chronic pancreatitis; F-H: 1D ^1H magnetic resonance spectroscopy spectra of pancreatic juice of patients with pancreatic cancer, other 7 similar spectra of pancreatic juice of patients with pancreatic cancer are not shown.

long-term alcohol consumption can cause pancreatic cancer. Riediger *et al.*^[31] found that the association between alcohol and pancreatic cancer was not apparent during epidemiological investigation, which is in accord with our results.

We applied MRS to study pancreatic juice obtained from patients with chronic pancreatitis and pancreatic cancer, and separated the various components of different amino acids in human pancreatic juice by 1D and 2D ^1H spectra. Recently, many analyses on the components of pancreatic juice have focused on the aspect of proteomics. Our study in the field of metabolomics is auxiliary to proteomics, and goes a step further in the study of pancreatopathy.

It is difficult to differentiate between pancreatic cancer and chronic pancreatitis with a mass in the head of the pancreas solely by MRI, because both these diseases have similar clinical behaviors and imaging features. There is no noninvasive method that can be successfully used to diagnose these diseases. Biopsy is frequently used to diagnose pancreatic cancer or chronic pancreatitis with a mass in the head of the pancreas but has some disadvantages, e.g. false negative results, many complications (bleeding, seepage of bile and pancreatic juice), and risk of tumor metastasis. ERCP causes some injury, but it is better than biopsy, because it causes fewer complications. ERCP, when combined with MRS technology, helps obtain more information on metabolites, which cannot be obtained from biopsy or MRI, and is likely to be used to distinguish pancreatic cancer from alcoholic chronic pancreatitis with a mass in the head of pancreas on the basis of ^1H MRS spectra of pancreatic juice.

There are some limitations to this study. Lack of control groups and the content of metabolites in the pancreatic juice being small meant we could not perform a quantitative analysis, and only obtained some qualitative results. The excreta of patients with pancreatic cancer or chronic pancreatitis may not only have different components but also differ in quantity.

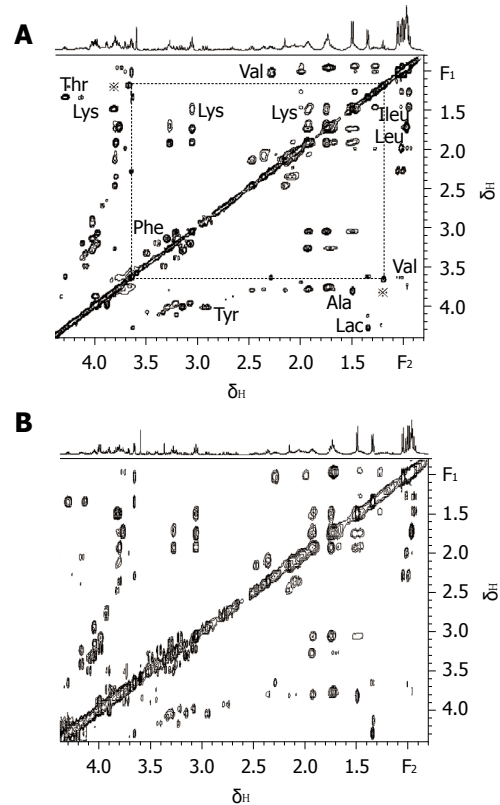


Figure 4 2D total correlation spectroscopy spectra of pancreatic juice with chronic pancreatitis and pancreatic cancer. A: Total correlation spectroscopy (TOCSY) spectra of pancreatic juice from patients with alcoholic chronic pancreatitis; B: TOCSY spectra of pancreatic juice from patients with pancreatic cancer. Ala: Alanine; Ile: Iso-leucine; Lac: Lactate; Leu: Leucine; Lys: Lysine; Phe: Phenylalanine; Thr: Threonine; Tyr: Tyrosine; Val: Valine.

In conclusions, MRS is a powerful tool that can be applied to the study of pancreatic juice obtained from patients by ERCP, and does not cause injury. The triplet peak, which is at the chemical shift of 1.19 ppm in 1D ^1H -MRS data of pancreatic juice obtained from the patients with alcoholic chronic pancreatitis, was identified as resonance of the methyl of ethoxy group ($\text{CH}_3\text{CH}_2\text{O}-$). The ethoxy group may be associated with alcohol metabolism in the pancreas, and is likely to be used to distinguish pancreatic cancer from alcoholic chronic pancreatitis with a mass in the head of the pancreas. In view of small numbers, further confirmation of the results in a larger number of patients is required.

COMMENTS

Background

Pancreatic cancer is a malignant neoplasm of the pancreas, and it has a high death rate. One of the medical dilemmas is to distinguish pancreatic cancer from chronic pancreatitis with a mass in the head of the pancreas; both diseases have similar clinical behavior and imaging features. Exploring the markers to distinguish the diseases is very important to the therapies of patients in clinic.

Research frontiers

CA19-9, *K-ras* gene, p53 anti-oncogene, and p53 protein are widely used as markers of pancreatic cancer, but they are not sensitive and show low specificity. Searching characteristic markers of the diseases is still the goal of tireless pursuit. Magnetic resonance spectroscopy (MRS) has high sensitivity and

resolution, allows in vitro testing of metabolites, and has been widely used in the field of metabolomics. MRS will be the most potent tool to help differentiate between pancreatic cancer and chronic pancreatitis.

Innovations and breakthroughs

It is difficult to differentiate between pancreatic cancer and chronic pancreatitis with a mass in the head of the pancreas solely by Magnetic Resonance Imaging (MRI). Biopsy is frequently used to diagnose the diseases but has many complications and risk of tumor metastasis. Endoscopic retrograde cholangio-pancreatography (ERCP) combined with MRS helps obtain more information on metabolites, which cannot be obtained from biopsy or MRI. We separated the various components of different amino acids in human pancreatic juice. The triplet peak which is at the chemical shift of 1.19 ppm in 1D ¹H MRS spectra was identified as resonance of the methyl of ethoxy group (CH₃CH₂O-), and it may be the characteristic metabolites of the patients with alcoholic chronic pancreatitis.

Applications

This study provides basic data and experimental methods for the diagnosis of pancreatic diseases. The ethoxy group is likely to be used to distinguish pancreatic cancer from alcoholic chronic pancreatitis with a mass in the head of the pancreas.

Terminology

Chemical shift is a basic concept in MRS, the peak appearing in the ¹H MRS spectra with different chemical shift means the nuclear of proton spins with different frequency. The same rotation frequency of nuclear of protons only shows one peak in the ¹H MRS spectra. The triplet peak at the chemical shift of 1.19 ppm is caused by the interaction of nearby nuclear of protons. In fact, it is split by one peak.

Peer review

In my opinion, the article is acceptable for publication.

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Ku80 gene G-1401T promoter polymorphism and risk of gastric cancer

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TT were 65.6%, 22.8% and 11.6% in gastric cancer cases, respectively, and 75.8%, 17.6% and 6.6% in controls, respectively. There were significant differences between gastric cancer and control groups in the distribution of their genotypes ($P = 0.03$) and allelic frequencies ($P = 0.002$) in the Ku80 promoter G-1401T polymorphism.

CONCLUSION: The T allele of Ku80 G-1401T may be associated with the development of gastric cancer.

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Key words: Ku80; Gastric cancer; Polymorphism; Promoter; Carcinogenesis

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Abstract

AIM: To evaluate the possible relationship between the Ku80 gene polymorphism and the risk of gastric cancer in China.

METHODS: In this hospital-based case-control study of gastric cancer in Jiangsu Province, China, we investigated the association of the Ku80 G-1401T (rs828907) polymorphism with gastric cancer risk. A total of 241 patients with gastric cancer and 273 age- and sex-matched control subjects were genotyped and analyzed by polymerase chain reaction-restriction fragment length polymorphism.

RESULTS: The frequencies of genotypes GG, GT and

INTRODUCTION

Gastric cancer is one of the most frequent malignancies in many countries, accounting for 8.7% of all cancers and 10.4% of all cancer deaths in the year of 2000^[1]. In China, gastric cancer remains the leading cause of cancer-related mortality among men and women^[1,2]. It is estimated that about 39% of gastric cancer cases occur in Chinese population^[1,2]. The environmental factors, diet, tobacco, alcohol and *Helicobacter pylori* infection are well-known causes of gastric cancer in China^[3-5]. However, only a fraction of individuals exposed to these factors

develop gastric cancer, suggesting that individual susceptibility to gastric cancer should be different. Currently, the genomic etiology of gastric cancer is of great interest but largely unknown.

DNA damage drives the formation and development of malignant tumors that ameliorate this damage, and its sequelae can be categorized as either gatekeeper or caretaker tumor suppressors, depending on their mode of action^[6]. Nonhomologous end joining (NHEJ) repairs DNA double-strand breaks (DSBs) by joining ends without using a homologous template strand and has been described as a caretaker^[7,8]. Many studies have shown that NHEJ is the predominant repair system in humans, which included the DNA ligase IV and its associated protein XRCC4, and the three components of the DNA-dependent protein kinase (DNA-PK) complex, Ku70, Ku80, and the catalytic subunit PKcs^[9]. The *Ku80* gene, also known as XRCC5, is an important and specific member of NHEJ. Ku70 and Ku80 form a heterodimer called Ku that is well known for its role in NHEJ pathway^[10].

Ku acts as a regulator of transcription by interacting with the recombination signal binding protein J κ and the nuclear factor (NF)- κ B p50 homodimer to up-regulate p50 expression, which may regulate the proliferation of gastric cancer cells^[11]. Gastric cancer cells with a low level of constitutive NF- κ B had a lower expression level of Ku70 and Ku80, which was reflected in the lower nuclear levels of Ku proteins, than the wild-type cells and the cells transfected with control vector^[12,13]. In addition, several studies reported that gastric cancer patients with a lower Ku80 expression level had a slightly prolonged survival after neoadjuvant chemotherapy^[14-16].

Genetic polymorphisms in *Ku80* genes influence DNA repair capacity and change predisposition of several cancers, including colorectal^[17], bladder^[18] and oral cancers^[19]. In addition, in these hospital-based case-control studies of other cancers, it was reported that the frequency of GT/TT type of the *Ku80* gene at promoter G-1401T (rs828907) was significantly higher in cases than in controls^[17-19]. Thus, we assumed that the specific polymorphism of *Ku80* gene may also contribute to gastric cancer. To test the hypothesis that the promoter G-1401T polymorphism is associated with the risk of gastric cancer, we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RELP) to genotype this polymorphism in a hospital-based case-control study of 241 patients with gastric cancer and 273 age- and sex-matched cancer-free controls. The results of this research will lead to a better understanding of the role of SNPs in the *Ku80* genes in gastric cancer carcinogenesis. Such knowledge may eventually lead to the development of better preventive measures for gastric cancer.

MATERIALS AND METHODS

Study population

The case-control study consisted of 241 patients with gastric cancer and 273 cancer-free control subjects. The

gastric cancer patients were confirmed histologically. Genetically unrelated cancer-free individuals were recruited as controls who were selected by matching for age and gender during the same period. All subjects were Han Chinese from the eastern region of China and randomly selected from the Department of General Surgery of the First Affiliated Hospital of Nanjing Medical University between 2005 and 2009. All patients and control subjects voluntarily participated in the study, completed a self-administered questionnaire and donated 5 mL of blood samples. The questionnaire included questions on sex, age, residence, diabetes, hypertension and smoking status. Smoking was defined as ≥ 10 cigarettes per day. This research protocol was approved by the Institutional Review Board of Nanjing Medical University.

Genotyping analysis

Genomic DNA was isolated from peripheral blood lymphocytes using standard phenol-chloroform extraction, as previously described^[20,21]. PCR-RELP assay was used to type the *Ku80* G-1401T (rs828907) polymorphisms. In brief, the primers of the *Ku80* G-1401T polymorphism were 5'-TAGCTGACAACCTCACAGAT-3' (forward) and 5'-ATTCAGAGGTGCTCATAGAG-3' (reverse)^[19], which generated a 252-bp fragment. The PCR reaction was performed in a total volume of 20 μ L containing 2 μ L 10 \times PCR buffer, 1.25 mmol/L MgCl₂, 0.1 mmol/L dNTPs, 0.25 μ mol/L each primer, 200 ng of genomic DNA and 1 U of *Taq* DNA polymerase (MBI Fermentas). The PCR was performed at 94°C for 5 min and followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final elongation at 72°C for 10 min. The restriction enzyme *Bfa*I (New England BioLabs) was used to distinguish the PCR product, and the genotypes were discriminated on 3% agarose gel and visualized by staining with 0.5 μ g/mL ethidium bromide. The wild-type G-allele produced a single 252-bp fragment, and the polymorphic T-allele produced 2 fragments of 81-bp and 171-bp. Approximately, 10%-15% of the samples were randomly selected for repeated assays, and the results were 100% concordant.

Statistical analysis

Continuous variables are presented as mean \pm SD and compared by unpaired Student's *t* test. Continuous variables departing from the normal distribution were presented as median and interquartile range and analyzed by Mann-Whitney *U*-test. Discrete variables were represented as frequencies and percentages and evaluated by the Pearson's χ^2 test. Pearson's χ^2 test was also used to compare the distribution of the *Ku80* genotypes between cases and controls. The association between the *Ku80* G-1401T polymorphism and the risk of gastric cancer was estimated by odds ratio (OR) and 95% CI using multivariate logistic regression. *P* < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1 Baseline characteristics of cases and controls *n* (%)

Characteristics	Cases (<i>n</i> = 241)	Controls (<i>n</i> = 273)	<i>P</i>
Sex (male)	181 (75.1)	193 (70.7)	0.43
Age (yr)	57.9 ± 12.9	56.9 ± 14.1	0.52
Smoking	61 (25.3)	39 (14.3)	0.014
Residence (rural)	111 (46.1)	142 (52.0)	0.32
Hypertension	21 (8.7)	28 (10.3)	0.58
Diabetes	15 (6.2)	22 (8.1)	0.51

Table 2 Genotype of *Ku80* G-1401T polymorphism in cases and controls *n* (%)

Genotype	Cases	Control
GG	158 (65.6)	207 (75.8)
GT	55 (22.8)	48 (17.6)
TT	28 (11.6)	18 (6.6)

$\chi^2 = 7.26$, *df* = 2, *P* = 0.03.

