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REVIEW

Role of ischaemic preconditioning in liver regeneration following major liver resection and transplantation

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Abstract

Liver ischaemic preconditioning (IPC) is known to protect the liver from the detrimental effects of ischaemic-reperfusion injury (IRI), which contributes significantly to the morbidity and mortality following major liver surgery. Recent studies have focused on the role of IPC in liver regeneration, the precise mechanism of which are not completely understood. This review discusses the current understanding of the mechanism of liver regeneration and the role of IPC in this setting. Relevant articles were reviewed from the published literature using the Medline database. The search was performed using the keywords "liver", "ischaemic reperfusion", "ischaemic preconditioning", "regeneration", "hepatectomy" and "transplantation". The underlying mechanism of liver regeneration is a complex process involving the interaction of cytokines, growth factors and the metabolic demand of the liver. IPC, through various mediators, promotes liver regeneration by up-regulating growth-promoting factors and suppresses growth-inhibiting factors as well as damaging stresses. The increased understanding of the cellular mechanisms involved in IPC will enable the development of alternative treatment modalities aimed at promoting liver regeneration following major liver resection and transplantation.

Key words: Liver regeneration; Ischaemic reperfusion; Ischaemic preconditioning; Hepatectomy; Transplantation

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INTRODUCTION

Ischaemic-reperfusion injury (IRI) is an inevitable phenomenon that results following major liver surgery, including partial hepatectomy and liver transplantation. As a consequence, parenchymal cell injury and liver dysfunction[1,2] of varied severity leads to significant morbidity and mortality post-surgery[3-5], in particular, in patients with liver cirrhosis and steatosis[6-9]. In addition, IRI significantly impairs liver regeneration following hepatectomy[10,11].

Due to the inevitability of ischaemia and reperfusion in liver surgery, various investigators have attempted to elucidate methods to limit the detrimental effects of IRI and improve liver function and regeneration of the remnant liver[12,13]. These include hypothermic perfusion of the liver[14], intermittent liver inflow occlusion[15,16] and ischaemic preconditioning (IPC)[17]. Liver IPC is an endogenous mechanism consisting of a short period of vascular occlusion followed by reperfusion that renders the liver more tolerant to subsequent prolonged episodes of ischaemia. Besides having protective effects on IRI following major liver resection and transplantation[17-19], it has been suggested that IPC is also beneficial in liver regeneration[20]. This article describes the current understanding of the liver regeneration cascade as published and gives a balanced review on the mechanisms by which IPC influences liver regeneration.

MECHANISMS OF LIVER REGENERATION

Liver regeneration is a complex and multi-factorial process that is mediated by interactions between regenerative cytokines, growth factors and metabolic demand of the liver following surgery and IRI.

The regenerative cytokine network and the priming pathway

During the first few hours following IRI, regenerative cytokines are produced that render the resting hepatocytes responsive to growth factors required for cellular division and proliferation[21,22]. This period is known as the "priming phase". Various mediators have been implicated as possible triggers of the regenerative cytokine network including gastrointestinal lipopolysaccharide (LPS)[23,24], Toll-like receptor-myeloid differentiation factor 88 (Myd88) signaling pathways[25-27], components from the complement cascade[28], nitric oxide (NO)[29-31] and prostaglandins[32].

Studies have identified tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) as important regenerative cytokines[21,33,34]. Akerman and co-workers showed that

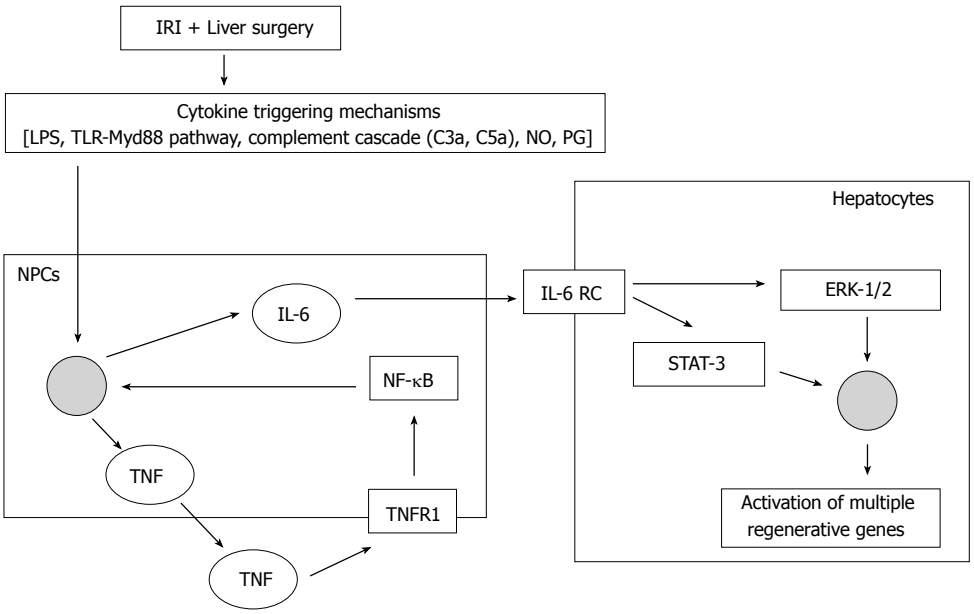

Figure 1 Current proposed mechanisms of the priming pathway of liver regeneration. IRI: Ischaemic reperfusion injury; LPS: Lipo-polysaccharide; TLR: Toll-like receptor; MyD88: Myeloid differentiation factor 88; NO: Nitric oxide; PG: Prostaglandins; NPCs: Non-parenchymal cells; TNF: Tumour necrosis factor; TNFR1: TNF receptor type I; NF-κB: Nuclear factor-kappaB; IL-6: Interleukin-6; IL-6 RC: IL-6 receptor complex; ERK-1/2: Extracellular regulated kinases 1/2; STAT-3: Signal transducer and activator of transcription-3.

anti-TNF antibodies led to delayed DNA synthesis in the regenerating rat liver and inhibited the increase in IL-6 levels following partial hepatectomy[35]. The initiation of liver growth by TNF-α was shown to be dependent on its binding to TNF-receptor type 1 (TNF-R1)[36]. Mice deficient of TNF-R1 exhibited a delay in liver regeneration and increased mortality following liver resection, which was subsequently reversed by recombinant IL-6 injection[37]. However, IL-6 lacking mice demonstrated impaired liver regeneration despite the presence of TNF-α, suggesting that TNF-R1 signaling results in the release of IL-6[38]. IL-6 deficient mice not only showed impaired ability to regenerate, but also had increased IRI following liver resection[39]. However, Wuestefeld *et al*[40] reported that mice deficient in IL-6 and its common signal transducer, glycoprotein 130 (gp130) had no defects in DNA replication following partial hepatectomy. The groups of Zimmers and Blindenbacher have suggested that the levels of serum IL-6 present following liver resection in mice are critical in modulating its regenerative effects[41,42]. This may account for the difference in results observed in studies attempting to determine the effect of IL-6 in liver regeneration. It has been suggested that other mediators such as stem cell factor and oncostatin M may play a role in enhancing the effects of IL-6 on hepatocyte regeneration[43-45].

Non-parenchymal liver cells [Kupffer cells and sinusoidal endothelial cells (SECs)] are involved in the priming phase (Figure 1)[46]. Following stimulation from cytokine triggering mechanisms, TNF-α binds to TNF-R1 on non-parenchymal liver cells and stimulates the production of IL-6[47], *via* the activation of the transcription factor nuclear factor-kappa B (NF-κB)[48-50]. Ping and colleagues demonstrated that the secretion of regenerative cytokines, such as IL-6 by rat liver SECs was mediated by the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt signaling pathway[51], *via* NF-κB activation[50]. The transcription factor NF-κB is also known to be an important component of pro-survival cellular signaling responses, and hence its activation will not only stimulate IL-6 production but also activate survival genes[52,53].

IL-6 acts directly on hepatocytes by binding to the IL-6 receptor complex and induces the translocation of signal transducer and activator of transcription-3 (STAT-3) to the nucleus. This initiates a cascade of events that leads to progression of the cell cycle, culminating in the synthesis of DNA and subsequent cellular mitosis[54,55].

Growth factors and growth-factor signaling systems in liver regeneration

Following this priming phase, cell cycle progression is then dependent on growth factors, such as hepatocyte growth factor (HGF), transforming growth factor-alpha (TGF-α) and epidermal growth factor (EGF)[56,57]. The two main growth-promoting signaling systems involved in liver regeneration are the HGF and its receptor (Met) and the epidermal growth factor receptor (EGFR) and its relatively large family of ligands. The effect of growth factors and their corresponding signaling systems may be dependent on the metabolic state of the hepatocytes and the presence or absence of other effectors[58].

HGF is produced by non-parenchymal cells of the rat liver following liver injury[59-61] and partial hepatectomy[62], and acts on its receptor on hepatocytes. Several authors have demonstrated that HGF is crucial in promoting liver regeneration following partial hepatectomy and transplantation in animal models[63-67]. HGF administration to recipients of reduced-size liver grafts in rats illustrated early regeneration and provided hepatoprotection against rejection-related injuries[64,68,69]. These essential signals are regulated by HGF, *via c-met*, the gene on the HGF-receptor.

EGF is mainly produced in the salivary glands in rodents and plays an important role in hepatocyte proliferation by binding to the EGFR on hepatocytes[57]. Sialoadenectomy-induced decrease in circulating EGF in mice and rat models resulted in impaired liver regeneration following partial hepatectomy, which was reversed by the administration of EGF[70,71]. In addition, combined administration of

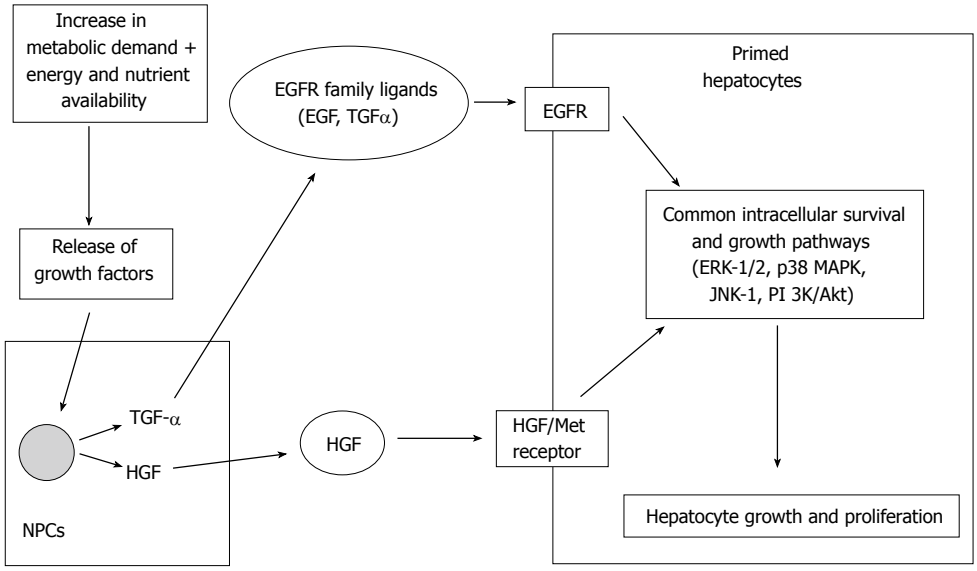

Figure 2 Potentiation of growth factor signaling pathways involved in cell cycle progression in liver regeneration. NPCs: Non-parenchymal cells; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF-α: Transforming growth factor-α; EGFR: Epidermal growth factor receptor; ERK-1/2: Extracellular regulated kinases 1/2; JNK: c-jun-NH2-terminal kinase; PI 3K/Akt: Phosphatidylinositol 3-kinase/Akt-signal pathway.

EGF and insulin increases the DNA synthesis following liver resection in cirrhotic rats[72]. Results from these studies suggest that EGF has a direct effect on hepatocyte proliferation.

TGF-α is produced in non-parenchymal cells, mainly the Kupffer cells. Mead and Fausto demonstrated that TGF-α may function as a physiological inducer of hepatocyte DNA synthesis during liver regeneration by an autocrine mechanism by binding to EGFR in both rat and culture models[73]. Besides TGF-α, there are many ligands for EGFR, including EGF, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF) and epiregulin[57]. Although TGF-α expression increases following partial hepatectomy in mice, TGF-α lacking mice do not display diminished liver regeneration[74,75]. This may be due to the fact that ligands such as EGF can also stimulate EGFR and activate common intracellular growth signaling pathways. Studies have shown that other growth factors, such as HB-EGF[50,76,77], amphiregulin[78], insulin and glucagon[79,80] may also play a role in liver regeneration.

Although these two growth-promoting signaling systems are largely independent, some integration may exist. Scheving *et al*[81] demonstrated that EGFR kinase inhibition by PKI166 (selective, potent inhibitor of EGFR kinase) blocked the mitogenic effects of HGF in cultured rat hepatocytes, suggesting EGFR may regulate HGF-mediated hepatocyte proliferation. Nevertheless, various regenerative pathways are initiated by HGF/*c-met* and the EGFR signaling mechanisms (Figure 2), which include the activation of mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinases 1 and 2 (ERK-1/2; aka p42/44 MAPKs), c-jun-NH2-terminal kinases 1 and 2 (JNK-1/2; aka p46/p54 SAPK) and p38 MAPKs. Collectively, these MAPKs have been shown to play essential roles in cell growth, transformation differentiation and apoptosis[82-84].

ERK-1/2 activation has been shown to correlate with hepatocyte proliferation in animal studies and *in vitro* models[85-87]. Besides being responsive to growth factor signals, studies have demonstrated that ERK-1/2 activity can also be induced by cytokines such as TNF-α[88]. Hence, the ERK-1/2 may be a signaling pathway that integrates both growth factor and cytokine signaling. Serandour *et al*[89] demonstrated that a combination of EGF and TNF-α induced hepatocyte proliferation by 30%, compared to EGF alone in a hepatocyte-liver epithelial cell co-culture model. This study suggested that TNF-α mediated extracellular matrix remodeling was required for continued hepatocyte replication and proliferation. Hence, this study reiterates the importance of the interaction of cytokines and growth factors in the liver regeneration cascade. Although ERK-1/2 has been identified to have a key role in hepatocyte growth, inhibition of ERK-1/2 does not significantly alter proliferation of regenerating rat hepatocytes. However, inhibition of p38 MAPKs results in decreased DNA synthesis, suggesting that p38 MAPKs activation is prerequisite for hepatocyte proliferation[90]. JNK-1 and p38 MAPKs are involved in the regulatory control of the induction of nuclear proteins, such as cyclin D1. The activation of cyclin D1 is one of the earliest steps in the pathway of resting cells to enter the pre-replicative phase of the cell cycle.

Metabolic demand of the liver

The increased metabolic demand imposed on the remnant liver following partial hepatectomy are likely to be interconnected with the activation of the mechanisms involved in DNA replication to sustain liver function. This is likely to be dependent on energy levels and nutrient availability.

Mitochondria are the predominant source of the high energy phosphates that are essential for energy-dependent processes in cells. Mitochondrial activity has been shown to be correlated with the recovery of liver function and subsequent regeneration[91,92]. Maruyama *et al*[93] showed the recovery of liver weight following hepatectomy was proportional to the energy (ATP) levels of the remnant liver in rats.

Amino acid deprivation has been shown to inhibit the regeneration process in rat livers following liver resection[94]. Nelsen *et al*[95] showed that selective amino acid deprivation in culture and protein deprivation in mice impaired hepatocyte cyclin D1 expression and that

transfection of cyclin D1 promoted cell cycle progression under these conditions. Hence, amino acids regulate hepatocyte proliferation *via* cyclin D1.

The absence of bile acids in the intestine has been shown to delay liver regeneration following partial hepatectomy in rats[96]. Following liver surgery or injury, bile flow is stimulated[97] which results in the release of bile from the gallbladder and its return through the entero-hepatic circulation exposes the remnant hepatocytes to an increase in relative bile acid flux. This early phase of bile acid overload and subsequent bile acid signaling is necessary for normal liver regeneration. However, liver-specific functions such as synthesis of clotting factors and albumin and the continuous formation of bile are impaired following partial hepatectomy and results in transient cholestasis[98]. The formation of bile is dependent on the active secretion of bile salts and other biliary constituents into the bile canaliculus by specific bile acid and organic anion transporters[99]. Gerloff *et al*[100] demonstrated that the expression of two ATP-dependent transporters [bile salt acid pump (*Bsep*) and multi-organic anion transporter (*Mrp2*)] was unchanged or slightly increased following partial hepatectomy in rats and this provided a potential mechanism by which the regenerating liver cells maintained or increased bile secretion. The authors in this study also suggested that the down-regulation of certain transporters [sodium-taurocholate cotransporter (*Ntcp*) and organic anion transporting polypeptides (*Oatp1 and Oatp2*)] could be a protective mechanism against the potentially hepatotoxic bile salts[100]. The differential regulation of hepatobiliary transporters during the regeneration process are likely to be mediated by cytokines such as TNF-α[101]. Recently, Huang *et al*[102] showed that bile acid activation of nuclear receptor-dependent signaling pathways regulated the regeneration process by sensing the liver's functional capacity following partial hepatectomy in mice. When inadequate function causes bile acids to build up, the resultant nuclear receptors activation not only induces negative feedback pathways that protect hepatocytes from bile acid toxicity but also increases the capacity of the liver to manage the overload by promoting liver growth[102].

Results from recent studies have implicated the mammalian target of rapamycin (mTOR) complex as a sensor of nutrient-energy levels and its downstream mediators, such as p70 S6 kinase, are thought to regulate protein translation and cell growth[103,104]. Inhibition of the mTOR complex leads to diminish DNA replication following partial hepatectomy in mice and rat models[105,106]. The activity of p70 S6 kinase has been shown to increase following partial hepatectomy[106] and mice lacking the S6 kinase demonstrated diminished hepatocyte proliferation[107]. Hence, energy is required for energy-dependent signaling pathways and processing the available nutrients for the regeneration process to proceed.

ISCHAEMIC PRECONDITIONING IN LIVER REGENERATION

Since IPC was first described by Murry *et al*[108], this strategy has been developed more widely and is currently practiced in major liver surgery in several centers. The mechanism of IPC is thought to be divided into two phases; early (classical/acute) pre-conditioning and delayed pre-conditioning. The hepatoprotective effects of early preconditioning occur within minutes after reperfusion and are maintained for 1 to 2 h[109]. This phase is thought to be mediated by pre-existing substances. The "second window of protection", delayed pre-conditioning, re-appears 24 to 72 h following IPC. The underlying mechanism is thought to rely on the modification of gene expression resulting in protein production as its effectors[110]. Various substances have been implicated as key effectors in liver IPC including adenosine[111-114], protein kinase C[115-117], NO[118-121], heat-shock proteins[122,123], tyrosine kinases[124], MAPKs[117], oxidative stress[125,126] and NF-κB[127,128].

The beneficial effects of IPC on the liver following IRI include decrease in severity of liver necrosis[129], anti-apoptotic effects[130], preservation of liver microcirculation[131,132] and improvement in survival rate[119], and more recently, its role in liver regeneration is currently being evaluated. For IPC to influence liver regeneration, its key effectors must be involved in either promoting or up-regulating mediators that are involved in the regeneration cascade or exert an inhibitory effect on growth-inhibitory factors of liver regeneration or *vice versa* (Figure 3).

Up-regulation of the regenerative cytokine network

Tumour necrosis factor-alpha and interleukin-6: The initiation of the regenerative response is dependent on the early activation of TNF-α and IL-6 responsive transcription factors[38,54]. Studies evaluating the effect of IPC in stimulating the release of TNF-α and IL-6 have produced conflicting findings. Tsuyama *et al*[133] reported that IPC prolonged survival, suppressed liver necrosis induced by IRI and inhibited the release of TNF-α at 1 h, and IL-6 at 2 and 5 h of the reperfusion phase in mice. IPC has also been shown to reduce TNF-α production in normothermic and cold ischaemic conditions[125,134,135].

In contrast, Bedirli and others demonstrated that IPC inhibited the production of TNF-α, but not IL-6 during the late (24 and 48 h) phases of reperfusion in rats following partial hepatectomy[136]. This study suggested that IL-6 is an important mediator in IPC-treated rats in promoting hepatocyte proliferation following liver resection[136]. Using a TNF gene-deleted mice model, Teoh *et al*[137] demonstrated that pre-treatment with low dose IL-6 prior to hepatic ischaemia conferred equivalent hepatoprotection and earlier cell cycle entry as IPC compared to non-IPC-treated mice at 2 h of reperfusion. Taken together, these studies emphasize the importance of IL-6 as a hepatoprotective and pro-proliferative mediator during the early and late phases of reperfusion following IRI. Although IPC attenuates the late onset prolonged release of TNF-α (up to 44 h) that mediates liver IRI in rats[138,139] and mice[140], IPC itself is associated with the early increase (10 min) in liver and serum TNF-α following hepatic ischaemia[140]. Injection of low dose TNF-α 30 min prior to liver ischaemia conferred similar hepatoprotection as IPC[140]. Both IPC and pre-treatment with low dose TNF-α injection were shown to stimulate earlier and more

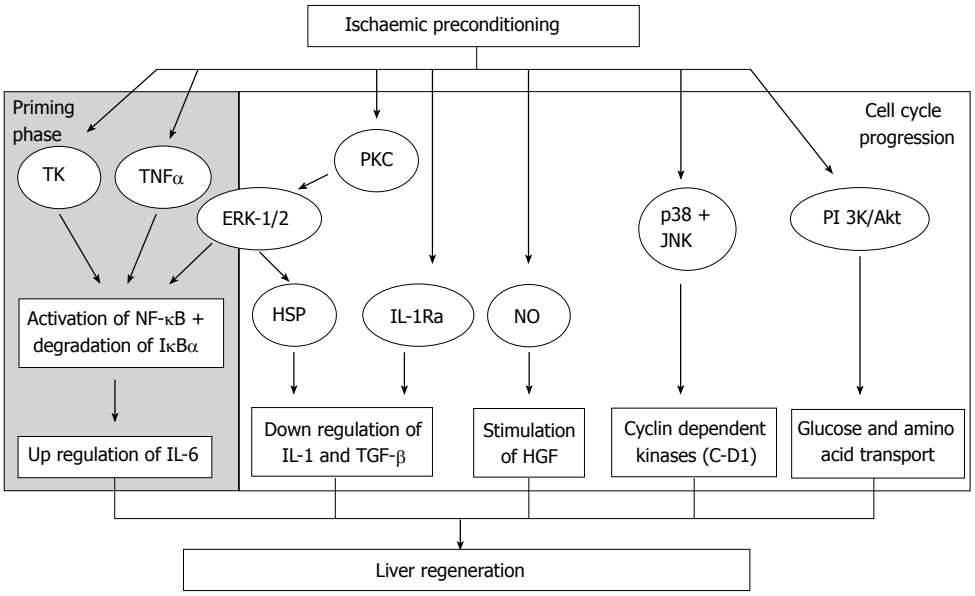

Figure 3 The effect of ischaemic preconditioning and its effectors on the signaling pathways of liver regeneration. TK: Tyrosine kinase; PKC: Protein kinase C; NF-κB: Nuclear factor-kappaB; IκBα: Inhibitory binding protein for NF-κB; ERK-1/2: Extracellular regulated kinases 1/2; HSPs: Heat shock proteins; NO: Nitric oxide; IL1-RA: Interleukin-1 receptor antagonist; HGF: Hepatocyte growth factor; ROS: Reactive oxygen species; PI 3K/Akt: Phosphatidylinositol 3-kinase/Akt-signal pathway; JNK: c-jun-NH2-terminal kinase; C-D1: Cyclin D1; IL-1: Interleukin-1; TGF-β: Transforming growth factor-β.

vigorous cell cycle entry following liver IRI compared to naïve mice during the first 24 h of the reperfusion period[140].

The differences reported among studies investigating the influence of IPC on the regenerative cytokine network may be related to the differences in experimental models and protocols employed. In addition, both TNF-α and IL-6 are known to be important components in other cellular signaling responses. For example, high levels of serum TNF-α following liver ischaemia in rats is thought to mediate liver IRI[138,139]. Investigators have suggested that the mechanism of action of IL-6 in promoting liver regeneration appear to be separate from those involved in the modulation of IRI[39,141]. The hepatoprotective mechanisms against IRI may involve the anti-inflammatory properties of IL-6 and the down regulation of TNF-α production[39,142,143]. This could potentially explain the differences in results obtained.

Nevertheless, the influence of IPC in promoting the early release of TNF-α and the up regulation of IL-6 during the reperfusion phase, and subsequent modulation of the hepatocellular proliferation process should not be discounted. In addition, these conflicting results may indicate that IPC can potentiate hepatocyte proliferation *via* at least two different pathways; liver regeneration *via* a TNF-α/IL-6-dependent pathway and a mitogen-induced proliferative pathway that does not require TNF-α or IL-6[144,145].

Down-regulation of inhibitory mediators of liver regeneration

Hepatocyte growth is controlled by a balance of both growth-promoting and growth-inhibiting factors[65,146]. While those cytokines and pathways previously described promote liver regeneration following liver resection, there are various mediators that are involved directly or indirectly in inhibiting the liver regeneration process.

Interleukin-1 and transforming growth factor-beta as inhibitors of liver regeneration: IL-1 is a mediator of acute inflammation[147] and is a significant down-regulator of hepatocyte proliferation[148]. IL-1β delays and inhibits hepatocyte proliferation in both culture models[149] and following partial hepatectomy in rats[148]. Other investigators have implicated IL-1α as a mediator of IRI and an inhibitor of liver regeneration[150]. Besides antagonizing the stimulatory effects of growth factors such as HGF[150], IL-1 strongly inhibits hepatocyte DNA synthesis leading to impaired liver regeneration in primary culture models[149]. TGF-β is another inhibitor of hepatocyte DNA synthesis[151-153] and antagonizes the stimulatory effects of HGF during liver regeneration in both *in vitro* and experimental hepatectomy models[152,154]. This anti-regenerative effect of TGF-β is thought to be modulated by the induction of oxidative stress in hepatocytes[155,156].

Results from several studies have suggested that IPC antagonizes the effects of these inhibitory cytokines. Previous studies have shown that the release of IL-1 is potentially influenced by NO during IRI in a variety of cell types[157-159]. In IPC-treated rats that underwent reduced size liver transplantation, an increase in IL-1α levels were noted following NO synthesis inhibition[150]. This practice abolished the benefits of IPC on hepatic IRI, oxidative stress and liver regeneration[3,150]. Furthermore, the detrimental effects of NO inhibition were not observed when rats subjected to this treatment were subsequently treated with an IL-1 receptor antagonist (IL-1-RA)[150]. IL-1α has also been shown to be involved in pulmonary injury following liver IRI. IPC mediated by NO, reduced IL-1α release and protected against pulmonary damage[160]. Data from these studies suggests that IPC, through increased NO availability, inhibits the release of IL-1, thereby protecting the liver graft and the lungs against liver IRI and preventing inhibition of liver regeneration. Hence, one proposed mechanism by which IPC promotes liver regeneration is by inhibiting the release of growth-inhibitory cytokines from Kupffer cells, such as IL-1, which is dependent on up-regulation of the NO pathway.

The mechanism by which IPC inhibits IL-1 production may also be related to the induction of intracellular

stress proteins[161,162], such as heat shock protein 70 (HSP 70). IPC induces over-expression of HSP 70 in isolated hepatocytes[163], and reduces liver IL-1 synthesis under normothermic conditions[164]. Besides expressing cytoprotective effects[165,166], HSP induction leads to the down-regulation of IL-1 synthesis in pancreatic[161,167] and lung[162,168] cell studies. The induction of HSP mediated by IPC may be independent of the NO pathway as the increase in HSP by IPC was not modified when NO synthesis was inhibited[150]. These results suggest that IPC potentiates the overexpression of HSP, *via* an NO independent pathway, leading to hepato-protection against IRI and ameliorates liver regeneration due to decrease inhibitor production.

Another proposed mechanism by which IPC promotes liver regeneration could be due to the stimulation of IL-1-RA. IL-1-RA is an acute phase protein that has been shown to inhibit the effects of IL-1α and IL-1β by competing for type I and type Ⅱ IL-1 receptors[169]. This leads to a decrease in the inflammatory response[169] and abrogates liver IRI *in vivo*[170]. A recent study on gene expression profiling on patients undergoing partial hepatectomy revealed that IPC stimulated the expression of the IL-1-RA gene[171]. Hence, liver over-expression of IL-1-RA following IPC directly inhibits the effect of high IL-1 concentrations induced by IRI and results in reduced liver injury and necrosis[171]. Although no study has formally assessed the effect of IL-1-RA on markers of liver regeneration, IL-1-RA could be indirectly involved in the regeneration process by antagonizing the inhibitory effects of IL-1 on hepatocyte proliferation.

Inhibitory binding protein for NF-κB (IκB-α) and NF-κB activity: The transcriptional activities of NF-κB are tightly controlled by its inhibitory proteins, especially IκB-α[172]. The phosphophorylation and subsequent degradation of IκB-α leads to the liberation of NF-κB proteins allowing binding to a variety of promoters and triggers gene expression. Data from studies examining the role of NF-κB as an effector of IPC have demonstrated conflicting results. IPC facilitated the activation of transcription factor NF-κB in an *in vivo* murine model, and this was parallel to the degradation of its inhibitory protein, IκB-α[173]. Ricciardi and co-workers found that IPC increased IκB-α phosphorylation and NF-κB concentration prior to cold ischemia in pig liver grafts. Data from this study suggested that the underlying mechanism involved was related to the activation of second messengers of tyrosine kinase[127]. Another proposed mechanism for NF-κB activation and degradation of IκB-α could be mediated by TNF-α. The release of TNF-α by IPC and pre-treatment with low-dose TNF-α was shown to increase IκB-α degradation and increase NF-κB DNA binding in a mouse model[140]. However, Li *et al*[174] observed that IPC inhibited the activity of the transcription factor NF-κB during the early reperfusion phase (1 and 2 h), and this was accompanied by diminished TNF-α expression and reduced IRI in liver transplantation rat model.

One explanation for this contradictory evidence might be the different experimental models and methodology used. Nevertheless, these results indicate that IPC does attenuate the nuclear levels of the transcription factor NF-κB. It is possible that other mediators may have an effect in determining the increase or inhibition of NF-κB activity and its corresponding cellular signaling responses.

Increased production of growth factors

Hepatocyte growth factor (HGF): Franco-Gou *et al*[175] demonstrated an increase in both liver and plasma HGF levels following IPC in reduced size liver transplantation rat model, and this was associated with an increase in hepatocyte proliferation. The modulation of HGF levels by IPC could be mediated by the generation of NO and its effect on TGF-β and HGF concentrations. IPC reduced the levels of TGF-β with an associated increase in HGF levels[150]. Similar results were seen with NO preconditioning treatment[150]. This suggests that IPC reduced TGF-β levels with a parallel increase in HGF and subsequent hepatocyte proliferation, possibly mediated by NO.

HGF may also exhibit the ability to promote hepatocyte survival. In a liver IRI rat model, pre-treatment with HGF inhibited the production of reactive oxygen species and its damaging effects[176]. Similar results were observed in a hypoxia-reoxygenation-induced oxidative stress model in hepatocytes[177]. These results suggest that the anti-apoptotic effect of HGF could pave the way for hepatocyte proliferation following IRI.

Activation of downstream mitogen-activated protein kinases (MAPKs)

Extracellular signal-regulated kinases 1 and 2 (ERK-1/2): ERK-1/2 is predominantly activated by growth-promoting factors. Studies have shown that protein kinase C plays a pivotal role in the activation of ERK-1/2 signaling pathway[163,178] that participates in the preservation of hepatocytes[163]. A number of reports have indicated that protein kinase C was critical for the development of IPC in rat, rabbit and human myocardiocytes[179,180]. Gao and associates showed that the activation of protein kinase C and its downstream ERK-1/2 mediators were increased in IPC-treated *in vivo* and *in vitro* models[163]. Data from this study also suggested that the expression of HSP70 was reduced and the protective effect of IPC was diminished when ERK-1/2 activity was reduced by a MAPK inhibitor (PD-98059). HSP70 expression and the cytoprotective effect of IPC were also reduced by a protein kinase C inhibitor (chelerythrine) in both *in vitro* and *in vivo* settings. Hence, this suggests that IPC increased the activation of ERK-1/2, *via* protein kinase C. The increased activation of protein kinase C-dependent ERK-1/2 by IPC may also increase the expression of HSP70. The up-regulation of ERK-1/2 by IPC may help in promoting liver regeneration in both the priming phase and growth factor signaling pathways.

p38 mitogen-activated protein kinases and c-jun-NH2-terminal kinase 1: Both p38 MAPKs and JNK-1 are known to modulate proliferative or apoptotic signaling pathways[181]. The activation of MAPKs and its corresponding downstream signaling pathway is regulated by specific stimuli and is also dependent on cell type. The co-activation of NF-κB protects the hepatocyte from

apoptosis and is involved in the priming of hepatocytes to enter the cell cycle[22,182].

Carini and co-investigators demonstrated that hypoxic preconditioning activated the p38 signaling pathway in rat hepatocytes subjected to hypoxia-re-oxygenation injury *in vitro*[117]. Teoh *et al*[173] demonstrated that proliferating hepatocytes were identified earlier in IPC-treated livers in a murine model of partial liver ischaemia, and this corresponded with the earlier activation and sustained maintenance of p38 and JNK-1. This suggests that IPC stimulus could prime quiescent hepatocytes to enter the cell cycle early, hence, setting up a regenerative response to compensate for hepatocyte injury by IRI.

Activation of survival and proliferative pathways

Phosphatidylinositol 3-kinase (PI 3-kinase)/Akt signaling pathway: The activation of the PI 3-kinase/Akt cascade has been shown to have a positive impact on cell survival and proliferation[183-186], inhibition of apoptosis and encourage the uptake of glucose and amino acids following stimulation by various growth-promoting factors in certain cells[187-189]. Data from the Izuishi group showed significant Akt activation following IPC in an *in vivo* model and suggested that this might contribute to the up regulation of glucose and amino acid transport after IRI required for liver regeneration[190].

Activation of nuclear proteins

Cyclin D1: Cyclin and cyclin-dependent kinases are involved in cell cycle regulation[191], in particular, the D group cyclins[192]. Cyclin D1 is a nuclear protein required for cell cycle progression in the G_1 phase[192-194], and controls hepatocyte proliferation[195]. IPC-treated livers showed earlier expression of cyclin D1 protein that corresponded with enhanced entry of hepatocytes into the cell cycle[173]. Cai and co-workers demonstrated that IPC stimulated cyclin D1 mRNA and protein expression during the early reperfusion phase in IPC-treated rat livers[196]. The early production of cyclin D1 could be mediated by the activation of the p38 MAPK pathway[182]. The cyclin D1 promoter region includes binding sites for NF-κB[197] and NF-κB activation is evident in hepatocytes during the early phase of regeneration following partial hepatectomy[48,198]. There is a close link between the activation of NF-κB by degradation of IκB-α cyclin D1 activation and cell cycle progression. IPC can modulate these transcription factors and increase cell proliferation, which is possibly one of the protective mechanisms against IRI.

Energy metabolism

Results obtained from an experimental model of 70% hepatectomy indicated that liver regeneration was closely correlated to the ATP levels of the liver remnant[93]. Studies have shown that IPC in normothermic conditions preserved the adenine nucleotide pool[199,200] and this is thought to be a consequence of the down regulation of cellular metabolism[199,201]. In a rat model of hypothermic transplant preservation injury, hepatocytes exposed to IPC had higher ATP concentrations and increased protein synthesis[202]. This improvement in energy metabolism is thought to contribute to hepatocyte viability following IRI[202]. Besides improvement in energy metabolism, Yoshizumi *et al*[203] also demonstrated an increase in bile production in IPC-treated rats. However, Franco-Gou and colleagues demonstrated similar energy metabolism (ATP, adenine nucleotides, ATP/ADP ratio and energy charge) in the IPC- and non-IPC-treated rat livers[175]. The difference in results obtained could be related to the difference in experimental models used. However, the role of IPC in modulating liver energy metabolism, which involves the preservation of ATP should not be discounted.

CLINICAL IMPLICATIONS OF ISCHAEMIC PRECONDITIONING IN LIVER SURGERY

The use of IPC as a surgical strategy to limit the detrimental effects of IRI during liver surgery has been extensively researched. Encouraging findings in animal studies in both warm ischaemia[203] and transplantation models[119], led to the first human trial which demonstrated that IPC reduced the severity of post-operative liver injury as well as alleviating endothelial cell injury[17]. Further human liver resection studies have shown that IPC reduces post-operative serum aminotransferase levels in both steatotic[204] and cirrhotic[205] livers and improves post-reperfusion haemodynamic stability[206] (Table 1). Although IPC is protective against IRI[207,208], it did not influence the morbidity and mortality rates in human studies[204,209,210]. In the transplant setting, Jassem *et al*[211] reported lower serum aminotransferase levels and shorter intensive care stay in IPC-treated cadaveric donor allografts. Other studies have not shown IPC to be beneficial in terms of graft function. Azoulay *et al*[212] found that although IPC protected cadaveric liver grafts against IRI, this beneficial effect was counter-balanced by decreased early graft function. Cescon and colleagues demonstrated similar protective effects against IRI, but IPC showed no clinical benefit (primary graft function and survival rates) in liver transplantation from deceased donors[213]. Although gaining popularity, the incongruous evidence of the clinical effects of IPC has precluded its widespread adoption in liver transplantation units.

Following major liver resection and IRI, the ability of the liver to regenerate is crucial to maintain liver function. This also has implication in live donor orthotopic liver transplantation and transplantation of segmental liver grafts. Since Yamada and co-workers first demonstrated that IPC significantly increased the regenerative capacity of the remaining hepatocytes in a rat model of IRI[20], various investigators have attempted to elucidate the role of IPC on liver regeneration using both culture and animal model studies as described above. At present, although there is no clinical trial published on the effect of IPC on liver regeneration, there are studies evaluating methods of monitoring liver regeneration. Special radiological imaging techniques currently available not only show volume of regeneration, but also determine functional ability of the remnant and regenerated liver[214,215]. This is another step towards assessing the role of IPC in liver regeneration in the clinical scenario.