RESULTS

Baseline characteristics

The frequency distributions of selected characteristics of the cases and controls are presented in Table 1. There was no significant difference between the cases and controls in sex (male: 75.1% *vs* 70.7%, *P* = 0.43) and age (57.9 ± 12.9 years *vs* 56.9 ± 14.1 years, *P* = 0.52), indicating that the matching for the subjects was successful. More smokers were found among gastric cancer cases compared with controls (25.3% *vs* 14.3%, *P* = 0.014). No significant differences were noted in residing in the rural area (46.1% *vs* 52.0%, *P* = 0.32), hypertension (8.7% *vs* 10.3%, *P* = 0.58) and diabetes (6.2% *vs* 8.1%, *P* = 0.51).

Genotype distributions and allele frequencies

Table 2 shows the distribution of the genotypic for the *Ku80* G-1401T (rs828907) between gastric cancer patients and controls. The genotypic frequencies in both gastric cancer and control groups were in agreement with those predicted by Hardy-Weinberg equilibrium (*P* = NS). The distribution of the *Ku80* G-1401T genotypes (GG, GT and TT) was markedly different between cases (65.6%, 22.8%, and 11.6%) and controls (75.8%, 17.6%, and 6.6%, *P* = 0.03). A significantly different distribution of the *Ku80* G-1401T genotype was demonstrated among the cases and controls. As shown in Table 3, the frequency of T allele was significantly higher in gastric cancer patients than in control subjects (23.0% *vs* 15.4%, *P* = 0.002).

Stratified analyses for the variant *Ku80* genotype in cases and controls

The multivariate logistic regression analysis was further used to evaluate the association between the G-1401T polymorphism and gastric cancer stratified by risk factors including age, sex, smoking and residence under control (Table 4). Adjusted OR (for age, sex, smoking status,

Table 3 Allele distribution of *Ku80* G-1401T polymorphism in cases and controls *n* (%)

Allele	Cases	Controls
G	371 (77.0)	462 (84.6)
T	111 (23.0)	84 (15.4)

$\chi^2 = 9.73$, *df* = 1, *P* = 0.002.

residence, diabetes and hypertension) with 95% CI for mutant genotypes was all described. In statistical analyses stratified by the median age of controls (58 years), the increased risk associated with the GT/TT genotypes tended to be more evident in the younger subjects aged < 58 years (adjusted OR = 1.97, 95% CI: 1.05-2.90). However, we did not note a statistically significant inverse association with gastric cancer risk in older subjects aged ≥ 58 years (adjusted OR = 1.31, 95% CI: 0.88-1.96). The adjusted OR for the GT/GT genotypes was 1.81 (95% CI: 1.28-2.52) in male subjects and 1.33 (95% CI: 0.81-2.24) in female subjects. We did not note a statistically significant inverse association with gastric cancer risk in both non-smokers (adjusted OR = 1.48; 95% CI: 1.08-2.02) and smokers (adjusted OR = 2.52; 95% CI: 1.25-5.18). In urban subjects, there was significant evidence of an increased risk of gastric cancer in the variant genotypes (adjusted OR = 1.88; 95% CI: 1.26-2.76), while the association was not statistically significant in rural subjects (adjusted OR = 1.48; 95% CI: 0.99-2.15).

DISCUSSION

In this hospital-based, case-control study, we assessed the potential association between the *Ku80* G-1401T polymorphism and the presence of gastric cancer in Chinese population. To our best knowledge, this is the first study linking the *Ku80* G-1401T polymorphism with gastric cancer risk. Our data showed that the *Ku80* -1401 G to T variant was associated with the increased risk of gastric cancer.

Gastric cancer is a genetic disease developing from a multifactorial, multigenetic and multistage process^[22,23]. It was widely accepted that both genetic and environmental factors may be involved in the etiology of gastric cancer^[24]. During the multistage carcinogenesis, Ku80 may be involved in multiple important cellular processes. To date, several studies have reported abnormal expression of Ku80 protein in various cancers^[13,25-28]. Over-expression of Ku80 increased the capability of cancer acquired resistance to radiation and chemical drugs^[29-31], while suppression of Ku80 expression decreased cellular proliferation, colony formation and inhibited tumorigenicity in a xenograft model^[32]. As an important component of NHEJ, Ku80 and Ku70 form a heterodimer, which acts as a regulatory subunit of the DNA-dependent protein kinase complex DNA-PK by increasing the affinity of the catalytic subunit PRKDC to DNA^[17]. The *Ku80* gene plays an important and specific role in removing DSBs. Chang *et al*^[18]

Table 4 Stratification analyses of the association between *Ku80* polymorphism and risk of gastric cancer *n* (%)

Variable	Cases (<i>n</i> = 241)		Controls (<i>n</i> = 273)		Adjusted OR (95% CI) ¹	<i>P</i>
	GG	GT + TT	GG	GT + TT		
Age (yr) (median)						
< 58	76 (62.3)	46 (37.7)	109 (76.8)	33 (23.2)	1.97 (1.05-2.90)	0.01
≥ 58	82 (68.9)	37 (31.1)	98 (74.8)	33 (25.2)	1.31 (0.88-1.96)	0.3
Sex						
Male	118 (65.2)	63 (34.8)	149 (77.2)	44 (22.8)	1.81 (1.28-2.52)	0.01
Female	40 (66.7)	20 (33.3)	58 (72.5)	22 (27.5)	1.33 (0.81-2.24)	0.46
Smoking status						
Smokers	39 (63.9)	22 (36.1)	32 (82.1)	7 (17.9)	2.52 (1.25-5.18)	0.051
Non-smokers	119 (66.1)	61 (33.9)	175 (74.8)	59 (25.2)	1.48 (1.08-2.02)	0.054
Residence						
Urban	85 (65.4)	45 (34.6)	102 (77.7)	29 (22.3)	1.88 (1.26-2.76)	0.025
Rural	73 (65.8)	38 (34.2)	105 (73.9)	37 (26.1)	1.48 (0.99-2.15)	0.16

¹Adjusted for age, sex, smoking status, hypertension, diabetes and residence.

found evidence that the *Ku80* G-1401T variant was associated with increased risk of bladder cancer in a central Taiwanese population. A recent study, involving 362 patients with colorectal cancer and 362 age- and gender-matched healthy controls, showed that the T allele *Ku80* G-1401T conferred a significantly ($P = 0.0069$) increased risk of colorectal cancer^[17]. These observations were consistent with the findings previously described by other investigators from Asian populations^[19].

To further investigate the association between the *Ku80* promoter G-1401T polymorphism and the risk of gastric cancer, we conducted this hospital-based case-control study in a Chinese population which incorporated the information on exposure to smoking, residence and other potential confounding factors (age and sex) that were frequency matched between cases and controls and further adjusted in the analysis. In our study, a significant difference of the *Ku80* G-1401T genotype distribution was found between gastric cancer cases and controls. The frequency of T allele was significantly higher in gastric cancer patients than in control subjects.

The precise mechanisms underlying the relationship between *Ku80* polymorphism and stomach carcinogenesis remain unclear. Although the *Ku80* promoter G-1401T genetic variation does not directly lead to amino acid coding change, presumably, it is plausible that this SNP influences the expression level or stability of the *Ku80* protein by the alternative splicing, intervention, modification, determination or involvement. It is similar to another important member of NHEJ, XRCC4. A few reports provided evidence that its SNPs located on the promoter region are significant in various cancers^[33,34].

Our data also showed that the association between increased gastric cancer risk and the mutant genotypes (GT + TT) was more evident in younger subjects aged < 58 years than in older subjects. We also found an interaction between genotype and sex. The adjusted OR was 1.81 (95% CI: 1.28-2.52) for GT/TT genotype compared with GG genotype among male subjects. But the OR (adjusted OR = 1.33; 95% CI: 0.81-2.24) was not statistically significant among female subjects. Our findings

were inconsistent with previous observations by Yang *et al.*^[17] and Chang *et al.*^[18]. The reason for the different observations remains unclear.

In addition, we did not note a statistically significant inverse association with gastric cancer risk in both non-smokers (adjusted OR = 1.48; 95% CI: 1.08-2.02) and smokers (adjusted OR = 2.52; 95% CI: 1.25-5.18). But Yang *et al.*^[17] reported that the GT and TT genotypes, in association with smoking, conferred an increased risk (adjusted OR = 2.537; 95% CI: 1.398-4.601) for colorectal cancer. Similarly, Chang *et al.*^[18] and Hsu *et al.*^[19] found a significantly decreased risk of bladder cancer (adjusted OR = 2.053; 95% CI: 1.232-3.419) and oral cancer in smokers with GT or TT genotypes^[18,19]. The results are inconsistent with our findings. The reason for the different observations remains unclear. Several studies have reported that smoking is associated with free radical-induced DNA damage and strand breaks^[26], and tobacco smoke contains some potential carcinogens including polycyclic aromatic hydrocarbons, tobacco nitro-amines, aromatic amines and BPDE, which form DNA bulky adducts and DNA strand breaks^[27,35].

The stratified analyses by residence revealed that the association was significant in variant genotypes in urban subjects (OR = 1.88; 95% CI: 1.26-2.76) but not in rural subjects (adjusted OR = 1.48; 95% CI: 0.99-2.15). The different results may be explained, at least in part, between rural and urban subjects. Environmental factors, including air, soil, diet, occupation and lifestyle, may be responsible for the different observations between rural and urban subjects. It was plausible, considering the better environment in rural areas^[36].

The potential limitations of the present study should be stressed. Firstly, in this hospital-based case-control study, we selected controls from individuals with a variety of nonmalignant diseases. These may cause the possibility of selection bias and confound the results. Nevertheless, the frequencies of *Ku80* G-1401T polymorphism variant alleles were similar to those reported in the NCBI Website in the Asian population studies. T allele frequencies of *Ku80* promoter G-1401T are 15.4% in our

control group and 17.4% for Asian population in NCBI. The genotype distribution of controls in our study met Hardy-Weinberg equilibrium conditions. Secondly, the sample size of the present study was relatively small, which may limit the statistical power. Finally, our study was conducted in Chinese population. Caution should be exercised when extrapolating the data to other ethnic groups.

In conclusion, we found a significant difference in the Ku80 G-1401T polymorphism distribution between the patients with gastric cancer and the control group. The T allele of the Ku80 G-1401T was found more frequently in patients with gastric cancer and it may be associated with an increased risk of gastric cancer, suggesting that the polymorphism of Ku80 G-1401T, involved in the gastric tract carcinogenesis, may be a useful marker for primary prevention and anticancer intervention. Further studies are needed to determine the exact nature of this relationship.

COMMENTS

Background

The Ku80 gene is an important and specific member of NHEJ. Genetic polymorphisms in Ku80 genes (G-1401T) influence DNA repair capacity and change the predisposition of several cancers, including colorectal, bladder and oral cancer. Whether genetic variants are involved in the risk of gastric cancer in a Chinese population is unknown.

Research frontiers

In this study, the frequency of the Ku80 G-1401T GT/TT genotypes was significantly higher in the gastric cancer patients than in control subjects. This is the first analysis of the association between genetic predisposition and gastric cancer risk in Chinese population.

Innovations and breakthroughs

The Ku80 G-1401T polymorphisms may modulate the development of gastric cancer in a Chinese population.

Applications

The Ku80 G-1401T GT/TT genotypes can be used as biomarkers for selecting patients from the individuals at high risk for gastric cancer in China. Identifying such susceptibility polymorphisms may lead to the development of tests that allow more focused follow-ups of high-risk groups.

Terminology

The Ku80 gene, also known as XRCC5, is an important and specific member of NHEJ. As an important component of NHEJ, Ku80 and Ku70 form a heterodimer, which acts as a regulatory subunit of the DNA-dependent protein kinase complex DNA-PK by increasing the affinity of the catalytic subunit PRKDC to DNA.

Peer review

The quality of the work and the methodology are sound. The conclusions are appropriate, although it seems unlikely that these findings represent a major breakthrough.

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Effects of penethyclidine hydrochloride on rat intestinal barrier function during cardiopulmonary bypass

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Abstract

AIM: To test the ability of penethyclidine hydrochloride (PHC) to attenuate intestinal injury in a rat cardiopulmonary bypass (CPB) model.

METHODS: Male Sprague-Dawley rats were randomly divided into six groups (eight each): sham-operated control; sham-operated low-dose PHC control (0.6 mg/kg); sham-operated high-dose PHC control (2.0 mg/kg); CPB vehicle control; CPB low-dose PHC (0.6 mg/kg); and CPB high-dose PHC (2.0 mg/kg). Blood samples were collected from the femoral artery 2 h after CPB for determination of plasma diamine oxidase (DAO), D-lactate and endotoxin levels. Spleen, liver, mesenteric lymph nodes and lung were removed for biochemical analyses. Intestinal tissue ultrastructure was examined by electron microscopy.

RESULTS: In the sham-operated groups, high- and low-dose-PHC had no significant impact on the levels of DAO, D-lactate and endotoxin, or the incidence of

intestinal bacterial translocation (BT). Serum levels of DAO, D-lactate, endotoxin and the incidence of intestinal BT were significantly increased in the surgical groups, compared with the sham-operated groups (0.543 ± 0.061 , 5.697 ± 0.272 , 14.75 ± 2.46 , and $0/40$ vs 1.038 ± 0.252 , 9.377 ± 0.769 , 60.37 ± 5.63 , and $30/40$, respectively, all $P < 0.05$). PHC alleviated the biochemical and histopathological changes in a dose-dependent manner. Serum levels of DAO, D-lactate, and endotoxin and the incidence of intestinal BT in the high-dose PHC group were significantly lower than in the low-dose PHC group (0.637 ± 0.064 , 6.972 ± 0.349 , 29.64 ± 5.49 , and $14/40$ vs 0.998 ± 0.062 , 7.835 ± 0.330 , 38.56 ± 4.28 , and $6/40$, respectively, all $P < 0.05$).

CONCLUSION: PHC protects the structure and function of the intestinal mucosa from injury after CPB in rats.

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Key words: Penethyclidine hydrochloride; Intestinal mucosa injury; Cardiopulmonary bypass

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INTRODUCTION

Cardiopulmonary bypass (CPB) is essential during some cardiovascular surgical procedures; however, it can cause

peripheral hypoperfusion as a result of non-pulsatile flow, low blood pressure, hemodilution, and other non-physiological conditions. Furthermore, an increase in intestinal permeability and bacterial translocation (BT) has been demonstrated, not only in animal models, but also in patients during CPB^[1-3]. Perioperative gastrointestinal integrity is therefore now recognized as an important factor determining the outcome of cardiac surgical procedures^[4]. In general, changes in mucosal permeability and morphology during CPB reflect the degree of damage to intestinal mucosal barrier function.

Previous *in vitro* studies have demonstrated that tropane alkaloids can stabilize the cell membrane and prevent oxidative stress. The new anticholinergic drug, penheyclidine hydrochloride (PHC), has been evaluated for its protective effects on the cardiovascular system^[5-7]. Its selective blocking of M1, M3 and N receptors means that PHC has few M2 receptor-associated cardiovascular side effects. It has been shown to reduce endotoxin-stimulated acute lung injury and to attenuate liver damage during CPB in a rat model^[8-10].

Based on the potential roles of PHC as an antioxidant and a cell membrane stabilizer, we hypothesized that its administration might reverse CPB-associated intestinal damage. Zhan *et al.*^[11] have suggested that PHC concentrations of 0.18-3.60 mg/kg were found to be safe, and we therefore tested this hypothesis in a rat CPB model, using high- (2.0 mg/kg) and low-dose (0.6 mg/kg) PHC.

MATERIALS AND METHODS

Animals and treatments

Forty-eight male Sprague-Dawley rats (weighing 300-450 g, 18-22 wk old) were randomly assigned to one of six groups (eight each): sham-operated control; sham-operated control + low-dose PHC (0.6 mg/kg) (sham L-PHC); sham-operated control + high-dose PHC (2 mg/kg) (sham H-PHC); CPB + vehicle (control); CPB + low-dose PHC (0.6 mg/kg) (L-PHC); and CPB + high-dose PHC (2.0 mg/kg) (H-PHC). PHC (Lisite Pharmacology Co. Chendou, China, No. 080301) was dissolved in absolute ethanol and diluted in saline (final concentration of ethanol < 1.0%), and added to the priming solution for CPB. All animals received humane care in compliance with the Principles of Laboratory Animal Care. The experimental protocol was approved by the local animal use and care committee at the General Hospital of Shenyang Commend, China.

Surgical procedure

None of the sham-operated control groups underwent CPB. After PHC injection, the respiratory rate (RR), heart rate (HR), blood pressure (BP) and electrocardiography (ECG) were continually monitored. The rat CPB model was established as previously described, with some modifications^[12]. In brief, rats were anesthetized by intraperitoneal administration of 10% chloral hydrate (0.3 mL/100 g body weight) to provide stable anesthesia,

while maintaining spontaneous ventilation during the entire operative procedure. All subsequent procedures were performed under aseptic conditions.

After surgical-level anesthesia was achieved, the left femoral artery was cannulated using a 22-gauge Teflon heparinized catheter. Arterial pressure was monitored and blood samples were collected for gas analysis, using a blood gas analyzer (GEM Premier 3000; Mallinckrodt, Lexington, MA, USA). Following administration of heparin (250 U/kg), an 18-gauge catheter was inserted into the right jugular vein and advanced to the right atrium. A 22-gauge catheter was cannulated into the tail artery, to serve as an arterial infusion line for the CPB circuit. The mini-CPB circuit comprised a venous reservoir, a specially designed membrane oxygenator, a roller pump, and sterile tubing with inner diameters of 4 mm for the venous line and 1.6 mm for the arterial line. The CPB circuit was primed with a total volume of 15 mL of synthetic colloid solution. The perfusion flow rate was gradually adjusted to sustain a mean arterial pressure of 60-80 mmHg. The gas flow (95% O₂, 5% CO₂) was initiated at around 50-75 mL/kg per minute and adjusted to maintain blood gas analysis parameters within the physiological range. When the flow rate reached 80-100 mL/kg per minute, it was maintained for 60 min. At the end of CPB, the flow rate was reduced stepwise to achieve hemodynamic stabilization. Throughout the experiment, central body temperature was monitored with a rectal probe and kept at 37.5 ± 1.0°C using a heat lamp placed above the animal and CPB equipment. The mean arterial pressure was maintained at 60-80 mmHg.

Specimen collection

Rats from each group were sacrificed by decapitation, and arterial blood (2.0 mL) and terminal ileums were sampled at 2 h after CPB. Plasma was prepared by centrifugation at 3000 g for 5-10 min at 4°C and stored at -70°C for determination of serum diamine oxidase (DAO), D-lactate, and endotoxin. Intestinal (ileum) tissue samples were obtained for electron microscopy.

Intestinal permeability

The permeability of the intestinal mucosa was assayed by measuring D-lactate and DAO levels in plasma. Plasma D-lactate levels were measured by enzymatic spectrophotometric assay using a centrifugal analyzer at 30°C, as described previously^[13]. Plasma DAO activities were also determined by enzymatic spectrophotometry, as described previously^[14]. D-lactate, D-lactate dehydrogenase, NAD⁺, O-dianisidine, cadaverine dihydrochloride and DAO were purchased from Sigma Chemical Company (Milan, Italy).

Plasma endotoxin determination

The endotoxin content of the plasma sample was assayed using the *Limulus* amoebocyte lysate test using the Endochrome K test kit (Cochran, Vienna, Austria). In brief, heparinized plasma was diluted 1:10 in pyrogen-free water

Table 1 Physiological data (mean \pm SD, $n = 8$)

	Groups	Pre-CPB	CPB 30 min	CPB 60 min	Post-CPB 2 h
MAP (mmHg)	Controls	86.33 \pm 16.82	84.26 \pm 14.55	85.45 \pm 10.36	83.63 \pm 11.24
	CPB + vehicle	84.50 \pm 7.05	62.14 \pm 15.23 ^{a,c}	67.08 \pm 19.12 ^{a,c}	72.18 \pm 17.39
	CPB + L-PHC	85.45 \pm 9.36	66.58 \pm 11.26 ^{a,c}	69.78 \pm 14.05 ^{a,c}	76.15 \pm 13.65
	CPB + H-PHC	87.54 \pm 10.43	65.73 \pm 9.85 ^{a,c}	70.85 \pm 12.36 ^{a,c}	77.84 \pm 12.36
HR (beats/min)	Controls	325 \pm 34	320 \pm 25	315 \pm 20	324 \pm 15
	CPB + vehicle	315 \pm 30	286 \pm 28	305 \pm 42	310 \pm 37
	CPB + L-PHC	305 \pm 26	315 \pm 34	302 \pm 38	304 \pm 27
	CPB + H-PHC	310 \pm 20	296 \pm 25	301 \pm 35	305 \pm 30
PH	Controls	7.40 \pm 0.02	7.41 \pm 0.03	7.39 \pm 0.02	7.40 \pm 0.02
	CPB + vehicle	7.41 \pm 0.03	7.38 \pm 0.05	7.35 \pm 0.06	7.39 \pm 0.02
	CPB + L-PHC	7.38 \pm 0.04	7.43 \pm 0.02	7.41 \pm 0.03	7.36 \pm 0.04
	CPB + H-PHC	7.42 \pm 0.01	7.42 \pm 0.03	7.38 \pm 0.04	7.43 \pm 0.02
BE (mmol/L)	Controls	-1.96 \pm 0.45	-2.36 \pm 0.75	-1.54 \pm 0.85	-1.95 \pm 0.54
	CPB + vehicle	-1.86 \pm 0.35	-1.36 \pm 0.26	-1.60 \pm 0.84	-2.30 \pm 1.50
	CPB + L-PHC	-1.75 \pm 0.26	-1.55 \pm 0.96	-1.95 \pm 0.63	-2.04 \pm 0.72
	CPB + H-PHC	-1.85 \pm 0.36	-2.05 \pm 0.90	-1.74 \pm 0.42	-2.45 \pm 0.14
HCT (%)	Controls	41.10 \pm 1.85	40.20 \pm 1.53	40.60 \pm 1.46	39.80 \pm 1.35
	CPB + vehicle	41.80 \pm 3.73	26.45 \pm 4.24 ^{a,c}	21.54 \pm 3.71 ^{a,c}	27.82 \pm 3.66 ^{a,c}
	CPB + L-PHC	42.20 \pm 2.45	27.65 \pm 3.65 ^{a,c}	23.05 \pm 5.30 ^{a,c}	29.53 \pm 5.45 ^{a,c}
	CPB + H-PHC	42.45 \pm 2.50	28.76 \pm 5.38 ^{a,c}	22.46 \pm 4.25 ^{a,c}	28.75 \pm 4.56 ^{a,c}

Controls summarize the results from sham-operated rats treated with saline or penehyclidine hydrochloride. ^a $P < 0.05$ vs baseline; ^c $P < 0.05$ vs controls. CPB: Cardiopulmonary bypass; MAP: Mean arterial pressure; HCT: Hematocrit; BE: Buffer excess; HR: Heart rate.

and kept heated at 75°C for 10 min to remove non-specific inhibitors. A quantitative chromogenic kinetic method was used, as specified by the manufacturer, using a Thermo microplate reader (Tecan Spectra, Salzburg, Austria). The method had a detection limit of 0.75 pg/mL at a 1:10 plasma dilution.

Bacteriological cultures

A midline incision was made using a sterile technique. Mesenteric lymph nodes (MLNs), portal vein, and samples from the liver, spleen, and lung were harvested and weighed prior to determination of bacterial growth. The samples were homogenized in test tubes containing 3 mL Brain Heart Infusion Broth (Difco, Detroit, IL, USA). The supernatant (0.2 mL) was cultured for growth of aerobic, microaerophilic and anaerobic bacteria. All media for aerobic cultures were incubated at 37°C for at least 3-5 d, while organ samples for anaerobic bacteria were cultured at 37°C for 7 d. Enteric Gram-negative bacteria were identified using the API 20 system (BioMérieux SA, Marcy-l'Etoile, France) and *Lactobacillus acidophilus* by API 50 CH (Analytab Products Inc., Plainview, NY, USA). All other aerobic, microaerophilic and anaerobic microbes isolated were identified by standard procedures. The numbers of living bacteria were calculated and expressed as the numbers of living organisms per gram of organ tissue.

Transmission electron microscopy

For transmission electron microscopy, ileum tissues were removed immediately from anesthetized rats 2 h after CPB, and then fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in PBS (pH 7.3) for 2 h at room temperature (25°C). The tissues were washed with PBS, fixed

with 1% osmium tetroxide for 2 h, washed again, and then embedded in Araldite 6005. Tissue sections were cut with a Leica EM FCS (Vienna, Austria) ultramicrotome. Tissue sections (1 μ m) were initially stained with toluidine blue-Azur II to select the region of interest for subsequent procedures. Thin sections (60-70 nm) were stained with uranyl acetate and lead citrate and examined and photographed using an H-7200 transmission electron microscope (80 kV; Oberkochen, Germany). Electron microscopy pictures were evaluated twice by two independent histologists with at least 10 years of experience, who were blinded to our study.

Statistical analysis

All experimental data were expressed as mean \pm SD and analyzed using a SPSS for Windows v. 13.0 (Chicago, IL, USA). One-way ANOVA was used for comparisons among various treatment groups. *Post-hoc* comparisons were analyzed using least significant difference test or Dunnett's T3 test. $P < 0.05$ was considered to be statistically significant.

RESULTS

PHC reverses the increase in intestinal mucosal permeability after CPB

There were no obvious changes in the RR, HR, BP or ECG at any time points (0, 30, 60, or 120 min) after anesthesia in the sham-operated groups (Table 1). Hemodynamic changes in the vehicle control, L-PHC and H-PHC groups are shown in Table 1. As shown in Table 2, DAO and D-lactate levels increased significantly in vehicle-treated CPB rats, compared with the sham group ($P < 0.05$), which effect was largely reversed by treatment with

Table 2 Plasma diamine oxidase, D-lactate and endotoxin levels (mean \pm SD, $n = 8$)

	DAO (U/L)	D-lactate (mg/L)	Endotoxin (pg/mL)
Controls	0.543 \pm 0.061	5.697 \pm 0.272	14.75 \pm 2.46
CPB + vehicle	1.038 \pm 0.252 ^a	9.377 \pm 0.769 ^a	60.37 \pm 5.63 ^a
CPB + L-PHC	0.998 \pm 0.062 ^{a,e}	7.835 \pm 0.330 ^{a,e}	38.56 \pm 4.28 ^{a,e}
CPB + H-PHC	0.637 \pm 0.064 ^{a,c}	6.972 \pm 0.349 ^{a,c}	29.64 \pm 5.49 ^{a,c}

Controls summarize the results from sham-operated rats treated with saline or penhyclidine hydrochloride (PHC). Data are shown as medians, $n = 8$ rats for each group. CPB: Cardiopulmonary bypass; H-PHC: High-dose PHC; L-PHC: Low-dose PHC; DAO: Diamine oxidase. The level of significance was set at $P < 0.05$. ^a $P < 0.05$ vs baseline; ^c $P < 0.05$ vs CPB group; ^e $P < 0.05$ vs H-PHC group.