Table 1 Previous published human studies on the results of ischaemic preconditioning following liver resection and transplantation

Study group	Sample[1]	Surgery	IPC[2]	Ischaemia and reperfusion time (min)[3]	Parameters assessed	Outcome of IPC
Clavien *et al*[17] (2000)	24 (12)	Liver resection	10I + 10R	IPC and control (TI: 30)	PT, Bilirubin, ALT, AST, Histology, Caspase-3 and 8 activity, SEC apoptosis, Blood loss, Transfusion, ITU stay, LOS	Protective against IRI Beneficial in patients with steatosis
Clavien *et al*[204] (2003)	100 (50)	Liver resection	10I + 10R	IPC (TI: 36 ± 5.9, Op: 225 ± 73), Control (TI: 35 ± 6.8, Op: 240 ± 92)	PT, Bilirubin, ALT, AST, Histology, Hepatic ATP, Blood loss, Transfusion, ITU stay, LOS	Protective against IRI Beneficial in younger patients, those with steatosis and longer periods of occlusion
Li *et al*[205] (2004)	29 (14)	Liver resection	5I + 5R	IPC (TI: 18 ± 3.6, Op: 191.3 ± 74.9), Control (TI: 17.4 ± 2.3, Op: 208.2 ± 45.3)	Bilirubin, ALT, AST, Histology, Caspase-3 activity, SEC apoptosis, LOS	Protective against IRI, mainly HCC patients with cirrhosis Shorter hospital stay
Nuzzo *et al*[209] (2004)	42 (21)	Liver resection	10I + 10R	IPC (TI: 54 ± 19, Op: 321 ± 92), Control (TI: 36 ± 14, Op: 339 ± 112)	PT, Bilirubin, ALT, AST, Transfusion, Morbidity, Mortality	Reduces operative bleeding Protective against IRI
Chouker *et al*[206] (2004)	68 (22)	Liver resection	10I + 10R	IPC (TI: 32 ± 6.3, Op: 251 ± 46), Control without PR (TI: NA, Op: 52 ± 30), Control with PR (TI: 35 ± 11, Op: 257 ± 83)	ALT, AST, Fluid loss, Transfusion, [4]Cardiovascular status	Protective against IRI Improves haemodynamic stability
Chouker *et al*[207] (2005)	75 (25)	Liver resection	10I + 10R	IPC (TI: 35.5 ± 2.7, LR: 32.2 ± 2.0), Control without PR (TI: NA, LR: 39 ± 4.5), Control with PR (TI: 35.6 ± 2.6, LR: 33.2 ± 2.3)	IL-6, IL-8, Cytochrome c, Adhesion molecules [B_2-integrins (CD18)], Histology (neutrophil infiltration)	Protective against IRI by attenuating neutrophil activation and IL-8 release
Chouker *et al*[208] (2005)	73 (25)	Liver resection	10I + 10R	IPC (TI: 35.12 ± 13.6, LR: 31.50 ± 9.1), Control without PR (TI: NA, LR: 34.77 ± 16.5), Control with PR (TI: 34.2 ± 10.9, LR: 32.13 ± 10)	PT, ALT, AST, α-GST	Protective against IRI Prevented early rise of α-GST
Koneru *et al*[210] (2005)	62 (34)	Transplant	5I + 5R	IPC (CI: 384 ± 92, WI: 41 ± 5.8), Control (CI: 415 ± 87, WI: 37 ± 5.6)	INR, Bilirubin, ALT, AST, Histology (apoptosis, hepatocyte swelling), LOS, Survival (6 mo)	No beneficial effect
Azoulay *et al*[212] (2005)	91 (46)	Transplant	10I + 10R	IPC (CI: 436 ± 116, Op: 441 ± 119), Control (CI: 461 ± 96, Op: 462 ± 98)	PT, Bilirubin, ALT, AST, Histology, Graft function, Morbidity, Mortality	Better ischaemic tolerance Decreased early graft function
Jassem *et al*[211] (2005)	23 (9)	Transplant	10I + 10R	IPC (CI: 620 ± 190, WI: 43.9 ± 13), Control (CI: 665 ± 280, WI: 40.4 ± 9)	AST, INR, Lactate, ITU stay, Histology (neutrophil infiltration, platelet deposition), Graft function	Protective against IRI Reduces inflammatory response Shorter ITU stay
Cescon *et al*[213] (2006)	47 (23)	Transplant	10I + 15R	[5]IPC [TI: 388 (259-830), Op: 440 (225-725)], Control [TI: 383 (279-695), Op: 465 (280-1015)]	PT, Bilirubin, ALT, AST, Histology (neutrophil, lymphocyte infiltration, iNOS, apoptosis), Graft function, Survival (1 yr)	Protective against IRI No clinical benefit

[1]Patients stated in brackets are the number of patients who had ischaemic preconditioning treatment; [2]IPC was performed by portal triad clamping in all these studies; [3]Ischaemia and operative times are presented as mean ± SD unless otherwise stated; [4]Cardiovascular status refers to mean arterial pressure, central venous pressure, heart rate, stroke volume index, systemic vascular resistance index, fluid infusion and catecholamines requirements; [5]Ischaemia and reperfusion times in this study were presented as median (range). I: Ischaemia; R: Reperfusion; IPC: Ischaemic preconditioning group; PR: Pringle maneuver; TI: Total ischaemia time; CI: Cold ischaemia time; WI: Warm ischaemia time; Op: Total operative time; LR: Liver resection time; ITU: Intensive therapy unit; LOS: Length of hospital stay; PT: Pro-thrombin time; INR: International normalized ratio of pro-thrombin time; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; α-GST: Alpha-Gluthathione S-Transferase; iNOS: Inducible form of nitric oxide synthase; ATP: Adenosine triphosphate; HCC: Hepatocellular carcinoma; IL: Interleukin; SEC: Sinusoidal endothelial cell; IRI: Ischaemia-reperfusion injury.

With the increasing laboratory evidence of protection against IRI and improved liver regeneration by IPC, several aspects of this strategy could be developed pharmacologically that may be more clinical applicable than IPC itself. This is especially in cases with a background of chemotherapy-induced steatohepatitis[216] and cirrhosis[217,218]. Pharmacologic agents targeting mediators of IPC that can be potentially developed include HGF, IL-6 and IL-1-RA. However, several issues such as the timing of administration of these agents, therapeutic doses and immunological response of the recipient need to be determined. Further understanding of the mechanistic pathways of IPC may pave the way for the development of these agents that are capable of conferring protection against IRI and promote liver regeneration.

CONCLUSION

Liver regeneration is of clinical significance in view of the increasing number of major liver resections and the increasing use of marginal donor liver and split-liver allografts for transplantation. Successful patient outcome often depends on liver regeneration, particularly in patients with cirrhotic and steatotic livers. Regeneration of the liver following IRI and major liver surgery is a complex process that involves the integration of a network of cytokines, growth factors, kinases, transcription factors and metabolic demands of the liver.

In comparison with the evidence available on the effect of IPC on IRI, its role in liver regeneration is still undetermined. However, current research has demonstrated that the beneficial effects of IPC on liver regeneration is mediated by up regulating growth-

promoting factors, suppressing growth-inhibitory factors and preserving energy levels for regeneration. Nevertheless, more studies are still required to further delineate the underlying pathophysiology of IPC and impact on mediators of liver regeneration. It is also important to determine whether the beneficial effect of IPC in the laboratory setting is reproducible in clinical practice. By understanding the underlying mechanisms by which IPC influences liver regeneration, other strategies as alternatives to IPC, could be developed to modulate the regenerative pathways in the clinical setting and improve outcomes of patients following major liver resection and transplantation. The assessment of IPC on liver regeneration in human studies is clearly the next step.

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REVIEW

Clinical role and importance of fluorescence *in situ* hybridization method in diagnosis of *H pylori* infection and determination of clarithromycin resistance in *H pylori* eradication therapy

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Abstract

H pylori is etiologically associated with gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Eradicating *H pylori* may convert rapidly the outcome of related diseases with the use of more accurate diagnostic molecular tests. Indeed some of the tests cannot give the evidence of current infection; *H pylori* can be detected by noninvasive and invasive methods, the latter requiring an endoscopy. Eradication failure is a big problem in *H pylori* infection. Recently, clarithromycin resistance in *H pylori* strains is increasing and eradication therapy of this bacterium is becoming more difficult. Molecular methods have frequently been applied besides phenotypic methods for susceptibility testing to detect clarithromycin resistance due to mutations in the 2143 and 2144 positions of 23S rRNA gene. Fluorescence *in situ* hybridization (FISH) method on paraffin embedded tissue is a rapid, accurate and cost-effective method for the detection of *H pylori* infection and to determine clarithromycin resistance within three hours according to the gold standards as a non-culture method. This method can also be applied to fresh biopsy samples and the isolated colonies from a culture of *H pylori*, detecting both the culturable bacillary forms and the coccoid forms of *H pylori*, besides the paraffin embedded tissue sections. This technique is helpful for determining the bacterial density and the results of treatment where clarithromycin has been widely used in populations to increase the efficacy of the treatment and to clarify the treatment failure *in vitro*.

Key words: *H pylori*; Fluorescence *in situ* hybridization method; Clarithromycin resistance

INTRODUCTION

H pylori is etiologically associated with gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma[1-3]. The prevalence of *H pylori* infection is 70%-90% in developing countries and 25%-50% in developed countries[2,4]. Person-to-person spread is the most probable mode of transmission. Fecal-oral and oral-oral transmission are also reported[5]. *H pylori* has been classified as a class I carcinogen by International Agency for Cancer Research (IACR)[6]. *H pylori* can be detected by noninvasive and invasive methods, the latter requiring an endoscopy. Noninvasive testing for *H pylori* can be done by measuring exhaled ^{13}C urea breath test (UBT), by serology, by stool antigen tests, by stool PCR and by analyzing body materials such as saliva and urine[2,7]. Invasive tests include endoscopy, with biopsy of the affected region followed by histopathologic examination of stained specimens to demonstrate the presence of the bacterium, rapid urease test, biopsy PCR and culture of the bacterium[1]. UBT, histopathology and culture of the organism, although not easily and routinely performed, are considered the gold standard for the diagnosis of *H pylori* infection[1,8].

Seven and fourteen days of triple therapy are recommended to eradicate *H pylori* according to Maastricht 2-2002 Consensus report. The triple therapy comprises a proton pump inhibitor in combination with two antibiotics, including amoxicillin, clarithromycin, or metronidazole[9-11]. Clarithromycin is a key component of most treatment recommendations to eradicate *H pylori*[12]. Resistance of *H pylori* to clarithromycin is regarded as a particular dilemma, since this drug is a part of both established therapy regimens. Thus, macrolide resistance is a frequent cause for failure of *H pylori* eradication therapy[10]. In industrialized countries approximately 10% of the

H pylori strains are clarithromycin-resistant. In developing countries, resistance rates to clarithromycin are higher, varying between 25% to 50%, while they are 5%-10% in the USA and as high as 10% in Europe[10,13,14]. Recently, the clarithromycin resistance rates are reported 16.8% and 52%-56% in Turkey[15-17]. In routine clinical laboratories the detection of clarithromycin resistance for *H pylori* is mainly based on phenotypic methods performed after culture: agar diffusion for the E-test or the agar dilution method, which is preferred as a reference method. However, there are some disadvantages of these methods such as results are not available until 48-96 h after inoculation of the agar plates[10,18]. Because *H pylori* is a fastidious organism, there are also some problems in application of culture and antibiotic susceptibility testing. Therefore easy, cheap and practicable methods are required for the detection of *H pylori* and determination of resistance, which is very important before the treatment if the resistance reaches 15%-20% in the area[7,19]. When the bacterial culture cannot be used routinely, the patient should benefit from the determination of macrolide resistance using non-invasive genotype-based methods[18].

Resistance of *H pylori* to clarithromycin is mainly due to major point mutations within an adenine-to-guanine transition at positions A2142G, A2143G and A2144G and to an adenine-to-cytosine transversion at positions A2142C and A2143C, which are included in the peptidyltransferase-encoding region of the 23S rRNA[9,11,20-23]. Mutations A2142G and A2143G are the most often observed, with the A2142C mutation being less common[24]. Other point mutations A2115G, G2141A, and T2717C have also been reported, though they appear to be very rare[18,25-27].

Detection of point mutations conferring resistance to clarithromycin for *H pylori* by molecular methods may constitute a more reliable approach and is attracting more attention[18]. Some molecular-based methods have been developed. PCR-based methods have been used to determine susceptibility to clarithromycin from biopsy specimens or cultured *H pylori* strains and stool specimens[7,28-30]. Fluorescence *in situ* hybridization (FISH) method for detection of *H pylori* and determination of its genotypic macrolide susceptibility in gastric biopsy specimens is a cultivation independent, reliable, sensitive and specific method[9,10,24]. Detection of *H pylori* and clarithromycin resistance genotype simultaneously in gastric biopsy specimens by FISH method may be a good tool for research in the future for the drug resistance mechanisms and to search for the eradication failure in developing countries, such as clarithromycin and similar antibiotics[23,31].

CLARITHROMYCIN ACTIVITY AND MECHANISM OF RESISTANCE

Clarithromycin activity

Clarithromycin is a bacteriostatic antibiotic, which belongs to a group of macrolides binding to peptidyl transferase loop of domain V of the 23S rRNA molecule. This binding interferes with protein elongation, and thus effectively blocks bacterial protein synthesis. The antibacterial activity of clarithromycin is similar to that of other macrolides, but clarithromycin is better absorbed in the gastric mucus layer, more acid-stable, and therefore more effective against *H pylori*[13,26,32]. Resistance to clarithromycin is thought to develop when substitutions in one amino acid at or near this binding site on the ribosome prevent the drug from binding, thereby making it ineffective[13].

Clarithromycin resistance

Resistance to clarithromycin in *H pylori* is caused by point mutations in three adjacent 23S rRNA nucleotides, namely 2142, 2143 and 2144. In *H pylori* these substitutions cause decreased affinity of the ribosomes for several macrolides, resulting in increased resistance. It can be induced by an adenine (A) to guanine (G) substitution at one of these positions or an adenine (A) to cytosine (C) substitution solely at position 2142. The A2142G and A2142C were significantly more frequently present in isolates with a higher minimal inhibitory concentration (MIC) for clarithromycin (> 64 mg/L), whereas the A2143G substitution was often found in isolates with a lower MIC (< 64 mg/L). Occasionally, other 23S rRNA mutations have also been reported for *H pylori*; some of them are associated with high-level resistance, while others are associated with low-level resistance[26,32]. Occasionally three described mutations, in which the adenine residues at positions 2143 and 2144 are replaced by guanine (A2143G and A2144G) or cytosine (A2143C), are localized within the peptidyl transferase region of the 23S rRNA gene[9,11,22,31].

H pylori contains two 23S rRNA genes and mutations are generally found in both copies, however, heterogeneity has been described. Heterogeneity still results in clarithromycin resistance, but it generally appears to be associated with lower resistance levels than in homogenic isolates. The higher prevalence of homogeneity over heterogeneity in *H pylori* may reflect a high efficiency of DNA recombination. The mutation in one copy of the 23S rRNA may be easily transferred to the other 23S rRNA gene by efficient homologous DNA recombination under selective pressure, conferring higher levels of clarithromycin resistance[11,22,24,26,31,32]. As expected, clarithromycin resistance coincides with resistance to other macrolides. The A2142G and A2142C mutations are linked to high-level cross-resistance to all macrolides, whereas the A2143G mutation gives rise to high-level resistance to erythromycin and intermediate-level resistance to clindamycin and streptogramin[26,32].

DETECTION OF CLARITHROMYCIN RESISTANCE

Molecular tests

The association between point mutations in the 23S rRNA gene and macrolide resistance in *H pylori* potentially provides a new approach for diagnosing macrolide resistant *H pylori* strains[33]. Numerous molecular-based methods are now available to assess clarithromycin in *H pylori*, such as PCR-RFLP, PCR-OLA, PCR-DEIA, PCR-

LipA, PCR-PHFA, 3M-PCR, real-time PCR hybridization assay, FISH, FRET, DNA sequencing by conventional and real-time (pyrosequencing) techniques. Most assays are polymerase chain reaction (PCR)-based using different methods to study the amplicons. The PCR-based molecular techniques are quicker than microbiological susceptibility testing, and more importantly, they can be performed directly on gastric biopsies and gastric juice[33].

Restriction fragment length polymorphism (RFLP) is a simple method based on the occurrence of restriction site within the amplicon. This assay allows for the detection of the previously mentioned 23S rRNA mutations using the restriction endonucleases, *Mbo*II (A2142G) *Bbs*I (A2142G), *Bsa*I (A2143G) and *Bce*AI (A2142C). As the PCR-RFLP was initially not able to detect the A2142C mutation, a 3'-mismatch reverse primer PCR method (3M-PCR) was developed[23,25,27]. However, the mutations are identified by the absence of a band, which is a less preferable endpoint than a positive endpoint[27].

Other methods, such as PCR-DNA enzyme immunoassay (DEIA), PCR oligonucleotide ligation assay (OLA), preferential homoduplex formation (PHFA) and PCR-line probe assay (LipA), include an additional hybridization step after the PCR. The PCR products were hybridized with labeled oligonucleotide probes under highly stringent conditions and hybrids were subsequently detected with specific antibodies or streptavidin-alkaline phosphatase[13,18,22,27,34,35]. The PHFA has been applied to direct detection of *H pylori* and clarithromycin resistant mutants in gastric juice samples[27]. The PHFA uses double labeled amplicons. Many of the assays are based on the principle of reverse hybridization with labeled probes for up to seven mutations and the wild type, immobilized either in microtitre wells (DEIA) or on nitrocellulose (LİPA). In these assays, PCR products are hybridized to the probes under highly stringent conditions and the resultant hybrids are detected colorimetrically. In the DEIA, the detection system is an enzyme linked immunoabsorbent assay with a labeled anti-double stranded DNA monoclonal antibody. Other more complex microtitre plate based systems such as the OLA use labeled capture and reporter probes[35].

Recently, several real-time PCR hybridization assays have been developed. The real-time PCR technique, which is powerful advancement of the basic PCR method, is developed based on amplification of a fragment of the 23S rRNA gene of *H pylori* followed by the melting curve analysis by biprobes and hyprobes[18,36]. In these assays a 23S rDNA fragment is amplified in the presence of a fluorescent-labeled mutation and anchor probe. Biprobes are sequence-specific probes labeled with the fluorophore Cy5. When the probe hybridizes to the target sequence, Cy5 is excited by the energy transfer from SybrGreen I, resulting in an increase of emitted light[29,36-38]. After completion of the PCR, the temperature is increased to determine the melting point of the mutation probe. The temperature at which the fluorescent signal drops indicates the point at which the mutation probe dissociates (melting point). When there are mismatches present in the target sequence, lower melting temperatures are obtained compared to the matched hybrid. This technique is simple and quick, and if applied directly to gastric tissue, results can be obtained within 3 h[13,27,29,34-38].

A technique named fluorescence resonance energy transfer (FRET) can be applied. In the first article in 1999, a DNA double strand specific fluorophor SYBR Green I and a second fluor dye Cy5 on a probe were used to test *H pylori* strains. This method was then applied to gastric biopsies[26].

In contrast with all the above approaches, DNA sequencing provides the gold standard reference method for mutation detection although it is not technically feasible or cost effective for routine laboratory determination of *H pylori* resistance markers. Nevertheless, knowledge of nucleotide sequences has proved invaluable for validation of the various assays mentioned above, particularly where a resistant phenotype is not associated with any of the more common mutations[35].

A recent development in rapid sequencing based on the principle of pyrosequencing, a real time DNA sequence analysis of short (25-30 bp) DNA stretches, has been applied to rapid identification of *H pylori*. Available data suggest this new technique can offer an accurate and rapid method for sequence analysis of PCR amplicons providing easily interpreted results within hours[35].

Fluorescence in situ hybridization

In situ hybridization (ISH) uses a labeled probe to detect and localize specific RNA or DNA sequences in a tissue or on a chromosome. ISH relies on DNA's ability to re-anneal, or hybridize, with a complimentary strand when at the correct temperature. "*In situ*" means "in the original place" in Latin, so ISH involves a labeled nucleic acid probe hybridizing with a DNA or RNA sequence *in situ* (in the cells) so that the location of the sequence of interest can be detected in the cells, tissue, or chromosome. Like Northern and Southern blots, ISH indicates the presence of a particular RNA or DNA sequence, but ISH differs from blots in that the labeled probe reveals the actual location of the sequence in the cells. The probe can be either radioactively labeled and detected by autoradiography or fluorescently labeled (abbreviated FISH) and detected by immunocytochemistry. The specificity of the probe depends on the permeability of the cells, the type of probes, the labeling technique, and the hybridization conditions, so specificity of ISH can be adjusted according to the desired results[39,40].

Fluorescence *in situ* hybridization (FISH) is to identify the presence of specific chromosomes or chromosomal regions through hybridization or fluorescence-labeled DNA probes to denatured chromosomal DNA[41]. FISH uses fluorescent molecules to vividly paint genes on chromosomes. This technique is particularly useful for gene mapping and for identifying chromosomal abnormalities. FISH involves the preparation of short sequences of single stranded DNA, called probes, which are complementary to the DNA sequences that researchers wish to paint and examine. These probes hybridize, or bind, to the complementary DNA and, because they are labeled with fluorescent tags, allow researchers to see the location of those sequences of DNA[41,42]. This technique allows the detection of whole bacteria in their natural habitat by fluorescence microscopy of prepared

specimens. Fluorescent signals indicate the presence of complementary chromosomal DNA; the absence of fluorescent signals indicates absence of complementary chromosomal DNA[43]. Unlike most other techniques used to study chromosomes, which require that the cells be actively dividing, FISH can also be performed on non-dividing cells, making it a highly versatile procedure[41,42,44].

Despite their small size, bacteria are accessible to the tools of cytology, such as immunofluorescence microscopy for localizing proteins in fixed cells with specific antibodies, fluorescence microscopy with the green fluorescent protein for localizing proteins in live cells, and FISH for localizing chromosomal regions and plasmids within cells[45].

The bacterial FISH technology is based on the specific DNA-DNA hybridization of defined oligonucleotides with the abundant copies of ribosomal RNA of a bacterial species (16S rRNA, 23S rRNA). The oligonucleotides, which are labeled with fluorescent dyes, penetrate the bacterial cells and bind to their target sequence. This technique allows the detection of whole bacteria in their natural habitat by fluorescence microscopy of prepared specimens, i.e. the gastric mucosa of infected humans or from animal models[43,46].

FISH is a rapid, accurate and also cost-effective method for the detection of *H pylori* and determination of macrolide resistance in cultured *H pylori* colonies. It can also be used directly on biopsy specimens for histopathological and microbiological examination[12,47]. In this assay intact *H pylori* are hybridized with fluorescent-labeled *H pylori*-specific 16S and 23S rRNA probes. The labeled bacteria were subsequently visualized by fluorescence microscopy. This assay allows detection of *H pylori* and clarithromycin resistance simultaneously. Moreover, this assay does not require DNA preparation and can directly be applied to gastric biopsy samples[9,23,26,27,32,35,48].

For *H pylori* a species-specific detection is performed by the 16S rRNA-specific oligonucleotide Hpy-1, labeled in green. Simultaneously to the species detection a genotypic antibiotic resistance determination is possible (labeled in red). The resistance against the macrolide clarithromycin, which is a major antibiotic used in the triple therapy against *H pylori* infections, is based on three defined point mutations in the 23S rRNA. These point mutations can be targeted specifically with the ClaWT, ClaR1, ClaR2, and ClaR3 probes. Different mutations correlate with different MICs of the antibiotics, ClaR1 > 64 mg/L, ClaR2, and ClaR3 between 8 and 64 mg/L[46,47].

CONCLUSION

The gold standard for accurate diagnosis of an *H pylori* infection is either culturing of the pathogen or concordant positive results obtained by histology and the rapid urease test or the ^{13}C-urea breath test (UBT)[24]. After culturing for the pathogen of gastric biopsies, probably most laboratories use disk diffusion or E-test for the determination of macrolide resistance. Both methods require further sub-culturing for several days and cannot identify the type of point mutations present in the strain[12].

The major advantage of FISH is the fact that the rRNA-targeted fluorescence-labeled oligonucleotide probes can be used for accurate determination of macrolide susceptibility, thus providing the clinician with important information with which to make a proper treatment recommendation[24,49]. Whilst the E-test is a phenotypic clarithromycin resistance measurement, FISH is an established genotypic technique for the detection of *H pylori* and discrimination between the clarithromycin-susceptible wild type and clarithromycin-resistant mutants[11,49]. FISH is a reliable fast method for the detection of clarithromycin-resistant *H pylori* mutants, and results are available within 3 h after an endoscopy. The probes are commercially available, and the method is cost-effective and can be applied in any laboratory without the need for special equipment or facilities, except for a fluorescence microscope[11,47,49].

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ESOPHAGEAL CANCER

Alterations of glutathione S-transferase and matrix metalloproteinase-9 expressions are early events in esophageal carcinogenesis

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Abstract

AIM: To investigate the role of glutathione S-transferase (GST) and matrix metalloproteinase-9 (MMP-9) expressions in the development and progression of reflux esophagitis-Barrett's metaplasia-dysplasia-adenocarcinoma sequence in the esophagus.

METHODS: GST and MMP-9 expressions were analyzed in 51 paraffin-embedded tissue samples by immunohistochemistry including patients with reflux esophagitis (n = 7), Barrett's metaplasia (n = 14), Barrett and esophagitis (n = 8), Barrett and dysplasia (n = 7), esophageal adenocarcinoma (n = 8) and a control group without any histological changes (n = 7). Immunostaining was determined semiquantitatively. Statistical analysis with one-way ANOVA, LSD test and correlation analysis were performed. P value of < 0.05 was considered significant.

RESULTS: GST expression was significantly higher while MMP-9 expression was significantly lower in control group compared to Barrett's metaplasia and the other groups. No major changes were observed between Barrett, esophagitis, and Barrett and concomitant esophagitis. Barrett and concomitant dysplasia, and adenocarcinoma revealed a significant lower expression of GST and higher levels of MMP-9 compared to all other groups. Adenocarcinoma showed almost no expression of GST and significantly higher levels of MMP-9 than Barrett and concomitant dysplasia. Alterations of GST and MMP-9 were inversely correlated (r = - 0.82).

CONCLUSION: Decreased GST and increased expression of MMP-9 in Barrett's metaplasia-dysplasia-adenocarcinoma sequence as compared to normal tissue suggest their association with esophageal tumorigenesis. Loss of GST and gain of MMP-9 in Barrett with dysplasia compared to non-dysplastic metaplasia indicate that these alterations may be early events in carcinogenesis. Quantification of these parameters in Barrett's esophagus might be useful to identify patients at higher risk for progression to cancer.

Key words: Glutathione S-transferase; Matrix metalloproteinase-9; Barrett's metaplasia; Esophagus; Adenocarcinoma; Dysplasia

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INTRODUCTION

Esophageal cancer is still one of the most widespread diseases, and the early diagnosis of esophageal carcinoma correlates closely with improvement in prognosis. Barrett's esophagus (BE) is a precancerous condition of the lower esophagus in which the normal stratified squamous epithelium is replaced with specialized metaplastic columnar epithelium. Barrett's mucosa represents a type of epithelium that is completely different from the normal esophageal mucosa. BE is the main precancerous condition in the development of esophageal adenocarcinoma[1,2].

BE is diagnosed in up to 20% of patients with documented chronic gastroesophageal reflux disease (GERD). Follow-up studies have shown that BE has a 30- to 125-fold increased risk of developing into an adenocarcinoma, which emerges at a rate of approximately one cancer per 100 patient-years[3]. Barrett's adenocarcinoma displays the most rapidly increasing incidence for gastrointestinal tract cancer in the Western world. Diagnosis of Barrett's adenocarcinoma is usually made late, and consequently, is associated with poor prognosis[4-6].

Carcinogens are one of the inducing etiological factors for esophageal adenocarcinoma. Glutathione S-transferase (GST), a family of detoxification enzymes, plays an important role in the prevention of cancer by detoxifying numerous potentially carcinogenic compounds,

which can cause oxidative damage to cells[7]. Therefore, a reduction in these anti-oxidant enzymes can increase the risk of carcinogenesis[8]. Decreased GST enzyme activity has been reported in BE, and an inverse correlation was demonstrated between GST enzyme activity and tumor incidence in the gastrointestinal tract[9,10]. It has been suggested that down-regulation of GST expression could be an early event in the development of BE[11].

The degradation of the extracellular matrix (ECM), including the basement membrane, which is a specialized matrix composed of type Ⅳ collagen, laminin, entactin, proteoglycans and glycosaminoglycans, is an important feature of cancer cell invasion, and proteolytic enzymes play an important role in this event[12].

Several human solid tumors have been reported to have increased levels of proteolytic enzymes in cancer tissue, strongly suggesting that proteases may be important in tumor invasion and metastasis. With respect to the gastrointestinal tract, we have previously demonstrated that proteolytic enzymes may have a role not only in the process of gastric[13] or colorectal cancer invasion[14], but also in the progression of gastrointestinal precancerous changes into cancer[15].

Matrix metalloproteinases (MMPs) degrade components of the ECM and connective tissue surrounding the tumor cells and the basement membrane. MMPs are classified as gelatinases, collagenases, stromelysins, membrane-type matrix metalloproteinases, based mainly on the *in vivo* substrate specificity of the individual MMP. It was initially believed that MMPs, *via* breakdown of the physical barrier, were primarily involved in tumor invasion[16]. There is growing evidence, however, that the MMPs have an expanded role, as they are important for the creation and maintenance of a microenvironment that facilitates growth and angiogenesis of tumors at primary and metastatic sites[17,18].

Type Ⅳ collagen is an important protein of the basement membrane. Type Ⅳ collagenase, matrix metalloproteinase-9 (MMP-9) (gelatinase B), has been reported to be especially important in the process of tumor invasion and metastasis[19,20]. Several MMPs (gelatinase A: MMP-2; stromelysin: MMP-3; matrilysin: MMP-7; metalloelastase: MMP-12; collagenase-3: MMP-13) are expressed by tumor cells in esophageal squamous cell and adenocarcinomas, suggesting that these MMPs are responsible for tumor aggressiveness and prognosis in human esophageal carcinomas[21-24].

In the specific case of MMP-9, increased expressions have been observed in gastric cancer[25-27] and esophageal squamous cell carcinoma[28-30], but its behaviour in esophageal adenocarcinoma and in preinvasive lesions of esophageal carcinogenesis is still uncertain.

On the other hand, GST and MMP-9 as actors either in cancer prevention or in carcinogenesis have not been evaluated in the same experimental setting. Therefore, the aim of the present study was to investigate the role of GST and MMP-9 expressions using immunohistochemical analysis in the development and progression of reflux esophagitis-BE-dysplasia-adenocarcinoma sequence in the esophagus.

MATERIALS AND METHODS

Tissue specimens were obtained endoscopically from in- and outpatients with upper abdominal complaints at the 2nd Department of Medicine, Semmelweis University, Budapest.

Informed consent was obtained from all patients involved in the study, and a local ethical permission has been obtained. The patients comprised of 33 males and 18 females. The median age was 64 years with a range from 22 to 83 years. The endoscopic specimens were fixed in formalin and embedded in paraffin wax, sliced serial step sections of 4 μm thickness. GST and MMP-9 immunohistochemical expressions were analyzed in a total of 51 paraffin-embedded tissue samples by immunohistochemistry including patients with reflux esophagitis (n = 7) (4 males, 3 females, mean age 61 years, range 36-68 years); BE (n = 14) (9 males, 5 females, mean age 66 years, range 48-69 years); BE and esophagitis (n = 8) (6 males, 2 females, mean age 67 years, range 55-71 years); BE and dysplasia (n = 7) (4 males, 3 females, mean age 68 years, range 52-72 years); and esophageal adenocarcinoma (n = 8) (6 males, 2 females, mean age 71 years, range 64-83 years). Esophageal biopsies from patients with functional dyspepsia without any histological changes were used as controls (n = 7) (4 males, 3 females, mean age 49 years, range 22-56 years).

GST immunohistochemistry

The 4 micron thick tissue sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubation for 30 min at room temperature in 3% hydrogen peroxide. After washing the sections 3 times in PBS for 5 min, non-specific blocking was done with 1% BSA-PBS solution for 10 min at room temperature. Next, the slides were incubated with diluted polyclonal rabbit anti-human GSTP1 antibody (1 μL GSTP1 antibody and 150 μL PBS) (Clone: A3600, DAKO) at 37℃ for 60 min in a humidified chamber. After washing the specimens 3 times in PBS, signal conversion was carried out with the LSAB2 system (DAKO) as described in the manual. Finally, haematoxylin co-staining was performed.

MMP-9 immunohistochemistry

After deparaffinization in xylene and rehydration through graded ethanol, endogenous peroxidase activity was blocked by incubation for 30 min at room temperature in 3% hydrogen peroxide. After washing the sections 3 times in PBS for 5 min, non-specific blocking was carried out with 1% BSA-PBS solution for 10 min at room temperature. Next, the slides were incubated with optimally diluted monoclonal anti-human MMP-9 antibody (Clone: 36020.111, R&D Systems) at 37℃ for 60 min in a humidified chamber. After washing the samples 3 times in PBS, signal conversion was carried out with the LSAB2 system (DAKO) as described in the manual. Finally, haematoxylin co-staining was performed.

Immunohistochemical analysis of GST and MMP-9

Known immunohistochemically-positive tissue sections

Table 1 GST immunohistochemical expression according to a semiquantitative score in various types of mucosal lesions of the esophagus

Histology	Score (mean ± SD)
Normal epithelium (Control group)[a] (n = 7)	2.85 ± 0.24
Reflux esophagitis (n = 7)	1.14 ± 0.24
Barrett's metaplasia (n = 14)	1.60 ± 0.34
Barrett's metaplasia and reflux esophagitis (n = 8)	1.12 ± 0.35
Barrett's metaplasia and dysplasia[b] (n = 7)	0.58 ± 0.37
Adenocarcinoma[b,c] (n = 8)	0.18 ± 0.25

[a]$P < 0.00001$ *vs* the other groups; [b]$P < 0.005$ *vs* the other groups (normal epithelium, reflux esophagitis, barrett's metaplasia, barrett's metaplasia and Reflux esophagitis); [c]$P < 0.05$ *vs* barrett's metaplasia and dysplasia.

Table 2 MMP-9 immunohistochemical expression according to a semiquantitative score in various types of mucosal lesions of the esophagus

Histology	Score (mean ± SD)
Normal epithelium (Control goup)[b] (n = 7)	0.28 ± 0.39
Reflux esophagitis (n = 7)	1.71 ± 0.39
Barrett's metaplasia (n = 14)	1.46 ± 0.41
Barrett's metaplasia and reflux esophagitis (n = 8)	1.75 ± 0.26
Barrett's metaplasia and dysplasia[a] (n = 7)	2.16 ± 0.25
Adenocarcinoma[a,c] (n = 8)	2.62 ± 0.35

[b]$P < 0.00001$ *vs* the other groups; [a]$P < 0.05$ *vs* the other groups (normal epithelium, reflux esophagitis, barrett's metaplasia, barrett's metaplasia and Reflux esophagitis); [c]$P < 0.05$ *vs* barrett's metaplasia and dysplasia.

were used as positive controls, and negative control sections were processed immunohistochemically after having replaced the primary antibody by PBS. None of the control sections exhibited immunoreactivity. Immunostaining was determined semiquantitatively, as previously described[31]. Essentially, the intensity of staining for GST and MMP-9 under a light microscope was graded from 0 to 3, denoting no staining or light, moderate, or intense staining. An immunohistochemical staining score was calculated for each histologic area by multiplying the staining intensity level (0 to 3) by the proportion of cells in each area staining with the given intensity. The immunohistochemical staining score for an area with 100% of cells with intense staining, for example, would be 1 × 3, equalling 3, whereas an area with 50% cells with moderate staining and 40% without any staining would have a score of 0.5 × 2 plus 0.4 × 1, equalling 1.4. Two independent investigators without knowledge of the clinical outcomes evaluated the degree of immunohistochemical staining intensity. There was less than 5% variance between the results of the two counts.

Statistical analysis

Statistical analysis with one-way ANOVA, LSD test and correlation analysis were performed by the Statistica for Windows 4.3 program package. P value of < 0.05 was considered significant.

RESULTS

The immunohistochemical expression scores of GST and MMP-9 in various types of mucosal lesions of the esophagus (n = 51) are shown in Tables 1 and 2.

Expression of GST (Table 1) in normal esophageal epithelium (control group) was significantly higher compared to BE and the other groups ($P < 0.00001$), while no major changes were observed between BE, esophagitis, and BE with concomitant esophagitis.

BE with concomitant dysplasia, and adenocarcinoma revealed a significantly lower expression of GST compared to all other groups ($P < 0.005$). Adenocarcinoma showed almost no expression of GST and a significantly lower expression than BE and concomitant dysplasia ($P < 0.05$).

The semiquantitative score of MMP-9 (Table 2) in the normal esophageal epithelium (control group) was significantly lower compared to BE and the other groups ($P < 0.00001$); while no major changes were observed between BE, esophagitis, and BE with concomitant esophagitis.

Significantly higher expression levels of MMP-9 have been observed in BE with concomitant dysplasia and adenocarcinoma compared to all other groups ($P < 0.05$). Finally, MMP-9 expression was significantly higher in adenocarcinoma compared to BE and concomitant dysplasia ($P < 0.05$).

GST and MMP-9 were expressed mainly within the cytoplasm and cytoplasmic membranes of the esophageal epithelium in dysplastic or adenocarcinoma cells (Figures 1 and 2). Immunoexpressions of GST and MMP-9 in the esophageal tissues were inversely correlated (r = - 0.82; P = 0.001) (Figure 3).