Table 3 Numbers of animals pretreated with vehicle or penhyclidine hydrochloride with positive bacteriological cultures from blood (portal vein), mesenteric lymph nodes, liver, spleen and lungs after induction of cardiopulmonary bypass

	Portal vein	MLN	Liver	Lungs	Spleen	Total
Controls	0	0	0	0	0	0/40
CPB + vehicle	7	8	6	4	5	30/40 ^{a,c}
CPB + L-PHC	3	4	3	2	2	14/30 ^{a,c}
CPB + H-PHC	2	3	1	0	0	6/40 ^a

Controls summarize the results from sham-operated rats treated with saline or penhyclidine hydrochloride (PHC). MLN: Mesenteric lymph node; CPB: Cardiopulmonary bypass; H-PHC: High-dose PHC; L-PHC: Low-dose PHC. ^a $P < 0.05$ vs controls; ^c $P < 0.05$ vs L-PHC group.

PHC in a dose-dependent manner ($P < 0.05$). H-PHC had significantly greater effects on the values of DAO and D-lactate than L-PHC (Table 2, $P < 0.05$).

PHC prevents CPB-induced BT

Bacteriological cultures from all sham-operated animals were negative. The incidence of *Escherichia coli*-positive cultures was significantly increased in the CPB-vehicle group, while pretreatment with PHC seemed to prevent systemic dissemination. The incidence of intestinal BT to the MLNs, spleen, liver, lung and blood was significantly higher in the CPB-vehicle group, compared with that in the sham groups (Table 3, $P < 0.05$), which was largely reversed by treatment with PHC in a dose-dependent manner (Table 3, $P < 0.05$). The plasma endotoxin level was increased in the CPB-vehicle group compared with the sham groups (Table 2, $P < 0.05$) and PHC decreased the plasma endotoxin levels in a dose-dependent manner (Table 2, $P < 0.05$).

PHC prevents CPB-induced damage to the intestinal mucosa ultrastructure

Transmission electronic microscopy demonstrated normal intestinal ultrastructure in the sham-operated group, including regularly aligned microvilli in the intestinal epithelium, integral mitochondria and rough endoplasmic reticulum (RER) and distinct junction complexes

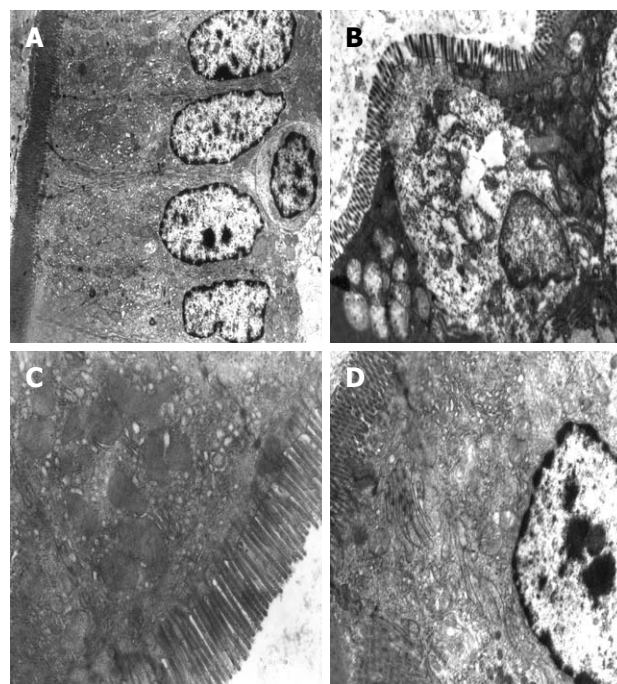


Figure 1 Effect of cardiopulmonary bypass on the ileal epithelial cells. A: Control group ($\times 5800$), regularly-aligned microvilli in the intestinal epithelium, intact mitochondria and rough endoplasmic reticulum (RER), and distinct junctional complexes were observed; B: Cardiopulmonary bypass group ($\times 7200$), epithelial damage was demonstrated by swollen mitochondria and loss of cristae, and tight junctions were disrupted; C: Mitochondrial swelling with damage to mitochondrial cristae and vacuolar degeneration were present. The nuclear structure was incomplete; D: Microvilli in the intestinal epithelium were regularly aligned, and the structure of the tight junctions became tight. However, mild swelling of mitochondria and RER were observed.

(Figure 1A). In the vehicle control group, the microvilli were reduced in number, and showed irregular lengths and arrangements. The mitochondria were swollen with cracked and vacuolated cristae. Some RER structures were destroyed, and the intercellular spaces between epithelial cells were widened. The structure of the tight junctions became shortened (Figure 1B). Mitochondrial swelling with damage to mitochondrial cristae and vacuolar degeneration was present in the L-PHC group. The nuclear structure was incomplete (Figure 1C). In the H-PHC group, microvilli in the intestinal epithelium were regularly aligned, and the tight junction structure became tight. However, mild swelling of the mitochondria and RER were seen (Figure 1D). These results indicated dose-dependent effects of PHC on the reduction of cellular damage after CPB.

DISCUSSION

The present study focused on intestinal barrier injury after CPB and the potential of PHC as a therapeutic agent. We demonstrated that application of PHC during CPB preserved intestinal barrier function in a dose-dependent manner, and histological evidence is provided to support these biochemical results.

DAO reduces the concentration of polyamines re-

quired for cell proliferation. DAO is localized to the small intestine and placenta, which are both organs with rapid cell turnover rates. In humans, DAO activity is especially high in the upper portion of the small intestinal villi, and has therefore been used as an index of small intestinal mucosal mass and integrity. Serum DAO levels have been found to increase markedly when the small intestine is strangulated, and elevations are thus believed to reflect small intestine mucosal ischemia^[15]. Tsunooka *et al.*^[4,16] have demonstrated simultaneous increases in serum DAO activity and peptidoglycan concentrations during clinical CPB, suggesting the occurrence of small intestinal mucosal ischemia and BT.

D-Lactate is habitually tested for in the intensive care unit. Mammals only have one type of enzyme: L-lactate dehydrogenase. L-Lactate is a marker of cell hypoxemia, and its levels correlate with survival in patients with septic shock^[17,18]. Microorganisms however, particularly bacteria, are equipped with D-lactate dehydrogenase and produce D-lactate during fermentation, and D-lactate therefore acts as a marker of bacterial infection. D-Lactate has also recently been proposed as a sensitive, specific and early marker of translocation in gut ischemia^[19].

In the current study, all the rats survived the CPB procedures. DAO and D-lactate activities remained low in the sham-operated animals (based on previously reported normal DAO value of 0.46 ± 0.087 U/L, and D-lactate value of 5.245 ± 0.653 mg/L^[13,14]). However, significant increases in DAO, D-lactate and endotoxin levels were found in the vehicle-treated CPB group. The incidence of intestinal BT to the MLNs, spleen, liver, lung and blood was also significantly higher in the vehicle-treated CPB group, compared with that in the sham groups. These results indicate the occurrence of severe intestinal barrier injury after CPB. Transmission electron microscopic examination of intestinal tissues further confirmed the intestinal barrier injury in this rat CPB model.

There is increasing interest in developing PHC as a novel therapeutic agent. PHC is a new anticholinergic drug derived from hyoscyamine, which has few M2 receptor-associated cardiovascular side effects, because of its selective blocking of M1, M3 and N receptors. Recent clinical results have demonstrated that PHC has curative effects in soman poisoning and pulmonary dysfunction associated with chronic obstructive pulmonary disease^[6,9,20]. In addition to improving microcirculation, PHC can inhibit lipid peroxidation, attenuate the release of lysosomes, and depress microvascular permeability^[9,20]. Moreover, it can significantly decrease brain nuclear factor (NF)- κ B expression in cerebral ischemia/reperfusion (I/R) injury. Furthermore, PHC can improve acute lung injury stimulated by endotoxin and attenuate liver damage during CPB in a rat model^[11].

In the present study, PHC lowered DAO, D-lactate and endotoxin levels in PHC-treated rats in a dose-dependent manner, suggesting its potential clinical application. The incidences of intestinal BT to MLNs, spleen, liver, lung and blood were lower in PHC-treated CPB rats than

in untreated CPB ones, suggesting that the use of PHC resulted in an overall decrease in bacteria. In intestinal mucosal injury, PHC can efficiently inhibit NF- κ B expression in intestinal mucosal I/R injury. More importantly, PHC can improve the microcirculation, inhibit lipid peroxidation, attenuate the release of lysosomes, and decrease microvascular permeability, leading to the inhibition of inflammation^[21].

However, our research was subject to some limitations. PHC treatment was only performed prior to CPB; although this pretreatment was effective, this study did not establish the efficacy of PHC given after CPB. Future studies are required to assess the effects of postoperative treatment with PHC. Additional research is also needed to establish the optimal time of PHC administration.

In conclusion, the present study demonstrated that PHC protected rat intestine from morphological and functional mucosal injury after CPB. These results suggest that PHC could be clinically useful for the treatment of intestinal injury induced by CPB.

COMMENTS

Background

An increase in intestinal permeability and bacterial translocation has been demonstrated not only in animal models, but also in patients during cardiopulmonary bypass (CPB). Previous *in vitro* studies have demonstrated that tropane alkaloids could stabilize the cell membrane and prevent oxidative stress. The new anticholinergic drug penethyclidine hydrochloride (PHC) has been evaluated for its protective effects on the cardiovascular system.

Research frontiers

PHC can improve microcirculation, inhibit lipid peroxidation, attenuate the release of lysosomes, and decrease microvascular permeability, leading to the inhibition of inflammation. The effects of PHC on intestinal barrier function during CPB have not been unequivocally addressed. We hypothesized that the administration of PHC could reverse CPB-associated intestinal damage.

Innovations and breakthroughs

The selectivity of PHC in blocking M1, M3 and N receptors means that it has few M2 receptor-associated cardiovascular side effects. It can reduce endotoxin-stimulated acute lung injury and attenuate liver damage during CPB in a rat model. The present study demonstrated that the application of PHC during CPB preserved intestinal barrier function in a dose-dependent manner, and provided histological findings to support these biochemical results.

Applications

The present study demonstrated the ability of PHC to protect rat intestine from morphological and functional mucosal injury after CPB. These results suggest that PHC could be clinically useful in the treatment of intestinal injury induced by CPB.

Terminology

PHC is a new anticholinergic drug with antioxidant and cell membrane-stabilizing activities.

Peer review

The authors have demonstrated a protective role for PHC in preventing intestinal breakdown during CPB. The conclusion was reached that the administration of PHC could prevent intestinal damage and its sequelae following CPB surgery. This manuscript describes an interesting and well-performed study with convincing results.

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***p53* gene therapy in combination with transcatheter arterial chemoembolization for HCC: One-year follow-up**

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Abstract

AIM: To evaluate the efficacy and safety of combination therapy with recombinant adenovirus *p53* injection (rAdp53) and transcatheter hepatic arterial chemoembolization (TACE) for advanced hepatocellular carcinoma (HCC).

METHODS: A total of 82 patients with advanced HCC treated only with TACE served as control group. Another 68 patients with HCC treated with TACE in combination with recombinant adenovirus-*p53* injection served as *p53* treatment group. Patients were followed up for 12 mo. Safety and therapeutic effects were evaluated according to the improvement in clinical symptoms, leukocyte count, Karnofsky and RECIST criteria. Survival rate was calculated with Kaplan-Meier method.

RESULTS: The total effective rate was 58.3% for *p53* treatment group, and 26.5% for control group ($P < 0.05$). The incidence of gastrointestinal symptoms was lower in *p53* treatment group than in control group ($P < 0.05$). The 3-, 6- and 12-mo survival rates were significantly higher for *p53* treatment group than for

control group ($P < 0.01$). The combination treatment was well tolerated with such adverse events as fever (51.5%, $P = 0.006$) and pain of muscles and joints (13.2%, $P = 0.003$), which were significantly higher than the chemotherapy. Except for these minor adverse effects, no severe vector-related complications were identified. With respect to the efficacy, patients in *p53* treatment group had less gastrointestinal symptoms ($P = 0.062$), better improvement in tumor-related pain ($P = 0.003$), less downgrade of leukocyte counts ($P = 0.003$) and more upgrade of Karnofsky performance score ($P = 0.029$) than those in control group. The total effective rate (CR + PR) for *p53* treatment group and control group was 58.3% and 26.5%, respectively, with distributions of different effect in two groups ($P = 0.042$). The survival rates were 89.71%, 76.13%, and 43.30% for *p53* treatment group, and 68.15%, 36.98%, and 24.02% for control group, respectively, 3, 6 and 12 mo after treatment, suggesting that the survival rates are significantly higher for *p53* treatment group than for control group ($P = 0.0002$).

CONCLUSION: The rAd-*p53* gene therapy in combination with TACE is a safe and effective treatment modality for advanced HCC.

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Key words: Adenovirus *p53*; Clinical trial; Hepatocellular carcinoma; Transcatheter hepatic arterial chemoembolization; *p53* gene therapy

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INTRODUCTION

Gene therapy is a potentially new treatment modality for cancer patients and an engineered recombinant replication-defective adenovirus can express the tumor suppressor gene *p53* (rAd-p53) with encouraging clinical responses^[1-3]. rAd-p53 has been recently approved by the State Food and Drug Administration of China as the very first gene therapy product for head and neck squamous cell carcinoma (HNSCC)^[4].

Hepatocellular carcinoma (HCC) is one of the major cancers in China with a poor prognosis due to its occult onset, rapid infiltrating growth and complicating liver cirrhosis. No effective treatment modality is available for it at present. Although transcatheter hepatic arterial chemoembolization (TACE) is currently one of the most popular treatment modalities for unresectable advanced HCC, the long-term survival rate of such patients remains low with a reported 5-year survival rate of 17%^[5]. In this study, the safety and efficacy of rAd-p53 therapy in combination with TACE were examined in patients with advanced HCC.

MATERIALS AND METHODS

rAd-p53

rAd-p53 is a recombinant human serotype 5 adenovirus in which the E1 region is replaced by a human wild-type *p53* expression cassette. The *p53* gene is driven by a Rous sarcoma virus promoter with a bovine growth hormone poly (A) tail. The recombinant adenovirus is produced in human embryonic kidney 293 cells and manufactured by Shenzhen SiBionoGenTech Co. Ltd (Shenzhen, China) and marketed under the trade name of Gendince®. Before *p53* gene therapy, a vial of rAd-p53 is taken out from a refrigerator in which the temperature is about -20°C. When thawed, the solution, diluted with 1 mL NS, is sucked into a 5-mL syringe for intra-tumor injection.