DISCUSSION

Despite advances in diagnosis and therapy, esophageal adenocarcinoma remains an aggressive and usually lethal tumor. BE is the main precancerous condition in the development of esophageal adenocarcinoma; however, its pathogenesis is poorly understood. BE typically progresses from metaplasia with atypia to dysplasia and adenocarcinoma. It is of great clinical importance to correctly identify changes with a high risk for malignant transformation, as high-grade dysplasias and early adenocarcinomas in patients with BE have a high chance for cure[32]. The identification of high-risk lesions in BE by histologic evaluation has drawbacks, especially regarding sampling errors and frequent intra- and inter-observer discrepancies in the histopathologic grading/staging of these lesions. Several new biomarkers are being tested to help in better determining the risk of cancer development.

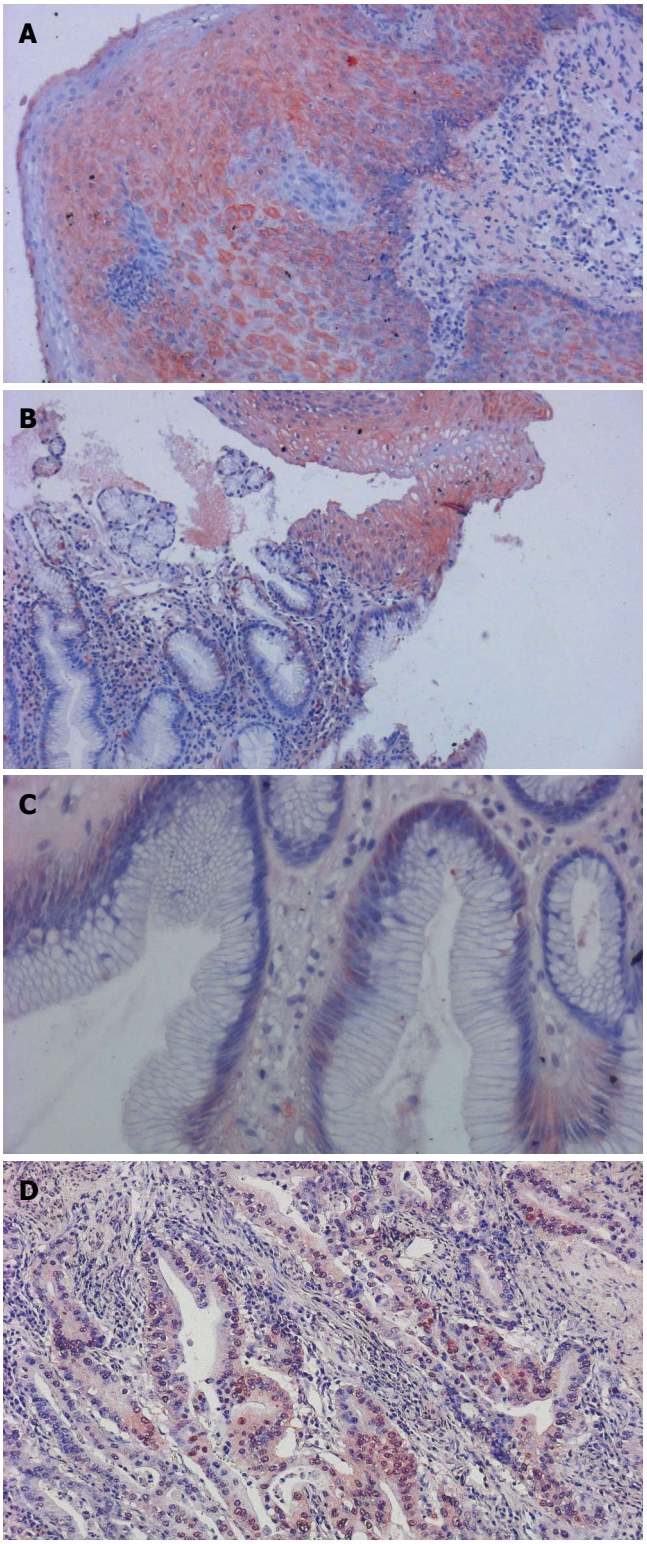

Figure 1 Expression of GST in different esophageal tissues. **A**: GST strong positive staining was observed in the normal esophagus (200 ×); **B**: Normal esophageal epithelium (top) with Barrett's metaplasia (bottom) (200 ×); **C**: Normal esophageal epithelium shows strong positive immunostaining compared to the weaker GST expression in Barrett's metaplasia (400 ×); **D**: Adenocarcinoma showing almost no expression of GST (200 ×); GST was mainly expressed within the cytoplasm.

Although most of the biological markers need to be evaluated further, at present, aneuploidy status, p16 and p53 gene abnormalities, or allelic losses are the most extensively documented alterations[33].

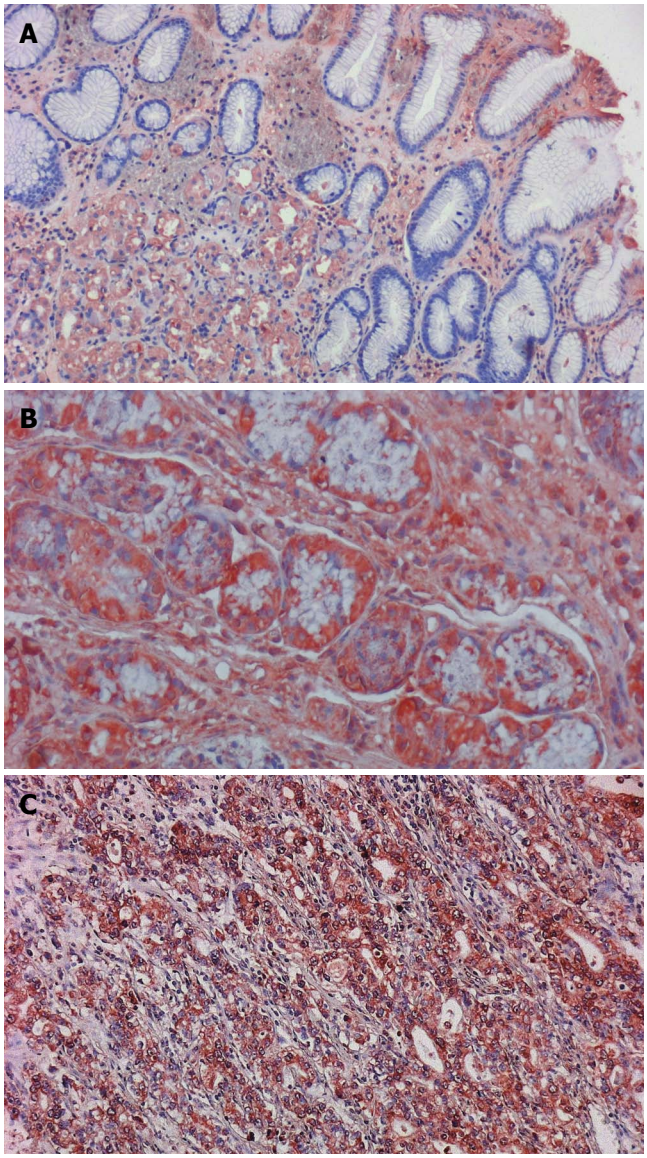

Figure 2 Expression of MMP-9 in different esophageal tissues. Strong positive immunostaining of MMP-9 in (**A**) Barrett's metaplasia (400 ×), (**B**) dysplasia (400 ×) and (**C**) adenocarcinoma (200 ×) of the esophagus. Cytoplasm of the metaplastic and dysplastic cells and cytoplasmic membranes of the esophageal adenocarcinoma cells were stained brown. Barrett's metaplasia with concomitant dysplasia and adenocarcinoma show the most intensive expression of MMP-9.

Immunostaining with a variety of antibodies provides a better understanding of the process of malignant transformation and helps to identify early markers of malignant transformation in BE[34].

Given the lack in the literature of the evaluation of GST and MMP-9 expressions in the same experimental setting, we evaluated the behaviour of detoxification enzyme GST, and one member of the matrix metalloproteinases family, MMP-9, in the development and progression of normal epithelium, reflux esophagitis, BE, dysplasia and adenocarcinoma sequence in the esophagus.

A number of findings in our study confirmed that GST is involved in esophageal carcinogenesis and progression. We have demonstrated that GST expression was significantly higher in normal esophageal epithelium compared to the other groups. On the other hand, BE with dysplasia, and adenocarcinoma revealed a significantly

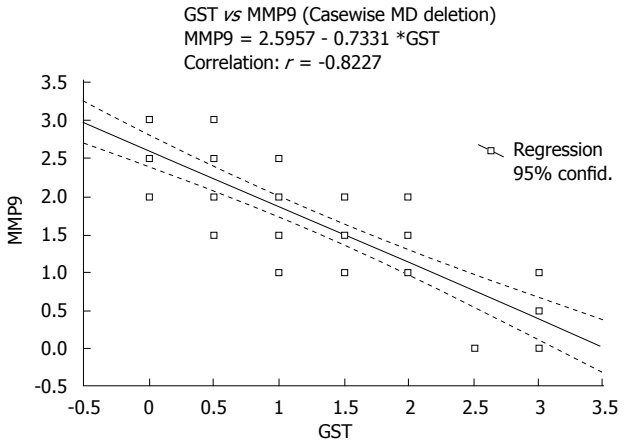

Figure 3 The correlation between immunohistochemical expressions of GST and MMP9 in different esophageal tissues. Immunohistochemical expressions of GST and MMP9 were inversely correlated (r = - 0.82; P = 0.001).

lower expression of GST, while adenocarcinoma expressed almost no GST.

Our findings are similar to the results reported by van Lieshout *et al*[9] and Cobbe *et al*[11]. They reported that the expression of GST appeared to be reduced in BE compared to normal esophageal squamous epithelium. In contrast to the van Lieshout *et al*[9] and Cobbe *et al*[11] studies, we also demonstrated that BE with concomitant dysplasia, and adenocarcinoma revealed a significantly lower expression of GST. Brabander *et al*[10] also found that GST expression was highest in the basal layer of normal esophageal squamous epithelium and lowest in adenocarcinoma cells, with BE cells showing intermediate staining intensity.

These results suggest that decreased GST expression could be an early event in the development of BE and may contribute to the risk of development and progression of adenocarcinoma in BE. The observed reduction in GST expression in BE may, therefore, contribute to the increased risk in this tissue.

Degradation of the ECM and basement membrane by tumor cells is a critical step in the process of tumor invasion and metastasis. MMP-9 is one member of the matrix metalloproteinases family, which is capable of degrading several components of the ECM. Increased expression of MMP-9 has been found in various carcinomas. With respect to the gastrointestinal tract, increased MMP-9 expressions have been observed in gastric[25-27] and colorectal cancer[35-38]. In the specific case of the esophagus, increased expression of MMP-9 has been demonstrated in esophageal squamous cell carcinoma[28-30], but its role and behaviour in esophageal adenocarcinoma and BE is not well established.

The relatively small number of patients in our study can be explained by the known data about the epidemiology of BE and esophageal adenocarcinoma in Hungary; since only 4% of patients with esophageal cancers were diagnosed to have adenocarcinoma and its proportion remained stable over the observed last decade, it seems that contrary to North American and Western European countries, the prevalence of adenocarcinoma has been, until now, very low in Hungary[39].

In the present study, immunohistochemical analysis revealed a progressive increase in the expression of MMP-9 with increasing severity of esophageal lesions. MMP-9 expression was significantly lower in normal esophageal epithelium compared to other groups. BE with concomitant dysplasia revealed a significantly higher expression of MMP-9 compared to BE, reflux esophagitis or BE with concomitant esophagitis. We observed that MMP-9 expression was significantly higher in adenocarcinoma compared to BE or BE with concomitant dysplasia. These results suggest that over-expression of MMP-9 plays an important role in the progression to esophageal adenocarcinoma, and MMP-9 protein may serve as a marker for invasiveness. Our results indicate that the activation of MMP-9 may be an early event in esophageal carcinogenesis.

Our findings are relevant from both, biological and clinical points of view. Despite the advance in preoperative and postoperative medical care of esophageal carcinoma patients, their prognosis has improved only marginally. Therefore, it would be useful to have additional biomarkers to help clinicians better determine the risk of esophageal cancer development. In esophageal cancer, novel targeted treatments are still in an early phase of development. It can be speculated that the relevance of MMP-9 in esophageal carcinogenesis may also support a possible therapeutic approach[40]. Indeed, this can be obtained directly by inhibition of MMP-9. Phase II-trials with the matrix metalloproteinase inhibitor prinomastat in patients with esophageal adenocarcinoma are under evaluation[41].

The present study showed that expressions of GST and MMP-9 were reversely or negatively correlated, thus suggesting a concomitant down-regulation and up-regulation, respectively, of these systems. GST plays an important protective role in the prevention of cancer by detoxifying potentially carcinogenic compounds, while MMP-9 should be considered an aggressive factor, playing a crucial role in the progression of esophageal carcinogenesis.

In conclusion, our results demonstrate a significantly lower expression of GST and a significantly higher expression of MMP-9, respectively, in the BE-dysplasia-adenocarcinoma sequence as compared to normal esophageal tissue. The simultaneous down-regulation of GST and up-regulation of MMP-9 strongly suggest their association with esophageal tumorigenesis and particularly, their specific role in the biology of esophageal adenocarcinoma. Loss of GST and gain of MMP-9 in BE with concomitant dysplasia compared to non-dysplastic BE indicate that these alterations may be early events in esophageal carcinogenesis. Together with other biological markers, quantification of these parameters in BE might be useful to identify patients at higher risk for progression to adenocarcinoma, to prevent tumor development and to improve prognosis.

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p53-expressing conditionally replicative adenovirus CNHK500-p53 against hepatocellular carcinoma *in vitro*

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Abstract

AIM: To develop a conditionally replicative gene-viral vector system called CNHK500-p53, which contains dual promoters within the E1 region, and combines the advantages of oncolytic virus and gene therapies for hepatocellular carcinoma (HCC).

METHODS: CNHK500-p53 was constructed by using human telomerase reverse transcriptase (hTERT) promoter to drive adenovirus *E1*a gene and hypoxia response element (HRE) promoter to drive adenovirus *E1*b gene. p53 gene expressing cassette was inserted into the genome of replicative virus. Viral replication experiments, cytopathic effect (CPE) and methyl thiazolyl tetrazolium (MTT) assay were performed to test the selective replication and oncolytic efficacy of CNHK500-p53.

RESULTS: Immunohistochemistry verified that infection with CNHK500-p53 was associated with selective replication of adenovirus and production of p53 protein in telomerase-positive and hypoxia-inducible factor-dependent HCC cells. p53 protein secreted from HepG2, infected with CNHK500-p53 was significantly higher than that infected with nonreplicative adenovirus Ad-p53 *in vitro* (388 ± 34.6 μg/L *vs* 76.3 ± 13.17 μg/L). Viral replication experiments showed that replication of CNHK500-p53 and CNHK500 or WtAd5, was much stronger than that of Ad-p53 in tested HCC cell lines. CPE and MTT assay indicated that CNHK500-p53 selectively replicated in and killed HCC cells while leaving normal cells unaffected.

CONCLUSION: A more efficient gene-viral system is developed by combining selective oncolysis with exogenous expression of p53 against HCC cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor worldwide, accounting for 500 000 new cases annually. The majority of patients presenting with advanced disease are not candidates for liver transplantation, surgical resection, or regional therapy. In 60% to 80% of patients with HCC, underlying liver cirrhosis and hepatic dysfunction complicate its treatment. Systemic treatments have minimal effects with significant toxicity, and cannot improve patient survival[1]. The search for alternative treatment modalities has revived the concept of using oncolytic viruses to treat cancer[2,3]. In this respect, conditional replicative adenoviruses (CRAds) appear to be attractive anticancer agents that are currently evaluated in clinical trials[4,5]. CRAds exert intrinsic anticancer activity through selective replication and lysis in cancer cells. In addition, release of CRAd progeny by infected tumor cells provides a potential to amplify the oncolytic effect by lateral spread through solid tumors.

Recent studies have shown that telomerase activity may serve as a general marker of cancer cells. Its activity in normal cells is restricted to fetal tissue, whereas it is elevated in tumors[6]. Although some tumors could activate a yet unknown alternative mechanism of telomere extension, the majority (> 85%) of human HCC cells acquire immortality by expressing telomerase reverse transcriptase (hTERT)[7]. It has been shown that hTERT expression is regulated at the transcriptional level, thereby providing a promising tool for tumor-specific gene expression.

Hypoxia occurs in virtually allsolid tumors as they outgrow their blood supply. Hypoxia augments cellular levels of hypoxia-inducible factor (HIF), a transcription

factor that regulates target genes through the binding of hypoxia response elements (HRE). Activation of the HIF pathway enables cancer cells to survive and proliferate in a hypoxic environment and contributes to a more aggressive phenotype[8,9]. Therefore, the HIF/HRE system of gene regulation, which is active under hypoxia or as a result of genetic alterations during cell transformation, is particularly attractive to specific target solid tumors.

Besides viral oncolysis, CRAds can be exploited as vectors of gene therapies by advanced virology and viral vector design to enhance their oncolysis. Many malignant neoplasms have lost the function of p53. Although many oncolytic adenoviruses use p53-dependent pathways to cause cell death, several studies have shown that replicating adenoviruses kill cells more rapidly when expressing p53[10,11].

Here we have constructed a novel gene-viral vector system called CNHK500-p53, which uses hTERT promoter to drive adenovirus *E1*a gene and HRE promoter to drive adenovirus *E1*b gene. In addition, human p53 gene was cloned into the downstream of E1A of adenovirus. E1A gene is essential for adenoviral replication, and adenovirus can hardly propagate without it. Telomerase and hypoxia are two important features of human solid tumors. Making use of these two promoters, CNHK500-p53 will replicate only in telomerase positive cancer cells undergoing hypoxia in theory. We tested the replication ability and oncolytic activity of CNHK500-p53 in HCC cell lines *in vitro*.

MATERIALS AND METHODS

Vectors, cell lines and cell culture

pXC1 (wild-type adenovirus plasmid) and pBGHE3 (a plasmid-containing right arm of adenovirus type 5 with deletion of 188-1339 bp sequence.) were purchased from Microbix Biosystems Ltd (Toronto, Canada). pGEM-3ZF and pGEM-3ZF-p53 were purchased from Promega Ltd, USA. Human HCC cell lines HepG2, Hep3B, normal human liver cell line L02, normal human fibroblast cell lines MRC-5 and BJ were purchased from the American Type Culture Collection (Manassas, VA). Human HCC cell lines SMMC-7721, Bel-7402 and wild-type adenovirus 5 (WtAd5) were obtained from Second Military Medical University (Shanghai, China). Human embryonic kidney 293 cell line was obtained from Microbix Biosystems (Toronto, Canada). Hep3B, HepG2, SMMC-7721, Bel-7402 and human embryonic kidney 293 cells were cultured in DMEM (Life Technologies, Rockville, MD). L02 was cultured in RPMI 1640 medium. BJ was cultured in modified Eagle's medium (MEM). All the media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 4mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured under a 5% CO_2 atmosphere at 37℃.

Construction of adenovirus vectors

Complete cDNA sequence of p53 gene was amplified by PCR from plasmid pGEM-3ZF-p53 by using the upstream primer VT182 (5'CCG GAA TTC (*EcoRI*) GCC ATG GAG GAG CCG CAG TCA GA3') and downstream primer VT183(5'CGC GGA TCC (*BamHI*) TTA TCA GTC TGA GTC AGG CCC TTC TG3'). Synthetic DNA sequence was released with endonucleases *EcoRI* and *BamHI* (New England Biolabs, Beverley, MA) and ligated into plasmid pClon15 (made by ourselves, which contains the sequence of mouse cytomegalovirus promoter + multiple clone site + SV40 poly A) to generate pClon15-p53. pClon15-p53 was digested with endonucleases *AgeI* and *NotI* (New England Biolabs), a 1917-bp fragment containing mouse cytomegalovirus promoter + p53 gene + SV40 polyA was excised and inserted into *AgeI* and *NotI* sites of pSG500, which was constructed in our previous study and contained hTERT promoter core sequence with three extra E-boxes downstream and HRE promoter[12]. The plasmid resulting from the insertion of p53 gene cassette into the pSG500 in orthograde orientation was designated as pSG500-p53. pSG500-p53 and pSG500 were transfected by Lipofectamine 2000 (Life Technologies) into 293 cells together with pBHGE3. Viral plaques appeared 9-14 d after cotransfection and were sublimated three times. Recombinant adenoviruses, extracted using QIAamp DNA blood mini kit (Qiagen, Valencia, CA), were verified by PCR and named CNHK500-p53 and CNHK500. Ad-p53 was used as a control, which is a nonreplicative adenovirus vector carrying a SV40 early promoter-driven human p53 expression cassette[13]. A similar procedure was used by replacing the p53 gene with a 1538-bp fragment of the green fluorescent protein (GFP) expression cassette, obtained from plasmid pCA13-GFP (Takara Ltd, Japan), to derive conditionally replicative adenovirus CNHK500-GFP and nonreplicative adenovirus Ad-GFP. The nonreplicative adenovirus Ad-blank was used as a control.

Production and purification of adenovirus

Viruses were purified by CsCl density purification and propagated in 293 cells. After 72 h, the detached cells were harvested by centrifugation at 1000 × *g* for 5 min at 4℃, resuspended in 10 mL cold PBS (free Ca^{2+} and Mg^{2+}), and then lysed with three cycles of freeze and thaw. Lysate was collected by centrifugation at 1500 × *g* for 10 min at 4℃, and the supernatant was placed on a gradient prepared with equal parts of CsCl in PBS and then centrifuged at 15000 × *g* for 2 h at 12℃. The virus band was removed and placed in a preformed CsCl gradient by ultracentrifugation for 18 h and dialyzed into 10 mmol/L Tris-HCl (pH 7.4) containing 10 mmol/L $MgCl_2$ and 10% glycerol. Titers of the purified adenovirus were determined by plaque assays of the tissue culture infectious dose 50 methods and shown as plaque forming unit per milliliter (pfu/mL). All viral preparations were free of endotoxin.

Viral replication assay

Monolayer cells, including logarithmically growing Hep3B, HepG2, SMMC-7721 (10^5 cells/well), and contact-inhibition BJ, L02 (10^6 cells/well) were cultured in six-well dishes overnight and infected with CNHK500-p53, WtAd5, Ad-p53 at a multiplicity of infection (MOI) of 5.0 pfu/cell. Virus inocula were removed after 2 h. The cells then were washed twice with PBS and incubated at 37℃ for 0, 12, 24, 48, or 96 h. Lysates of cells were prepared with three cycles of freeze and thaw. Serial dilutions of

the lysates were titered on human embryonic kidney 293 cells with the tissue culture infectious dose 50 methods, normalized with that at the beginning of infection, and reported as multiples.

Western blot analysis of E1A and E1B protein

HepG2, Hep3B and BJ were seeded in 6-well plates at a density of 5×10^5 cells/well and infected with CNHK500-p53 or wtAd5 at a MOI of 1 after 24 h of incubation. Two days after viral infection, cells were harvested and lysed with M-PER mammalian protein extraction reagent (PI-ERCE, Rockford, IC). Concentration of the extracted protein was measured with a biophotometer (Eppendorf AG, Hamburg, Germany). Total proteins (20 μg) were separated on 10% SDS-polyacrylamide gel, electroblotted onto PROTRAN nitrocellulose transfer membrane (Schleicher & Schuell Inc, Dassel. Germany) and blocked with 5% fat-free milk in Tris-buffered saline (TBS: 10 mMTris, pH 7.5, 0.9% NaCl) containing 0.1% Tween-20 (TBST) at room temperature for 1 h. The membrane was incubated with either rabbit polyclonal antibody against Ad-E1A protein (Santa Cruz Biotechnology) or rat anti-Ad5 E1B 55k monoclonal antibody overnight at 4℃ and repeatedly washed in TBST. After incubation for 1 h with appropriate secondary horseradish peroxidase-conjugated anti-bodies and extensive washing with TBST, immunocomplexes on the membrane were detected with LumiGLOTM reagent and visualized with Kodak BiomaxMR film. To detect the expression of E1A and E1B under hypoxic condition, CNHK500-p53 infected cells were exposed to 0.1% hypoxia for 16 h before harvest.

ELISA determination of p53 gene expression

HepG2 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured for 24 h, followed by infection with CNHK500-p53 and Ad-p53 at a MOI of 0.1. On days 3, 5, 7, and 10 post-infection, the supernatants of cell cultures were collected and assayed for p53 gene expression levels using the ELISA kit of p53 (Chemicon International, Temecula, CA) and the manipulation was done according to the manufacturer's instructions.

Evaluation of oncolytic activity of virus vector

Cytopathic effect (CPE): Hep3B, HepG2, Bel-7402 (2×10^4 Cells/well) and BJ (6×10^4 cells/well) were dispensed in 24-well plates. The culture solution was removed on the second day, and 1 mL serum free DMEM and virus were added to each well. The multiplicity of infection (MOI) of each well was 0.01, 0.1, 1, 10 and 100, respectively. The culture plate was then incubated for 90 min in a 37℃ incubator under the condition of 5% CO_2 in DMEM containing 5% serum.

Methyl thiazolyl tetrazolium (MTT) assay: MTT assay was performed to determine cell viability at various viral MOIs. HepG2, Hep3B and BJ cells were plated at a density of 1×10^4 cells/well in 96-well plates (Falcon) and 24 h later, the cells were infected with CNHK500-p53 at serial MOIs from 0.001 to 100. After 7 d of incubation, cell viability was measured by MTT assay using a non-radioactive cell proliferation kit (Roche Molecular Biochemicals) according to its protocol, and the spectrophotometrical absorbance of samples was measured with a microplate reader model 550 (BIO-RAD Laborato-ries, Tokyo, Japan) at 570 nm with a reference of 655 nm. Percentage of cell survival was calculated using the formula: % cell survival = (OD value of infected cells/OD value of uninfected control cells) × 100%. Eight replicate samples were taken at each MOI and each experiment was repeated at least 3 times. IC_{50} of CNHK500-p53, CNHK500, and WtAd5 was calculated in HepG2 and BJ 7 d after infection. Statistical analysis was performed using Student's *t* test for differences among groups. $P < 0.05$ was considered statistically significant.

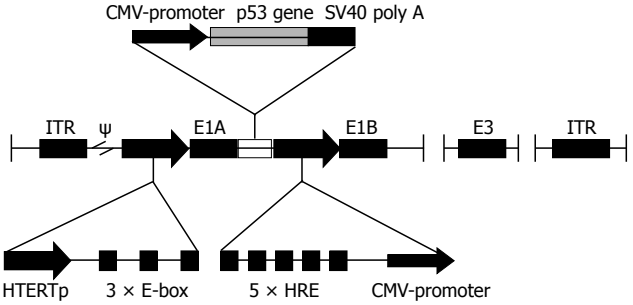

Figure 1 Schematic diagram of the CNHK500-p53 adenoviral construct. A 310-bp fragment of human telomerase reverse transcriptase (hTERT) promoter with three E-boxes (CACGTG) downstream of the core sequence replaced the endogenous E1A promoter (digested with *NotI* and *XhoI*) to control the expression of E1A. A 241-bp fragment of hypoxia response element (HRE) promoter replaced the endogenous E1B promoter to control the expression of E1B. A 1805-bp fragment of transgene expression cassette containing cytomegalovirus (CMV) promoter + p53 + SV40 poly A was inserted into the downstream of E1A (digested with *AgeI* + *NotI*) to generate CNHK500-p53. ITR, inverted terminal repeat; ψ, the adenovirus 5 packing signal.

Replication between CNHK500-GFP and Ad-GFP

HepG2, Bel-7402 (1×10^5 cells/well), and BJ (1×10^6 cells/well) were inoculated into six-well plates, respectively. When the cells were confluent, CNHK500-EGFP or Ad-EGFP was added to each well at the MOI of 1 and then washed with PBS 2 h later. Cells were then coated with 1.25% agarose. On days 3, 7 and 10, the cells were observed under a fluorescence microscope and significant changes were photographed. Fluorescence microscopy was performed with routine methods with fluorescence in isothiocyanate (FITC), with the excitement and emission wavelength being 475 nm and 490 nm, respectively.

RESULTS

To make a conditional replicative gene-viral vector, we adopted a design as shown in Figure 1, in which the adenovirus *E1*a gene was placed under the control of hTERT promoter plus three extra E-boxes, and *E1*b gene was controlled by HRE promoter, p53 gene-expressing cassette was inserted between E1A and HRE promoter. CNHK500-p53 was successfully made and verified by PCR. We were able to produce the adenovirus with high titers (2×10^{10} pfu/mL).

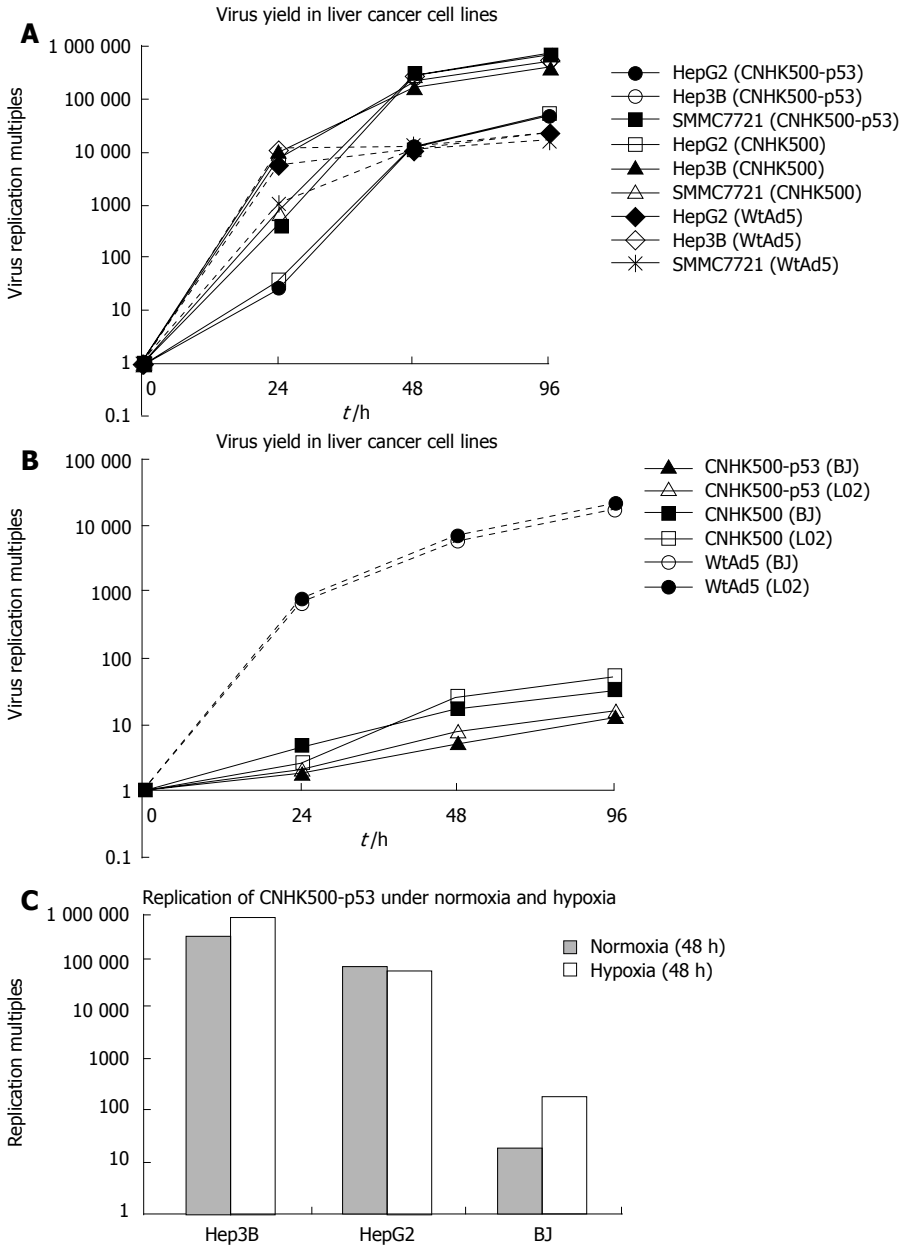

Figure 2 Selective replication of CNHK500-p53 *in vitro*. **A**: Human HCC cell lines HepG2, Hep3B, and SMMC-7721 were infected with CNHK500-p53 at a MOI of 5. Cells and media were harvested, and lysates were prepared from each group at diverse time points 0 h, 24 h, 48 h, and 96 h. Viral titers were measured with the tissue culture infectious dose 50 method, normalized with that at the beginning of infection, and shown as multiples. CNHK500-p53 replicated similarly as CNHK500 and WtAd5 in all of the tested telomerase-positive cancer cells; **B**: Comparison of replication capability of CNHK500-p53, CNHK500 and WtAd5 in telomerase-negative normal cell lines. At diverse time points 0 h, 24 h, 48 h, and 96 h after infection, cells and medium were harvested, and viral titers were measured as described previously. In all of the tested normal cell lines, the replication capability of CNHK500-p53 and CNHK500 was severely attenuated than that of WtAd5; **C**: Forty-eight hours after infection with CNHK500-p53 in HepG2, Hep3B and BJ, CNHK500-p53 showed enhanced replication ability both in HepG2, Hep3B and in BJ under hypoxia condition, but was higher in HepG2, Hep3B than in BJ.

Selective replication of CNHK500-p53

Selective replication of the new recombinant adenovirus CNHK500-p53 was evaluated using telomerase-positive HCC cell lines Hep3B, HepG2, SMMC-7721 and telomerase-negative normal cell lines BJ, L02. In HepG2, Hep3B and SMMC-7721, the replicative multiples increased to 47 230-, 459 837- and 669 251- fold respectively after 96 h of CNHK500-p53 replication, similar to those of CNHK500 and WtAd5 (Figure 2A). However, in normal cell lines BJ, L02, the replicative multiples of CNHK500-p53 and CNHK500 were only 12.8-, 16.3- and 31-, 53- fold at 96 h, and attenuated as much as 1354-, 1325- and 559-, 407.5- fold when compared with WtAd5 (Figure 2B). CNHK500-p53 showed enhanced replication ability both in HepG2, Hep3B and in BJ under hypoxia condition, but was higher in HepG2 and Hep3B cells than in normal BJ cells (Figure 2C). As hTERT promoter regulates *E1*a gene, E1A protein could be detected in telomerase positive hepatocellular cells HepG2 and Hep3B, but not in telomerase negative normal cells BJ. Under normoxic condition, E1B protein could hardly be detected due to poor activity of HRE promoter. When HCC cells were exposed to hypoxia, E1B protein was induced as a result of increased activity of HRE promoter (Figure 3A).

Expression of p53 produced by CNHK500-p53

To verify that the p53 expressed by CNHK500-p53 could secrete efficiently into the media, the conditioned media from 5×10^4 HepG2 cells infected with CNHK500-p53 or Ad-p53 at a MOI of 0.1 were collected and analyzed for the presence of p53 protein by ELISA. The quantity of p53 expressed by CNHK500-p53 and Ad-p53 on d 3, 5, 7, and 10 post-infection is shown in Figure 3B, indicating that p53 expression in CNHK500-p53 was 5.1 times more than that in Ad-p53 in HepG2 on d 7. Western blot analysis revealed a clear band of M_r 53 000 in the conditioned media after HepG2 cells were infected

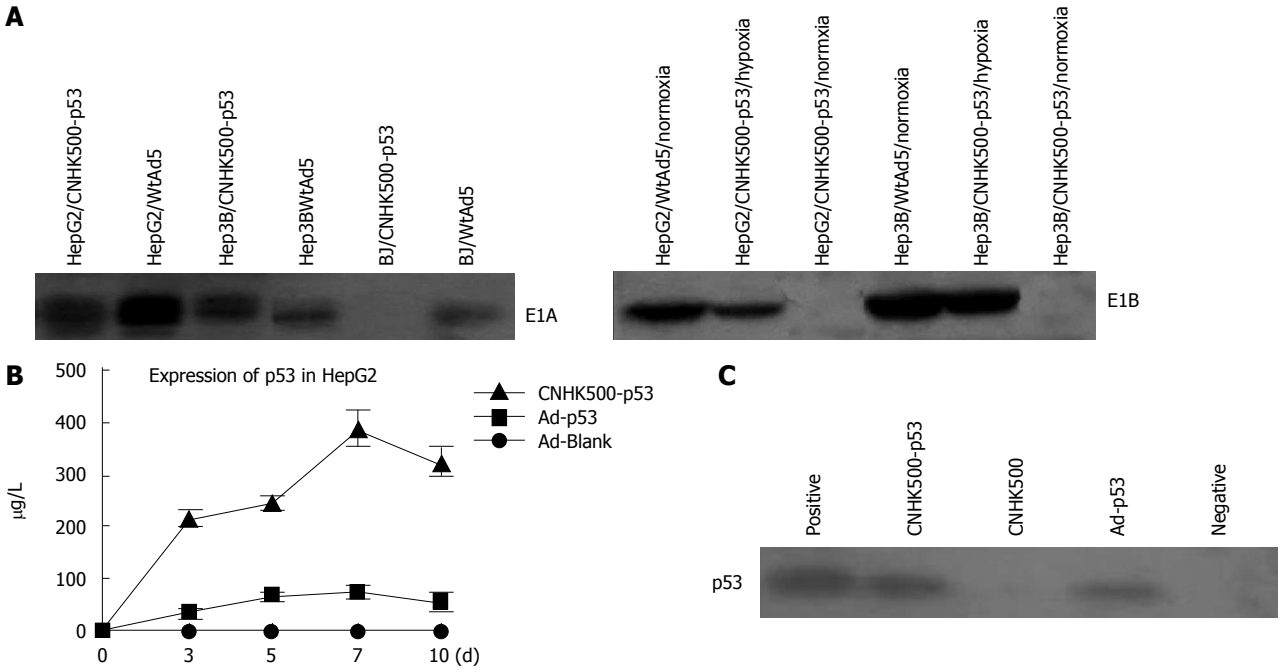

Figure 3 **A**: E1A and E1B expression identified by Western blot demonstrating that all HCC cells infected with CNHK500-p53 or WtAd5 were positive for E1A expression, however, normal BJ cells were negative for E1A expression when they were infected with CNHK500-p53, and positive only when they were infected with WtAd5, while E1B of CNHK500-p53 was only expressed under hypoxia condition in HepG2 and Hep3B, and expressed both under normal and hypoxia condition with WtAd5; **B**: ELISA assay showing that p53 protein secreted from a HepG2, infected with CNHK500-p53, was significantly higher than that infected with nonreplicative adenovirus Ad-p53 and Ad-Blank *in vitro* ($P < 0.05$); **C**: Western blot showing enhanced p53 expression in HepG2 infected with CNHK500-p53.