Patients and trial design

One hundred and fifty patients (83 men and 67 women) with advanced HCC were enrolled in this study from March to July 2004. Patients with Child C disease^[6], tumor thrombus in the main portal trunk, or extrahepatic metastasis were excluded. These exclusion criteria were implemented to ensure at least a 3-mo life span in the enrolled patients so as to have enough time to follow up. All patients did not receive local ethanol injection, microwave coagulation, systemic chemotherapy or radiotherapy before and after TACE or gene therapy. All tumors were diagnosed according to pathologic examination, distinctive findings on computed tomography (CT), conventional angiography, magnetic resonance imaging (MRI), or serum tumor markers [alpha-fetoprotein (AFP) or ferritin]. The patients were divided into gene treatment group ($n = 68$) with a mean age of 43 years (range 20-72 years) and control group ($n = 82$) with a mean age of 45 years (range 18-75 years). No patient was classified as stage I or II while 91 patients were classified as stage

Table 1 Characteristics of enrolled patients with hepatocellular carcinoma

Characteristics	Gene group ($n = 68$)	Control group ($n = 82$)	Statistic analysis
Age	43.5 (20 - 72)	45.7 (18 - 75)	NS
Sex (M/F)	43/25	40/42	NS
Child class A	41	43	NS
Child class B	27	39	NS
UICC TNM classification			
Stage I and II	0	0	NS
Stage III	31 (46.5%)	60 (73.2%)	NS
Stage IV	37 (54.4%)	22 (26.8%)	NS
Size of main tumors			
≥ 5 cm	53 (77.9%)	61 (74.3%)	NS
< 5 cm	15 (22.1%)	21 (25.7%)	NS

NS: No statistical difference.

III and 59 patients as stage IV according to the International Union against Cancer TNM classification^[7].

Patients who gave their informed consent to receive Ad-p53 gene therapy served as gene treatment group, while those not willing to receive gene therapy served as control group. Patients in gene treatment group underwent rAd-p53 gene therapy and TACE while those in control group received only TACE. Although this was a retrospective nonrandomized study, no statistical difference was observed in baseline between the two groups. The characteristics of the two groups are illustrated in Table 1.

Procedure of rAd-p53 intra-tumor injection

The patients in gene treatment group were placed in a supine, prone or lateral position on the CT scanning bed and asked to hold their breath after an inhalation. The slice for puncture was carefully determined, the puncture site on the surface of body as well as the needle-traveling depth and angle within the body were determined. The bed was moved to the slice and a marker for puncture was made on the body surface according to the laser beam emitted from the gantry. The bed was then moved out and the puncture site was sterilized. After local anesthesia, a 19-G needle was inserted into the puncture site according to the determined angle and depth as the operator asked the patient to hold his or her breath after an inhalation. Finally, another scan was performed to make sure that the tip of the needle was within the tumor, and the rAd-p53 gene was injected into the tumor in a multi-point fashion. Usually, this procedure is repeated according to the patient's clinical condition and the interval between two procedures is about 1 wk. At each injection, 1-4 rAd-p53 injections are administered at a viral dose of $1-4 \times 10^{12}$ VP (viral particles) according to the diameter of the lesion, and the intra-tumor injection usually lasts 1-2 min.

TACE

TACE was performed through the femoral artery using the Seldinger technique with local anesthesia. Arteriography of the celiac trunk and superior mesenteric artery was performed to visualize the arterial vascularization of liver and evaluate portal vein patency. An angiographic

catheter was inserted into the right or left hepatic artery where the target tumor was located. TACE agents, involving embolic agent (Lipiodol) and anticancer drugs, were injected through the right or left hepatic artery. In both groups, the dose of Lipiodol, ranging 3-20 mL, was determined according to the tumor location, tumor size, number of tumors, and functional hepatic reserve. Anticancer drugs used were 5-Fluorouracil (800-1000 mg) and vinorelbine (30-40 mg). TACE was repeated according to the patient's clinical condition at a 1-mo interval.

Follow-up protocol

Clinical symptoms, leukocyte counts and Karnofsky index evaluation were recorded before and after treatment. After treatment, CT scan or MRI was performed every three months with or without contrast enhancement to evaluate the features of Lipiodol deposit and the therapeutic effect according to the response evaluation criteria for solid tumors^[8]. If elevated tumor markers (AFP and ferritin), diminished Lipiodol, or enlarged lesions or new nodules were observed, the patients were readmitted for angiography and treatment. The starting point of survival analysis was regulated as the day of initial treatment. The Kaplan-Meier method was used to analyze the survival rates in the two groups.

Statistical analysis

Statistical analysis was performed to assess the baseline, leukocyte counts, Karnofsky index, clinical symptoms and survival curve between the two groups using the SPSS 11.0. $P < 0.05$ was considered statistically significant.

RESULTS

Two hundred and fifty-one p53 intra-tumor injections were performed for 83 lesions in 68 patients of gene treatment group. Of the 68 patients, 9 received one injection, 13 received two injections, 15 received three injections, 20 received four injections, 7 received five injections, 3 received six injections and 1 received seven injections. One hundred and ninety-two (mean 2.82 procedures) and 167 (mean 2.03 procedures) procedures of TACE were performed in gene treatment and control groups, respectively. Arterial portal vein shunt (AVS), arterial hepatic vein shunt (APS) or/and portal vein involvement, signs that meant a high invasion and a poor prognosis were found in 27.9% (19/68) patients of gene treatment group and 36.6% (30/82) patients of control group, respectively, during the TACE. Although the patients with tumor thrombus in the main portal trunk were excluded, some of them developed vascular invasion because of tumor progression after they were enrolled in this study. No difference was observed in the incidence of malignancy signs such as AVS, APS or portal vein involvement between the two groups.

Safety

The clinical symptoms were carefully recorded after treatment (Table 2). Overall, rAd-p53 gene therapy in combination with TRCE was well tolerated. The most

Table 2 Clinical symptoms after treatments

Group	Fever	Gastrointestinal symptoms	Palliation of mass-associated pain	Pain of muscles or joint
Gene group	35 (51.5) ^a	20 (29.4) ^b	30 (44.1) ^c	9 (13.2) ^d
Control group	24 (29.3)	28 (34.1)	21 (25.6)	1 (1.2)

$\chi^2 = 7.679$, ^a $P = 0.006$; $\chi^2 = 4.001$, ^b $P = 0.062$; $\chi^2 = 5.674$, ^c $P = 0.017$; $\chi^2 = 8.626$, ^d $P = 0.003$.

Table 3 Changes in leukocytes before and after treatment

Group	Change degree ($\times 10^9/L$)			n (%)
	< 4.0	< 3.0	< 2.0	
Gene group	12 (25.0)	4 (8.3)	2 (4.2)	18 (37.5)
Control group	8 (13.3)	20 (33.3)	11 (18.3)	39 (65.0)

Rank sum tests (Wilcoxon test), $T = -3.018$, $P = 0.003 < 0.05$.

frequent adverse event occurred in patients receiving rAd-p53 gene therapy in combination with TACE was the flu-like symptom associated with fever. Of the 68 patients in gene treatment group, 35 (51.5%) had a fever at 38-39.5°C, usually occurred 3-10 h after p53 intra-tumor injection and decreased after physis cooling, and 9 (13.2%) had pain of muscles or joints which often faded away (Table 2). No other severe gene therapy-associated complications were encountered in this study.

Efficacy

The clinical symptoms were carefully recorded after treatment (Table 2). The patients in gene treatment group had less gastrointestinal symptoms such as nausea, vomiting, abdominal pain or belling than those in control group. The palliative rate of mass-associated pain one week after treatment was 44.1% (30/68) for patients in gene treatment group, higher than that for those in control group.

Before and one week after treatment, the number of leukocytes was calculated (Table 3). Statistical analysis showed that the number of leukocytes was smaller in gene treatment group than in control group ($P = 0.003$).

Karnofsky index was changed in gene treatment group one month after treatment (Table 4). Generally speaking, the patients in gene treatment group had a higher Karnofsky index than those in control group ($P = 0.029$).

The therapeutic effect was evaluated following the response evaluation criteria for solid tumors after treatment. CR, PR, NC and PD in the two groups are listed in Table 5. The total effective rate (CR + PR) was 58.3% and 26.5% for the gene treatment group and control group, respectively ($P < 0.05$). Chi-square test showed that the distributions of therapeutic effect were statistically different ($P = 0.042$, Figures 1 and 2).

The patients were followed up for 12 mo. The number of withdrawal patients in gene treatment group and control group was 4 and 7, respectively. The survival rate was 89.71% (standard error 0.036), 76.13% (standard error 0.052), and 43.30% (standard error 0.061),

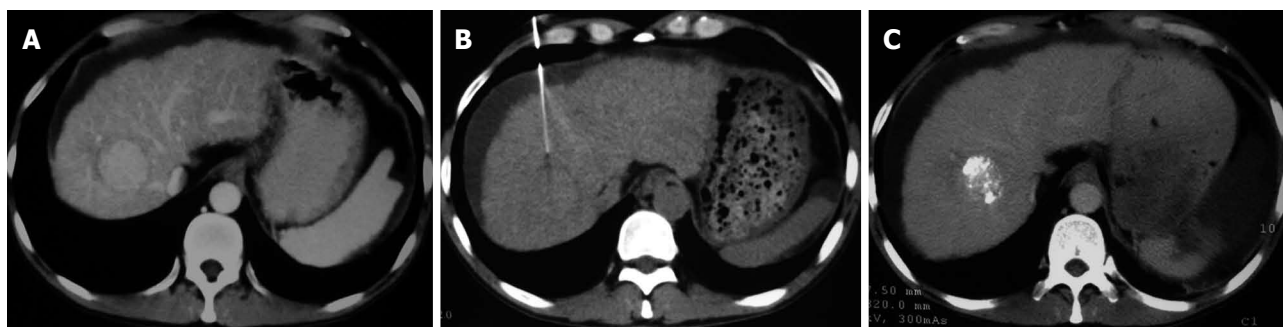


Figure 1 Contrast computed tomography showing a nodule (3.5 cm in diameter) in the right upper liver lobe manifested as homogenous enhancement (A); computed tomography scan (b) demonstrating the course of fine needle biopsy under computed tomography guidance with the diagnosis of hepatocellular carcinoma confirmed (B); computed tomography follow-up (c) revealing lipiodol deposit in the mass and splenic infarction after spleen embolization (C) in a 52-year-old man with multiple hepatic nodules, liver cirrhosis, splenomegaly and elevated alpha-fetoprotein.

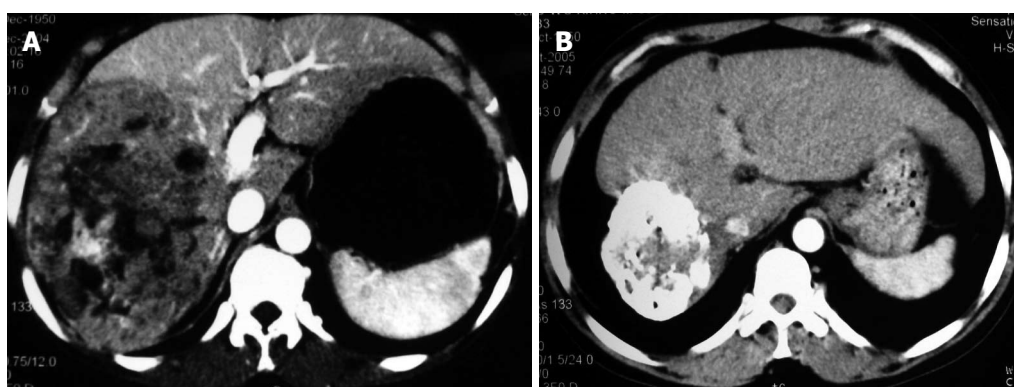


Figure 2 Contrast computed tomography scan showing a 15 cm × 11.5 cm hepatocellular carcinoma in the right liver lobe manifested as a heterogenous lower density, partial enhancement and well-differentiated contour (A) and computed tomography follow-up displaying the significant regression of a 8.5 cm x 6 cm lesion with compact lipiodol deposit in a 72-year-old man after 3 p53 gene injections and 4 courses of transcatheter hepatic arterial chemoembolization.

Table 4 Changes in Karnofsky index before and after treatment

Group	Upgrade > 20 points	Upgrade > 10 points	No changes	Downgrade > 10 points	Total upgrade [n (%)]
Gene group	14	28	18	8	42 (61.8)
Control group	12	24	18	28	36 (43.9)

$\chi^2 = 4.752$, $P = 0.029$.

respectively, for the patients in gene treatment group 3, 6, and 12 mo after treatment. The survival rate was 68.15% (standard error 0.051), 36.98% (standard error 0.054), and 24.02% (standard error 0.049), respectively, for those in control group 3, 6, and 12 mo after treatment. Log-rank test showed that the survival rate for the two groups was significantly different ($P = 0.0002$, Figure 3).

DISCUSSION

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a very high morbidity and mortality. Since TACE was introduced as a palliative treatment of unresectable HCC, it has become one of the most common

Table 5 Therapeutic effect evaluated following response evaluation criteria for solid tumors 2 mo after treatment

Group	n	CR	PR	NC	PD	Effective rate (CR + PR)
Gene group	68	0	46	15	7	67.60%
Control group	82	0	42	27	13	51.20%

$\chi^2 = 4.137$, $P = 0.042 < 0.05$. CR: Complete response; PR: Partial response; NC: No change; PD: Progressed disease.

interventional therapies^[9-12]. However, its therapeutic effect is also limited due to the lack of appropriate and reliable embolic agents, and the infiltrative or hypovascular nature, too large or small in size^[13-15]. Another limitation of TACE is the need for repeated treatment, thus resulting in deterioration of liver function^[16]. So, lots of efforts have been made to explore other new therapies in order to achieve the better efficacy of multiple treatments. PEI or RFA gene therapy in combination with TACE may improve the survival rate of HCC patients and decrease the risk of liver failure^[17-19]. In this study, p53 gene therapy in combination with TACE could overcome the downside of TACE and improve the prognosis of HCC patients.

The p53 tumor suppressor gene is a gene guardian and loss of p53 is responsible for the lack of apoptotic signals

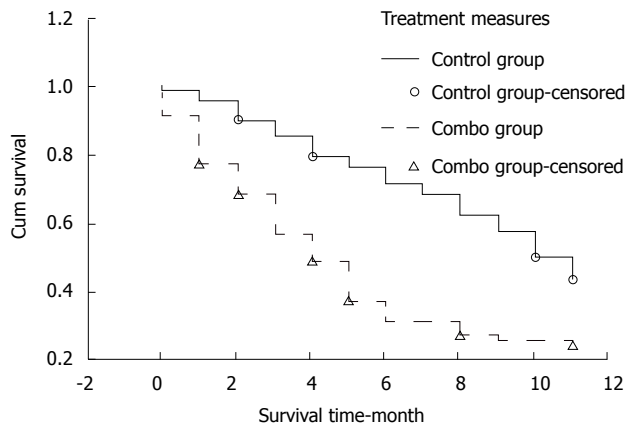


Figure 3 Survival curves for patients following treatment.

in tumor cells and thus for their uncontrolled proliferation and recurrence^[20]. Many human tumors carry mutations in the *p53* gene^[21,22] and mutant or absent *p53* gene is associated with the resistance to radiotherapy and apoptosis-inducing chemotherapy^[23]. It has been shown that *p53* gene therapy in combination with radiotherapy or chemotherapy can control local tumor, suggesting that it is superior to either radiotherapy or chemotherapy alone^[24,25]. It was reported that the incidence of *p53* mutation is 61% in HCC^[22]. Chen *et al*^[26] also reported that mutations in the *p53* gene are frequently detectable in recurrent HCC and the interval between surgical resection and recurrence of HCC is significantly longer in patients with the wild-type *p53* gene than in those with mutant *p53* gene mutations, strongly suggesting that the mutant *p53* gene plays a role in pathogenesis of HCC. Jeng *et al*^[27] demonstrated that the biological behavior of the mutant *p53* gene is strongly related to the invasiveness of HCC and may also influence the postoperative course of HCC. Many scholars suggest that immunopositivity of the mutant *p53* gene plays a role in predicting the prognosis of patients with HCC after resection^[27-29].

The rAd-*p53* gene has been approved in China under the trade name of Gendicine for the treatment of head and neck squamous cell carcinoma (HNSCC). In one of the trials^[3], 75% tumors experienced complete regression following 8 wk of therapy involving 1 injection per week, which was significantly higher than that in control group, and combined chemotherapy and radiotherapy improved the treatment efficacy of over 3-fold. Although its recommended indications are limited in HNSCC according to the specification, good treatment efficacy can be achieved in HCC patients when rAd-*p53* is used^[30]. In the current study, Gendicine was used in treatment of HCC to evaluate its effect in order to provide some evidence for its off-table use in treatment of HCC.

As for the safety of rAd-*p53* used in treatment of advanced HCC, just fever at 38-39.5°C was observed in our study, which was returned to normal after symptomatic treatment. In addition, some patients suffered from pain of muscles or joints and its cause is still controversial. However, no severe complications caused by Gendicine

were observed. Although these adverse events have been observed in clinical practice, they can be well tolerated by most patients with no severe physical and mental harm.

The patients receiving *p53* gene therapy had less severe post embolization syndrome than others after TACE. Gastrointestinal symptoms, such as nausea, vomiting and abdominal pain or belling, were less frequently observed in gene treatment group than in control group. The decreased number of leukocytes in gene treatment group was a pleasing phenomenon. However, its mechanism remains to be studied. The Karnofsky index was significantly higher, suggesting that the life quality of patients is largely improved in gene treatment group. It could be concluded that the rAd-*p53* gene therapy could reduce the side effects of chemical drugs and Lipiodol embolization. Also, it was noticed that many patients in gene treatment group had a compact Lipiodol deposit manifested as a high homogeneous density occupying the majority of tumor mass (Figures 1 and 2). Compact deposit means tumor necrosis. Further study is needed to observe whether *p53* gene therapy is related to the better deposit of Lipiodol in lesions.

Theoretically, *in-vitro* *p53* protein can bring about specific anti-tumor cells into effect in such ways as induction of apoptosis or necrosis, incentive of body immune response, regulation of cell cycle, *etc.* Two months after treatment, the distributions of therapeutic effect in the two groups were statistically different and the effective rate (CR + PR) was higher for *p53* gene treatment group than for control group, suggesting that *p53* gene therapy can enhance the efficacy of TACE, radiotherapy and chemotherapy.

Kaplan-Meier analysis showed that the survival rate was higher for gene treatment group than for control group. Because no other control study is available, the outcome of *p53* gene therapy for such a large number of patients was not compared with that in other studies. The 1-year survival rate was lower in our study than in another study (67% *vs* 81%)^[31], which may be attributed to the different baselines, in which our enrolled patients might have a larger lesion and a poorer liver function reserve.

Although it seems that the higher survival rate in gene treatment group may be attributed to the longer mean TACE time in patients of gene treatment group than in those of control group (2.82 *vs* 2.03), it was the clinical improvement after *p53* gene therapy that made the patients in gene treatment group have more chance to receive repeated TACE. On the other hand, no difference was found in the incidence of malignancy DSA signs between the two groups. However, these signs appeared later with a lower incidence in gene treatment group than in control group, which is an interesting phenomena, and further study with a larger sample size is needed to confirm it.

Usually, the rAd-*p53* gene begins to express *p53* protein 3 h after intra-tumor injection, reaches its peak on day 3, and then gradually decreases according to the specification of Gendicine®. On day 5 after injection, the expression decreases to 30%. Because most of the chemotherapeutic drugs can affect DNA or RNA duplication or expression, cell cycle or nucleic acid metabolism would likewise affect the expression of *p53* gene in

tumor tissue. In this study, TACE was started 3-4 d after p53 injection when the p53 protein was highly expressed in tumor tissue, indicating that these anti-tumor drugs do not interfere with the expression of p53. However, the optimal interval remains to be further studied.

In conclusion, rAd-p53 gene therapy in combination with TACE is well tolerated and its anti-tumor efficacy is superior to that of TACE alone in terms of the survival rate and improved symptoms of HCC patients. Further clinical study with a large sample size is warranted to optimize the administration procedure and assess the impact of anti-p53 antibody on its therapeutic effect.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the major cancers in China with a poor prognosis due to its occult onset, rapid infiltrating growth and complicating liver cirrhosis. Although transcatheter arterial chemoembolization (TACE) has been used in treatment of HCC for years, its effect is often unsatisfactory.

Research frontiers

Among the actively studied novel treatment modalities for HCC, the majority of experts hold that comprehensive or combination ones are most promising. In addition, gene therapy with p53 (rAd-p53) is a potentially new treatment modality for cancer.

Innovations and breakthroughs

TACE in combination of rAd-p53 injection has a synergistic effect on HCC and its strategy is gene addition. Tumor with mutant of the rAd-p53 gene is a better candidate for p53 therapy. However, this treatment is also effective in those with inactivated wild-type p53, a common condition in tumors. Injection of rAd-p53 can lead to apoptosis of tumor cells and TACE can result in necrosis of tumor tissue.

Applications

The results of this study demonstrate that TACE in combination with rAd-p53 with is well tolerated and its anti-tumor efficacy is superior to that of TACE alone with respect to the survival rate and improved symptoms. Further study with a large sample size would provide an alternative treatment modality for HCC.

Terminology

p53 gene is a tumor suppressor gene which can prevent the formation of tumors. Mutations in p53 are found in most tumor types and contribute to complex molecular events leading to tumor formation. Recombinant adenovirus is one of the viral vectors which are commonly used to deliver genetic materials into cells. Gene therapy for diseases is to insert, alterate, or remove such materials in cells.

Peer review

This is a well-designed study in which the authors analyzed the clinical effect of rAd-p53 injection and TACE on advanced HCC. The data show that the combination therapy is a safe and effective treatment modality for advanced HCC, and can significantly improve the survival rate of HCC patients.

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Celiac disease and microscopic colitis: A report of 4 cases

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Abstract

Celiac disease (CD) is an autoimmune disorder of the small intestine that occurs in genetically predisposed people at all ages. However, it can be associated also to other immunopathological disorders, and may be associated with abnormal histology in segments of the gut other than the small bowel including colonic inflammation. While guidelines for endoscopic investigation of the jejunum are well defined, no indication is defined for colonic investigation. We describe four cases of concurrent CD and microscopic colitis (MC) diagnosed at our department over a 10-year period and analyzed the main features and outcomes of CD in this setting. The symptoms of these patients were improved initially by a gluten-free diet before the onset of MC symptoms. Two of the patients were siblings and had an atypical form of CD. The other two patients with CD and MC also presented with fibrosing alveolitis and were anti-Saccharomyces cerevisiae antibody positive. The co-existence of immune-mediated small bowel and colonic inflammatory and pulmonary diseases are not well-known, and no systematic approach has been used to identify the lifelong patterns of these immune-based diseases. Patients can develop, or present with CD at any stage in life, which can co-exist with other gastrointestinal diseases of (auto-) immune origin. In addition, the fa-

miliar co-existence and prevalence of MC in patients with a prior diagnosis of CD are unclear. Clinicians managing celiac disease should be aware of these associations and understand when to consider colon investigation.

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Key words: Collagen colitis; Lymphocytic colitis; Celiac disease; Fibrosing alveolitis; Anti-saccharomyces cerevisiae antibody

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INTRODUCTION

Celiac disease (CD) is an immune-mediated disorder, an autoimmune enteropathy, triggered by the ingestion of gluten in genetically susceptible persons. The disease primarily affects the gastrointestinal tract and is characterized by chronic inflammation of the small bowel mucosa that may result in atrophy of intestinal villi, malabsorption, and a variety of clinical manifestations. Of genetic factors, the strongest recognized association is with HLA-DQ2 and/or -DQ8: 95%-100% of the patients carry these molecules. Dietary gluteins interact with these HLA molecules to activate an abnormal mucosal immune response and induce tissue damage. Most affected individuals experience remission after gluten is excluded from their diet.

The diagnosis of CD is established by serologic testing, biopsy evidence of villous atrophy, and improvement of symptoms on a gluten-free diet. Avoidance of gluten

exposure is crucial for CD patients to reduce the risk of complications so the follow-up serological assessment of treatment effectiveness should be added to be sure of a good compliance.

There are atypical forms of CD. For example, silent CD is found in individuals who are asymptomatic but have a positive serologic test and villous atrophy on biopsy, and latent CD is defined by a positive serology but no villous atrophy on biopsy. These individuals are asymptomatic, but later may develop symptoms and/or histological changes^[1]. The late concordance in the appearance of CD in monozygotic twins also suggests that the disorder may remain in the latent stage for a long time^[2,3]. Small bowel villous atrophy with crypt hyperplasia and recovery of the lesion on a gluten-free diet suggest that villous atrophy comprises only the end stage of the clinical course of the disease and that CD clearly develops gradually from mucosal inflammation to crypt hyperplasia and finally to overt villous atrophy.

A typical feature of CD, in addition to mucosal changes, is gluten-dependent serum IgA class autoantibodies against transglutaminase 2 (TG2). These serum autoantibodies, endomysial and TG2, are powerful tools in disclosing CD with overt villous atrophy. Furthermore, positive serum celiac autoantibodies can predict impending CD in many patients evincing normal small bowel mucosal villous architecture. Hence, patients having “false-positive” celiac autoantibodies in serum are in fact at risk of developing overt CD. Some patients with positive serum endomysial or tissue TG2 antibodies may still seroconvert negatively during follow-up. However, it is well recognized that serum celiac autoantibodies in some cases fluctuate before a patient eventually develops overt CD after a longer follow-up period. The reason for this still remains obscure.

Transglutaminases are a family of 8 currently known calcium-dependent enzymes that catalyze the cross-linking or deamidation of proteins and are involved in important biological processes such as wound healing, tissue repair, fibrogenesis, apoptosis, inflammation, and cell-cycle control. Therefore, they play an important role in the pathomechanisms of autoimmune, inflammatory, and degenerative diseases, many of which affect the gastrointestinal system. Transglutaminase 2 is prominent, since it is central to the pathogenesis of CD, and modulates inflammation and fibrosis in inflammatory bowel and chronic liver diseases^[4]. Respiratory disease and subclinical pulmonary abnormalities are the recognized complications of both CD and inflammatory bowel disease (IBD) but the mechanisms of lung disease in CD differ from that in IBD and support the hypothesis of a common mucosal defect in lung and small intestine in CD that allow increased permeability^[5].

Lymphocytic colitis (LC), together with collagenous colitis (CC), is included under the umbrella term “microscopic colitis” (MC), in which chronic gastrointestinal symptoms, including diarrhea, abdominal pain, fecal urgency, incontinence, and nausea, are not associated with endoscopic or radiological alterations. It is not known whether LC and CC are two different diseases or distinct manifestations of the same clinical condition. Data on pathophysiology conflict and different hypotheses refer to genetic pre-

disposition, immune dysregulation, autoimmunity, bile acid malabsorption, infection, and drug effect. Familial occurrence of MC has been identified in some families^[6-13]. The central role of an altered immune system in MC pathogenesis is supported by the association with several conditions in which an immune dysregulation is involved, such as CD, rheumatoid arthritis, and hypo- and hyperthyroidism. Up to now, it has not been clear whether CC (or LC) is a distinct entity or only an epiphenomenon of another disease that leads to thickening of the collagen layer. However, whether MC (both CC and LC) is an autoimmune disease has not been conclusively established^[14]. Diagnosis of MC can be established only by colonic biopsies and subsequent histopathological examination, when an increase in inflammatory infiltration and/or a thickening of the collagen layer are found. A number of papers have documented an association between CD and MC^[15-18]. However, the prevalence of MC in patients with a prior diagnosis of CD is unclear, but it does feature prominently in several series of patients with CD who have persisting symptoms despite gluten exclusion. When continuing gluten ingestion, inadvertent or covert, has been excluded, colon investigation should be considered as part of the investigation of these patients. The link may be genetic, at least in part. Both types of MC are known to resolve spontaneously in a majority of cases. Data are limited regarding pharmacological therapies, but budesonide appears best documented as showing an efficacy against CC and MC^[19].