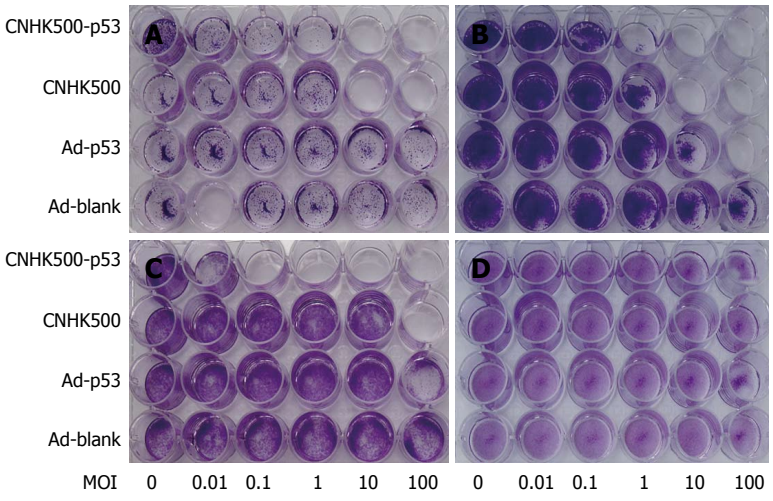

Figure 4 Cytopathic effects associated with CNHK500-p53, CNHK500, Ad-p53, and Ad-Blank infection in HCC cell lines HepG2 (**A**), Hep3B (**B**), Bel-7402 (**C**), and normal cell line BJ (**D**). Seven days after virus infection, all the cells were stained with crystal violet and photographed. Comparison with other viruses, CNHK500-p53 showed the strongest selective cytolysis against HCC cell lines. Infection with CNHK500-p53 at MOI of 0.1-1 was sufficient to induce its lytic effects in Bel-7402, HepG2, and Hep3B, although the sensitivity varied among the cell types. In contrast, no apparent cytopathic effects were observed in BJ even at MOI of 100 after CNHK500-p53 infection, which was similar to other viruses.

with CNHK500-p53 or Ad-p53 at a MOI of 1 on d 3, suggesting that the protein in the media was p53 protein (Figure 3C).

Selective cytolysis of CNHK500-p53

CPE was used to determine whether CNHK500-p53 infection induces selective cell lysis. HCC cell lines (HepG2, Hep3B, and Bel-7402) and the normal cell line (BJ) were infected with CNHK500-p53, CNHK500, Ad-p53, and Ad-Blank at various MOIs, fixed in methanol and stained with crystal violet 7 d after infection to visualize viable cells. CNHK500-p53 showed the strongest selective cytolysis effect among the viruses and killed all cancer cell lines in a dose-dependent fashion (Figure 4). Infection with CNHK500-p53 at a MOI of 0.1-1 was sufficient to induce its lytic effects, although the sensitivity varied among the cell types. In contrast, no apparent CPE was observed in BJ cells 7 d after CNHK500-p53 infection. Cytotoxicity of CNHK500-p53 was also assessed by MTT assay. HepG2, Hep3B and MRC-5, BJ cells were infected with CNHK500-p53 at various viral MOIs. As shown in Figure 5A, CNHK500-p53 induced more rapid cell death in HCC cells than in normal BJ cells 7 d after infection. Furthermore CNHK500-p53 showed a different ability to kill HepG2 and Bel-7402 with the IC_{50} being 0.012 and 0.28 of MOI. On the contrary, the IC_{50} in normal BJ cells was as high as 352.1 of MOI, suggesting that more than 29 341- or 1257-fold of CNHK500-p53 was needed to kill half BJ compared with HepG2 and Bel-7402. Comparison with CNHK500 and WAd5 infection, CNHK500-p53 not

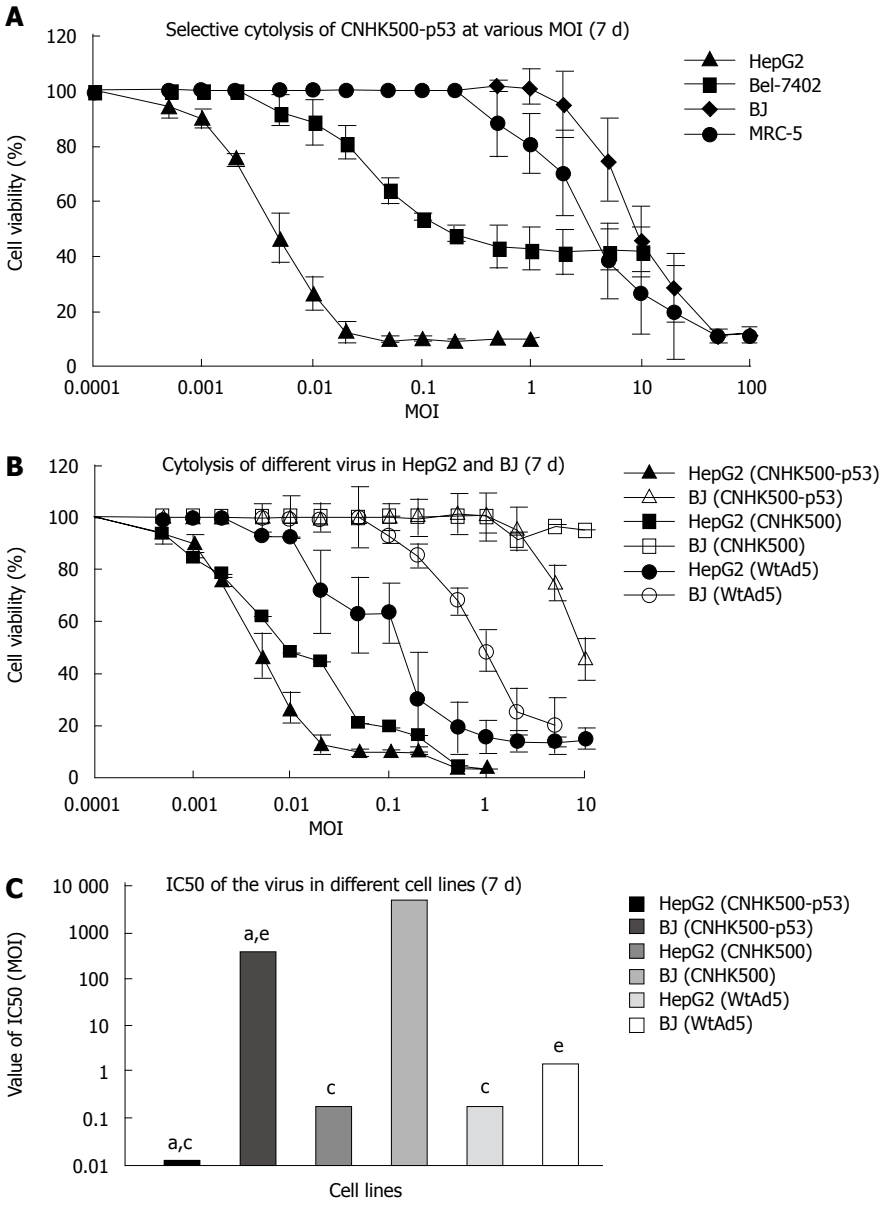

Figure 5 Oncolytic efficacy induced by CNHK500-p53 infection evaluated by MTT assay. Statistical analysis was performed using Student's *t* test for differences among groups. Statistical significance was defined as $P < 0.05$. **A**: HCC cell lines HepG2, Bel-7402 and normal cell lines BJ, MRC-5 were infected at different MOI of 0.001-100 pfu/cell of CNHK500-p53. Cell viability was measured by MTT assay 7 d after infection. CNHK500-p53 induced a more powerful oncolysis in HCC cell lines than in normal cell lines ($P < 0.05$); **B**: Seven days after infection with virus, CNHK500-p53 not only showed more powerful cytolysis than CNHK500 and WtAd5 in HepG2, but also less profound cytolysis than WtAd5 in BJ ($P < 0.05$); **C**: IC50 of CNHK500-p53, CNHk500, and WtAd5 demonstrated significant differences among groups with $^{a,c,e}P < 0.05$.

only demonstrated more apparent cytolysis against HepG2 than CNHK500 and WtAd5 but less profound cytotoxicity than WAd5 against BJ (Figure 5B and C).

Replication of gene-viral system and expression of GFP gene

To determine the selective replication of CNHK500-GFP, HCC cell lines including HepG2, Bel-7402 and normal cell line BJ were observed under fluorescent microscope at different time points after being infected with CNHK500-GFP and Ad-GFP. By conducting GFP expression, CNHK500-GFP demonstrated a greater replicative ability than Ad-GFP in HCC cell lines, with no significant difference in normal cell line. After 3, 7 and 10 d of infection with CNHK500-GFP and Ad-GFP, only a few scattered cells emitted fluorescence in normal cell line. However, in HCC cell lines, CPE such as deformation and aggregation appeared 3 d after infection, and the fluorescence emission spread from a single cell to many cells within a large area 7 d after infection. Bel-7402 cells were particularly sensitive to CNHK500-GFP, many cells died with GFP degradation and fluorescence extinction 10 d after infection (Figure 6), showing selective replication of CNHK500-derivative and correct insertion of GFP gene.

DISCUSSION

Besides conventional approaches, CRAds specifically killing tumor cells while sparing normal cells have been introduced as new agents for cancer therapy in the past decade[14,15]. The efficacy of CRAds against cancer, including HCC, is however, limited by several factors, mainly including tumor specificity and oncolysis[16].

With the advancement in molecular biology and understanding of the function of viral genes, it has become possible to genetically re-engineer viruses to make them selectively kill tumor cells over normal tissue. At present, tumor specificity has been achieved in oncolytic adenoviruses mainly (1) by altering viral genes that attenuate replication in normal tissue but not in tumor cells such as ONYX015 with E1B 55KD deleted[15],

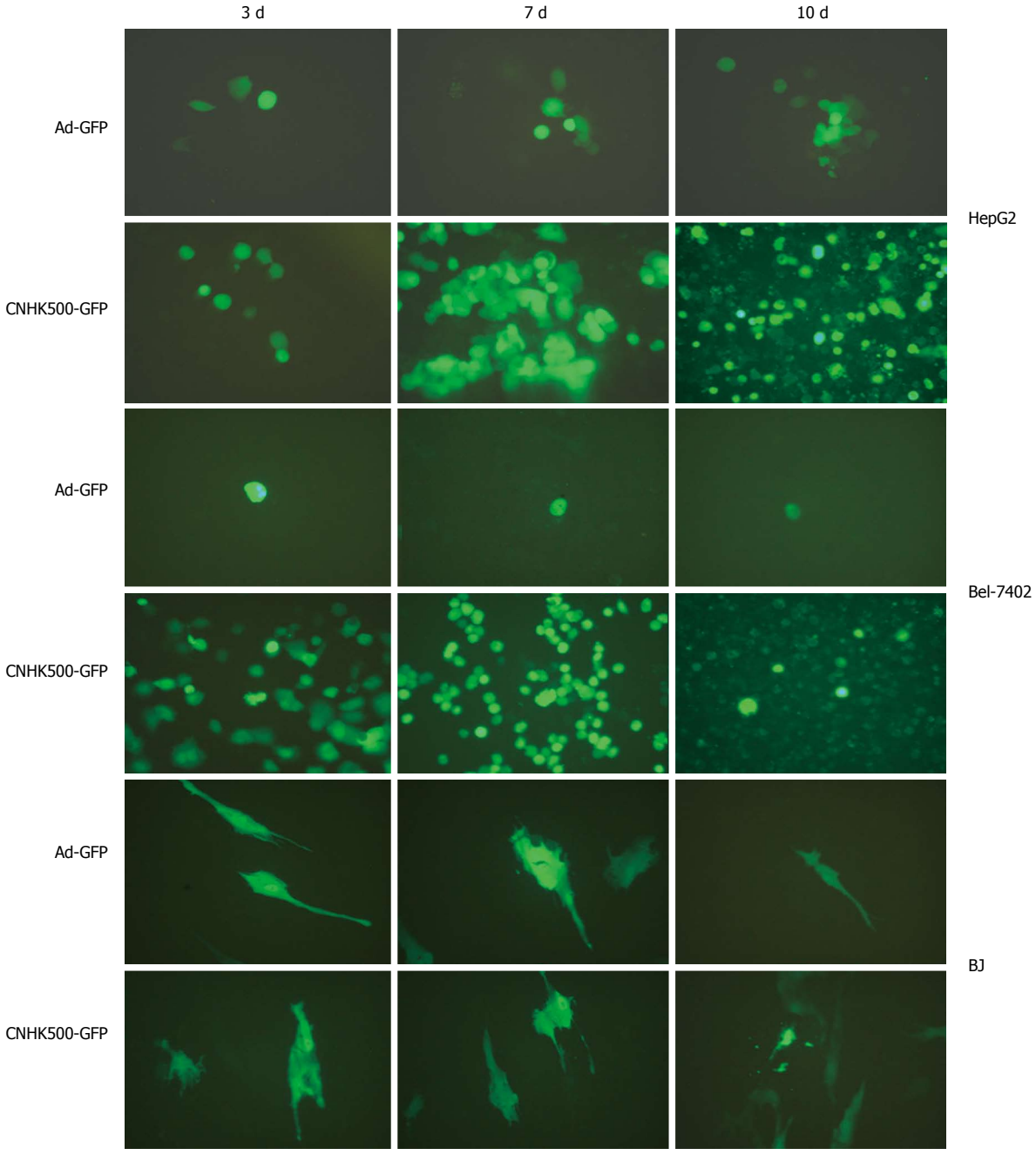

Figure 6 Fluorescent photos of HCC cell lines HepG2, Bel-7402 and normal cell line BJ infected with replicative virus CNHK500-GFP and nonreplicative virus Ad-GFP (200 magnifications).

(2) by placing viral genes that initiate viral replication under the control of promoter sequences that are active in tumor cells such as using AFP promoter to restrict viral replication in AFP-producing HCC[17,18], and (3) by modifing viral coat proteins that function in host cell infection.

Studies showed that adenovirus-induced oncolysis can benefit from combined gene therapy[11,19]. Cancer gene therapy typically involves delivery of tumor suppressor, enzyme/pro-drug gene, cytotoxic/pro-apoptotic gene, immunogene, anti-angiogenic gene directly into tumor cells[20]. After more than two decades of study, the tumor suppressor p53 gene is widely regarded as the "genome guardian." It has been estimated that at least half of all human malignancies, including HCC, are related to a mutation of the p53 gene[21].

In our previous study, we constructed a CRAd containing dual promoters within the E1 region, designated as CNHK500[12], in which the viral *E1*a gene is regulated by hTERT promoter and *E1*b gene by HRE promoter. Since telomerase is highly activated in most malignant tumors but inactive in normal somatic cells[22], CNHK500 can selectively replicate in telomerase-positive cancer cells. At the same time, its replication ability is further attenuated in normal cells as hypoxia microenvironment seldom exists among normal tissues. However, it may propagate very well in solid tumors because HRE promoter is transcriptally activated due to hypoxia, a unique feature

of human solid tumors[23]. A further study suggested that CNHK500 is tumor-selective *in vitro* and *vivo* when compared with CNHK300 and WtAd5[24]. To enhance the oncolytic potency of CNHK500, we constructed a new CRAd CNHK500-p53 by combining oncolytic virotherapy with gene therapy, which expresses functional p53 during viral replication in HCC cells as verified by PCR, Western blot, and ELISA assay. We evaluated the efficacy of CNHK500 and CNHK500-p53 against human HCC cell lines *in vitro*.

We found that exogenous expression of p53 by CNHK500-p53 could lead to enhanced oncolytic potency compared with its parent CNHK500 on most HCC cell lines, while the ability of selective replication was not significantly different between them. The superior efficacy of CNHK500-p53 was independent of the cellular p53 genetic background. It was reported that the expressed p53 gene appears to exert its anticancer activities by one or more of the following mechanisms: (1) simultaneously triggering apoptotic pathways in tumor cells by a transcription-dependent mechanism in cell nuclei[25,26] and by a transcription-independent mechanism in mitochondria[27] and Golgi apparatus[28]; (2) activating immune response factors such as natural killer cells[29] to exert "bystander effects"; (3) inhibiting DNA repair and antiapoptosis functions in tumor cells[30]; (4) down-regulating the expression of multidrug resistance genes[31] to revert the resistance of tumor cells against radio- and chemotherapies as well as the vascular endothelial growth factor gene[32] to block the blood supply to tumor tissue and matrix metalloproteinase[33] to suppress tumor cell adhesion, infiltration, and metastasis; (5) blocking the transcription of survival signals in tumor cells[34,35], thus inhibiting the growth of tumor cells in any stage of the cell cycle. Hence, dysfunctional p53 of HCC cells might delay conditionally replicative adenovirus-induced cell death, thus limiting conditionally replicative adenovirus efficacy.

In conclusion, CNHK500-p53 has the selective replicative ability in HCC cell lines and a higher oncolytic efficacy than its parent CNHK500 *in vitro*. The enhanced oncolytic efficacy may be related to the expression of p53 gene carried by CNHK500-p53. Further experiments are needed to warrant its potential therapeutic effect against HCC.

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We are grateful to Laboratory of Viral and Gene Therapy, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China, for its help in constructing recombinant adenoviruses.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most frequent and lethal malignancies worldwide especially in China. According to the reports of American cancer society (ACS) in 2005, the 5-year survival rate of HCC is only about 8.3%. Therefore, development of effective alternative approaches is needed. Replication-selective virus-mediated gene therapy holds great promise for the treatment of cancer, including HCC.

Research frontiers

So far, 656 cancer gene therapy clinical protocols are in different phase of evaluation worldwide. Unfortunately, successful delivery and targeted expression of therapeutic gene into cancer cells still are very difficult to achieve. The main problem is due to the very low *in vivo* transduction and expression efficacy of available vectors. In this respect, conditionally replicative adenoviruses appear as attractive vectors. Combination of oncolytic virotherapy and gene therapy may be an effective alternative approach against cancer.

Innovations and breakthroughs

In order to construct tumor-specific conditionally replicative adenoviruses, adenovirus E1A gene expression was driven by the hTERT promoter and E1B gene by the hypoxia response (HRE) promoter through genetic engineering, which assures adenovirus replication only in telomerase-positive cells exposed to hypoxia. Besides, human p53 gene was cloned into the downstream of E1A of adenovirus to enhance oncolysis. It was different from other study in adenovirus reconstruction methods. Meanwhile, the recombinant adenovirus was verified by PCR assay, and showed tumor-specific replication and enhanced oncolysis against HCC cell lines *in vitro*.

Applications

The results of our present study demonstrate that p53-expressing conditionally replicative adenovirus has the selectively replictive ability in HCC cell lines and a higher oncolytic efficacy than non-p53-expressing conditionally replicative adenovirus *in vitro*. The enhanced oncolytic efficacy may be related to the expression of p53 gene. Further experiments are needed to warrant its potential therapeutic effect against HCC.

Terminology

CRAds: conditional replicative adenoviruses, recombinant adenoviruses modified to selectively replicate in cancer cells; Oncolytic virotherapy: one of the cancer therapies by obtaining a virus that replicates and preferentially kills cancer cells, leaving the surrounding normal tissues relatively intact; Cancer gene therapy: Cancer gene therapy can be defined as transfer of nucleic acids into tumor or normal cells to eradicate or reduce tumor mass by direct killing of cells, immunomodulation or correction of genetic errors, and reversion of malignant status. Initially started with lots of optimism and enthusiasm, cancer gene therapy has shown limited success in treatment of patients.

Peer review

This is an interesting manuscript. In this manuscript, Hing-Chuan Zhao *et al* take advantage of the selective expression of hTERT and HIF in tumoral cells to express under their respective promoters E1A and E1B assuring adenovirus replication only in telomerase cancerous cells exposed to hypoxia. The data are clearly presented. They show the selective replication and expression of adenoviral proteins as well as the selective cytopathic effect of their adenovirus.

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COLORECTAL CANCER

Early apoptosis and cell death induced by ATX-S10Na (Ⅱ)-mediated photodynamic therapy are Bax- and p53-dependent in human colon cancer cells

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Abstract

AIM: To investigate the roles of Bax and p53 proteins in photosensitivity of human colon cancer cells by using lysosome-localizing photosensitizer, ATX-S10Na (Ⅱ).

METHODS: HCT116 human colon cancer cells and Bax-null or p53-null isogenic derivatives were irradiated with a diode laser. Early apoptosis and cell death in response to photodynamic therapy were determined by MTT assays, annexin V assays, transmission electron microscopy assays, caspase assays and western blotting.

RESULTS: Induction of early apoptosis and cell death was Bax- and p53-dependent. Bax and p53 were required for caspase-dependent apoptosis. The levels of anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-x_L, were decreased in Bax- and p53-independent manner.

CONCLUSION: Our results indicate that early apoptosis and cell death of human colon cancer cells induced by photodynamic therapy with lysosome-localizing photosensitizer ATX-S10Na (Ⅱ) are mediated by p53-Bax network and low levels of Bcl-2 and Bcl-x_L proteins. Our results might help in formulating new therapeutic approaches in photodynamic therapy.

Key words: Photodynamic therapy; ATX-S10Na (Ⅱ); Apoptosis; Bax; p53

Mitsunaga M, Tsubota A, Nariai K, Namiki Y, Sumi M, Yoshikawa T, Fujise K. Early apoptosis and cell death induced by ATX-S10Na (Ⅱ)-mediated photodynamic therapy are Bax- and p53-dependent in human colon cancer cells. *World J Gastroenterol* 2007; 13(5): 692-698

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INTRODUCTION

Photodynamic therapy (PDT) is a therapeutic procedure involving the use of tissue-penetrating laser light after the administration of tumor-localizing photosensitizers, and is used for the efficient treatment of a variety of solid and superficial cancers[1]. Tumor cell death in response to PDT is induced *via* apoptosis and/or necrosis, and depends on various conditions, such as tumor cell type, intensity of laser irradiation, and subcellular localization and concentration of the photosensitizer[2-4]. Localizing photosensitizers in cytoplasmic organelles generate reactive oxygen species by photochemical reactions, resulting in induction of cell damage[1,5,6]. Cell damage modes and the initial subcellular targets are related to the localization sites of the photosensitizers, specifically, mitochondria and lysosomes[7]. Mitochondria-localizing photosensitizers, such as silicon phthalocyanine (Pc) 4, cause rapid dissipation of the mitochondrial membrane potential and result in the release of cytochrome *c*[8]. In contrast, the hydrophilic chlorine photosensitizer ATX-S10Na (Ⅱ), localizes mainly in lysosomes[9,10] and activates apoptotic pathways *via* mitochondrial destabilization following the photodamage of lysosomes[11]. Lysosomal proteases released by lysosomal photodamage, in turn activate caspases directly and/or indirectly subsequent to mitochondrial damage[12]. Nonetheless, the mechanisms by which lysosome-localizing photosensitizers activate apoptotic pathways are not fully understood.

Members of the p53 tumor suppressor gene family play various roles in response to DNA damage, such as cell cycle regulation, DNA repair, and induction of apoptosis[13,14]. Expression of the wild-type p53 induced by chemotherapy or radiation increases the sensitivity to apoptosis, whereas a mutated or deleted p53 alters the sensitivity[15]. Furthermore, p53 regulates pro-apoptotic Bcl-2 family proteins[16,17], and these proteins localize to mitochondria and heterodimerize through a BH3 domain with anti-apoptotic Bcl-2 family members, such as

Bcl-xL[18,19]. Bax modulates the mitochondrial pathway of apoptosis by allowing the efflux of apoptogenic proteins. The shift in the balance of the pro-apoptotic and anti-apoptotic Bcl-2 family members regulates the translocation of cytochrome *c* from mitochondria to cytosol[20]. Although various studies indicate the involvement of Bax and p53 in PDT-mediated apoptosis[21-24], there are conflicting reports about whether the induction of apoptosis correlates with cell death.

The aim of the present study was to elucidate the roles of Bax and p53 in response to lysosomal photodamage induced by ATX-S10Na (Ⅱ)-PDT, which might contribute to more effective clinical use of PDT in cancer therapy. We used an established human colon cancer cell line, HCT116, which expresses wild-type Bax and p53, and derivative lines of HCT116 that differ from the parental line by virtue of a selective knockout of either Bax or p53[25,26].

MATERIALS AND METHODS

Reagents

ATX-S10Na (Ⅱ), 13, 17-bis (1-carboxypropionyl) carbamoylethyl-8-ethenyl-2-hydroxy-3-hydroxyiminoethylidene-2, 7, 12, 18-tetramethylporphyrin sodium salt was provided by Photochemical Co. (Okayama, Japan), and was dissolved in phosphate-buffered saline (PBS).

Cell cultures

Bax-null or p53-null derivatives of the wild-type HCT116 cell line, generated by targeted homologous recombination to create homozygous deletion, were a generous gift from B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD, USA)[27,28]. Cells were maintained in McCoy's 5A medium (GIBCO-BRL, Bethesda, MD, USA) supplemented with 10% fetal calf serum (Thermo Trace, Melbourne, Australia) and 1% penicillin/streptomycin (GIBCO-BRL) in a 37℃, 5% CO_2, fully humidified incubator and passaged twice weekly.

PDT protocols

Exponentially growing cells were seeded in 96-well microplates or 35-mm dishes to approximately 30% confluence 48 h before PDT. ATX-S10Na (Ⅱ) was added to the culture medium to a final concentration of 20 μg/mL 24 h before PDT. Medium was replaced with fresh medium, and the cells were irradiated with a diode laser (Hamamatsu Photonics, Hamamatsu, Japan) at a wavelength of 670 nm. The energy fluence rate was 0.167 W/m^2 as measured using LaserMate power meter (Coherent, Auburn, CA, USA). Exposure for 5 min resulted in an incident energy fluence of 5 J/cm^2.

MTT assays

Cytotoxicity of PDT was determined by colorimetric assay with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt reagent (Cell Counting Kit-8, Wako Pure Chemical Industries, Osaka, Japan). Following PDT, cells were incubated in 96-well microplates for 24 h. Ten microliters of Cell Counting Kit-8 reagent[27] were added to each well, and cells were incubated at 37℃ for 4 h. After thorough mixing, the absorbance of each well was measured at 450 nm with an Ultramark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Annexin V assays

Cells were collected by trypsinization and washed with ice-cold PBS, suspended in 500 μL annexin V binding buffer containing 5 μL of propidium iodide (PI) and 5 μL of annexin V-FITC (BioVision, Mountain View, CA, USA), and incubated for 15 min at room temperature in the dark. Fluorescence was measured on a BD LSR flow cytometer (Becton Dickinson, NY, USA) and processed with Cell Quest software (Becton Dickinson) for analysis.

Cell cycle analysis

Cells were collected and washed in cold PBS, fixed in 70% ethanol pre-chilled at -20℃, washed, resuspended in 25 μg/mL of PI with 100 μg/mL RNase A, and incubated for 30 min at 37℃. Fluorescence was measured on a BD LSR flow cytometer. Data were analyzed using the MODFIT 2.0 program (Verity Software).

Transmission electron microscopy assays

Ultrastructural appearances of apoptotic cells were confirmed by electron microscopy. Following PDT, cells were prefixed with 2% glutaraldehyde, post-fixed with 1% osmic acid, dehydrated in graded ethanol, embedded in resin, and cut into sections on an ultramicrotome. The cells were examined by a transmission electron microscope (TEM) (H-7500, Hitachi, Tokyo, Japan).

Quantification of caspases 3, 8 and 9 activity

Activities of caspases 3, 8 and 9 were measured by Caspase Fluorometric Assay kits (R&D Systems Inc., Minneapolis, MN, USA) according to the instructions provided by the manufacturer. Briefly, cells in 35-mm dishes were washed twice with ice-cold PBS and lysed in 100 μL of lysis buffer. Next, 50 μL of cell lysates were transferred to a 96-well plate containing reaction buffer, and were incubated for 2 h at 37℃ with 5 μL of caspase 3-, 8- or 9-specific fluorescent substrate DEVD-AFC, IETD-AFC and LEHD-AFC, respectively. Plates were read with an ARVOsx-2 fluorescence microplate reader (Wallac, Turku, Finland) using an excitation light of 400 nm and an emission light of 505 nm.

Preparation of protein extracts and immunoblotting

Cells in 35-mm dishes were washed twice with ice-cold PBS, lysed in 100 μL of lysis buffer [50 mmol/L HEPES (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 150 mmol/L sodium chloride, 5 mmol/L EDTA with protease inhibitors pepstatin A (2 μg/mL), aprotinin (10 μg/mL), leupeptin (10 μg/mL) and phenylmethylsulfonyl fluoride (100 μg/mL)] and sonicated. The lysates were centrifuged at 10000 × *g* at 4℃ for 10 min. The supernatant was recovered and protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA). Twenty microgram of proteins were loaded on an SDS-polyacrylamide gel. After electrophoresis, proteins were electrotransferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% nonfat milk

in Tris-buffered saline with 0.05% Tween-20 (TBS-T) for 2 h at room temperature, and then probed with primary antibodies for 1 h at room temperature or overnight at 4℃. Horseradish peroxidase (HRP)-labeled secondary antibodies were used for signal detection and blots were visualized with Enhanced Chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and recorded on an X-ray film. For cytosolic fractionation, cells were harvested in digitonin lysis buffer (75 mmol/L NaCl, 1 mmol/L NaH_2PO_4, 8 mmol/L Na_2HPO_4, 250 mmol/L sucrose and 190 μg/mL of digitonin), supplemented with protease inhibitors, and incubated on ice for 5 min. Samples were centrifuged at 10000 × *g* at 4℃ for 30 min and the resulting supernatant was used for Western blotting. Antibodies were as follows: mouse monoclonal p53 (DO-1) (Calbiochem, San Diego, CA, USA), mouse monoclonal Bcl-2 (100) and rabbit polyclonal Bax (N-20), Bcl-x$_{S/L}$ (S-18) and β-tubulin (H-235) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal cytochrome *c* (7H8.2C12) (BD Pharmingen, San Diego, CA, USA), and HRP-labeled secondary anti-mouse and anti-rabbit antibodies (Santa Cruz). The image of the specific protein band on the membrane was scanned, and the intensity of the image was analyzed by an NIH Image J program version 1.31.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were evaluated by *t*-test. *P* values < 0.05 were considered statistically significant.

RESULTS

Bax- and p53-dependent cell death and apoptosis in response to ATX-S10Na (Ⅱ)-PDT

To determine the role of Bax and p53 in ATX-S10Na (Ⅱ)-PDT, we first examined the cells' phototoxicity by MTT assays 24 h after laser irradiation. The extent of ATX-S10Na (Ⅱ) phototoxicity was dependent on the intensity of laser irradiation (Figure 1A) and the concentration of ATX-S10Na (Ⅱ) (data not shown). Wild-type HCT116 cells were significantly more sensitive to ATX-S10Na (Ⅱ)-PDT than Bax-null or p53-null cells at 5 J/cm^2 (Figure 1A). Laser irradiation greater than 10 J/cm^2 resulted in global phototoxic cell death, thereby preventing the accumulation of cells with PDT-mediated regulatory responses. Thus, laser irradiation at 5 J/cm^2 was chosen for further studies. Next, to determine the role of early apoptosis in Bax and p53 dependence of phototoxicity, we examined ATX-S10Na (Ⅱ)-PDT-mediated apoptosis by flow cytometry with annexin V and cell cycle analysis that determines a population of cells with sub-G_1 DNA content 3 or 6 h after laser irradiation. The percentage of early apoptotic, annexin V-positive and PI-negative, cells was significantly reduced in Bax-null or p53-null cells compared with wild-type HCT116 cells (Figures 1B and D). In contrast, the percentage of late apoptotic or necrotic, annexin V-positive and PI-positive, cells was not significantly different (Figure 1C). These results indicate that Bax and p53 play a central role in early apoptosis induced by ATX-S10Na (Ⅱ)-PDT. TEM studies revealed that untreated cells have thickened mitochondria and numerous cytoplasmic vesicles. In contrast, when treated with PDT, the sections contained many apoptotic cells with condensed chromatin, apoptotic bodies in the cytoplasm and cell membrane budding (Figure 1E). The percentage of apoptotic cells was higher in the wild-type than Bax-null or p53-null cells 6 h after laser irradiation.

Roles of Bax and p53 in caspase-dependent ATX-S10Na (Ⅱ)-PDT-induced apoptosis

Since caspases are early effectors for triggering PDT-mediated apoptosis[28,29], we examined the roles of Bax and p53 in caspase activation by ATX-S10Na (Ⅱ)-PDT. Caspase-3 activity increased in intensity in laser irradiation- and time-dependent manners within 24 h following PDT in wild-type HCT116 cells (data not shown). As shown in Figure 2, activities of caspase-3 and -9 apparently increased in wild-type HCT116 cells 6 h after laser irradiation. In contrast, it was significantly inhibited in Bax-null or p53-null cells. These results indicate that the caspase-dependent apoptotic process induced by ATX-S10Na (Ⅱ)-PDT was Bax- and p53-dependent. Activation of caspase-8 was slightly increased compared with caspase-9, indicating that the mitochondrial pathway of apoptosis is the major process in response to ATX-S10Na (Ⅱ)-PDT. In Bax-null HCT116 cells, caspase-9 activity was significantly but not completely inhibited, and the protein level of cytochrome *c* released from mitochondria was reduced but not absent (Figure 3), indicating that the mitochondrial pathway of apoptosis is induced by ATX-S10Na (Ⅱ)-PDT, even though Bax was absent. In p53-null cells, caspase-9 activity was slightly inhibited, and the protein level of cytochrome *c* released from mitochondria was not reduced (Figure 3), indicating that p53 is required for ATX-S10Na (Ⅱ)-PDT-mediated apoptosis.

Decreased levels of anti-apoptotic proteins Bcl-2 and Bcl-x$_L$ in response to ATX-S10Na (Ⅱ)-PDT play a role in early apoptosis, and are Bax- and p53-independent

Previous studies indicated that the levels of anti-apoptotic proteins Bcl-2 and Bcl-x$_L$ were reduced and pro-apoptotic proteins Bcl-x$_S$, Bak and Bad, but not Bid, were up-regulated in response to PDT[30-33]. Therefore, we determined the association of Bax and p53 with anti-apoptotic Bcl-2 family proteins in ATX-S10Na (Ⅱ)-PDT. Immunoblots demonstrated no significant changes in Bax and p53 expression (Figure 3). In contrast, Bcl-2 and Bcl-x$_L$ expression were decreased to similar levels in each cell type, indicating that Bax- and p53-independent downregulation of Bcl-2 and Bcl-x$_L$ plays a role in early apoptosis induced by ATX-S10Na (Ⅱ)-PDT.

DISCUSSION

Many studies have shown that PDT kills tumor cells *via* apoptosis and/or necrosis *in vivo* and *in vitro*. Although cell death in response to PDT depends on various conditions, the role of Bax or p53 in PDT remains controversial. This study was designed to determine the role of these proteins in ATX-S10Na (Ⅱ)-PDT by using an isogenic set

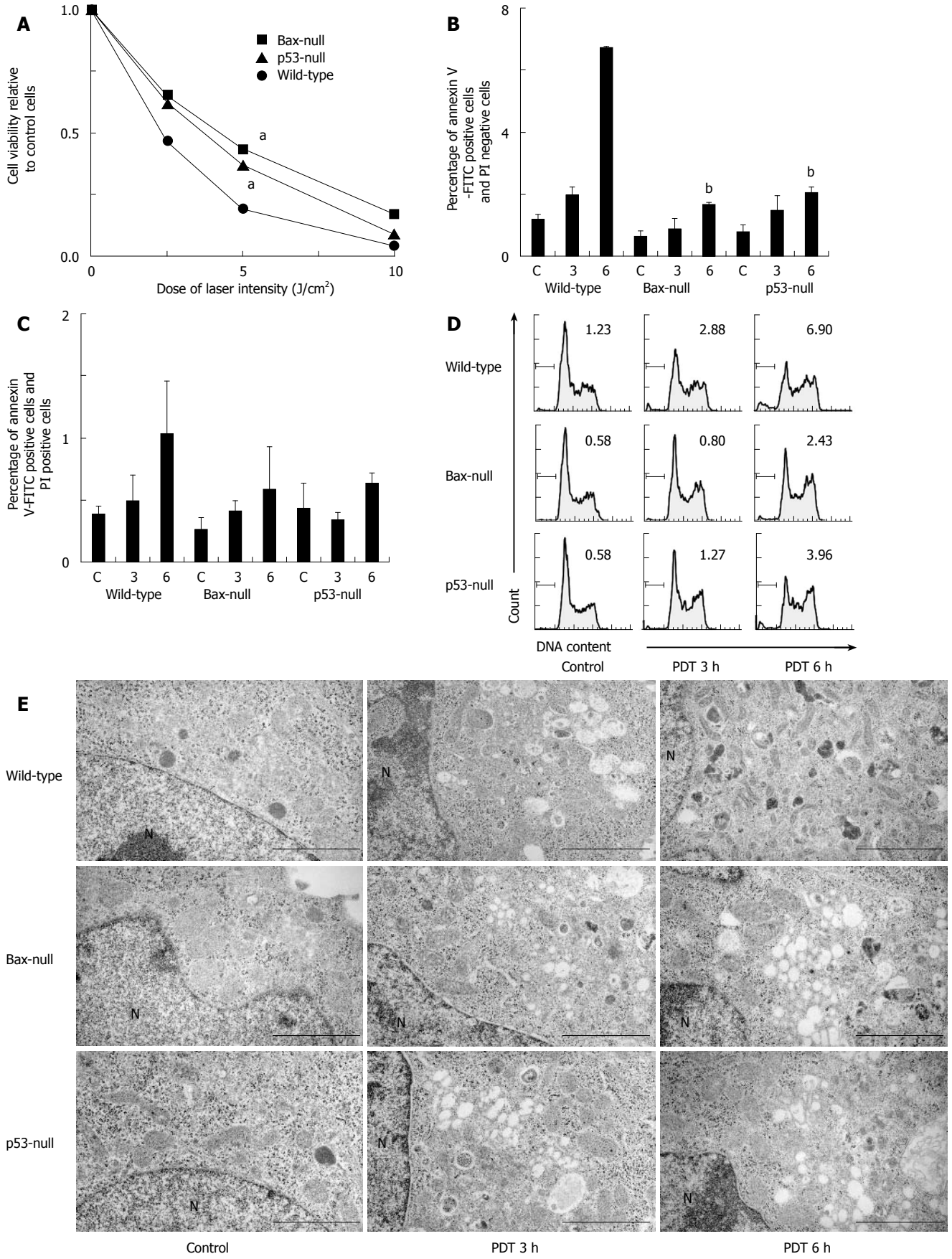

Figure 1 ATX-S10Na(Ⅱ)-PDT-mediated cell death and early apoptosis were Bax- and p53-dependent. Cells were treated with, or without, ATX-S10Na(Ⅱ)-PDT and harvested at each indicated time point following irradiation. **A**: Phototoxicity was determined by MTT assay 24 h after laser irradiation. Data are expressed as a ratio of cell viability relative to untreated control cells. Data represent the mean ± SD of three independent experiments ($^aP < 0.05$ *vs* wild-type cells). **B**, **C**: Early apoptotic changes (**B**) and late apoptotic or necrotic changes (**C**) were determined by annexin V apoptosis assays at 3 or 6 h after laser irradiation. Data are expressed as a percentage of annexin V-positive and PI-negative (**B**) or annexin V-positive and PI-positive cells (**C**). Data represent the mean ± SD of three independent experiments ($^bP < 0.05$ *vs* wild-type cells). **D**: Cell cycle distributions were determined by flow cytometry at 3 or 6 h after laser irradiation. Data are expressed as a percentage of sub-G_1 fraction. **E**: Morphological changes in response to ATX-S10Na(Ⅱ)-PDT were determined by transmission electron microscopy assays at 3 or 6 h following laser irradiation. Typical subcellular changes in response to PDT are shown (original magnification × 12 000). Scale bar: 15 μm, N: nuclei.

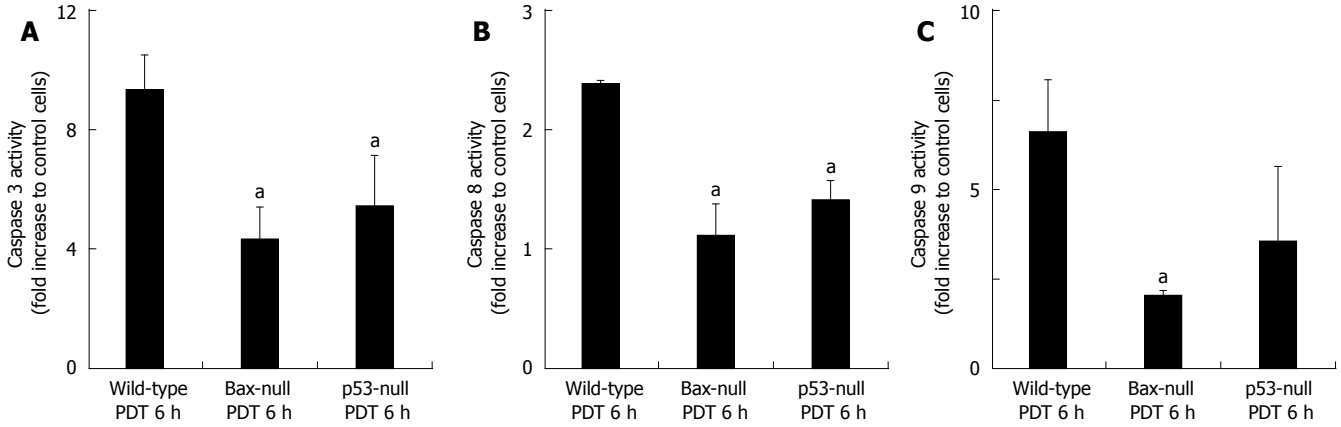

Figure 2 Induction of apoptosis by ATX-S10Na (Ⅱ)-PDT was caspase-dependent. Cells were treated with, or without, ATX-S10Na (Ⅱ)-PDT, harvested and lysed at 6 h after laser irradiation. Induction of caspase activity was assayed. **A**: caspase-3; **B**: caspase-8; **C**: caspase-9. Data represent the mean ± SD of three independent experiments ($^aP < 0.05$ *vs* wild-type cells).

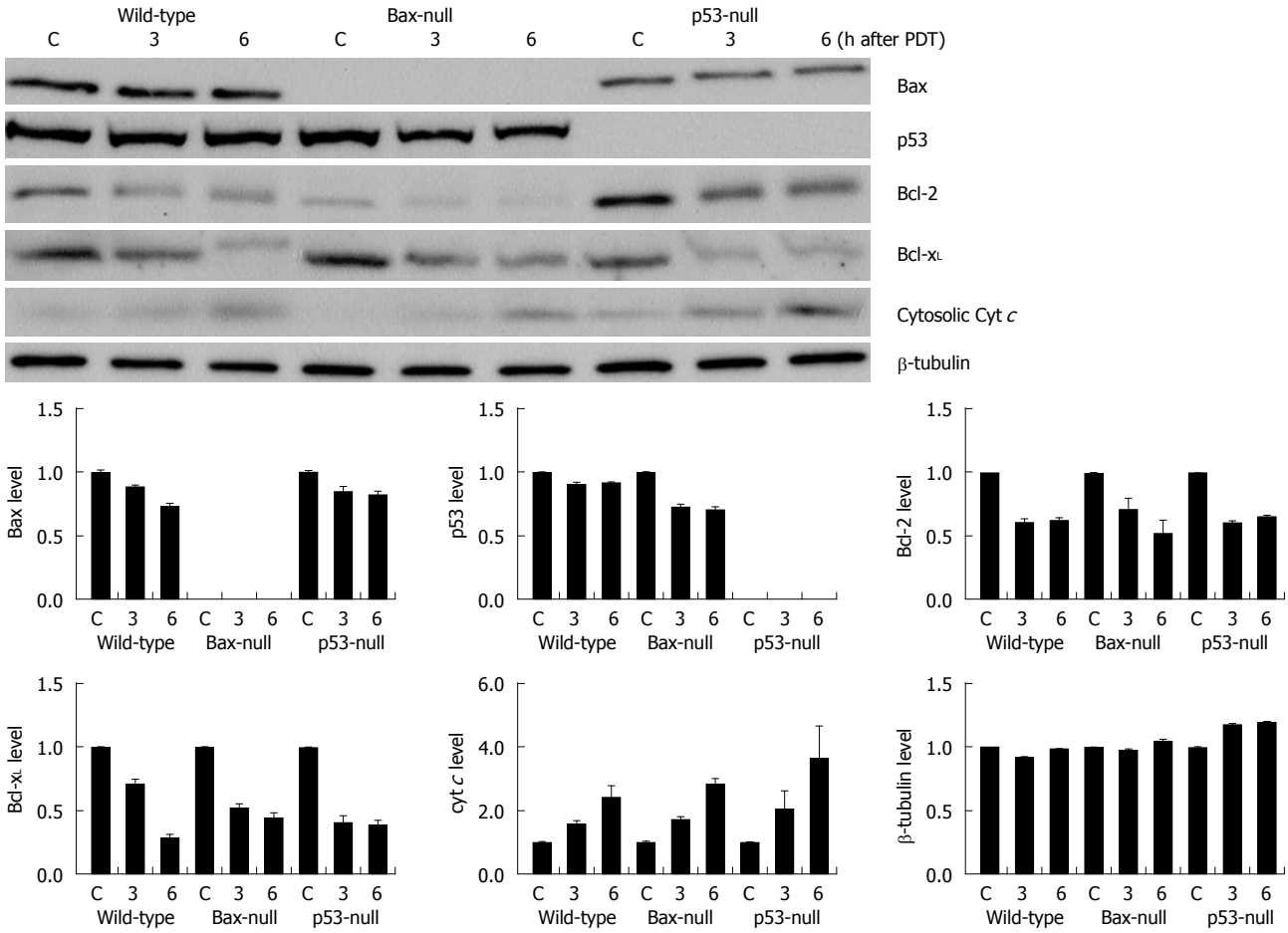

Figure 3 Low levels of Bcl-2 and Bcl-xL played a role in ATX-S10Na(Ⅱ)-PDT. Cells were treated with, or without, ATX-S10Na(Ⅱ)-PDT, harvested and lysed at 3 or 6 h after laser irradiation. Western blot analysis was performed. Each protein level was quantified by using NIH Image J program. Data represent the mean ± SD of three independent experiments.

of human colon cancer cell lines derived from HCT116. Our results indicated that ATX-S10Na (Ⅱ)-PDT induced Bax- and p53-dependent cell death and early apoptosis of human colon cancer cells. ATX-S10Na (Ⅱ)-PDT induced caspase-dependent apoptosis and reduced the levels of anti-apoptotic proteins Bcl-2 and Bcl-xL. Apoptosis was mediated mainly through a Bax-regulated mitochondrial pathway. Taken together, these results suggest that Bax and p53 play a central role in inducing early apoptosis and cell death by ATX-S10Na (Ⅱ)-PDT.

Various experiments have shown that the pro-apoptotic Bcl-2 family protein Bax plays a role in the mitochondrial pathway of apoptosis by translocating from the cytosol to the mitochondria[20]. Our present experiments revealed that

wild-type HCT116 cells were significantly more sensitive to ATX-S10Na (Ⅱ)-PDT than Bax-null cells, as determined by MTT assays (Figure 1). Induction of early apoptosis by ATX-S10Na (Ⅱ)-PDT was also Bax-dependent, as determined by the annexin V assay and cell cycle analysis (Figure 1). Bax was required for caspase activation and cytochrome *c* release, but the mitochondrial pathway of apoptosis was not completely inhibited in the absence of Bax (Figures 2 and 3). The protein level of Bax did not apparently change in the wild-type or p53-null cells (Figure 3). These findings suggest that Bax plays a central role in PDT-mediated cell death and the mitochondrial pathway of apoptosis. As Bax was a major mediator of p53-dependent apoptosis, early apoptosis was apparently processed without Bax activation. Contrary to our findings, earlier reports showed that DU-145 human prostate cancer cells that lack Bax expression do not show release of cytochrome *c* from mitochondria, loss of mitochondrial potential, caspase activation or apoptosis, but do have an altered sensitivity to overall cell death[24]. In contrast, a comparison of the PDT response of wild-type and Bax-null HCT116 human colon cancer cells showed reduced release of cytochrome *c* but it was not completely blocked in Bax-null cells. In both cell lines, caspase-dependent apoptosis was triggered and cell killing was equally sensitive; however, no significant differences in the activation of caspase-3 were found[34]. The authors concluded that the commitment to cell death after PDT occurs at a step prior to and irrespective of Bax activation. Differences between our results and the above findings may be due to cellular differences unrelated to Bax expression, intensity of laser irradiation or type of photosensitizer.

The tumor suppressor protein p53 plays an important role in response to various stress conditions[13,14]. Our present experiments revealed that wild-type HCT116 cells were significantly more sensitive to ATX-S10Na (Ⅱ)-PDT (Figure 1). In addition, induction of early apoptosis by ATX-S10Na (Ⅱ)-PDT was p53-dependent (Figure 1). p53 was required for caspase-3 activation (Figure 2). The mitochondrial pathway of apoptosis was not significantly inhibited in the absence of p53 (Figures 2 and 3). These findings suggest that p53 might play a role in PDT-mediated early apoptosis and cell death. Consistent with our findings, HL60 human promyelocytic leukemia cells that express wild-type p53 are more photosensitive than p53-deleted or mutated HL60 cells[21]. Introduction of the wild-type p53 gene into the HT29 colon cancer cells induced growth arrest but not cell death by PDT[23]. In non-isogenic colon carcinoma cell lines, increased photosensitivity of a wild-type p53 phenotype was observed compared with a mutated p53 phenotype[22]. Taken together, it is conceivable that Bax and p53 play central roles both, in cell death and in early apoptosis induced by ATX-S10Na (Ⅱ)-PDT.

The ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members helps determine the threshold for inducing mitochondrial-related apoptosis[20]. Overexpression of Bcl-2 in Chinese hamster ovary (CHO) cells inhibited apoptosis and partly protected against cell death induced by PDT[35]. Reduction of Bcl-2 protein levels by Bcl-2 antisense oligonucleotides in radiation-induced fibrosarcoma (RIF-1) cells resulted in sensitization to PDT-mediated apoptotic death[31]. Our experiments revealed that decreased levels of Bcl-2 and Bcl-xL were Bax- or p53-independent in response to ATX-S10Na (Ⅱ)-PDT. Cytochrome *c* release from mitochondria was not completely inhibited in the absence of Bax or p53 (Figure 3). These findings suggest that reduced levels of anti-apoptotic Bcl-2 family proteins, that are Bax- or p53-independent, played a role in cytochrome *c* release and apoptosis induced by ATX-S10Na (Ⅱ)-PDT. Low levels of these proteins could be the result of caspase activation or photochemical targets of PDT. PDT using mitochondrial photosensitizer phthalocyanine Pc 4, directly damaged Bcl-2 and Bcl-xL, and contributed in the induction of apoptosis[33,36].

In conclusion, Bax and p53 play a central role in the apoptotic process and cell death induced by ATX-S10Na (Ⅱ)-PDT in human colon cancer cells. Low levels of anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL, which are Bax- and/or p53-independent, play a role in the early apoptotic process. Bax and p53 can induce apoptosis and cell death in response to ATX-S10Na (Ⅱ)-PDT, which might help in the design of new therapeutic approaches.

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Survey of molecular profiling during human colon cancer development and progression by immunohistochemical staining on tissue microarray

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Abstract

AIM: To explore the molecular events taking place during human colon cancer development and progression through high-throughput tissue microarray analysis.

METHODS: We constructed two separate tissue microarrays containing 1.0 mm or 1.5 mm cylindrical samples acquired from 112 formalin-fixed and paraffin-embedded blocks, including carcinomas (n = 85), adenomatous polyps (n = 18), as well as normal para-cancerous colon tissues (n = 9). Immunohistochemical staining was applied to the analysis of the consecutive tissue microarray sections with antibodies for 11 different proteins, including p53, p21, bcl-2, bax, cyclin D1, PTEN, p-Akt1, β-catenin, c-myc, nm23-h1 and Cox-2.

RESULTS: The protein expressions of p53, bcl-2, bax, cyclin D1, β-catenin, c-myc, Cox-2 and nm23-h1 varied significantly among tissues from cancer, adenomatous polyps and normal colon mucosa (P = 0.003, P = 0.001, P = 0.000, P = 0.000, P = 0.034, P = 0.003, P = 0.002, and P = 0.007, respectively). Chi-square analysis showed that the statistically significant variables were p53, p21, bax, β-catenin, c-myc, PTEN, p-Akt1, Cox-2 and nm23-h1 for histological grade (P = 0.005, P = 0.013, P = 0.044, P = 0.000, P = 0.000, P = 0.029, P = 0.000, P = 0.008, and P = 0.000, respectively), β-catenin, c-myc and p-Akt1 for lymph node metastasis (P = 0.011, P = 0.005, and P = 0.032, respectively), β-catenin, c-myc, Cox-2 and nm23-h1 for distance metastasis (P = 0.020, P = 0.000, P = 0.026, and P = 0.008, respectively), and cyclin D1, β-catenin, c-myc, Cox-2 and nm23h1 for clinical stages (P = 0.038, P = 0.008, P = 0.000, P = 0.016, and P = 0.014, respectively).

CONCLUSION: Tissue microarray immunohistochemical staining enables high-throughput analysis of genetic alterations contributing to human colon cancer development and progression. Our results implicate the potential roles of p53, cyclin D1, bcl-2, bax, Cox-2, β-catenin and c-myc in development of human colon cancer and that of bcl-2, nm23-h1, PTEN and p-Akt1 in progression of human colon cancer.

Key words: Colon cancer; Immunohistochemistry; Tissue microarray

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http://www.wjgnet.com/1007-9327/13/699.asp

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent cancers in the Western world. At present time, with the development of living conditions and changes of life behaviors, CRC has become more and more frequent in China. The prognosis in advanced cases is poor, and more than one-third of the patients will die from progressive disease because the overall survival is about 40% (15%-65%) after 5 years[1]. The development and progression of CRC, like others cancers, are results of multiple genetic alterations, so investigation of molecular changes in tumors representing the entire disease spectrum may enhance our understanding of mechanism involved in CRC tumorigenesis. Because CRC is one of the first major epithelial cancers in which molecular alterations were

described to occur in a systematic fashion during disease progression, studies of single molecular markers have not been successful in defining the biology of this disease[2]. This has prompted investigators to explore multiple molecules regulating the tumorigenesis in an effort to identify biologically aggressive tumors and appropriately select patients for adjuvant systemic or targeted therapies. However, with the progression of molecular biology and the development of oncogene research, the cancer-related genes and genetic alterations have been found rapidly. The evaluation of the clinical utility of each of these genes would require multiple consecutive experiments with hundreds of tumors. This would be both time-consuming and labor-intensive.

As a new biological technique which allows rapid visualization of molecular targets in thousands of tissues specimens at a time, either at the DNA, RNA, or protein level, tissue microarray (TMA) can facilitate rapid translation of molecular discoveries to clinical applications[3]. So it brings us a high-throughput and rapid technique which can help us complete the time- and people-consuming work. Here, we constructed two tissue microarrays containing samples from different stages of human colon cancer, adenomatous polyps and corresponding normal para-cancerous colon tissues to survey the gene alterations that may contribute to clinical behaviors of the colon cancer. We decided to investigate the role of protein expressions which had been shown correlated with the development and progression as well as metastases of colon cancer in previous studies. In this study, 11 different proteins (p53, p21, cyclin D1, bcl-2, bax, β-catenin, c-myc, PTEN p-Akt1, Cox-2 and nm23-h1) expressions were assayed by using immunohistochemical (IHC) staining to consecutive formalin-fixed tissue microarray sections. The aim was to obtain a comprehensive survey of the frequency of the target molecular alterations and the relationship between the alterations and the clinicopathological features in human colon cancer.

A number of proteins have been associated with human carcinogenesis and may be relevant to CRC. Among these molecules, we chose 11 cancer-related genes (p53, p21, cyclin D1, bcl-2, bax, β-catenin, c-myc, PTEN p-Akt1, Cox-2 and nm23-h1) which are altered during the development and progression of CRC according to the previous study reports[4-11]. Among these molecules, p53, p21, cyclin D1, bcl-2 and bax play pivotal roles in cell cycle regulation and apoptosis. Akt/protein kinase B (PKB), which is included in phosphatidyl inositol-3-OH kinase (PI3K) signaling, controls many intracellular processes, such as the suppression of apoptosis and the promotion of the cell cycle[12]. PTEN on 10q23.3 encodes a dual-specificity phosphatase that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell-cycle arrest and apoptosis[13,14]. β-catenin is a member of the cadherin-catenin complex that mediates homotypic cell-cell adhesion[15]. It also plays a role in the *Wnt* signaling pathway through regulating target genes like *c-myc*. Cox-2 was elevated in human colon cancers, and the Cox-2 inhibitor, celecoxib, inhibited intestinal tumor multiplicity in a mouse model and reduced the number of adenomatous polyps in a familial adenomatous polyposis patient[16,17]. The human *nm23* gene, a candidate metastatic suppressor gene, consists of two genes, *nm23-h1* and *nm23-h2*. *Nm23-h1* aberration has been shown to be correlated with the metastatic potential of colorectal cancer in some studies[9,18]. More of these molecules were studied previously by conventional pathological or molecular biological technologies and the numbers of selected target molecules were lesser, but in this study we would assay 11 proteins at a time by IHC staining on TMA.

Many investigators and clinicians consider cancer of the colon and rectum to be two distinct diseases, thus, we chose to evaluate only the patients with colon cancer treated with surgery alone in an effort to optimize the homogeneity of the study population. In addition, all the tumor specimens selected according our data were from sporadic colon cancer patients.

MATERIALS AND METHODS

Materials

Demographic and clinical data were collected retrospectively. None of the patients received radiotherapy or chemotherapy before surgery. Formalin-fixed and paraffin-embedded tumors, adenomatous polyps and para-cancerous tissues specimens were from the archives of the Department of Gastroenterology, the First Affiliated Hospital of Soochow University and National Engineering Center for Biochip at Shanghai. All specimens were viewed by one pathologist (Jing Fang). The specimens that were interpretable for IHC included: (1) Eighty-five cancers including different grades, such as high (n = 11), moderate (n = 50), low differentiated (n = 24); (2) eighteen adenomatous polyps removed at colonoscopy; (3) nine para-cancerous colon tissues resected from colon tissues at least 5 cm apart from the corresponding cancer tissues.

Construction and sectioning of tissue microarray

The colon cancer microarray was constructed as previously described[3]. Briefly, fresh sections were cut from the donor block and stained with hematoxylin-eosin (HE), these slides were used to guide the samplings from morphologically representative regions of the tissues. A tissue array instrument (Beecher Instruments, Silver Spring, MD) was used to create holes in a recipient paraffin block and to acquire tissue cores from the donor block by a thin-walled needle with an inner diameter of 1.0 mm or 1.5 mm, held in an X-Y precision guide. The cylindrical samples were retrieved from the selected regions in the donors and extruded directly into the recipient blocks with defined array coordinates. After the construction of the array block, multiple 4-μm thick sections were cut with a microtom using an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ) (Figure 1).

Tissue loss was a significant factor for tissue array-based analysis with previously reported rates of tissue damage ranging from 15% to 33%[19-21]. In our analysis the rates of lost cases attributable to tissue damage were less than 5% for the different markers and damaged tissues

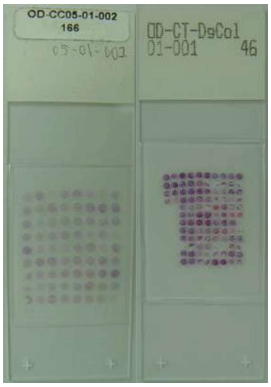

Figure 1 HE staining of 4-μm thick section of the tissue microarray.

were excluded from clinicopathological analyses of the respective markers.

IHC on formalin-fixed tissue microarray sections

IHC staining for the target genes to sections of the formalin-fixed samples on the tissue microarray was carried out by using the Envision ready-to-use methods. Slides were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water, and endogenous peroxidase activity was blocked by incubation with 30 mL/L H_2O_2 in methanol for 10 min at room temperature. Then sections were submitted to antigen retrieval in a pressure cooker containing 0.01 mmol/L natrium citricium buffer for 10 min. Slides were subsequently incubated in 100 mL/L normal goat serum for 20 min at room temperature. Sections were permeabilized in PBS-Triton and incubated overnight with primary antibody at 4℃. The antibodies were used in PBS-Triton with variable dilution. Mouse anti-human monoclonal antibodies to p53 (clone Do-7; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.), p21 (clone DCS-60.2; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.), bcl-2 (clone 100/D5; 1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd), bax (clone 2D2; 1:50; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd), β-catenin (clone CAT-5H10; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd), c-myc (clone 9E11; 1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd), Cox-2 (clone COX229; 1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd), nm23-h1 (1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd) and rabbit anti-human antibody to PTEN (FL-403; 1:100 dilution; Santa Cruz Biotechnology Inc, USA), p-Akt1 (1:50 dilution; Upstate, USA), cyclin D1 (clone SP4; 1:50 dilution; Shanghai Chang-Do Biotechnology Co. Ltd) were used. Each section was then incubated with Envision^{+TM}, peroxidase, mouse or rabbit (GeneTech) for 30 min. Finally, the sections were reacted with 0.02% 3, 3'-diaminoberzidine and 0.005% H_2O_2 in 0.05 mmol/L Tris-Hcl buffer and counterstained by hematoxylin.

The evaluation of the immunohistochemical staining was performed independently by two authors without knowledge of the clinicopathological information. P53, p21 and cyclin D1 immunoreactivities were observed in the nuclei of the cells, while bcl-2, bax, PTEN, p-Akt1, c-myc, Cox-2 and nm23-h1 in the cytoplasm. Only the immunoreactivity in the nucleus or cytoplasm of β-catenin was seemed as positive. The immunoreactive scores besides β-catenin and c-myc were determined by the sum of extension and intensity as reported previously[22] and were modified for some markers according to clinicopathological correlations. The intensity of the staining was scored using the following scale: 0, no staining of the tumor cells; +, mild staining; ++, moderate staining; and +++, marked staining. The area of staining was evaluated and recorded as a percentage: 0, less than 5%; +, 5%-25%; ++, 26%-50%; 3+, 51%-75%; and ++++, more than 75%. The combined score was recorded and graded as follows: -, 0-1; +, 2; ++, 3-5; +++, 6-7. More than 10% of the cancer cells showing elevated β-catenin labeling in the cytoplasm was recorded as positive expression. According to the immunostaining of c-myc in our study, we considered less than 40% cells expressed c-myc as negative.

Statistical analysis

Computerized statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 13.0. Clinical and histopathologic information and the results from the immunohistochemical studies of the tissue microarray were entered into a database. The variances of molecular expressions among different tissues and associations between molecular variables and clinicopathological data were analyzed with χ^2 test, but when the numbers of the cells in crosstables which had expected count less than 5 exceeded 25% or the minimum expected counts were less than 1, the Fisher's exact test was used. The relations among these molecules were analyzed by Spearman's bivariates correlation test. In all statistical analyses, a two-tailed P value $\leqslant$ 0.05 was considered statistically significant.

RESULTS

Clinicopathological data

Complete histological and clinical data of the patients were collected from patients' records. The median age for the study population was 58 years (range, 30-86 years). There were 24 patients with median age less than 58 years. There was a male predominance in the cancer patients (male: female ratio = 48:37). Thirty-two patients had positive lymph node metastasis, whereas 53 had negative. Fourteen patients had distant metastasis, and 71 had no distant metastasis. The clinical stages of these patients were strictly identified as A (n = 15), B (n = 33), C (n = 23) and D (n = 14) according to Dukes stage. After the second diagnostic assessment, there were 24 low, 50 moderately and 11 high differentiated tissues in these cancer tissue blocks according to the histological grades, while 18 tissues were diagnosed as adenomatous polyps and 9 were normal colon mucosa epithelium tissues.

Expression of p53, p21, cyclin D1, bcl-2 and bax

Owing to the short half-life of p53 protein, and its low expression levels in normal cells, wild-type p53 levels cannot be detected by IHC. In cancer cells, most p53 mutations lead to products that accumulate in the nuclei and can easily be detected by IHC. Positive immunostaining most

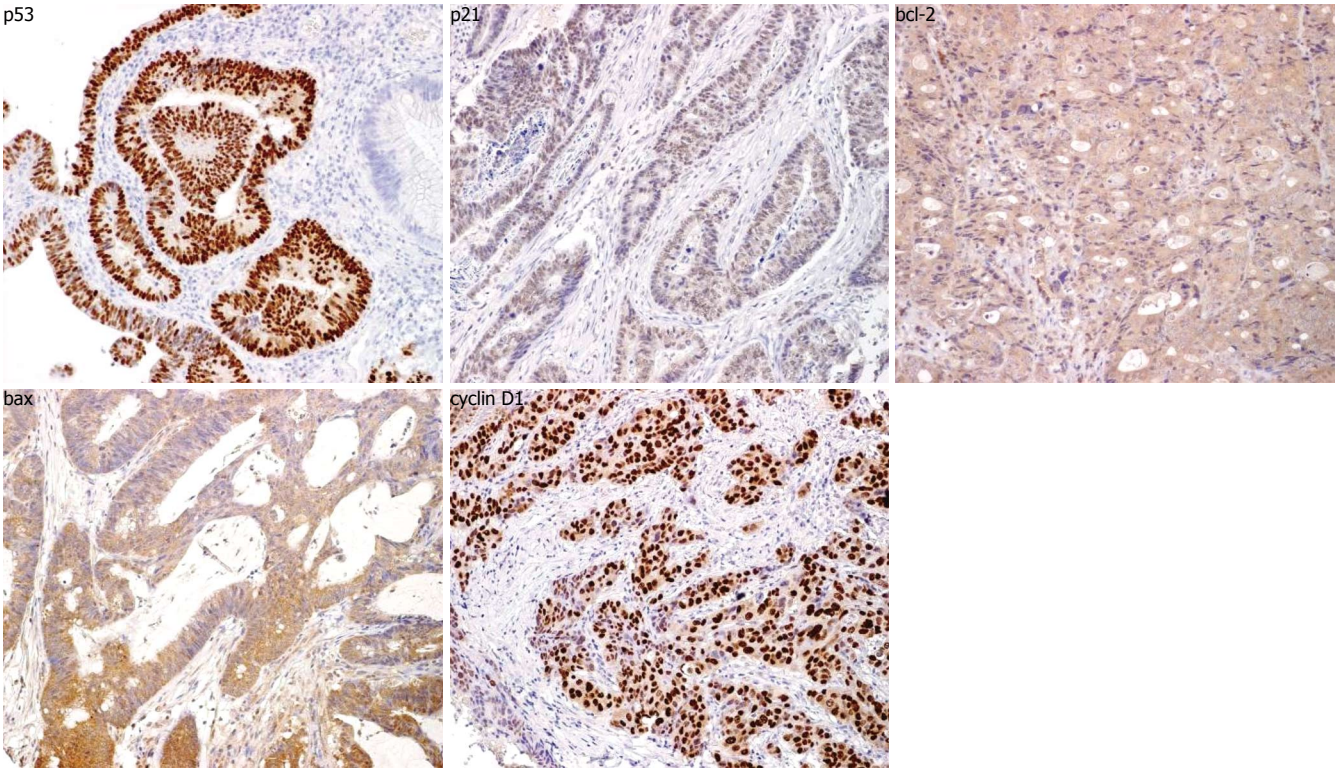

Figure 2 Immunophenotype of the investigated antigens (p53, p21, cyclin D1, bcl-2 and bax) in colon cancer (original magnification x 200). Positive stainings of p53, p21 and cyclin D1 were located in the cell nuclei, while those of bcl-2 and bax were in the cytoplasm.

Table 1 Comparison of IHC results (p53, p21, cyclin D1, bcl-2 and bax) among varying tissues

Groups	*n*	p53				*P*	p21				*P*	Cyclin D1				*P*	bcl-2				*P*	bax				*P*
		-	+	++	+++		-	+	++	+++		-	+	++	+++		-	+	++	+++		-	+	++	+++	
Cancer	85	39	6	19	21		19	9	28	26		17	16	30	22		5	1	46	32		21	9	39	16	
Adenomas	18	15	2	1	0	0.003[2]	8	5	4	1	0.000[1]	3	7	8	0	0.000[1]	1	1	7	8	0.001[2]	0	0	9	9	0.000[1]
Benign tissue	9	9	0	0	0		9	0	0	0		8	1	0	0		2	3	3	0		0	1	1	7	
Total	112	63	8	20	21		36	14	32	27		28	24	38	22		8	5	56	40		21	10	49	32	

[1]Chi-square test; [2]Fisher's exact test.

commonly represents accumulation of the stable protein product of a mutated *p53* gene that has lost its cell cycle-regulatory function. In this study, expression of p53 was identified in 46 of 85 (54%) colon cancer cell nuclei and 3 of 18 (16.66%) adenomas but absent in normal colon mucosa. Positive stainings of p21 and cyclin D1 were also located in the cell nuclei, while bcl-2 and bax were in the cytoplasm (Figure 2). The expression profiles of p21, cyclin D1, bcl-2 and bax are summarized in Table 1, which shows differences among these various tissues (cancer, adenomas, normal mucosa) regarding the IHC results of the cell cycle and apoptosis-associated protein. The relation between the immunohistochemical pattern and clinicopathological features is presented in details in Table 2. We found positive correlation between p53 and bcl-2 (r = 0.245, P = 0.010), while no significant correlation between p53 and bax (r = -0.081, P = 0.395).

Expression of Cox-2 and nm23-h1

Cox-2 and nm23-h1 were all correlated with the colon cancer progression as previously reported[8,9]. The protein expression of Cox-2 was detected in 81 of 85 (95%) colon cancer cytoplasm and 16 of 18 (88.88%) adenomas, and 6 of 9 (66.66%) normal mucosa (Figure 3). Sixty-two colon cancer patients showed a strong positive staining of nm23-h1 (score ++~+++) in the cell cytoplasm. The details of the two genes expression profiles are shown in Table 3 and the relations with clinicopathological parameters are presented in Table 4.

Expression of PTEN, p-Akt1, β-catenin, c-myc

Expressions of PTEN, p-Akt1, β-catenin, c-myc proteins were detected in the cell cytoplasm (Figure 4). Immunohistochemical results of PTEN, p-Akt1No showed no significant difference among the different tissues (data not shown). Significant difference in the expressions of β-catenin and c-myc was found among the benign mucosa, adenomas and malignant tissues (Table 5). β-catenin protein expression had a positive correlation with c-myc expression (r = 0.483, P = 0.000), thereby suggesting their

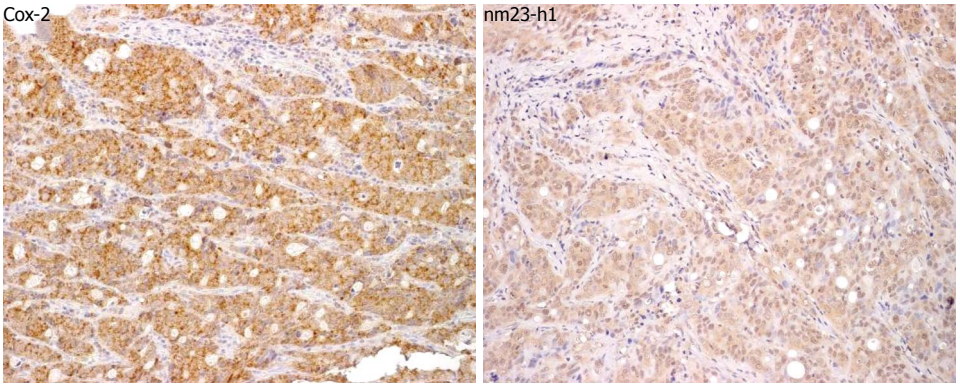

Figure 3 Immunophenotype of the investigated antigens (Cox-2 and nm23-h1) in colon cancer (original magnification x 200). The protein expressions of Cox-2 and nm23-h1 were detected in the cytoplasm.

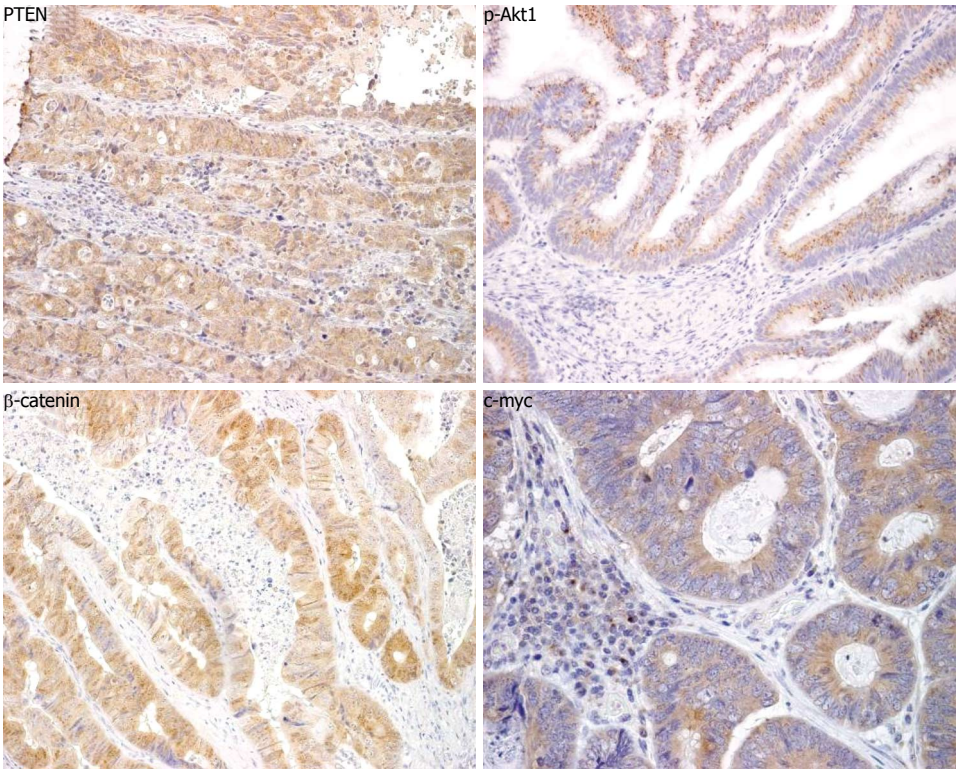

Figure 4 Immunophenotype of the investigated antigens (PTEN, p-Akt1, β-catenin and c-myc) in colon cancer (original magnification x 200). Expressions of PTEN, p-Akt1, β-catenin and c-myc were detected in the cell cytoplasm.

potent roles in *Wnt* pathway during the colon carcinogenesis. Correlations of the protein expression profiles with the clinicopathological features are shown in Tables 6 and 7.