We report here 4 cases with sequential development of CD and MC and discuss the possible connection of these co-existences.

CASE REPORT

Case 1

A 42-year-old female with a previous history of both cognitive and neurovegetative symptoms of depression, including depressed mood, anhedonia, feelings of worthlessness, low energy, troubled sleep, and poor concentration, was evaluated in the local medical center for complaints of watery diarrhea. She had longstanding lactose intolerance for which she was taking a lactose-free diet. As her mother had manifestations of CD, enteroscopy was performed. However, the first endoscopic and histological evaluation showed no duodenal mucosal alterations (Marsh 0). Six months later, endoscopic findings were persistent and duodenal biopsies were taken which were not diagnostic for CD, and biochemical laboratory tests were within normal ranges. She was then referred to our clinic and additional laboratory tests showed increased antibody titers against gliadin, endomysium, and tTG. Her psychiatric disease was controlled after treatment and then remained stable. She was given a gluten-free diet, which resolved her diarrhea and allowed her to regain her lost body weight. Five years later, the patient presented with watery diarrhea occurring 8-10 times daily and mild body weight loss. At the beginning, this condition was associated with urgency, nocturnal stools, abdominal cramping, nausea, mild body weight loss, and fatigue, and persisted

despite strict adherence to the gluten- and lactose-free diet. With the loss of patience of the diet and of her symptoms she broke her diet and clinical signs remained. Small bowel biopsies at upper endoscopy demonstrated nothing (Marsh 0) but the gluten panel was unambiguously positive. Stool cultures and *Clostridium difficile* toxin assay were negative. After consultation with dietitians, the patient was maintained on a gluten- and lactose-free diet for six months but with mild improvement. Colonoscopy was performed later, and biopsies from her colon demonstrated LC. Her symptoms responded partially to mesalazine treatment over the subsequent two months, at which point her medical therapy was changed to budesonide (9 mg/d). After she was put on budesonide with a strict diet, both abdominal complaints and psychiatric problems resolved (Figure 1).

Case 2

A 45-year-old woman with suspected irritable bowel syndrome was admitted to the hospital. Her bowel movements increased from one to six or eight a day with watery stools. She did not note any mucus or blood in the stool and could not identify any alleviating or aggravating factors. She consumed a normal diet, including meat, wheat, and dairy. Over-the-counter anti-diarrheal medications did not relieve her symptoms. She had no fevers, chills, or night sweats, but body weight loss. Her medical history included major depression for 10 years, which was controlled after treatment and remained stable at admission. Results of basic laboratory tests, including thyroid-stimulating hormone (TSH), complete blood count, blood chemistries, renal function, and liver function, were normal. Colonoscopy showed normal mucosa as far as the cecum. Colonic biopsy revealed a mildly expanded lamina propria and intraepithelial lymphocytosis with significantly thickening of the subepithelial collagen table. This set of features was consistent with CC, a variant of MC. Her symptoms were eventually controlled after a 6-mo course of oral budesonide (9 mg) and ongoing intermittent use of loperamide (Imodium). Six years later, similar problems with body weight loss caused her to be hospitalized at our clinic. A detailed previous history unraveled the familial connection with Case 1 and her mother with known CD. Psychiatric disease was controlled, so control and further GI investigations were organized. The histopathology report of colonic biopsy showed aspecific inflammation without MC. Further laboratory investigation revealed that the entire celiac antibody panel was positive. Results of duodenal biopsy did not reveal typical lymphocyte infiltration, crypt hyperplasia, and villous atrophy but normal mucosal architecture, without significant intraepithelial lymphocytic infiltration (Marsh 0). The diagnosis was latent CD, as the patient had abnormal antibody blood tests for CD but normal small intestines. After she was put on a strict gluten-free diet, both abdominal complaints and psychiatric problems resolved (Figure 1).

Case 3

A 56-year-old woman with a previous history of chronic (non-specific) colitis and fibrosing alveolitis was referred

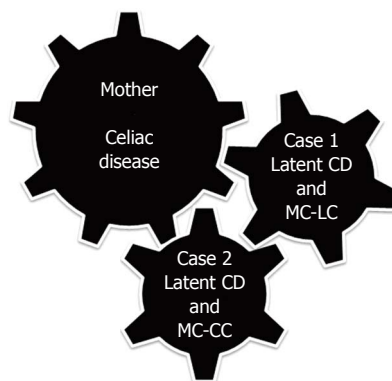


Figure 1 Family of cases 1 and 2.

to our hospital from an outside hospital because of continued signs and symptoms of CD that persisted despite self-reported adherence to a gluten-free diet. The patient reported abdominal pain, bowel distension, and body weight loss over the past few years. Diagnosis of CD was made 4 years ago, based on the small bowel biopsy results showing evidence of villous blunting with increased chronic inflammatory cells, positive laboratory tests, and typical gastrointestinal signs and symptoms with negative stool cultures and *Clostridium difficile* toxin assay. Repeated laboratory tests showed elevated antibodies against gliadin, endomysium, and tTG, and small bowel biopsy proved villous atrophy. The patient met with a nutritionist and implemented recommended dietary changes to eliminate gluten. Her symptoms temporarily improved with her bowel function returned to normal, but after a short time her symptoms recurred. Results of further tests excluded conditions known to complicate or coexist with CD, including bacterial overgrowth and lactose intolerance. Because of chronic watery stools, a colonoscopy was done with random biopsies from the colon for histological investigations. Based on the typical picture of prominent intraepithelial lymphocytes but no thickened collagenous layer, the pathologist diagnosed her with LC. She was started on strict gluten-free diet and budesonide with success. Five years later, she was free of complaints of CD and LC.

Case 4

A man at age 31, with a previous history of bronchial asthma, was investigated for abdominal pain, chronic watery diarrhea, and body weight loss with negative stool samples (both the cultures and *Clostridium difficile* toxin assay). Findings from an upper gastrointestinal endoscopy were normal, but distal duodenal biopsies showed subtotal villous atrophy, inflammatory infiltration of the lamina propria, and an increase in intraepithelial lymphocytes. Based on the histology and positive laboratory tests, CD was diagnosed and the patient was started on a gluten-free diet. Abdominal pain ceased but he did not gain body weight and diarrhea remained a problem. Compliance with a gluten-free diet was confirmed by the assessment of dietitians. Repeated biopsies of the duodenal mucosa showed mild improvement in villous atrophy but serology

Table 1 Summary of the cases

	Case 1	Case 2	Case 3	Case 4
Sex/birth (yr)	Female/1956	Female/1955	Female/1945	Male/1964
Small bowel histology	Normal small-bowel mucosal structure	Normal small-bowel mucosal structure	Partial villous atrophy (Marsh 3A) according to the modified Marsh criteria	Total villous atrophy (Marsh 3C) according to the modified Marsh criteria
HLA-DQ2	Present	Present	Present	Present
IgA TTG	+	+	+	+
IgG/IgA EMA	+/+	-/+	-/+	+/+
IgG/IgA Gliadin	+/+	-/+	-/+	+/+
IgG/IgA ASCA	-/-	-/-	+/+	+/+
Celiac disease	Latent	Latent	Manifest	Manifest
Colon histology	Lymphocytic colitis	Collagenous colitis	Lymphocytic colitis	Collagenous colitis
Therapy	GFD and budesonide	GFD and budesonide	GFD and budesonide	GFD and budesonide
Other disease	-	-	Fibrosing alveolitis	Fibrosing alveolitis

Laboratory tests and histology of the small bowel before gluten-free diet, colon histology after gluten-free diet, therapy, and concomitant lung disease. TTG: Tissue transglutaminase; EMA: Endomysial antibody; ASCA: Anti-saccharomyces cerevisiae antibody; GFD: Gluten-free diet; HLA: Human leukocyte antigen.

was negative. Four years later, a dietitian again confirmed adherence to a strict gluten-free diet and colonic biopsies showed no alteration. A barium follow-through showed mild jejunal and rather featureless ileal mucosa but no obstructive lesion of the small bowel, nothing abnormal was seen on an ultrasound scan of the abdomen. Because of bloody stools and in view of his worsening symptoms despite the gluten-free diet, repeated colonoscopy with random biopsies was done for histological investigations from ileal and colonic samples. Both proved a submucosal thickened collagen layer, thus the diagnosis of collagenous enterocolitis (with CD) was made. He was started on mesalazine and budesonide but without effect. The next step was methylprednisolone, initially 32 mg/d, and then the dose was decreased to 4 mg/d. This therapy was continued with corticosteroids for three months. Over the next year, his clinical condition improved, with resolution of his diarrhea and a body weight gain of 3 kg. Three years later, his symptoms recurred. Results of further tests excluded conditions known to complicate or coexist with CD, including bacterial overgrowth and lactose intolerance. Repeated biopsies excluded collagenous enterocolitis, thus fibrosing alveolitis was diagnosed by the pulmonologist based on the lung function, laboratory and radio-imaging tests, chest X-ray, and high-resolution CT scanning (HRCT). Because the abdominal symptoms of the patient were refractory to treatment, he was treated again with budesonide and his clinical condition improved.

The diagnoses of CD, MC, and fibrosing alveolitis in all cases were made according to the formally accepted criteria. Two independent pathologists certified the diagnosis of MC by verifying the subsequent sections and completing the check with additional investigations (intraepithelial lymphocytes, tenascin labeling of the collagen layer, mast cells, and other lamina propria cell components). Fibrosing alveolitis was proved by HRCT and upon the ATS/ERS clinical criteria. The laboratory tests were performed. In brief, the HLA-DQ alleles were determined from whole blood samples by PCR with sequence-specific primers, traditional IgG and IgA AGA were detected by ELISA (α -gliatest IgG and IgA; Eurospital, Trieste, Italy), anti-tTG was measured

by ELISA using recombinant human tissue transglutaminase as an antigen (EutTG, Eurospital), IgG and IgA EMA were investigated by indirect immunofluorescence using human umbilical cord cryostat sections prepared in our laboratory as a substrate, and both serum IgG and IgA levels in anti-Saccharomyces cerevisiae antibody (ASCA) were evaluated (separately) according to the manufacturer's protocol (ASCA IgG, ASCA IgA, QUANTA Lite, INOVA Diagnostics) (Table 1).

DISCUSSION

Is CD much ado about nothing? This report presents four cases of CD with MC. The symptoms of these patients were improved initially by a gluten-free diet before the onset of MC symptoms. Their history indicates and underlines that patients can develop, or present with CD at any stage in life and that CD can co-exist with other gastrointestinal diseases of an (auto-) immune origin. Patients with CD can fail to respond to the initial introduction of a gluten-free diet or have a recurrence of symptoms after initial improvement, despite maintaining gluten exclusion. The most feared causes of either scenario are complicating malignancy, notably enteropathy-associated T-cell lymphoma, or refractory sprue. Other causes of persistent symptoms with increased prevalence in CD include lactose intolerance, exocrine pancreatic insufficiency, bacterial overgrowth, and microscopic (lymphocytic or collagenous) colitis. Thus, in patients whose symptoms fail to respond or who later relapse, despite the exclusion of gluten from their diet, the possibility of additional pathology should be considered and colonoscopy should, therefore, be part of the follow-up in patients who present with chronic watery diarrhea, even if initial tests indicate only CD.

Relations between CD and ulcerative ileojejunitis, polymyositis, and fibrosing alveolitis have been previously described^[20,21], and it is of interest that an auto-immune pathophysiology has been implicated in each of these conditions. An association has been suggested between CD and diffuse interstitial lung disease of the hypersensitivity pneumonitis type in several reports from Europe^[22]. A case

of lymphocytic bronchoalveolitis and CD with improvement following a gluten free diet was also reported^[23].

Our patients with manifestations of CD and MC presented with fibrosing alveolitis and were ASCA (anti-yeast antibodies to yeast antigens that are found in bread and other cereal derived products) positive (both IgG and IgA types). Previously, ASCA positivity was shown to be evident in up to 40%-60% of CD patients and 13%-15% of MC patients, but its implication is disputed^[24]. A possible connection between alveolitis and ASCA is also not known. Only one case of a Japanese patient was published: lung biopsy specimens showed alveolitis and serum-precipitating antibody gave a positive reaction for an extract from *S. cerevisiae*^[25].

In conclusion, the co-existence of immune-mediated small bowel and colonic inflammatory diseases (i.e. CD and IBD) and pulmonary diseases is not well-known and no systematic approach has been used to identify the life-long patterns of these immune-based diseases^[26]. Such information may be useful for both disease prevention and treatment approaches. Clinicians managing CD should be aware of these associations and when to consider colon investigation.

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Pure red cell aplasia caused by pegylated interferon- α -2a plus ribavirin in the treatment of chronic hepatitis C

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INTRODUCTION

Pure red cell aplasia (PRCA) is a rare hematological disorder which is characterized by severe anemia, reticulocytopenia and almost complete absence of erythroid precursors in bone marrow^[1]. Patients typically present with symptoms of severe anemia in the absence of hemorrhagic phenomena. Common causes of PRCA include human parvovirus B19 infection, lymphoproliferative disorder, humoral or cellular immunity, production of erythropoietin-neutralizing antibody, and drugs such as ribavirin and standard interferon^[1-5]; however, to our knowledge, there is only one case report in the English literature of PRCA after pegylated interferon combination therapy for chronic hepatitis C^[6]. We report a second case of PRCA during combination therapy for chronic hepatitis C.