DISCUSSION

Colon carcinogenesis is characterized by distinct morphological, genetic and cellular events. Development and progression of colon cancer to metastasis and lethal state are believed to be driven by multiple genetic alterations, the nature of which has remained poorly understood. Several pathways, such as cell cycle and apoptosis regulation, *Wnt* and PI3K/Akt pathways and so on, have been suggested to be involved in the progression[5,22,23]. However, the specific molecular alterations are largely dependent on the genetic background of the individual tumor. Therefore, investigations of molecular changes in tumors representing the entire disease spectrum may enhance our understanding of mechanisms involved in colon tumorigenesis.

To efficiently investigate the various molecules potentially relevant for colon tumor biology and to determine their potential clinical significance, large-scale analysis of multiple molecules in the same tumor tissues is required. The newly evolved and recently validated tissue microarray technique allows such molecular profiling of cancer specimens by immunohistochemistry[24,25]. In 1998, Kononen *et al*[24] introduced tissue microarrays (TMAs) as a powerful technology to rapidly visualize molecular targets such as genes and gene products in thousands of tissue specimens at a time. Furthermore, the use of TMAs for immunophenotyping of malignant tumors has recently been validated by Hoos *et al*[25] who demonstrated immunohistochemical analysis to characterize the significance of alterations in the p53 pathway and other cell cycle-related molecules in a histopathologically well-characterized cohort of patients with Hurthle cell (HC) neoplasm. In this study, two tumor tissue microarrays were constructed that allowed us to investigate the pattern of protein expressions of multiple genes and the relationships between the gene alterations and biological behaviors of colon cancer. We found that

Table 2 p53, p21, cyclin D1, bcl-2 and bax status in relation to clinicopathological parameters

Clinicopathalogical parameters		Total	p53				P	p21				P	cyclin D1				P	bcl-2				P	bax				P
			-	+	++	+++		-	1+	2+	3+		-	1+	2+	3+		-	+	++	+++		-	+	++	+++	
Age	< 58	39	17	5	8	9	0.301[1]	10	4	13	12	0.966[1]	8	6	13	12	0.752[1]	3	1	21	13	0.654[2]	11	3	17	8	0.772[1]
	⩾ 58	46	22	1	11	12		9	5	15	14		9	10	17	10		2	0	25	19		10	6	22	8	
Gender	Male	48	24	2	10	12	0.608[1]	12	1	17	16	0.040[1]	11	9	16	12	0.891[1]	5	1	26	16	0.139[2]	14	6	20	8	0.579[1]
	Female	37	15	4	9	9		7	8	11	10		6	7	14	10		0	0	20	16		7	3	19	8	
Histological grade	Low	24	18	0	3	3		10	4	3	6		9	4	8	3		3	1	16	4		11	3	9	1	
	Moderate	50	17	3	13	17	0.005[2]	6	3	21	18	0.013[2]	5	9	20	16	0.082[2]	2	0	24	23	0.075[2]	8	6	25	11	0.044[2]
	High	11	4	3	3	1		3	2	4	2		3	3	2	3		0	0	6	5		2	0	5	4	
Lymph node metastasis	Negative	53	19	4	16	14	0.067[1]	9	4	19	19	0.199[1]	11	11	20	11	0.575[1]	2	0	29	21	0.407[2]	10	6	26	11	0.457[1]
	Positive	32	20	2	3	7		10	5	9	7		6	5	10	11		3	1	17	11		11	3	13	5	
Distant metastasis	Negative	71	30	6	18	17	0.292[2]	14	8	23	23	0.644[2]	12	16	24	19	0.100[2]	3	0	39	29	0.052[2]	17	7	32	15	0.694[2]
	Positive	14	9	0	1	4		5	1	5	3		5	0	6	3		2	1	8	3		4	2	7	1	
Dukes stage	A	15	5	2	6	2		3	2	5	5		2	7	4	2		0	0	9	6		2	2	8	3	
	B	33	10	2	9	12	0.061[2]	3	2	13	13	0.286[2]	7	4	15	7	0.038[2]	1	0	18	14	0.487[2]	6	3	16	8	0.716[2]
	C	23	15	2	3	3		8	4	5	5		3	5	5	10		2	0	12	9		9	2	8	4	
	D	14	9	0	1	4		5	1	5	3		5	0	6	3		2	1	8	3		4	2	7	1	

[1] Chi-square test; [2] Fisher's exact test.

Table 3 Comparison of IHC results of Cox-2 and nm23-h1among varying tissues

Histology	n	Cox-2				P	nm23-h1				P
		-	+	++	+++		-	+	++	+++	
Cancer	85	4	4	30	47	0.002	5	7	33	29	0.007
Adenomas	18	2	0	13	3		2	1	13	2	
Benign tissue	9	3	0	1	5		4	1	2	1	
Total	112	9	4	44	55		11	9	48	32	

Fisher's exact test.

Table 4 Cox-2 and nm23-h1 status in relation to clinicopathological data

Groups		n	Cox-2				P	nm23-h1				P
			-	+	++	+++		-	+	++	+++	
Age (yr)	< 58	39	3	1	17	18	0.239	4	3	21	10	0.272
	⩾ 58	46	1	3	13	29		1	4	22	19	
Gender	Male	48	1	2	17	28	0.628	5	5	24	14	0.157
	Female	37	3	2	13	19		0	2	19	15	
Histological grade	Low	24	3	3	12	6		3	6	13	2	
	Moderate	50	1	1	14	33	0.008	2	0	23	24	0.000
	High	11	0	0	4	7		0	1	7	3	
Lymph node metastasis	Negative	53	1	2	17	33	0.223	2	3	29	18	0.434
	Positive	32	3	2	13	14		3	4	14	11	
Distant metastasis	Negative	71	1	3	27	40	0.026	3	3	39	26	0.008
	Positive	14	3	1	3	7		2	4	4	3	
Dukes stage	A	15	0	1	3	11	0.016	1	1	11	2	0.014
	B	33	0	0	12	21		0	1	16	16	
	C	23	1	2	12	8		2	1	12	8	
	D	14	3	1	3	7		2	4	4	3	

Fisher's exact test.

Table 5 Comparison of the IHC results of β-caternin and c-myc among varying tissues

Histological characteristics	Total	β-caternin		P	c-myc		P
		-	+		-	+	
Cancer	85	15	70	0.034	16	68	0.003
Adenomas	18	5	13		4	14	
Benign tissue	9	5	4		6	2	
Total	112	25	87		26	84	

Fisher's exact test.

Table 6 PTEN and p-Akt1 status in relation to clinicopathological data

Clinicopathological parameters		Total	PTEN				P	p-Akt1				P
			-	+	++	+++		-	+	++	+++	
Age	< 58	39	3	2	20	14	0.860[2]	10	0	16	13	0.408[1]
	⩾ 58	46	2	2	22	20		9	3	19	15	
Gender	Male	48	4	4	24	16	0.167[2]	14	0	19	15	0.096[1]
	Female	37	1	0	18	18		5	3	16	13	
Histological grade	Low	24	4	2	14	4		11	3	6	4	
	Moderate	50	1	2	24	23	0.029[2]	8	0	26	16	0.000[2]
	High	11	0	0	4	7		0	0	3	8	
Lymph node metastasis	Negative	53	3	2	22	26	0.132[2]	7	1	26	19	0.032[1]
	Positive	32	2	2	20	8		12	2	9	9	
Distant metastasis	Negative	71	2	3	37	29	0.060[2]	13	3	31	24	0.275[2]
	Positive	14	3	1	5	5		6	0	4	4	
Dukes stage	A	15	0	1	7	7	0.281[2]	1	0	6	8	0.106[2]
	B	33	1	1	15	16		4	1	17	11	
	C	23	1	1	15	6		8	2	8	5	
	D	14	3	1	5	5		6	0	4	4	

[1] Chi-square test; [2] Fisher's exact test.

Table 7 β-catenin and c-myc status in relation to clinicopathological data

Clinicopathological parameters		Total	β-caternin		*P*	c-myc		*P*
			-	+		-	+	
Age	< 58	39	6	33	0.983[1]	9	29	0.117[1]
	⩾ 58	46	7	39		5	41	
Gender	Male	48	8	40	0.689[1]	10	38	0.237[1]
	Female	37	5	32		4	32	
Histological grade	Low	24	10	14		11	13	
	Moderate	50	3	47	0.000[2]	3	46	0.000[2]
	High	11	0	11		0	11	
Lymph node metastasis	Negative	53	4	49	0.011[1]	4	48	0.005[1]
	Positive	32	9	23		10	22	
Distant metastasis	Negative	71	8	63	0.020[1]	6	65	0.000[1]
	Positive	14	5	9		8	5	
Dukes stage	A	15	0	15		0	15	
	B	33	2	31	0.008[2]	2	31	0.000[2]
	C	23	6	17		4	19	
	D	14	5	9		8	5	

[1]Chi-square test; [2]Fisher's exact test.

the 1.0-1.5 mm cores used to create the arrays were easy to work with, included enough tumor tissue that histological relationships were easily evaluated, and focused attention on limited regions of tumor, thus ensuring high reproducibility of scoring. Furthermore, at the same time, we could study the morphous of cells and protein expression parallely and avoid the variance in results in different experiment conditions as seen in the conventional technology.

The cell cycle-regulatory machinery is a complex system of proteins regulating each other's activity and controlling the division of cells[26]. We selected p53, p21, cyclin D1 and bcl-2/bax for analysis as target proteins participating in the regulation of proliferation and apoptosis, which are known to be deranged in cancer cell cycles, and which have been shown to affect survival of colorectal carcinomas. The p53 tumor suppressor plays a pivotal role in cell cycle regulation and apoptosis. Mutations in the *p53* gene are among the most common mutations encountered in human malignancy. Wild-type p53 along with other cellular growth factors activate *p21* gene expression and the corresponding p21 protein triggers cell-cycle arrest in the G_1 phase[27]. In colorectal cancer cells, mutated p21 neither suppressed apoptosis nor affected cell survival[28]. In addition to cell-cycle control, p53 mediates programmed cell death through the bcl-2/bax apoptotic pathway[29]. In this study, we observed that p53 was undetectable in normal colon tissue and over-expressed in only three adenomas and 54% of the adenocarcinomas. When the expression of p53 with clinicopathological parameters was compared, only the significant relation with histological grade could be seen, thereby indicating that p53 may be involved in the late stage of colon carcinogenesis and in the malignant progression of colon cancer. As to the others genes, there were significant differences among the three groups (normal mucosa, adenoma and cancer) regarding the immunohistochemical results (cyclin D1, $P = 0.000$; bcl-2, $P = 0.001$; bax, $P = 0.000$). Comparing these markers with the clinicopathological features, we observed the correlation of histological grades with bax, and Dukes stage with cyclin D1. Thus, we can speculate that cyclin D1, bcl-2 and bax aberrations may involve in the colon cancer development and the decreased expression of bax can accelerate the cancer tissue further differentiate into advanced stage, and malignant colon tissue highly expressed cyclin D1 may acquire the invasive potent. In addition, though the difference of this protein expression between distant metastasis-positive group and -negative group was not significant ($P = 0.052$) in this study, colon cancer with alteration of bcl-2 expression may facilitate to metastasize to distance sites. In our study, p21 over-expression was found in 64 of 82 (78.04%) cancers and was correlated with advanced stage colorectal cancer, which is in agreement with a previous report[30]. Furthermore, IHC revealed that some cancer cells expressed both p21 and p53, suggesting that p21 can also be activated by a p53-independent mechanism. A previous study reported that p53 can inhibit bcl-2 gene expression by transcriptional activation of the pro-apoptotic bax gene. But the relationship between p53 and bax in this cohort tissue was not established. However, our results showed that the expression of p53 had positive correlation with bcl-2. The precise mechanism of regulation of bcl-2/bax pathway through p53 needs further study.

There are two distinct Cox isoenzymes, namely constitutive Cox-1 and inducible Cox-2. It was reported that Cox-2 elevated in human esophagus, skin, and colon cancers, and the Cox-2 inhibitor, celecoxib, inhibited intestinal tumor multiplicity in a mouse model and reduced the number of adenomatous polyps in a familial adenomatous polyposis patient[16,17]. Enhanced Cox-2 expression has been related to tumor differentiation, distant metastasis and Dukes stage[31,32]. In present study, Cox-2 immunoreactivity was increased in the colon carcinoma (91%) and adenomas (88%). However, the expression of Cox-2 was correlated with less advanced grade, fewer distant metastases and lower Dukes stage in these cohort colon cancer tissues. These results indicate that Cox-2 over-expression might be an early event during the tumorigenesis in the colon and its role in progression of colon cancer deserves further investigation. Although a reduced expression of nm23-h1 has been shown to be correlated with a high metastatic potential in some human cancers, such as colorectal cancers, conflicting data have been reported[9,33]. In our study, expression of nm23-h1 was detected in 79 of 85 (92.94%) cancers, 16 of 18 (88.88%) adenomas and 4 of 8 (50%) normal tissues. The decreasing tendency of expression of nm23-h1 was found to be associated with the aggravation of differentiation, distant metastasis in the cohort patients. Therefore, the exact role of nm23-h1 in development and progression of the colon cancer needs further studies.

β-catenin is known to complex with E-cadherin to form intercellular junctions. It also participates in the *Wnt*-signaling pathway, which frequently is disrupted in colorectal carcinomas by adenomatous polyposis

coli (APC) or β-catenin mutations[15]. Translocation of β-catenin to the nucleus with transcription start of cyclin D1 and metalloproteinase 7 could lead to more aggressive colorectal carcinomas[34]. C-myc was also found to be involved in the *Wnt* pathway and seemed as a target gene of β-catenin/TCF[35]. It has been extensively documented that c-myc is over-expressed at RNA and protein levels at both early and late stages of the colorectal tumorigenesis[36]. Previous immunohistochemical studies with β-catenin showed distinct subcellular expression patterns in the colorectal carcinomas, adenomas, and benign epithelium, whereas benign tissue almost universally expressed β-catenin on the plasma membrane only, adenomas and carcinomas expressed β-catenin to varying degrees in the cytoplasm and in the nucleus as well[6,36,37]. This finding is not surprising, given the intracellular pathway described above. However, correlations between alterations of β-catenin expression in colorectal cancer and outcome variables have not been consistent[38-40]. In this study, different expressions of these two molecules were found among the varying types of tissues. β-catenin was expressed in 70 of 85 (82.83%) cancer tissues, 13 of 18 (72.22%) adenomas and only 4 of 9 (44.44%) para-cancerous normal mucosa. Similarly, positive expression of c-myc was detected in 68 of 84 (80.95%) colon cancers, 14 of 18 (77.77%) adenomas as well as 25% benign tissues. In addition to the results which showed positive correlation between the β-catenin and c-myc in our study ($r = 0.483$, $P = 0.000$), we can assume that over-expression of β-catenin in the cytoplasm and c-myc might be early events during the carcinogenesis in human colon *via* involving in the *Wnt* pathway which always distorts during colon tumorigenesis. However, the cancer tissues in the cohort with over-expression of β-catenin or c-myc exhibited less capacity to differentiate into advanced grade and invasive potential, which are contradicted to some previous reports[40,41]. The exact roles of β-catenin and c-myc aberration in progression of colon cancer requires further investigations.

The phosphatidylinositol-3 kinase (PI3K)/Akt is an important survival signal pathway that has been shown to be crucial in the regulation of balance between pro-apoptotic and survival (anti-apoptotic) signal[42]. The phosphorylated Akt level can monitor cell growth and resistance to apoptosis, indicating that activation of Akt plays an important role during the progression of colorectal carcinomas by helping promote cell growth and rescue cells from apoptosis. PTEN is a phosphatase that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell-cycle arrest and apoptosis[43]. One study reported that PTEN protein expression was abnormal when compared the cancer with benign tissue[44], but same phenomenon was not found among the cohort patients including in this study. However, at the same time, PTEN expression detected by us was found to be related to tumor histological grade and p-Akt1 was related to lymph node metastasis. In this study, the expression profiles demonstrated that though did not involve in the early development of tumorigenesis, these two molecules may conduce to the lately progression of colon cancer which showed lymph node or distant metastasis. Contradiction between the results of p-Akt1 expression in our study and a previous study[11] which reported that Akt over-expression occurred frequently during human colon carcinogenesis might be because the gene background (FAP, HNPCC or sporadic CRC) of the patients studied were not strictly identified and the antibody used which was a polyclonal antibody in our study.

In summary, tissue microarrays (TMAs) combined with immunohistochemical staining for colon cancer, adenomas and normal mucosa showed that TMAs technology with 1.0 to 1.5 mm core tissue adequately represents the immunohistochemical pattern of the colon tissue. It considerably reduces the cost and labor needed to process tissue slides and enrich the technical spectrum of histopathology. The over-expressions of p53, cyclin D1, bcl-2, Cox-2, β-catenin and c-myc and the low or no expression of bax might be involved in the colon tumorigenesis and PTEN, p-Akt1 may contribute to the progression of colon cancer at late stage. No or low expression of nm23-h1 in colon cancer may contribute to the cancer cells acquired the invasion and distant metastasis potential. Further study needs to investigate the precise mechanism of colon cancer development and progression.

COMMENTS

Background

Studies of single molecular marker have not been successful in defining the biology of the colon cancer, so we explored multiple molecules regulating the colon tumorigenesis by using tissue microarray combining with immunohistochemical staining which can help us complete the time- and people-consuming work.

Research frontiers

In this study, the most worthwhile hotspot I think was investigating 11 molecules expressions by using the tissue microarray which allows rapid visualization of molecular targets at a time in protein level.

Innovations and breakthroughs

Firstly I should mention that in this study, we explored 11 molecular expressions at a time during colon carcinogenesis which was seldom found in our country. In addition, the expressions of these molecules were detected by using tissue micoarray which is a new high-throughput biological technique.

Applications

Tissue microarray can facilitate rapid translation of molecular discoveries to clinical applications used, so we can speculate that the tissue microarray technology which is fast, convenient and economic may have potential dominant position in macro-scale detection of tissue specimens in cancer studies.

Terminology

In 1998, Kononen and Kalloiniemi developed tissue microarrays (TMAs) whereby an ordered array of tissue samples are placed on a single slide. Once constructed, the TMA can be probed with a molecular target (DNA, RNA or protein) for analysis by immunohistochemistry, fluorescence *in situ* hybridization (FISH) or other molecular detection methods, enabling high-throughput *in situ* analysis of specific molecular targets in hundreds or even thousands of tissue specimens.

Peer review

In this study, the authors investigated the expressions of 11 cancer related genes involving cell proliferation, cell cycle, apoptosis, as well as signal pathway in human colon cancer, adenoma and para-cancerous mucosa by using tissue microarray, a new biological technique, combining with immunohistochemical staining at a time. The results from this study may manifest the colon cancer situation at some level and have some reference values in our country.

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BASIC RESEARCH

Neural mechanism of acupuncture-modulated gastric motility

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Abstract

AIM: To investigate the acupuncture-modulated gastric motility and its underlying neural mechanism.

METHODS: Intragastric pressure and/or waves of gastric contraction in rats were recorded by intrapyloric balloon and changes of gastric motility induced by acupuncture stimulation were compared with the background activity before any stimulation. Gastro-vagal or splanchnic-sympathetic nerves were recorded or cut respectively for investigating the involvement of autonomic nerve pathways. Spinalization experiment was also performed.

RESULTS: Acupuncture-stimulation by exciting Aδ and/or C afferent fibers, could only modulate gastric motility. Acupuncture-stimulation on fore- and hind-limbs evoked a moderate gastric motility followed by increased vagus discharges with unchanged sympathetic activity, while the same stimulus to the acupoints in abdomen resulted in reversed effects on gastric motility and autonomic nervous activities. The inhibitory gastric response was completely abolished by splanchnic denervation, but the facilitative gastric response to stimulation of acupoints in limbs was not influenced, which was opposite to the effect when vagotomy was performed. The similar depressive effects were produced by the stimulation at the acupoints homo-segmental to the gastric innervation in the animals with or without spinalization. However, the facilitation induced by the stimulation at the acupoints hetero-segmental to the gastric innervation was not observed in the spinalized animals.

CONCLUSION: Facilitative effects of stimulating hetero-segmental acupoints are involved in the intact preparation of vagal nerves and spinal cord, while the inhibitory response induced by stimulating homo-segmental acupoints is involved in the intact preparation of sympathetic nerves. Only the acupuncture-stimulation with intensity over the threshold of Aδ and/or C afferent fibers can markedly modulate gastrointestinal motility.

Key words: Gastric motility; Acupuncture-stimulation; Intensity of acupuncture stimulation; Aδ-fiber; C-fiber; Autonomic nervous system; Supraspinal circuit

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INTRODUCTION

Acupuncture, a commonly used neuromodulating technique, has been widely accepted in the treatment of pain syndromes[1]. It modulates the functions of visceral organs by inducing activation of the somato-visceral reflexes and change of the autonomic nervous system[2-4].

Electro-acupuncture therapy is increasingly adopted in Western countries. Acupuncture involves stimulating specific somatic points on the body by puncturing the skin with a needle. Heat, pressure or impulses of electrical energy can also stimulate the points. It was reported that the nervous system, neurotransmitters and endogenous substances respond to electro-acupuncture. Abundant information is now available concerning the neurobiological mechanisms of acupuncture related with the neural pathways and neurotransmitters/hormonal factors that mediate autonomic regulation, pain relief and other therapeutics[5]. Being a kind of traditional technique with a long history, acupuncture has been used to treat a variety of diseases including pain. Electrophysiological studies showed that acupuncture might inhibit the neuron discharge induced by pain of both somatic and visceral sources at different levels of the central nervous system, which gives a good explanation for the clinic phenomena in which acupuncture produces quick effect on somatic and visceral pain and relatively slow and long post-effect. Though there are data relating the modulation of somatic afferent inputs on visceral nociception or dysfunction, evidence for the role of acupuncture in this process is not sufficient.

The Committee of NIH published a report on the indications of acupuncture in November 1997. The summary of the consensus statement indicates that there is clear evidence that needle acupuncture treatment is effective on postoperative and chemotherapy-induced nausea and vomiting during pregnancy and post-operative dental pain[6]. Recent investigations suggest that acupuncture modulations on visceral functional activities can be mediated via the autonomic nervous system[2,4,7-9].

In the present study, therefore, we investigated the modulation of gastric motility by manual/electrical acupuncture at representative meridian-acupoints in different parts of the body and its underlying mechanism involved in different afferent fibers, autonomic nervous system and the supraspinal center.

MATERIALS AND METHODS

Animal preparation

All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of China Academy of Chinese Medical Sciences. Experiments were performed on 48 adult male Sprague-Dawley rats weighing 250-300 g. The rats were fasted overnight with free access to water and anesthetized with an intraperitoneal injection of urethane (1.0-1.2 g/kg). The trachea was cannulated and a catheter was inserted into one of the jugular veins for infusion of necessary solutions or anesthetics. The abdomen was opened by midline, and then a small longitudinal incision was made in the duodenum about 2-3 cm from the pylorus. A small balloon made of condom was inserted via the incision into the pyloric area and fixed. Another catheter (1 mm in inner diameter) was also inserted into the same incision to drain digestive juices secreted from the stomach. The balloon was filled with about 0.2-0.5 mL warm water, which gave the pressure at about 80-150 mmH_2O. Pressure in the balloon was measured by a transducer (TP-400T, Nihon Kohden) through a thin polyethylene tube (1.5 mm in outer diameter) and then input into a polygraph amplifier (RM-6000, Nihon Kohden) and led to a data acquisition system (Power-Lab/8 s, AD Instruments) for further analysis. Demi-fasting gastric motor activity was recorded as a control for at least 1 h before any stimulation was applied.

Changes of gastric motility induced by the stimulation were compared with the background activity in terms of intragastric pressure and/or waves of gastric contraction. If the changes of gastric motility during stimulation were 20% more or less than the background activity, the response was considered to have an excitatory or inhibitory regulation.

Blood pressure in a common carotid artery and heart rate were continuously monitored. The rectal temperature was kept constantly around 37℃ by a feedback-controlled heating blanket.

Recording of neural activity, severance of autonomic nerves and transection of spinal cord

To investigate whether vagal or sympathetic pathways are involved in the stimulatory or inhibitory effects of acupuncture on gastric motility, subdiaphragmatic gastro-vagal or sympathetic postganglionic nerves coming from a celiac ganglion and innervating the gut branch were dissected under microscope. Discharges of the vagal or sympathetic nerves were put into a polygraph (RM-6000, Nihon Kohden) amplifier through bipolar platinum wire electrodes and input into a data acquisition system (Power-Lab) for further analysis. In experiments, gastro-vagal nerves were cut in subdiaphragmatic bilateral truncal vagotomy (an additional cervical bilateral vagotomy was also performed in several cases) or postgangliotic nerves innervating the gut branch were cut in sympathetomy (an additional bilateral splanchnicotomy was also performed in several cases) in 24 rats.

In spinalization experiments, laminectomy was performed in C_8-T_1 of 10 animals. Frozen physiological saline was used to produce a reversible cold block (five rats), or transected with knife to determine the involvement of supraspinal circuit in acupuncture-induced effect on gastric motility.

Acupuncture stimulation

A needle (0.3 mm in diameter) was inserted into the skin and its underlying muscles at different acupoints on the body. The needle was rotated clockwise and anti-clockwise at 2 Hz for 30 s. Based on the anatomical localization in rats as compared with that in human body, the stimulated acupoints included St-13 (acupoint of Stomach-Meridian in upper-chest), Li-11 (acupoint of Large intestine-Meridian in forelimb), Cv-6 (acupoint of conception-vessel) and St-21 (acupoint of Stomach-Meridian in abdomen), Bl-21 (T_{12} segment, acupoint of Bladder-Meridian in dorsum) and St-36 (acupoint of Stomach-Meridian in hindlimb).

In electroacupuncture experiments, acupoints were stimulated by a pair of needle-electrodes inserted 0.3 cm deep into the skin and electroacupuncture intensities were chosen as the multiples of the threshold for the activation of Aδ-fiber or C-fiber, including 0.8 (0.8-$T_{A\delta}$, non-noxious stimuli, 1.42 ± 0.36 mA) and 2-times of the Aδ-fiber reflex threshold (2-$T_{A\delta}$, slight nociceptive stimuli, 3.62 ± 0.44 mA), and 1.5 times (1.5-T_C, strong nociceptive stimuli, 7.68 ± 0.53 mA) of C-fiber reflex threshold.

The thresholds of Aδ-fiber and C-fiber reflexes were decided by electrical stimulation of the sural nerve territory, which could elicite a two-component reflex response in the ipsilateral biceps femoris muscle. The first-component had a low threshold (1.64 ± 0.33 mA) response with a short latency (13.2 ± 1.4 ms) and duration (24.2 ± 1.5 ms). The second-component had a longer latency (158.8 ± 10.7 ms) and duration (243.8 ± 31.5 ms) and a higher threshold (4.84 ± 0.67 mA). These electrophysiological features clearly suggested that the two-components were produced due to the different afferent volleys activated by sural nerve stimulation. According to our previous study, it seems that the first component with a low-threshold and a short-latency could be elicited by activating the Aδ-fibers, whereas the second with a higher threshold and a longer latency might result from the activation of the unmyelinated C-fiber afferents[10].

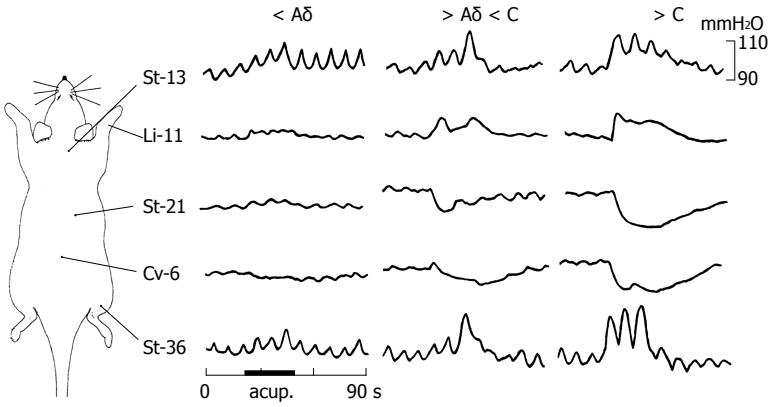

Figure 1 Gastric motility response to electro-acupuncture stimulation with different intensities. Gastric motility was recorded for 90 s, of which the first 20 s was the duration of spontaneous background of gastric activity, 21-50 s was the period of acupuncture stimulation at acupoints, poststimuli activity was recorded for 51-90 s. *Dash line* represents located abdominal acupoints.

Experimental procedures

The rats were kept in supine position and gastric motor activity was first analyzed visually to detect the waves of contractions. A standard protocol was employed for the determination of effects of acupuncture-stimulation on gastric motility response. A background gastric activity was recorded for 5-10 min followed by a test of the responses to manual or electro-acupuncture at the acupoints for 30 s. The post-stimuli response was recorded for another 5-10 min.

After a series of experiments was finished, the rats were sacrificed under deep anesthesia (urethane, 2 g/kg ip).

Statistical analysis

The data obtained before and after intervention between the two groups were compared statistically by independent *t*-test and paired *t*-test respectively. $P < 0.05$ was considered statistically significant. All data were expressed as mean ± SE.

RESULTS

Gastric motor characteristic

Gastric motor characteristics were observed in 42 rats. When the intrapyloric balloon pressure was increased to about 80-200 mmH_2O, the rhythmic waves of contractions in pyloric area were observed. When the pressure was maintained at about 100 mmH_2O by expanding the volume of the balloon with warm-water, rhythmic contractions occurred at a rate of four to six per minute and these rhythmically gastric contractions could be recorded in either the spinalized rats or the rats with intact central nervous system. The gastric contractions could also be recorded after denervation of gastric nerve branches of the vagal or splanchnic nerves.

Gastric motility response to electro-acupuncture stimulation with different intensities

The response of gastric motility to the activation of Aδ-/C-fibers was elicited by stimulating the ipsilateral sural nerve. The effects of acupuncture stimulation to Li-11 in the forelimb, St-13 in upper-breast, St-21 and Cv-6 in the abdomen and St-36 in the hindlimb on gastric motility were studied. As illustrated by an representative example in Figure 1 in combination with data in Figure 2,

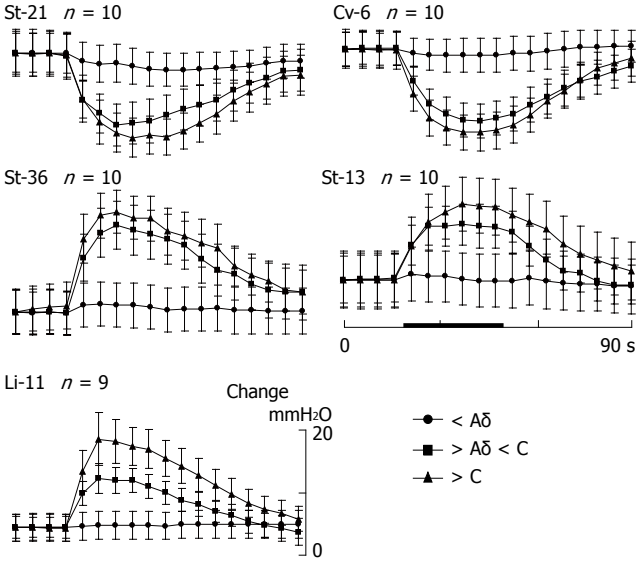

Figure 2 Quantitative analysis of gastric motility in strength-dependent response to electro-acu puncture-stimulation applied to the acupoints on different parts of body. "< Aδ": the intensity of the stimulation less than the threshold for activation of Aδ-fiber; "> Aδ < C": the intensity of the stimulation over the threshold for activation of Aδ-fiber, but less than the threshold for activation of C-fiber; "> C": the intensity of the stimulation over the threshold for activation of C-fiber.

the acupuncture stimulation with the intensity of 0.8-$T_{Aδ}$ (1.42 ± 0.36 mA) at the above five acupoints did not produce any significant influences on the gastric motility. The acupuncture stimulation with the intensity of 2-$T_{Aδ}$ (3.62 ± 0.44 mA) at the acupoints of Li-11, St-13 and St-36 elicited a mild-moderate facilitation on the gastric motility, whereas the acupuncture stimulation with the same intensity to the acupoints of St-21 and Cv-6 brought about a moderate depression of gastric motility. The acupuncture stimulation with the intensity of 1.5-T_C (7.68 ± 0.53 mA) at the acupoints of Li-11, St-13 and St-36 resulted in a strong excitatory gastric motility. In contrast, a strong inhibition on the gastric motility was induced by the same acupuncture stimulation at the acupoints of St-21 and Cv-6.

Electro-acupuncture stimulation could induce regulatory effects on gastric motility. Moreover, facilitation/depression on the gastric motility induced by acupuncture stimulation was intensity-dependent. Only the stimulation with an intensity over the threshold for activation of Aδ- and/or C-fibers could modulate gastric

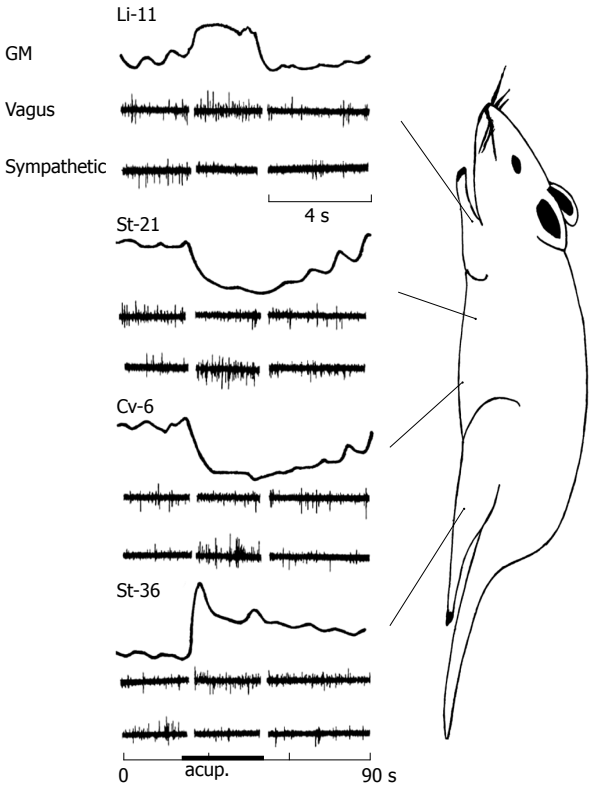

Figure 3 Gastric motility and autonomic nerve responses to acupuncture stimulation at different acupoints. In each acupoint stimulus, gastric motility (GM), activities of gastric vagal (vagus) and postganglionic sympathetic nerve branches to the stomach (symp), were simultaneously recorded.

motility ($P < 0.05$, $P < 0.001$) and the powerful effects were produced when the strongest electro-acupuncture stimulation, *i.e.* 1.5-Tc, was given ($P < 0.05$, $P < 0.001$).

Gastric motility and activity of autonomic nerves in response to acupuncture stimulation

Gastric motility in the most rats showed various responses to acupuncture stimulation at some representative acupoints on several meridians and different parts of the body. Manual acupuncture stimulation at either Li-11 in forelimb or St-36 in hindlimb led to a moderate facilitation of gastric motility with a rapid enhancement at the beginning of stimulation, followed by a tonic gastric contraction lasting throughout the period of acupuncture stimulation. The typical effects of acupuncture stimulations at Li-11 or St-36 acupoints on discharges of the branches of both gastric vagal and postganglionic sympathetic nerves to the stomach are shown in Figure 3. Acupuncture stimulation at either Li-11 ($n = 10$) or St-36 ($n = 16$) acupoints markedly increased vagal discharges (339.42 ± 133.2 spikes/min *vs* 381.6 ± 135.42 spikes/min, $P < 0.05$). The latency of increased discharges was about 2-3 s after the onset of stimulation. The facilitated response lasted for 30 s with a maximum response at about 10 s after the onset of acupuncture stimulation. In contrast to vagus response, spontaneous activity of the sympathetic nerve branch was not clearly influenced by acupuncture stimulation at either Li-11 ($n = 12$) or St-36 acupoints ($n = 12$) ($P > 0.05$). Generally, in the animals with high spontaneous activity of sympathetic nerves, the same acupuncture stimulation could mildly inhibit the discharges. However, the stimulation could produce a slight excitation in the animals with lower spontaneous sympathetic activity.

The same stimulus to the acupoints of either St-21 or Cv-6 in abdomen, resulted in high suppression with a rapid onset on gastric tonic motility, followed by an obvious inhibition on the rhythmic wave of contraction. The suppression maintained throughout the period of acupuncture stimulation. In the autonomic nerve participation, acupuncture stimulation at either St-21 ($n = 12$) or Cv-6 ($n = 10$) acupoints markedly increased sympathetic discharges (476.4 ± 150.78 spikes/min *vs* 638.82 ± 171.78 spikes/min, $P < 0.05$). In most cases, spontaneous vagal activity could also be inhibited slightly ($P > 0.05$, $n = 12$) or remained unchanged (Figure 3).

Effect of acupuncture stimulation on gastric motility involved in autonomic nerves

In order to discover their contribution to the facilitation/inhibition of acupoint-visceral reflex responses, denervation of the vagal or sympathetic nerves was performed in the present study.

The effect of acu-stimulation on gastric motility was examined in the rats after amputation of bilateral splanchnic nerves just beneath the diaphragm.

The inhibitory gastric response induced by acupuncture stimulation to acupoints of St-21 ($n = 9$), Cv-6 ($n = 9$) or Bl-21 ($n = 9$) was completely abolished after sympathectomy of the bilateral splanchnic nerves, but the facilitative gastric response induced by acupuncture stimulation to acupoint of Li-11 ($n = 9$) was not influenced significantly and St-36 ($n = 9$) was only less influenced by splanchnic denervation (Figure 4 and Figure 5A).

Bilateral subdiaphragmatic vagotomy was performed to determine the vagal involvement in the stimulatory or inhibitory effects of acupuncture on gastric motility (in some experiments, additional vagotomy of bilateral vagal nerves was also performed at the cervical level to observe the different results from those obtained in the rats after incomplete subdiaphragmatic vagotomy). As shown in Figure 4 and the cumulative results in Figure 5B, the bilateral vagotomy did not abolish the suppressive gastric response induced by acupuncture stimulation to the acupoints of either St-21 ($n = 9$), Cv-6 ($n = 9$) in abdomen, or Bl-21 in middle-dorsum ($n = 9$). However, the bilateral vagotomy completely depleted the facilitative gastric response provoked by acupuncture stimulation to the acupoints of Li-11 in forelimb ($n = 9$) or St-36 in hindlimb ($n = 9$).

Effect of acupuncture stimulation on gastric motility involved in supraspinal circuit

During acute spinalization at C_8-T_1 level, steady gastric motility could still be recorded. But for avoiding the spinal shock, 30-min period of rest was necessary.

In pre-spinalized animals, as shown in Figures 6 and 7, the gastric motility was inhibited by acupoints of St-21, Cv-6 or Bl-21, and facilitated by St-36 acupoint. Acupuncture stimulation at the acupoints of St-21 ($n =$

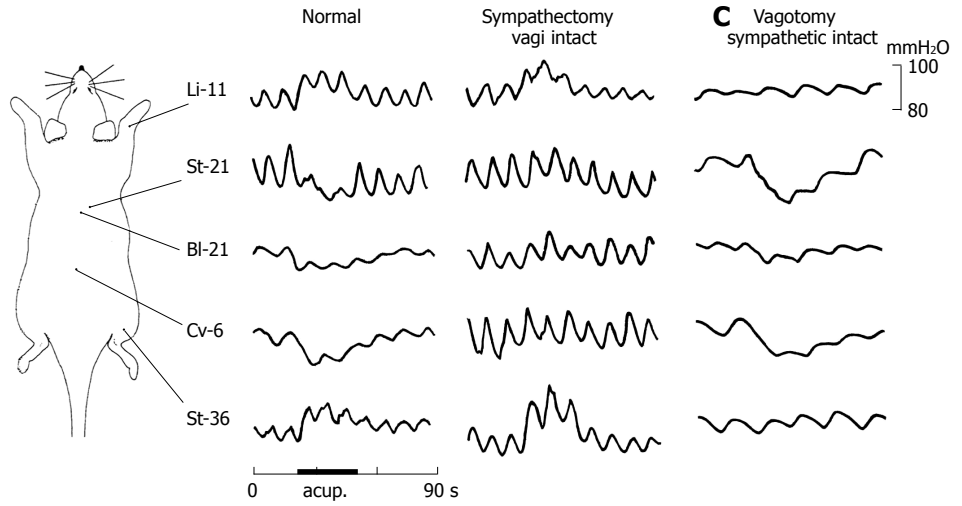

Figure 4 Effect of acupuncture stimulation on gastric motility in animals with intact autonomic nervous system, after sympathectomy of bilateral splanchnic nerves just beneath the diaphragm but intact vagi and bilateral subdiaphragmatic vagotomy but intact sympathetic nerve.

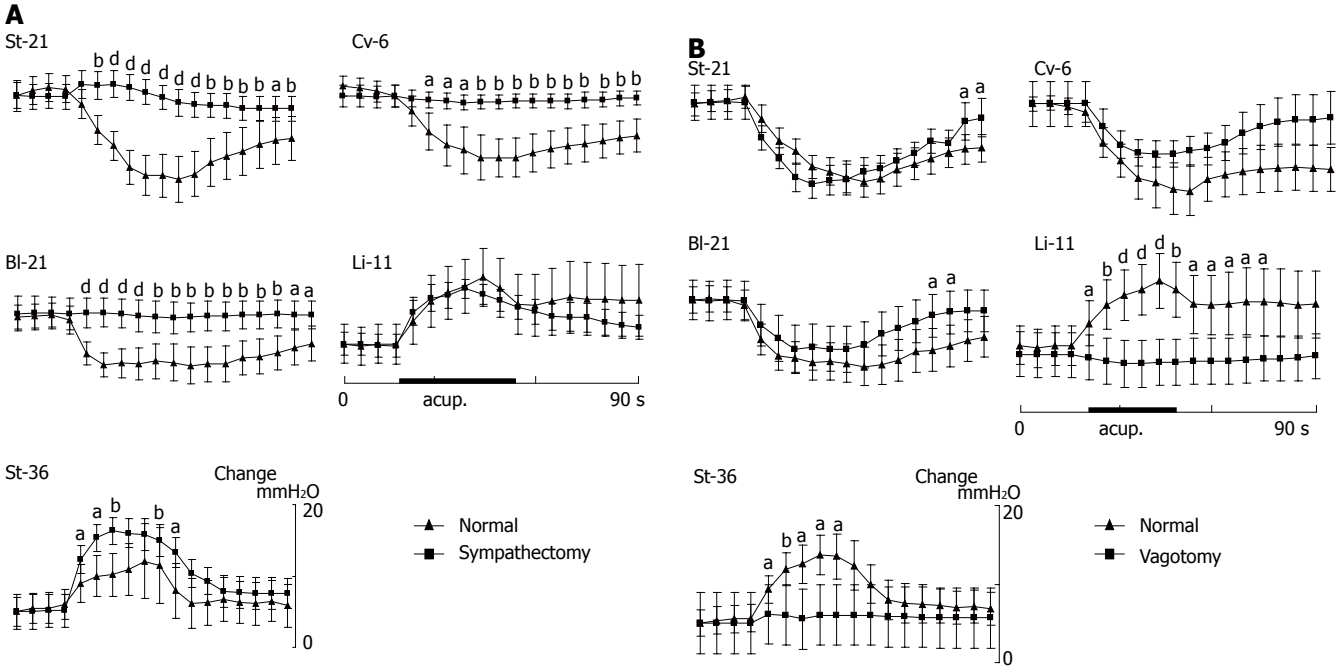

Figure 5 Analysis of the changes in gastric motility induced by acupuncture stimulation in rats after bilateral sympathectomy (**A**) of splanchnic nerves and vagotomy (**B**), respectively. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{d}P < 0.001$ *vs* animals with intact autonomic nervous system.

10), Cv-6 (n = 10) or Bl-21(n = 10) could inhibit gastric motility in the spinalized rats. On the contrary, gastric facilitative response induced by St-36 (n = 10) acupuncture stimulation disappeared completely after spinalization.

DISCUSSION

Our previous study showed that acupuncture-stimulation had facilitative or inhibitory effect on gastric motility depending on the different stimulated acupoints on different parts of the body[10]. In this study, we investigated how strong the stimulation and what autonomic nervous system can effectively modulate the gastric motility.

Modulation of gastric motility induced by acupuncture stimulation involved in activation of fine-diameter afferent fibers

The flexion reflex is usually described as an EMG discharge that mainly consists of two-components separated by periods of an EMG absence. The present results are very similar to those described by Falinower *et al*[11] and by our recent observation[12] in which sural nerve stimulation evoked a two-component reflex in the flexor muscles of the ipsilateral hindlimb. Based on accumulative electrophysiological and pharmacological evidence, the first-component is triggered by activities of small diameter Aδ-fibers, and the second-component is mediated by the unmyelinated C-fibers.

The present study demonstrated that electro-acupuncture stimulation with the strength below $T_{A\delta}$ could not effectively trigger regulatory effects on gastric motility, regardless of the locations of acupoints. Only electro-acupuncture stimulation with the strength exceeding the thresholds for activation of Aδ and/or C-fibers could profoundly modulate gastric motility. Moreover, the effects were strength-dependent and the powerful effects were

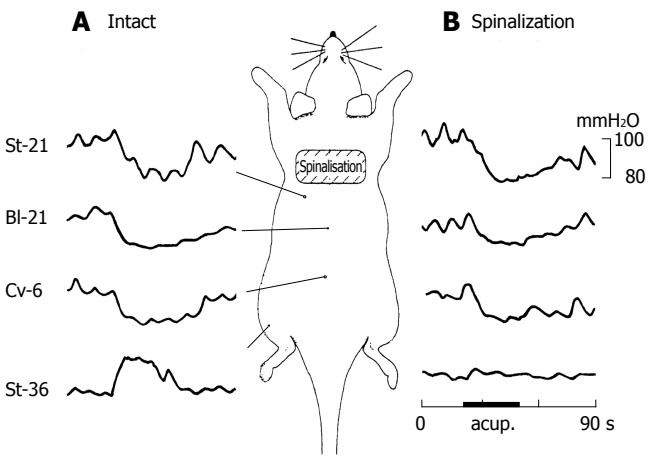

Figure 6 Effects of acupuncture stimulation involving the supraspinal circuit on gastric motility in normal (**A**) and spinalized (**B**) rats.

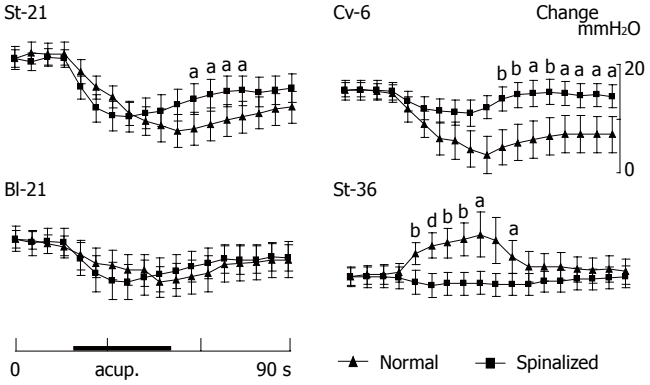

Figure 7 Effects of acupuncture stimulation at different acupoints on the regulation of gastric motility in normal and spinalized rats. Triangle-lines represent the pre-spinalized effects and square-lines indicate the post-spinalized effects. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{d}P < 0.001$ *vs* the control gastric motility of pre-spinalization.

observed when the strongest stimulation was given.

Electro-acupuncture stimulation has been known to excite various afferent fibers of rats including groups Ⅱ-Ⅲ[13], groups Ⅲ-Ⅳ[14] or groups Ⅱ-Ⅳ[15]. Koizumi *et al*[16] performed a systematical analysis of the relationship between the magnitude of cutaneo-intestinal reflex response and groups of stimulated afferent fibers. Their results indicate that stimulation of groups Ⅱ-Ⅲ cutaneous fibers in the T_{10} spinal nerve could not produce any changes in jejunal motility, while stimulation of groups Ⅱ-Ⅲ-Ⅳ fibers could always induce inhibitory responses. Stimulation of the sural afferent nerves of the hindlimb could elicit facilitative jejunal reflex, which was obtained when the stimulus intensity could activate group Ⅲ fibers and the maximal facilitation appeared when the stimulus intensity could activate group Ⅳ afferent fibers. Noguchi *et al*[17] reported that when electro-acupuncture stimulation intensity at hindlimb was less than 1.5-mA (only activation of group Ⅱ fibers), equivalently to 0.8-$T_{A\delta}$ (1.42 ± 0.36 mA) electro-acu puncyure-stimulation in our present study, there was no significant response of duodenal motility. When the intensity of electro-acupuncture stimulation exceeded 2.0-5.0-mA (activation of groups Ⅱ-Ⅲ and Ⅳ fibers), equivalently to 2-$T_{A\delta}$ (3.62 ± 0.44 mA) electro-acu-stimulation in our study, a slight increase was observed in duodenal motility. The maximal excitatory response of duodenal motility to electro-acupuncture stimulation was observed in 10-mA (activation of various afferent fibers) at hindlimb, which was equivalent to1.5-T_C (7.68 ± 0.53 mA) electro-acu puncyure-stimulation in our study. These studies demonstrated that stimulating groups Ⅲ-Ⅳ, particularly group Ⅳ or C afferent fibers of hindlimb, could produce an excitatory intestinal reflex response. On the other hand, stimulation of only group Ⅳ abdominal nerves produces an inhibitory intestinal reflex response.

These data suggest that only acupuncture stimulation with an intensity strong enough to excite Aδ (or group Ⅲ) and/or C (or group Ⅳ) afferent fibers, can lead to markedly excitatory/inhibitory modulation of gastro-intestinal motility.

Facilitation of gastric motility induced by acupuncture stimulation involved in vagal nerve and supraspinal circuit

The present study showed that acupuncture stimulation to the acupoints on face, neck, forelimbs, upper chest-dorsum and hindlinbs produced a facilitative response of gastric motility. These effects were involved in intact preparation of vagal nerves and the spinal cord, but did not require intact preparation of the splanchnic nerves.

Motor activity of the stomach, like other gastrointestinal organs, is modulated by the changes in vagal activity. It was reported that electro-acupuncture at acupoints of St-36 and Pc-6 (Neiguan, located in forelimb) enhances the gastric migrating myoelectrical complex in dogs by reducing the length of phase-I and increasing the length of phases Ⅱ and Ⅲ[18]. Ouyang *et al*[19] observed that electro-acupuncture at Pc-6 and St-36 substantially accelerates gastric emptying of liquid in dogs. The accelerating effect of electro-acupuncture on gastric emptying may be attributed to the improvement in gastric tone and antral contractile activity. Enhanced vagal activity by electro-acupuncture suggests a possible involvement of vagal pathways in its regulation of gastric motility. Xu[20] found that the regulatory effect of electro-acupuncture on gastric dysrhythmia in rabbits could be abolished after vagotomy. Sato *et al*[9] showed that acupuncture-like stimulation could elicit an excitatory gastric response when it is applied to the hindlimb. These findings suggest that acupuncture provokes a reflex that has cutaneous and muscle nerves as its afferent pathway, and the gastric vagus as its efferent pathway. In our study, acupuncture stimulation at St-36 could facilitate gastric motility by increasing the activity of gastric efferent nerves. However, this facilitation of gastric motility disappeared in spinalized rats. The facilitative duodenal response by electro-acupuncture stimulation to a hindpaw is a supraspinal reflex response involving vagal excitatory nerves, cutting the splanchnic nerve branches to the duodenal does not affect the enhanced duodenal response[17].

Facilitation in gastric motility and increase in parasympathetic activity by (electro-) acupuncture

stimulation to all acupoints except for those of abdomen and middle-dorsum (the acupoints of homo-segmental to the innervation of stomach) are accompanied with a decrease in sympathetic activity or a breakage of possible "sympathetic dominance"[21,22], suggesting that these effects require the participation of supraspinal center.

Inhibition of gastric motility induced by acupuncture stimulation involved in sympathetic nerve and propriospinal circuit

The present study showed that acupuncture stimulation to the acupoints on lower-chest, middle-dorsum and whole abdomen could induce an inhibitory response of gastric motility with an increase in the activities of sympathetic and/or a slight inhibition in the activity of vagal nerves innervating the stomach, suggesting that these effects are involved in intact preparation of sympathetic (splanchnic) nerves, but do not require intact preparation of bilateral vagal nerves and high spinal cord.

Sato *et al*[9] showed that the abdominal acupuncture-like stimulation could elicit suppressive response of gastric motility, indicating that acupuncture provokes a reflex that has cutaneous and muscle nerves as its afferent pathway, and the sympathetic gastric branches as its efferent pathway. This inhibition is accompanied with an increase in the activity of efferent nerves of sympathetic gastric branches and can be observed in spinalized rats. The inhibitory duodenal response to electro-acupuncture stimulation in abdomen is a propriospinal reflex response involving splanchnic excitatory nerves, cutting the vagal nerve branches to the duodenal does not affect the suppressed duodenal response[17]. Using duplex Doppler sonography, Choi *et al*[23] found that the frequency of intestinal motility is increased during acupuncture stimulation at St-36 acupoint. Acupuncture on the lower abdomen causes a transient relaxation of the stomach, which can be abolished by splanchnic ganglionectomy but not by truncal vagotomy[24].

In conclusion, acupuncture stimulation to the acupoints on face, neck, forelimbs, upper chest-dorsum and hindlinbs, which are distant to the region of gastric innervation, produces a facilitative response of gastric motility with increased activities of vagus and/or a slight inhibition in the activity of sympathetic nerves. These effects are involved in the intact preparation of vagal nerves and spinal cord, but do not require intact preparation of the splanchnic nerves. Acupuncture stimulation to the acupoints on lower-chest, middle-dorsum and whole abdomen, which are homo-segmental to the region of gastric innervation, induces an inhibitory response of gastric motility with increased activities of sympathetic nerves and/or a slight inhibition in the activity of vagal nerves. These effects are involved in intact preparation of sympathetic nerves, but do not require intact preparation of bilateral vagal nerves and high spinal cord. Only the acupuncture stimulation with its intensity greater than the threshold for activation of Aδ and/or C afferent fibers can induce markedly excitatory/inhibitory modulation of gastrointestinal motility.

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BASIC RESEARCH

Experimental study of therapeutic efficacy of Baicalin in rats with severe acute pancreatitis

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Abstract

AIM: To observe the therapeutic efficacy of Baicalin in rats with severe acute pancreatitis (SAP) and explore its therapeutic mechanisms.

METHODS: The SAP rat models were randomly divided into the model control group, Baicalin treatment group, octreotide treatment group and sham operation group. All groups were randomly subdivided into 3 h, 6 h and 12 h groups with 15 rats in each group. The survival, ascites volume and pathological changes of pancreas in all rats were observed at different time points after operation. The plasma amylase content and serum TNF-α, IL-6, malonaldehyde (MDA) and PLA_2 contents were also determined.

RESULTS: The survival was not obviously different between the treated groups, and was significantly higher in treated groups at 12 h compared to the model control group ($P < 0.05$, 15 *vs* 10). The ascites/body weight ratio at 3 h and 6 h was significantly lower in Baicalin treatment group compared to the model control group and octreotide treatment group ($P < 0.05$, 1.00 *vs* 2.02 and 1.43 and $P < 0.001$, 2.29 (1.21) *vs* 2.70 (0.80) and 2.08 (2.21), respectively). The contents of amylase, TNF-α, IL-6, MDA and PLA_2 were significantly lower in the treated groups than in the model control group ($P < 0.05$, 4342 *vs* 5303, 5058 *vs* 6272 in amylase, $P < 0.01$, 21.90 *vs* 36.30, 23.80 *vs* 39.70, 36 *vs* 54.35 in MDA and 56.25 *vs* 76.10 in PlA_2, or $P < 0.001$, 65.10 and 47.60 *vs* 92.15 in TNF-α, 3.03 *vs* 5.44, 2.88 *vs* 6.82, 2.83 *vs* 5.36 in IL-6, respectively). The pathological scores of pancreas in the treated groups were significantly lower than that in the model control group ($P < 0.05$, 9.00 *vs* 10.05, 6.00 *vs* 9.00, 8.00 *vs* 10.05), but no marked difference was found between the treated groups.

CONCLUSION: The Baicalin injection has significant therapeutic effects on SAP rats, its effects are similar to those of octreotide. The Baicalin injection is also cheap and has a big application range, quite hopefully to be used in clinical treatment of SAP.

Key words: Severe acute pancreatitis; Baicalin; Octreotide; Rats; Serum amylase; TNF-α; IL-6; Malonaldehyde; PLA_2

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INTRODUCTION

As one of the life-threatening severe diseases, severe acute pancreatitis (SAP) has acute onset, rapid progression, multiple complications, and its mortality has reached 20%-30%. The pathogenesis of SAP is still unknown, and no breakthrough has ever been made in its treatment. Currently, mainly somatostatin and its analog octreotide are effective drugs for SAP treatment in the clinic.

Octreotide, also named sandostatin, as an analog of

natural somatostain plays an important role in improving the survival rate of SAP animals, inhibiting secretion of pancreatin and alleviating multiple organ injury[1-3]. However, the expensive price, short half-life and inconvenient administration of these drugs have restrained their clinical popularization, especially in remote poor areas. So, it is necessary to find some cheap and highly effective drugs.

In "Qing Yi Tang" which is a representative prescription of Chinese medicine for SAP treatment, the enormous clinical practices also suggest its sound therapeutic effects on SAP[4]. Scutellaria baicalensis georgi is a main material in "Qing Yi Tang" while Baicalin (monomer) is its main active constituent. The intravenous administration with very low price can overcome the shortcomings of oral administration of "Qing Yi Tang", including poor absorption and inconvenience. The *in vitro* experiments of Baicalin have proved[5-7]: it has anti-bacterial, anti-viral and anti-inflammatory activities. It can also inhibit platelet aggregation and eliminate oxygen-free radicals. In animal experiments, Baicalin with choleretic effectcan can relieve fever, inhibit the thrombin-induced transforming process from fibringen to fibrin, lower endotoxin generation, treat and prevent endotoxemia-induced disseminated intravascular coagulation. In addition, the initial metabolite of Baicalin in body is baicalein that can more effectively inhibit pancreatin. All these pharmacologic effects can antagonize many processes during SAP onset. Its several effects are similar to those of somatostatin and its analog such as octreotide, but it has a broader application range. It is theoretically feasible to use it for SAP treatment.

Presently, to the best of our knowledge, there has not been any study report on Baicalin treatment of SAP internationally. In this experiment, we have established the SAP rat model, discussed the effects of Baicalin in treating SAP rats, compared its effects with those of octreotide and observed the therapeutic efficacy of Baicalin and explored its therapeutic mechanisms in order to provide the reliable basis for Baicalin treatment of SAP.

MATERIALS AND METHODS

Experimental animals and reagents

Clean grade healthy male Sprague-Dawley (SD) rats weighing 250-300 g were purchased from the Experimental Animal Center of Medical School, Zhejiang University, China. Sodium taurocholate and sodium pentobarbital were purchased from Sigma Company, USA. Octreotide was purchased from Swiss Pharmaceutical Company Novartis, and 5% Baicalin injection (China National Invention Patent Number ZL200310122673.6) was prepared by the first author with 305 mmol/L osmotic pressure. The TNF-α ELISA kit was purchased from Jingmei Bioengineering Corporation (China) and the calculation unit for content is pg/mL (ng/L). The IL-6 ELISA kit was purchased from Shanghai Shenxiong Biotech Company (China) and the calculation unit for content is pg/mL (ng/L). The serum malonaldehyde (MDA) kit was purchased from Nanjing Jiancheng Bioengineering Research Institute, China. The calculation units for content are respectively nmol/mL. The serum secretory phospholipase A_2 enzyme assay ELA kit (PLA_2) was purchased from R&D System Ins, USA and the calculation unit for content is U/mL.

Animal grouping

The improved Aho's method[8] was adopted to prepare SAP rat models *via* retrograde injection of 3.5% sodium taurocholate to the pancreatic duct through epidural catheter and duodenal papilla. The 135 SAP rat models were randomly divided into model control group, Baicalin treatment group and octreotide treatment group with 45 rats in each group; other 45 rats were selected as sham operation group, which only received laparotomy. All groups were then randomly subdivided into 3 h, 6 h and 12 h groups with 15 rats in each group.

Observation index

We examined the rat mortality at 3 h, 6 h and 12 h after operation and calculated the survival, observed the gross changes of pancreas and ascites volume. Ascites/body weight ratio was measured as follows: Dry gauze was used to wipe intra-abdominal hydrops; then, a scale was used to weigh the weights of gauze before and after its soaking; the difference between weights (g) was converted into ascites volume (mL); and ascites/body weight ratio was thus obtained. After mercy killing the rats anesthetized by sodium pentobarbital in batches, the samples of pancreas were collected, fixed according to the requirements, and the pathological changes of pancreas after hematoxylin-eosin (HE) staining were observed. The contents of plasma amylase and serum TNF-α, IL-6, MDA and PLA_2 were determined *via* blood sampling from heart. The full automatic biochemical analyzer was used to determine the plasma amylase level and the calculation unit for content is U/L. The serum TNF-α, IL-6 and PLA_2 levels were determined by ELISA method.

Pancreatic pathological score

A modified Schmidt's pathological score system was used (Table 1) and two pathologists in double-blind control condition performed the evaluation of severity of pancreatic tissue pathology. We modified the pathological score of pancreas, because the Schmidt's pathological score[9] was too difficult and complex to be used in our experiment.

Preparation methods of animal models

Fasting but water restraint was imposed on all rat groups 12 h prior to the operation. The rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (0.25 mL/100 g), then laid and fixed, and the routine shaving, disinfection and draping were performed. We first established the right external jugular vein transfusion passage to use the microinfusion pump for continuous transfusion (1 mL/h per 100 g) and then used 3.5% sodium taurocholate to prepare SAP model.

Model control group: After entering the abdomen *via* median epigastric incision, we confirmed the bile-pancreatic duct, hepatic hilus and common hepatic duct, disclosed the pancreas, identified the duodenal papilla inside the duodenum duct wall, and then used a No. 5 needle to drill a hole in the mesenteric avascular

Table 1 Improved Schmidt score of pathological changes of the pancreas

Edema		Acinar necrosis (necrotic cells/HPF)		Inflammation and perivascular infiltrate (intralobular or perivascular leukocytes/HPF)		Hemorrhage and fat necross (focus)	
Absent	0	Absent	0	0-1	0	Absent
Focal expansion of interlobular septae	1	1-4	1	2-10	1	1-2
Same as 1 + diffuse expansion of interlobar; Septae/diffuse expansion of interlobar septae	2	5-10	2	11-20	2	3-4
Same as 2 + expansion of interacinar septae	3	11-16	3	21-30	3	5-6
Same as 3 + expansion of intercellular spaces	4	> 16	4	> 30/microabscesses	4	> 7

area. After inserting a segmental eqidural catheter into the duodenum cavity *via* the hole, we inserted the bile-pancreatic duct toward the direction of the papilla in a retrograde way, used the microvascular clamp to nip the duct head temporarily and meanwhile used another microvascular clamp to temporarily occlude the common hepatic duct at the confluence of the hepatic duct. After connecting the anesthetic tube end with the transfusion converter, we transfused 3.5% sodium taurocholate (0.1 mL/100 g) by retrograde transfusion *via* the microinjection pump (made by Zhejiang University) at a speed of 0.2 mL/min, then stayed for 4-5 min after injection and removed the microvascular clamp and eqidural catheter. After checking for bile leakage, we sutured the hole in the lateral duodenal wall, used the disinfected cotton ball to absorb up the anesthetic in the abdominal cavity and closed the abdomen. During the laparotomy in the sham operation group, we performed pancreas and duodenum turning over, observed pathological changes of multiple organs and finally closed the abdomen.

Dosage and methods

In Baicalin treatment group, the animal experiments of 5% Baicalin injection were completed including the acute toxicity test and SAP rat treatment by small, middle and large dose. The large dose could achieve the best therapeutic effect (dose is 10 mg/h per 100 g) and the dosage referred to the result of the previous preliminary experiment. Ten minutes after successful modeling, Baicalin treatment group was first injected 5% Baicalin injection 10 mg/100 g *via* the external jugular vein passage, followed by continuous intravenous administration (10 mg/h per 100 g) by microinfusion pump; octreotide treatment group was first injected octreotide 0.2 μg/100 *g via* the external jugular vein passage, followed by continuous intravenous transfusion (10 mg/h per 100 g) by microinfusion pump at a transfusion speed of 0.2 μg/h per 100 g. All above dosages have been proved as effective dosages in the previous preliminary experiment. Both of the sham operation group and model control group were injected saline of equivalent volume at the corresponding time points after operation.

Statistical analysis

The values were presented as mean ± SD for nomal distribution variables or median and quartile range for highly skewed variables. The significance of differences among the four groups was tested using the Kruskal-Wallis test for highly skewed data and analysis of variance (ANOVA) for normal distribution data. Multiple comparisons were subjected to Bonfferoni correction test. The Chi-square test was used to evaluate equality of frequencies for discrete variables. Correlations were tested using the Spearman rank correlation coefficients. A P value less than or equal to 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS version 11.5 for windows.

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

Groups	3 h	6 h	12 h
Sham operation	0.28 (0.23)	0.44 (0.15)	0.39 (0.22)
Model control	2.02 (0.89)	2.62 (0.97)	2.70 (0.80)
Baicalin treatment	1.00 (1.30)	1.16 (0.73)	2.29 (1.21)
Octreotide treatment	1.43 (0.62)	2.15 (0.88)	2.08 (2.21)

RESULTS

Survival rate

The mortality rates of the model control group were 0% (0/15), 13.33% (2/15) and 33.33% (5/15) at 3 h, 6 h and 12 h, respectively, while those of Baicalin treatment group and octreotide treatment group were 0% at different time points. The whole sham operation group survived at different time points. The survival rate of the model control group was 66.67% (10/15) at 12 h, while the survival rate of each of Baicalin treatment group and octreotide treatment group was 100% at 12 h, indicating a marked difference ($P < 0.05$).

Comparison of ascites/body weight ratio among all groups

The ascites/body weight ratio of the model control group and treatment group significantly exceeded the sham operation group at different time points ($P < 0.001$), while there was no significant difference between the octreotide treatment group and model control group at different time points. Ascites/body weight ratio of the Baicalin treatment group was significantly less than the model control group ($P < 0.01$) and octreotide treatment group at 3 h ($P < 0.05$). Ascites/body weight ratio of the Baicalin treatment group was significantly less than the model control group and octreotide treatment group at 6 h ($P < 0.001$). However, there was no significant difference among the Baicalin treatment group, model control group and octreotide treatment group at 12 h (Table 2).

Table 3 Comparison of different indexes of blood [*M* (Q_R)]

Indexes	Sham operation			Model control			Baicalin treatment			Octreotide treatment		
	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h
Amylase (U/L)	1582 (284)	1769 (362)	1618 (302)	5303 (1373)	6276 (1029)	7538 (2934)	4342 (1496)	5130 (1591)	5571 (2307)	5419 (1670)	5058 (1314)	6531 (2280)
TNF-α (ng/L)	3.90 (3.20)	4.00 (1.70)	5.30 (3.00)	41.44 (37.72)	92.15 (23.12)	65.02 (26.81)	44.93 (45.84)	65.10 (27.51)	47.65 (25.52)	39.30 (30.60)	47.60 (16.50)	54.50 (41.40)
IL-6 (ng/L)	1.8460 (0.35)	1.74 (0.84)	2.04 (0.82)	5.44 (1.03)	6.82 (0.81)	5.36 (0.75)	3.03 (0.87)	2.88 (1.39)	2.83 (0.60)	2.65 (1.37)	3.08 (1.210)	2.46 (1.35)
MDA (nmol/mL)	9.90 (9.90)	16.50 (13.20)	16.50 (13.20)	36.30 (13.40)	39.70 (9.90)	54.35 (19.00)	21.90 (13.45)	23.80 (14.60)	36.00 (11.60)	29.60 (18.60)	33.00 (9.90)	40.30 (16.80)

Table 4 Comparison of serum PLA2 content (mean ± SD)

Groups	3 h	6 h	12 h
Sham operation	14.62 ± 3.02	17.49 ± 3.82	19.02 ± 5.07
Model control	76.10 ± 16.70	101.46 ± 14.67	105.33 ± 18.10
Baicalin treatment	56.25 ± 22.43	67.91 ± 20.61	66.86 ± 22.10
Octreotide treatment	74.37 ± 19.94	63.13 ± 26.31	53.63 ± 12.28

Comparison of plasma amylase content among all groups

The plasma amylase content in the model control group and two treatment groups significantly exceeded that in the sham operation group at different time points ($P < 0.001$). However, there was no significant difference between the Baicalin treatment group and octreotide treatment group at different time points. Although the plasma amylase content of the Baicalin treatment group was lower than that of the model control group at different time points, the difference did not reach statistical significance at 6 h and 12 h, but reached statistical significance at 3 h ($P < 0.05$). Although plasma amylase content was significantly less in the octreotide treatment group compared to the model control group at 6 h ($P < 0.05$), no significant difference was observed at 3 h and 12 h (Table 3).

Comparison of serum TNF-α content among all groups

Serum TNF-α content significantly exceeded in the model control group and treatment groups compared to the sham operation group at different time points ($P < 0.001$). There was no significant difference in serum TNF-α content among the model control group, Baicalin treatment group and octreotide treatment group at 3 h and 12 h. However, at 6 h, both Baicalin treatment group and octreotide treatment group had significantly less serum TNF-α content compared to the model control group ($P < 0.001$); and the octreotide treatment group had significantly less serum TNF-α content than the Baicalin treatment group ($P < 0.01$) (Table 3).

Comparison of serum IL-6 content among all groups

The serum IL-6 content at 3 h and 6 h was significantly higher in the model control group and treated groups than in the sham operation group ($P < 0.001$). Baicalin treatment group and octreotide treatment group had no significant difference in serum IL-6 content at all time points. The Baicalin treatment group and octreotide treatment group had significantly lower serum IL-6 content than the model control group at all time points ($P < 0.001$). However, the model control group had significantly higher serum IL-6 content than the sham operation group at 12 h ($P < 0.001$), and so had the Baicalin treatment group ($P < 0.01$), but no significant difference was found between the octreotide treatment group and the sham operation group (Table 3).

Comparison of serum MDA content among all groups

Serum MDA content significantly exceeded in the model group, Baicalin treatment group and octreotide treatment group compared to the sham operation group at different time points ($P < 0.05$). Serum MDA content was significantly less in the Baicalin treatment group than the model group ($P < 0.01$). Similarly, serum MDA content was significantly less in the octreotide treatment group than the model group at 6 h and 12 h ($P < 0.05$). However, serum MDA content was significantly less in the Baicalin treatment group compared to the octreotide treatment group at 12 h ($P < 0.05$) (Table 3).

Comparison of serum PLA_2 content among all groups

Serum PLA_2 content significantly exceeded in the model group and treatment groups compared to the sham operation group at different time points ($P < 0.001$). At 3 h, the Baicalin treatment group had significantly less serum PLA_2 content than the model group ($P < 0.01$), while there was no marked difference between the octreotide treatment group and the model group, and the Baicalin treatment group had significantly less serum PLA_2 content than the octreotide treatment group ($P < 0.01$). At 6 h and 12 h, the Baicalin treatment group and octreotide treatment group had significantly less serum PLA_2 content than the model group ($P < 0.001$). At 6 h, there was no marked difference between the Baicalin treatment group and the octreotide treatment group, whereas at 12 h, the octreotide treatment group had significantly less serum PLA_2 content than the Baicalin treatment group ($P < 0.001$) (Table 4).

Macroscopic and microscopic pathological changes of the pancreas

Sham operation group: Macroscopically, there were only some amber ascitic fluid within the abdominal cavity, and no pathological changes visible to naked eyes in other

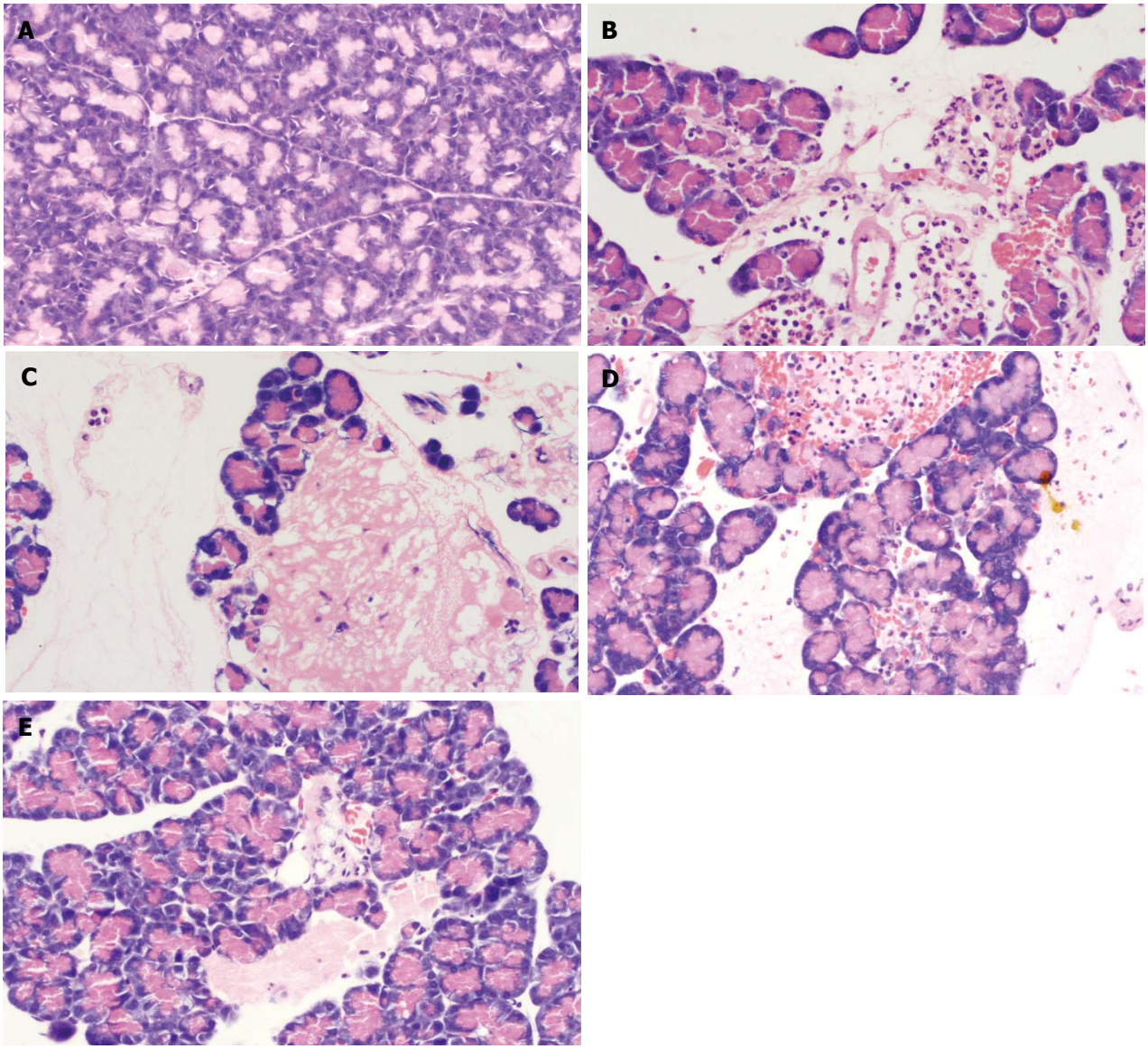

Figure 1 **A**: Sham operation group at 12 h (normal pancreas); **B**: Model control group at 12 h (Interacinar edema accompanied with piecemeal necrosis of acinus, a great deal of neutrophil infilatration among acini); **C**: Model control group at 12 h (Massive acinus necrosis); **D**: Baicalin treatment group at 12 h (interlobular and interacinar edema, focal necrosis of acinus, relatively much neutrophil infiltration among acini); **E**: Octreotide treatment group at 12 h (Interlobular edema accompanied with focal necrosis of acinus).

organs. The overall structure of the pancreas remained intact. There were no hemorrhagic changes in the pancreas, which was yellowish without volume reduction, and no significant abnormality in the pancreas, peripancreatic and epiploon at all time points. Microscopically, most remained normal with intact gland structure, mild interstitial edema occurred in very few cases, neutrophil infiltration was occasional, and no acinar cell and fat necrosis and hemorrhage were observed (Figure 1A).