CASE REPORT

A 69-year-old male was referred to our hospital for treatment of chronic hepatitis C. His past medical history was remarkable for megaloblastic anemia due to vitamin B12 deficiency. He received regular vitamin B12 injection therapy with a stable hemoglobin level of around 11 g/dL. At

Abstract

Pure red cell aplasia (PRCA) is a rare hematological disorder which is characterized by severe anemia, reticulocytopenia and almost complete absence of erythroid precursors in bone marrow. The pathophysiology of PRCA may be congenital or acquired. To our knowledge, there is only one case report in the English literature of PRCA after pegylated interferon combination therapy for chronic hepatitis C. We report a second case of PRCA after pegylated interferon combination treatment for chronic hepatitis C. The diagnosis of PRCA was confirmed by the typical findings of bone marrow biopsy. The possible etiologies of our case are also discussed in this paper.

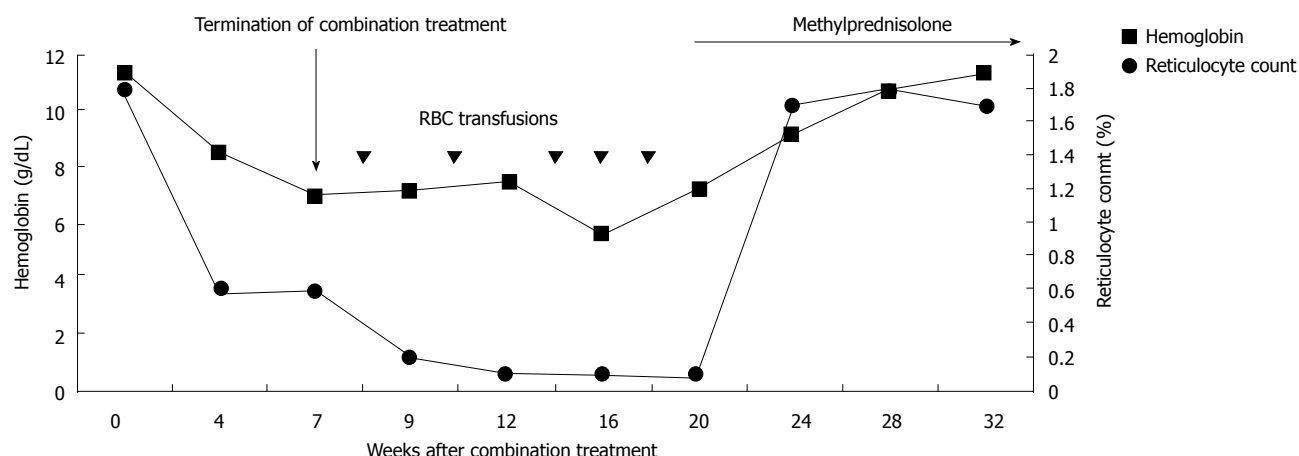


Figure 1 Hemoglobin level and reticulocyte count over time. RBC: Red blood cells.

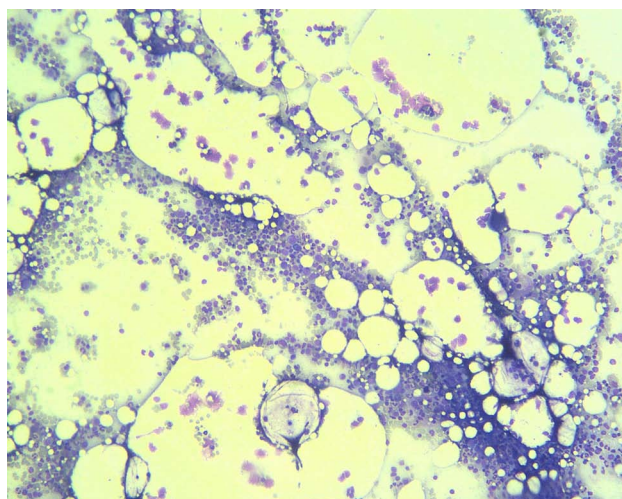


Figure 2 Photomicrograph of bone marrow biopsy showing overall hypocellularity of 10% (HE, x 40).

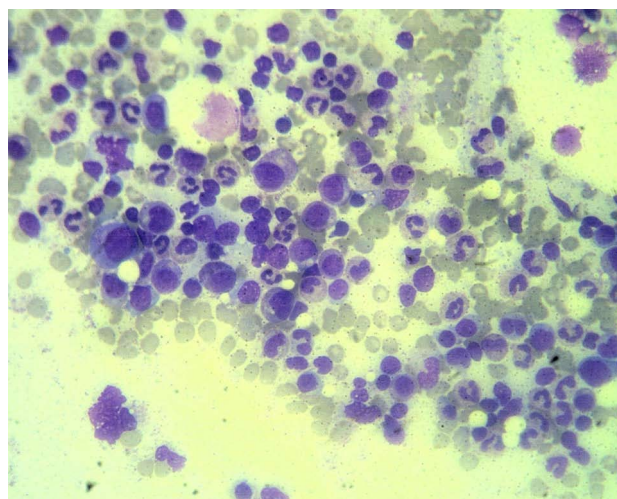


Figure 3 Photomicrograph of higher magnification showing normal maturation of myeloid precursors and absence of erythroid precursors in this field (HE, x 100).

initial presentation, elevated aminotransferase levels and positive antibody to hepatitis C virus were detected. HCV RNA level was 85000 IU/mL. The genotype was 2. The pretreatment hemoglobin level was 11.2 g/dL and the reticulocyte count was 1.8% (normal range 0.5-1.5%). Serum level of vitamin B12 was 288 pg/mL (normal range 272-1078 pg/mL). Serum level of folic acid was 11.0 ng/mL (normal range 5-26 ng/mL). Treatment with peginterferon alfa-2a 180 ug weekly and ribavirin 800 mg daily was started. After four weeks of treatment, the ribavirin dose was reduced to 600 mg due to a prominent decrease in the hemoglobin level to 8.4 g/dL. HCV RNA level at the 4th wk was undetected. Three weeks after dose reduction of ribavirin, the hemoglobin level continued to drop to 6.9 g/dL. The combination therapy was discontinued after 7 wk of treatment due to the patient's intolerance of anemia (Figure 1). Serum levels of indirect bilirubin, lactate dehydrogenase and haptoglobin level remained normal during combination treatment. Parvovirus serologies revealed positive immunoglobulin (Ig) G but negative IgM antibodies, which were consistent with past exposure.

He received follow up at a previous institution after termination of combination treatment. However, his anemia persisted and became transfusion-dependent. Blood transfusion with packed red blood cells was administered every month (Figure 1). Three months after discontinuation of combination treatment, the hemoglobin dropped to 5.5 g/dL and the reticulocyte count was 0.1% (normal range 0.5-1.5%). Levels of vitamin B12 and folic acid were within the normal range. Bone marrow biopsy revealed severe hypocellularity of 10% (Figure 2) with normal maturation of myeloid precursor cells (Figure 3). Erythroid precursor cells were markedly decreased with a ratio of myeloid to erythroid precursors of 10. The diagnosis of PRCA was made based on these histopathological findings. Oral methylprednisolone 15 mg daily was administered. After four weeks of oral methylprednisolone therapy, his hemoglobin level increased to 9.2 g/dL and the reticulocyte count increased to 1.7% (Figure 1). He needed no further blood transfusions in the following months.

DISCUSSION

Combination treatment of pegylated interferon α and ribavirin has become the standard therapy for patients with chronic hepatitis C infection^[7]. Patients with chronic hepatitis C receiving combination treatment develop anemia because of ribavirin-induced hemolysis^[8] and interferon-induced bone marrow suppression^[9]. The ribavirin-induced anemia is dose-dependent and reversible^[10]. Conversely, interferon directly suppresses the bone marrow synthesis of granulocytes, erythrocytes and megakaryocytes^[9]. PRCA is rarely encountered in chronic hepatitis C patients receiving combination treatment. To our knowledge, there is only one previous case report in the English literature of PRCA after combination treatment in patients with chronic hepatitis C^[6].

The pathophysiology of PRCA is heterogenous, which may be congenital or acquired^[1]. Diamond-Blackfan anemia is a congenital form of PRCA with genetic defects affecting erythropoietic lineage. Acquired causes of PRCA include human parvovirus B19 infection, lymphoproliferative disorder, humoral or cellular immunity, production of erythropoietin-neutralizing antibody, and drugs such as ribavirin and standard interferon^[1-5]. Other reported causes of PRCA include hepatitis A infection^[11], malignant thymoma^[12], and systemic lupus erythematosus^[13]. The diagnosis of PRCA is based on bone marrow findings such as severe hypocellularity, a markedly elevated myeloid: erythroid ratio, and severe decreased erythroid precursors^[6,14]. With regard to the treatment of PRCA, corticosteroid therapy is considered the treatment of first choice, although relapse is not uncommon^[15]. In the study of Clark *et al.*^[6], 80% of patients relapsed as the dosage of steroid was tapered during the first year after remission. In contrast, cyclosporine A is suggested when the long-term feasibility of maintenance is considered^[15].

As to the etiology of PRCA in our case, several possibilities should be considered. Firstly, although HCV infection itself has been associated with PRCA^[17], the HCV RNA in our case was undetected at the 4th wk of combination treatment. Furthermore, parvovirus serologies revealed positive IgG but negative IgM antibodies, which excluded parvovirus infection as the cause of PRCA in our case. Secondly, although ribavirin-induced PRCA has been reported previously^[3], the anemia in our case might not have been caused solely by ribavirin since the anemia persisted for 3 mon after discontinuation of ribavirin. Drug-induced PRCA generally resolves within 1-2 wk after removal of the causative agent^[18]. Thirdly, one might consider the possible association of underlying pernicious anemia with PRCA in this case. Pernicious anemia is generally considered an autoimmune disease resulting in deficiency of intrinsic factor and subsequent vitamin B12 deficiency^[19]. Coexistence of pernicious anemia and PRCA in the same patient has been reported in earlier literature^[20-22], suggesting that an immunological process is involved in the pathogenesis of PRCA in these cases. In our case, serum levels of vitamin B12 did not

alter significantly before and after combination treatment, thus excluding vitamin B12 deficiency as the cause of severe anemia after combination treatment. Therefore, it is reasonable to assume that pegylated interferon might have played a role in the pathogenesis of PRCA in our case. Several studies have also suggested that interferon may play a role in the development of acquired PRCA^[4,5,23]. However, further investigations may be needed to determine whether PRCA is caused by pegylated interferon alone or by combination treatment.

In conclusion, this case highlights the importance of considering PRCA when severe anemia associated with reticulocytopenia develops during the combination treatment of patients with chronic hepatitis C.

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Enucleation for gastrointestinal stromal tumors at the esophagogastric junction: Is this an adequate solution?

Nadia Peparini, Giovanni Carbotta, Piero Chirletti

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Abstract

The authors discussed the proposal by Coccolini and colleagues to treat gastrointestinal stromal tumors (GISTs) at the esophagogastric junction with enucleation and, if indicated, adjuvant therapy, reducing the risks related to esophageal and gastroesophageal resection. They concluded that, because the prognostic impact of a T1 high-mitotic rate on esophageal GIST is worse than that of a T1 high-mitotic rate on gastric GIST, enucleation may not be an adequate surgery for esophagogastric GISTs with a high mitotic rate in which the guarantee of negative resection margins and adjuvant therapies can be the only chance of survival.

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Key words: Gastrointestinal stromal tumor; Esophagogastric junction; Surgery; Resection; Enucleation

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TO THE EDITOR

We read with great interest the article by Coccolini and colleagues on the treatment of gastrointestinal stromal tumors (GISTs) at the esophagogastric junction^[1]. They stated the problems related to the choice of extended esophageal and gastroesophageal resection (i.e. a better guarantee of R0 resection but a higher prevalence of morbidity and mortality) or enucleation (i.e. a higher risk of microscopically positive margins but a better postoperative outcome).

The impact of microscopically negative margins on long-term survival remains controversial and there is no evidence that extensive resections are related to a better survival rate. The authors suggested that, for GISTs at the esophagogastric junction, enucleation and adjuvant therapies can be useful alternatives to avoid the high prevalence of morbidity and mortality associated with esophageal and esophagogastric resections. However, the 2009 edition of the TNM Classification of Malignant Tumors states that, in the absence of nodal metastasis, esophageal GISTs ≤ 2 cm (T1, i.e. tumors that may be treated with enucleation more frequently) are classified as stage I in the case of a low mitotic rate but as stage IIIA in the case of a high mitotic rate. This case is different from T1 gastric GISTs that are classified as stage I or stage II in the presence of a low or high mitotic rate, respectively^[2]. In the case of a high mitotic rate, the prognostic impact of a T1 esophageal GIST is worse than that of a gastric GIST with an identical size. Prospective, multicenter evaluation of the different treatment strategies for esophagogastric GISTs is sorely needed. However, enucleation may not be an adequate surgery for esophagogastric GISTs with a high-mitotic rate in which the guarantee of negative resection margins and adjuvant therapies can be the only chance of survival.

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Events Calendar 2011

January 14-15, 2011

AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011

Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011

Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011

9. Gastro Forum München, Munich,
Germany

February 4-5, 2011

13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011

Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011

APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011

Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011

2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011

International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011

Canadian Digestive Diseases Week,
Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011

Childhood & Adolescent Obesity:

A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011

42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011

Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011

British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011

41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011

Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011

UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011

MedicRes IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011

26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011

IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011

International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011

Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011

Pediatric Emergency Medicine:
Detection, Diagnosis and Developing

Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011

9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011

The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011

Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011

4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011

Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011

2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011

1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011

22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011

4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011

The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011

Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011

International Scientific Conference
on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011

ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011

XI Congreso Interamericano
de Pediatría "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium

178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne,
Martinstr. 29-37, 50667 Cologne,
Germany

September 10-11, 2011

New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011

ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
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September 30-October 1, 2011

Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011

Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise,
Papeete, French Polynesia

October 22-26, 2011

19th United European
Gastroenterology Week,
Stockholm, Sweden

October 28-November 2, 2011

ACG Annual Scientific Meeting &
Postgraduate Course,
Washington, DC 20001,
United States

November 11-12, 2011

Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku,
Tokyo 107-0052, Japan

December 1-4, 2011

2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 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>

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- 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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