Model control group: Macroscopically, pathological changes of the pancreas tail were a little more obvious than those of the pancreas head; 5 min after model induction, the pancreas manifested edema, hemorrhage and necrosis. The overall severity of the pathological changes at 3 h, 6 h and 12 h increased with time after modeling. In the 3 h group, small amount of ascitic fluid mostly slightly bloody visible to naked eyes, obvious hyperemia and edema of the pancreas, partly jellly-like edema, hemorrhage and necrosis were observed. Most ascitic fluid after 6 h and 12 h was bloody with the larger average amount than that at 3 h. The amount and characters of ascitic fluid increased and deepened with time after modeling; the degree and range of the pancreas edema, hemorrhage and necrosis became more obvious than those at 3 h; many saponified spots on peripancreatic great epiploon and peritoneum, jelly-like change, contour vanishing, quite obvious hemorrhage and necrosis changes of the pancreatic tissue were also observed. Microscopically, in the 3 h group, obvious pancreas interstitial hyperemia and edema, small amount of inflammatory cell infiltration, sporadic focal necrosis and interstitial hemorrhage occurred among which some were hemorrhagic or lytic necrosis; in the 6 h group, pancreas interstitial edema and hemorrhage, visible focal or lamellar necrosis, comparatively large area of inflammatory cell infiltration around were observed; and in the 12 h group, obvious pancreas interstitial edema, interstitial

Table 5 Comparison of pathological lesion score for pancreas (M (Q_R))

Groups	3 h	6 h	12 h
Sham operation	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Model control	8.00 (2.00)	9.00 (3.00)	10.50 (1.50)
Baicalin treatment	7.00 (1.50)	7.00 (3.00)	9.00 (4.00)
Octreotide treatment	7.00 (2.00)	6.00 (2.00)	8.00 (2.00)

hemorrhage, large area of necrosis, lobule contour damage with a large amount of inflammatory cell infiltration were observed (Figures 1B and C).

Treatment group: Macroscopically, in the 3 h group, the pancreatic tissue with hyperemia and edema changes, milder hemorrhage and necrosis than those of model control group were observed; in the 6 h and 12 h groups, compared with the model control group, relatively limited pancreas hemorrhage and necrosis, lighter ascitic fluid color, obviously less ascitic fluid, decreased distribution and area of saponified spot, milder pancreas hemorrhage and necrosis, and relatively integrated overall pancreas structure were observed. The pathological changes of pancreatic tissue of the octreotide treatment group resembled those of the Baicalin treatment group. Microscopically, the pathological changes of most cases in the treatment group were milder than those of the model control group at the corresponding time points, such as decreased degree of interstitial edema, reduced inflammatory cell infiltration, more clear cell structure than that of the model control group, reduced pancreas interstitial erythrocyte exudation, small amount of focal hemorrhage and necrosis with little lamellar hemorrhage and necrosis, and reduced hemorrhage and necrosis range. The pathological changes of pancreatic tissue of the octreotide treatment group resembled those of the Baicalin treatment group (Figures 1D and E).

Comparison of pancreas pathological score among all groups

Pancreas pathological scores of the model control group, Baicalin treatment group and octreotide treatment group significantly exceeded those of the sham operation group at different time points ($P < 0.001$). Pancreas pathological score of the Baicalin treatment group was significantly less than that of the model control group at 12 h ($P < 0.01$). Moreover, pancreas pathological score of the octreotide treatment group was significantly less compared to the model control group at 6 h and 12 h ($P < 0.01$). However, there was no significant difference between the Baicalin treatment group and the octreotide treatment group at different time points (Table 5).

DISCUSSION

Severe acute pancreatitis (SAP) induces inflammatory reactions of pancreatic tissue itself or even systemic reactions, which is by a large portion related to excessive generation and cascade reactions of inflammatory mediators, mainly including endotoxin, oxygen-free radical, PLA_2, bradykinin, complement, acute-phase protein, vasoactive amine, arachidonic acid metabolite, cytokine (lymphokine) and PAF[10-14]. This study mainly investigates the therapeutic effects of Baicalin and octreotide by observing plasma amylase content and serum TNF-α and IL-6 content.

Table 6 Comparison of therapeutic efficacy of octreotide and Baicalin

Therapeutic efficacy	Curative	
	Baicalin	Octreotide
Improve survival rate	++	++
Decrease ascites volume	++	+
Decrease amylase	+	+
Decrease TNF-α	+	++
Decrease IL-6	++	+
Decrease MDA	+	++
Decrease PLA_2	++	+
Protect pancreatic tissue	++	++

++: Represents significant effect; +: Represents normal effect; -: No effect.

TNF-α participates in onset and progression of early-phase inflammations of acute pancreatitis (AP), and is also related to AP severity[15,16]. Excessive generation of TNF-α, or imbalance between it and other cytokines will stimulate cascade reactions, induce generation of IL-1, IL-6, IL-8, *etc*, later generate inflammatory mediators, and aggravate cell damage. Current studies found serum TNF-α had two aspects in regulating apoptosis, namely inducing apoptosis and promoting inflammation healing when its concentration was low, while leading to necrosis of pancreatic acinar cell when its concentration was high[17].

IL-6, mainly generated by monocyte after induction of IL-1, TNF, *etc*, has intensive inflammation-causing activity[18-21]. IL-6 can both directly increase the permeability of vascular endothelial cell, and has synergistic effect with TNF-α, *etc* to constitute a network of inflammatory mediators[20]. IL-6 level in serum can reflect AP severity[18,23,24]. It is generally recognized that the PLA_2 content or activity rises when SAP occurs[25]. The abnormal release and activation of PLA_2 can change lecithin into hemolytic lecithin, cause lysis and breakdown of pancreatic cell membrane, and lead to autodigestion of the pancreas[26-28]. The excessive free radicals generated in body during SAP may cause the accumulation of MDA, a lipid oxidative product. MDA content in serum can indicate the level of free radical overproduction[29,30].

In addition, a great quantity of endotoxin can induce TNF-α, stimulate or promote cytokine release including IL-1β[31], IL-6, TNF, and further mediate activation of leucocyte and platelet in multiple organs, such as pancreas, kidney and lung, and release lysosome, oxygen-free radical and lipid inflammatory substance. The excessive cytokines and inflammatory factors can cause waterfall-like cascade reactions, induce iNOS expression all over the body, generate a great deal of NO, damage blood vessel endothelium, and cause tissue necrosis[32-36].

Octreotide, a medicine currently adopted in the clinic for SAP treatment, mainly achieves its therapeutic effects

by inhibiting secretion of pancreatin and other digestive enzymes, and loosening the oddi sphincter. The most important is that octreotide can block the excessive expression of inflammatory mediators and cytokines, reduce iNOS mRNA expression and NO synthesis, and then alleviate injury of multiple organs, such as pancreas and lung[37,38]. This experiment found that compared with the model control group, both the Baicalin and octreotide treatment groups could effectively reduce the generation of ascites, plasma amylase content, and serum TNF-α, IL-6, MDA and PLA_2 content, alleviate pathological changes of pancreatic tissue, and lower mortality of SAP rats.

Compared with the octreotide group, Baicalin can more significantly inhibit the generation of ascites and excessive release of IL-6 and PLA_2 (Table 6). In addition, Baicalin also has features, such as a broad range of pharmacological actions, low side effect, and low price[37,39]. Therefore, using Baicalin to treat SAP will be an effective and cost-effective therapy for SAP.

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BASIC RESEARCH

Antidiabetic effects of chitooligosaccharides on pancreatic islet cells in streptozotocin-induced diabetic rats

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Abstract

AIM: To investigate the effect of chitooligosaccharides on proliferation of pancreatic islet cells, release of insulin and 2 h plasma glucose in streptozotocin-induced diabetic rats.

METHODS: *In vitro*, the effect of chitooligosaccharides on proliferation of pancreatic islet cells and release of insulin was detected with optical microscopy, colorimetric assay, and radioimmunoassay respectively. *In vivo*, the general clinical symptoms, 2 h plasma glucose, urine glucose, oral glucose tolerance were examined after sixty days of feeding study to determine the effect of chitooligosaccharides in streptozotocin-induced diabetic rats.

RESULTS: Chitooligosaccharides could effectively accelerate the proliferation of pancreatic islet cells. Chitooligosaccharides (100 mg/L) had direct and prominent effect on pancreastic β cells and insulin release from islet cells. All concentrations of chitooligosaccharides could improve the general clinical symptoms of diabetic rats, decrease the 2 h plasma glucose and urine glucose, and normalize the disorders of glucose tolerance.

CONCLUSION: Chitooligosaccharides possess various biological activities and can be used in the treatment of diabetes mellitus.

Key words: Chitooligosaccharides; Diabetes mellitus; Two hours plasma glucose; Oral glucose; Tolerance test; Pancreastic islet cells; Streptozotocin

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INTRODUCTION

Diabetes mellitus (DM) is a highly prevalent disease all over the world. Its long-term tissue complications that affect small and large blood vessels are directly connected with the time of patients suffering from hyperglycemia. Chitosan is a polycationic copolymer consisting of β-1, 4-linked 2-acetamido- D-glucose and β-1, 4-linked 2-amino-D-glucose units. Crab and shrimp shell wastes are currently utilized as the major industrial source of biomass for large-scale production of chitosan. Chitosan which is biodegradable, non-toxic and biocompatible has been shown to be particularly useful in many fields[1], including food, cosmetics, biomedicine, agriculture and environmental protection. Furthermore, it can be used as a bioactive material due to its biodegradable, non-toxic and non-allergenic natures. However, chitosan shows its biological activity only in acidic medium because of its poor solubility at pH above 6.5 and low absorbability of non-digestible and high molecular polysaccharides. Therefore, recent studies on chitosan have attracted interest in converting it to chitooligosaccharides(COS), because COS not only are water-soluble but also possess versatile functional properties such as antitumor enhancing properties[2,3], immunostimulating effects[2,4], antimicrobial activity[5], free radical scavenging activity[6-8], protective effects against infections[9], arthritis controlling activity[10], plant disease controlling activity[11,12] and angiotensin I converting enzyme inhibitory activity[13]. However, little attention has been paid to its activity in diabetes mellitus and related mode of action.

In the present study, soluble chitooligosaccharides with low molecular weight were prepared by enzymatic hydrolysis of chitosan with chitosanase as previously described[14]. The purpose of this study was to examine the effect of chitooligosaccharides on proliferation of pancreatic islet cells and release of insulin *in vitro*.

MATERIALS AND METHODS

Materials

Chitosan (minimum 90% deacetylated, M_r: 500 000) was purchased from Jinan Haidebei Marine Bioengineering

Co., Ltd (Shangdong, China). Chitooligosaccharides were prepared by enzymatic hydrolysis of chitosan with chitosanase. NIT-1 cell line was from Institute of Medicine, Ocean University of China. Male Wistar rats (200 ± 20 g) were from Laboratory Animal Centre, Institute of Medicine, Ocean University of China. Streptozotocin (STZ) was from Sigma Chemical Co. Tissue-culture medium and reagents were from Gibco. All chemicals were purchased from Sigma Chemical unless otherwise stated.

Cell culture

Rat islets were isolated from the pancreas of male Wistar rats by collagenase digestion as previously described[15,16] and dispersed into single cells by shaking in a low calcium medium[17]. The viability of cells after isolation, determined using the fluorochrome-media-ted viability test (see below) was ⩾ 98%. The islets were placed on glass coverslips in 35-mm petri plates and cultured in RPMI-1640 medium containing 20% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin. NIT-1 cell line, a widely used β cell line for insulin secretion studies, was established from non-obese diabetic (NOD) mice transgenic for the SV40 T antigen under control of the insulin promoter, and cultured in DMEM containing 10% FBS and antibiotics (100 IU/mL of penicillin and 100 μg/mL of streptomycin). All cultures were kept at 37℃ in 950 mL/L O_2 and 50 mL/L CO_2. The medium was changed every two days. The islet cells cultured under these conditions spread out as a monolayer within 14 d and exhibited normal responses of insulin release to glucose stimulation, as described in previous studies[18,19].

Effects of COS on proliferation of pancreastic islet cells and insulin release

COS were dissolved in Dulbecco's modified Eagle's medium (DMEM) without FBS and then diluted with medium to form the five degrades (10, 100, 500, 1000, and 2000 mg/L). The structure and function of cultured islet cells were observed under inverted phase contrast microscope. MTT assay was used to estimate the cell viability. Pancreastic islet cells were digested by 0.25% trypsin until appearance of unicellular suspension. Then 100 μL suspension with the concentration of 5.0×10^4 cells/mL was transplanted into 96-well plates. Each well had three parallels. After incubated for 24 h, the medium was changed with COS at different concentrations. Cells were incubated with 0.5 mg/mL of MTT in the last 4 h of the culture period (48 h). The medium was then decanted, 200 μL DMSO was added to the wells, and the absorbance was determined at *492 nm* using an ELISA reader. The proliferation rate was calculated (PR = A/A0 × 100%). Furthermore, the effect of COS on the viability of pancreastic islet cells was also examined at 12, 24, 36, 48, 60, 72, 96, 120, 144, 168, 192 and 216 h to study the effect of COS on the growth phase of pancreastic islet cells in 9 d. At the same time, freshly dispersed islet cells were transplanted into 24-well plates at the concentration of 5.0×10^4 cells/mL, then divided into groups and stimulated with 100 mg/L COS for 14 d. The incubation medium was collected every 2 d and then stored at -20℃ until assay. Control group was maintained in basal RPMI 1640. Each well had five parallels. At the end of the stimulation period, insulin was measured using a radio-immunoassay kit for human insulin from Linco. The experiments were performed in triplicate.

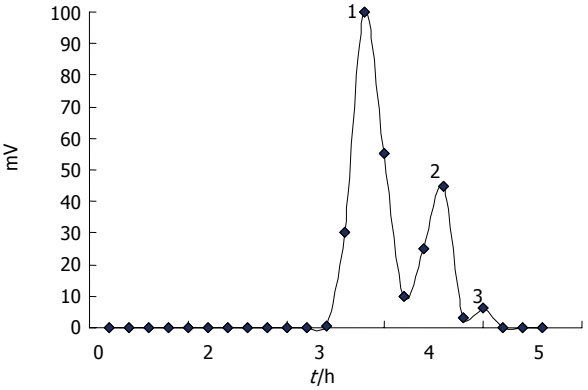

Figure 1 Chromatogram of chitooligosaccharids on Sephadex G-25 colum.

Effect of COS on streptozotocin-induced diabetic rats

Male Wistar rats were rendered diabetic by intraperitoneal injection of STZ at 65 mg/kg. The rats whose fasting blood glucose level was above 11.11 mmol/L were used for experiments after seven days. Then the rats were randomly divided into metformin treatment group, positive control group (DM) and COS treatment groups. Test samples were given intragastrically by gavage needle for 60 successive days. COS treatment groups were given chitooligosaccharides at the concentration of 250, 500, 1500 mg/kg daily. Normal control group and DM group received an equal volume of distilled water about 10 mL/kg. Metformin treatment group was given metformin at a concentration of 200 mg/kg daily. Two-hour plasma glucose (2hPG) and urine glucose were measured every 10 d. Oral glucose tolerance was examined after 60 d of feeding study. Plasma glucose was immediately measured in duplicate using a Beckman glucose analyzer Ⅱ (Beckman, Palo Alto, CA).

Statistical analysis

ANOVA was performed with Duncan's multiple range tests. SAS was used to compare the means (SAS Institute, Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant, $P < 0.01$ was considered very statistically significant.

RESULTS

Physicochemical properties and HPLC analysis of chitooligosaccharides

Chitooligosaccharides were prepared by enzymatic hydrolysis of chitosan. Components of the hydrolysis product were separated by Sephadex G-25 (Figure 1), and component 1 analyzed by TSK-GEL G3000PWXL had an average molecular weight of 1200 u, and a degree of deacetylation of 90% by the first derivative method of UV spectrometry.

Effects of COS on proliferation of pancreastic islet cells and insulin release

The influence of COS on pancreastic islet cell viability

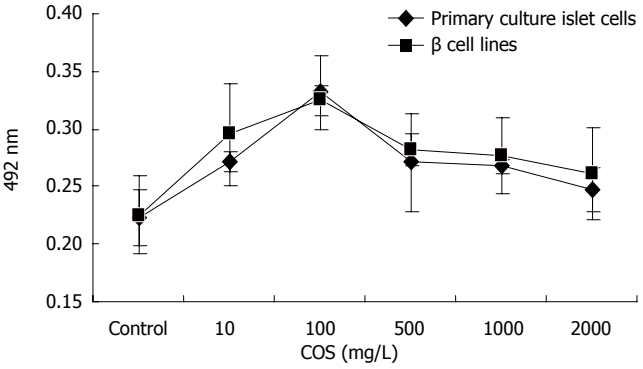

Figure 2 Effect of COS on proliferation of primary culture islet cells and β cell lines.

was assessed by MTT assay (Figure 2). Cell viability in the presence of different concentrations of COS was significantly higher than that in the absence of different concentrations of COS. The maximum stimulatory effect on cell viability was achieved at 100 mg/ L concentration among the five samples (Figures 3A and B), the proliferation rate was 148.43% and 143.71% respectively, and had a significant difference compared with the nomal control group (Figures 3C and D, $P < 0.01$), suggesting that COS could accelerate the proliferation of pancreastic islet cells in 9 d, and exert direct effects on pancreastic β cells, promote the growth of pancreastic islet cells to maximal density in 72 h, decease the time of latent phase and logarithm growth phase of pancreatic β cells (Figure 4A). The maximum stimulatory effect on cell growth phase was also achieved at 100 mg/L concentration among the five samples ($P < 0.01$)

As can be seen in Figure 4B, the results showed that exposure of primary cultured pancreatic cells to COS (100 mg/L) could continuously increase the secretion of insulin from the 6^{th} to the 14^{th} day, and had a significant difference compared with the normal control group ($P < 0.05$).

Effect of COS in streptozotocin-induced diabetic rats

All concentrations of chitooligosaccharides could decrease the 2hPG in 60 d (Figure 4C), the best effect was observed in the 500 mg/kg treatment group, with the 2hPG decreased to 16.14 mmol/L. The decrease rate was 47.48% compared with 2hPG before treatment.

The test of glucose tolerance was used to further evaluate the effects of different concentrations of chitooligosaccharides on improving the sensitivity of insulin. AS shown in Figure 4D, if the area under the DM group curve was regard as 100%, then AUC of the normal control group accounted for only 17.69% AUC of the DM group, while all concentrations of chitooligosaccharides could decrease AUC (Table 1). The best effect was observed in the medium dosage (500 mg/kg) treatment group, accounting for 68.69% of the area under the DM group curve ($P < 0.01$).

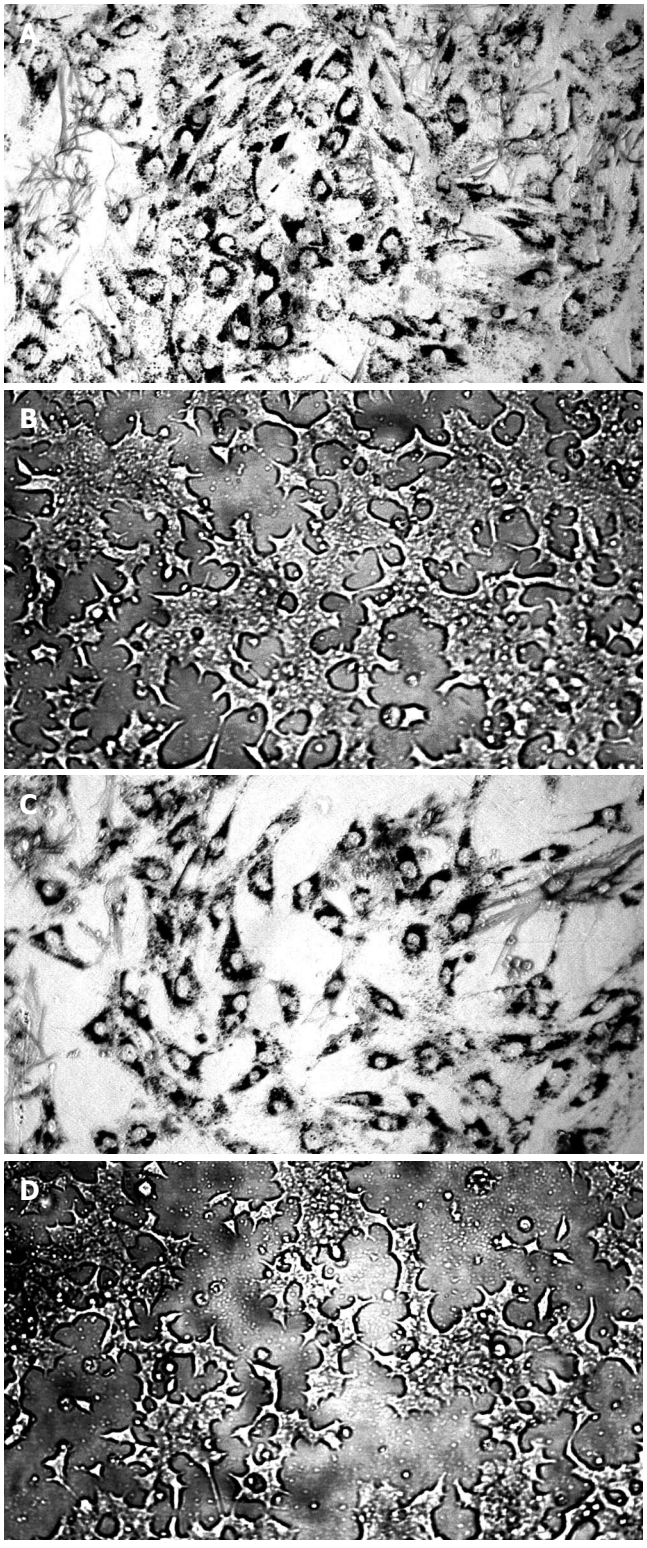

Figure 3 Photomicrograph of COS on the 48h proliferation of pancreatic islet cells and β cells (× 100) in control group (**A**) and 100 mg/L COS treatment group (**B**) of pancreatic islet cells, control group (**C**) and 100 mg/L COS treatment group (**D**) of β cell lines.

DISCUSSION

COS possess various biological activities and can be used in a number of industries. Unlike high molecular weight chitosan, COS which are readily soluble in water due to their shorter chain and free amino groups in D-glucosamine units and easily absorbed through the intestine, can quickly get into the blood flow and have systemic biological effects in the organism. In food industry, COS attract a greater interest as antimicrobial agents, antioxidants and enhancers of nutritional quality

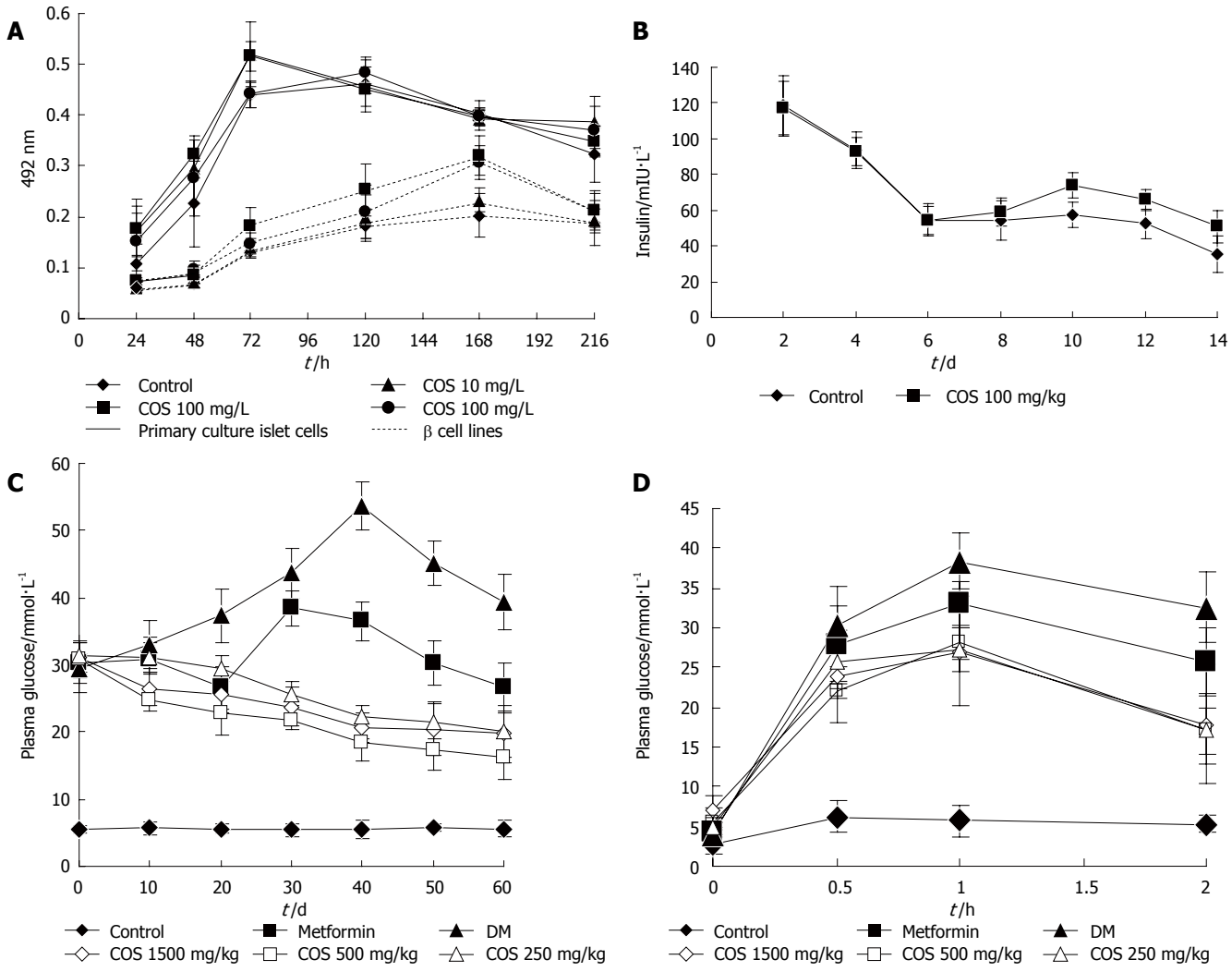

Figure 4 Effect of COS on growth phase of primary culture islet cells and β cell lines (**A**), insulin release from pancreatic islet cells (**B**), changes in 2 h plasma glucose (**C**), and in glucose tolerance test (**D**) after 60 d.

Table 1 Effect of COS on changes of plasma glucose in glucose tolerance test and AUC after 60 d (n = 9, mean ± SD)

Group	Concentration	Plasma glucose tolerance/mmol/L				AUC	AUC
	mg/kg	0 h	0.5 h	1 h	2 h	mmol/L	%
Control	-	2.75 ± 1.14[b]	6.27 ± 1.99[b]	5.78 ± 1.99[b]	5.34 ± 1.06[b]	21.66 ± 6.18[b]	17.69
Met	200	4.42 ± 1.72	28.01 ± 4.71	32.98 ± 2.98	25.64 ± 4.26	105.33 ± 3.74	86.05
DM	-	4.08 ± 1.40	30.20 ± 5.14	38.41 ± 3.41	32.54 ± 4.41	122.40 ± 12.05	100
COS-H	1500	7.01 ± 1.99	23.88 ± 5.86	27.04 ± 6.81[b]	17.88 ± 7.41[b]	85.79 ± 23.90[b]	70.09
COS-M	500	5.54 ± 1.77	22.05 ± 0.79[a]	28.17 ± 2.19[b]	17.01 ± 3.00[b]	84.08 ± 5.93[b]	68.69
COS-L	250	5.03 ± 0.51	25.62 ± 3.28	27.18 ± 2.72[b]	17.17 ± 4.42[b]	86.07 ± 8.06[b]	70.32

[a]$P < 0.05$, [b]$P < 0.01$ *vs* DM group.

of food[20,21], and also have various potential applications in agricultural industry[5]. Chemical and enzymatic methods are widely used to produce COS and chemical hydrolysis is more commonly used. However, chemical hydrolysis has some drawbacks to be commercialized, due to the production of some toxic compounds, higher risk associated with environmental pollution, and lower production yields. COS prepared by acid hydrolytic methods cannot serve as bioactive materials in general due to the possible contamination of toxic chemical compounds. Therefore, lack of proper technology for large-scale manufacture of COS with desired molecular weights has made its use difficult in human beings in the past years. Enzymatic processes which are generally carried out in batch reactors are preferred to chemical methods. This is due to minimized adverse chemical modifications of products during enzymatic hydrolysis and promotion of their biological activities. The properties of COS, such as DP, DA, charge distribution and nature of chemical modification in the molecules strongly influence its

observed biological activities. Therefore, molecular weight is considered a principal characteristic of COS that highly correlates to their biological activities. We have successfully obtained highly deacetylated (90%) chitooligosaccharides with a molecular weight of 1200 u by enzymatic hydrolysis and investigated its effect on the proliferation of pancreatic islet cells and release of insulin, lowering 2h plasma glucose and normalizing oral glucose tolerance *in vivo* and *in vitro*.

Type 2 diabetes characterized by peripheral insulin resistance followed by failure of compensation for pancreatic β cells and ultimately frank hyperglycemia[22], is traditionally considered discrete pathophysiological lesions. Proliferation of pancreatic β cells is slow and has a limited potential for regeneration (< 1% of the cells enter mitosis over 24 h in the adult state). Pancreatic β cells maintain the blood glucose concentration within a narrow range by modulating insulin secreting rate in response to the glucose levels in blood. Proper insulin secretion requires the coordinated functioning of numerous β cells that form pancreatic islets. This coordination depends on a network of communication mechanisms whereby β cells interact with extracellular signals and adjacent cells *via* connexin channels. The structure and function of primary culture islet cells and β cell lines were observed under inverted phase contrast microscope, and the viability of pancreatic islet cells and β cell lines was determined by MTT colorimetric assay indirectly in our study. In order to ensure adequate access of COS to pancreatic islet cell surfaces, we performed COS-stimulated insulin release experiments using monolayer cultures of rat islets. The results indicated that all concentrations of COS could markedly increase the viability of pancreatic islet cells. COS at the concentration of 100 mg/L effectively accelerated the proliferation of pancreatic islet cells and had direct effects on pancreastic β cells and release of insulin. Exposure of primary culture pancreatic cells to COS (100 mg/L) could continuously increase the secretion of insulin from 6 to14 d, as compared to the normal control group.

To study the specific role of COS in endocrine pancreatic function, we performed *in vivo* experiments of islet function in STZ-DM mice and measured the 2hPG and OGTT. Fasting plasma glucose and random plasma glucose used to be measured as the standard about the effect of lowering the plasma glucose. However neither fasting plasma glucose nor random plasma glucose can represent the actual glucose tolerance and insulin action[23-25]. The gold standards for assessment of insulin action and glucose homeostasis are the euglycemic, hyperinsulinemic and hyperglycemic clamp studies[26-28]. Clinically, single-value glucose determinations are probably the most commonly used marker of glucose homeostasis. Assessment of glucose tolerance in a clinical setting has traditionally been conducted using the glucose tolerance test[29,30]. Since 2 h plasma glucose is far more sensitive than fasting plasma glucose, we chose 2 h plasma glucose and oral glucose tolerance test as the standard to evaluate the effect of chitooligosaccharides in STZ -induced diabetic rats.

Our findings suggest that all concentrations of chitooligosaccharides can decrease 2hPG in 60 d. The best effect was observed in the medium dosage (500 mg/kg) treatment group, with the plasma glucose level decreased to 16.14 mmol/L compared to the DM group ($P < 0.01$). The medium dosage of chitooligosaccharides also had the best effect on glucose tolerance, accounting for 68.69% of the area under the DM group curve. The mechanism of chitooligosaccharides in decreasing the plasma glucose is perhaps due to the following reasons. Chitooligosaccharide is an alkaline, which can increase the pH values in the body fluid and the sensitivity of insulin. Chitooligosaccharides can regulate the function of the endocrine system, and reduce the secretion of insulin to normal, thus maintaining the normal metabolism of plasma glucose. Chitooligosaccharides can promote the proliferation of β cells and recovery of the function of damaged β cells.

Insulin is the primary hormone that regulates glucose uptake in mammals. In addition, a recent study demonstrated that insulin resistance itself itself at the level of β cells may contribute to the failure of pancreatic compensation, suggesting a theory for the pathogenesis of type 2 diabetes[31]. Indeed, mice with STZ -induced damage in β cells lose their ability to respond adequately to glucose stimulation and have progressive glucose intolerance. In the present study, we demonstrated that patients treated with COS had a normal β cell hyperplastic response to insulin resistance, with their 2hPG and urine glucose decreased, and the disorders of glucose tolerance normalized, indicating that COS play an important role in maintenance of glucose homeostasis *in vivo*.

In conclusion, COS possess various biological activities and can be used in the treatment of diabetes mellitus. COS can increase insulin secretion of pancreatic cells and improve the overgrowth of β cells and isolated pancreatic islet cells, decrease the 2hPG, and normalize the disorders of glucose tolerance in STZ-induced diabetic rats *in vivo*. Further study should be directed towards understanding their molecular mechanisms.

ACKNOWLEDGMENTS

We thank Mrs. Lie-Huan Chen for her help in revising this paper.

COMMENTS

Background

Chitin and chitosan can be isolated from different sources, crab and shrimp shell wastes are currently utilized as the major industrial source of biomass for the large-scale production of chitooligosaccharide (COS). Several methods have been recently used to prepare COS, and enzymatic preparation methods capture a great interest due to their safety and non-toxicity. COS possesses various biological activities and has been shown to be particularly useful in many fields. However, little attention has been paid to its activity in diabetes mellitus and related mode of action.

Research frontiers

Type 2 diabetes is characterized by peripheral insulin resistance followed by failure of compensation for pancreatic β cells and ultimately hyperglycemia. Type 2 diabetes is traditionally consodered discrete pathophysiological lesions. The proliferation of pancreatic β cells is slow with a limited potential for regeneration (< 1% of the cells enter mitosis over 24 h in the adult state). Pancreatic β cells maintain the blood glucose concentration within a narrow range by modulating insulin secreting rate in response to the glucose levels in blood. Proper insulin

secretion requires the coordinated functioning of numerous β cells that form pancreatic islets. This coordination depends on a network of communication mechanisms whereby β cells interact with extracellular signals and adjacent cells *via* connexin channels. Our study indicated that COS could increase insulin secretion of pancreatic cells and improve the overgrowth of β cells and isolated pancreatic islet cells, and decrease 2hPG, normalize the disorders of glucose tolerance of STZ-induced diabetic rats *in vivo*.

Innovations and breakthroughs

Soluble chitooligosaccharides with lower molecular weight were prepared by enzymatic hydrolysis of chitosan with t chitosanase. Enzymatic processes are generally carried out in batch reactors and are preferred to chemical methods. Our study indicated that COS could increase insulin secretion of pancreatic cells and improve the overgrowth of β cells and isolated pancreatic islet cells, decrease the 2hPG, and normalize the disorders of glucose tolerance of STZ-induced diabetic rats *in vivo*.

Applications

COS possesses various biological activities and can be utilized in a number of industries. Unlike high molecular weight chitosan, COS which is readily soluble in water due to its shorter chain and free amino groups in D-glucosamine units and easily absorbed through the intestine, can quickly get into the blood flow and has systemic biological effects in the organism. However, lack of proper technology for large-scale manufacture of COS with desired molecular weights has made its use difficult in human beings in the past years. Enzymatic processes which are generally carried out in batch reactors are preferred to chemical methods. This is due to minimized adverse chemical modifications of products during enzymatic hydrolysis and its biological activities. We have successfully obtained highly deacetylated (90%) chitooligosa-ccharide with a molecular weight of 1200 u by enzymatic hydrolysis and investigated its effect on the proliferation of pancreatic islet cells and release of insulin, lowering the 2 h plasma glucose and normalizing the oral glucose tolerance *in vivo* and *in vitro*. Our study indicate that COS could increase insulin secretion of pancreatic cells and improve the overgrowth of β cells and isolated pancreatic islet cells, and decrease the 2hPG, normalize the disorders of glucose tolerance in STZ-induced diabetic rats *in vivo*.

Terminology

Chitin, chitosan and chitooligosaccharides: Chitin is a natural polymer, the second most abundant organic resource on the earth next to cellulose. Chitosan is a polycationic copolymer consisting of β-1, 4-linked 2-acetamido-D-glucose and β-1, 4-linked 2-amino-D-glucose units. Both chitin and chitosan are copolymers of β (1-4) linked Nacetyl-β-glucosamine and glucosamine units. The proportion of N-acetyl-glucosamine units in total number of units determines the degree of deacetylation. The degree of deacetylation has an inverse relationship with the number of N-acetyl-glucosamine units, thus deacetylation of chitosan is achieved by removing Nacetyl group. In chitosan, the degree of deacetylation is higher than 50%. Chitooligosaccharide obtained by hydrolysis or degradation of chitosan, is not only water-soluble but also more effective than chitosan. NIT-1 cell line: NIT-1 cell line, a widely used β cell line in insulin secretion studies, is established from non-obese diabetic (NOD) mice transgenic for the SV40 T antigen under control of the insulin promoter; Streptozotocin (STZ): 2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose is actively transported into pancreatic β cells *via* the Glut-2 glucose transporter. It reacts at many sites in DNA but in particular at the ring nitrogen and exocyclic oxygen atoms of the DNA bases, predominantly producing 7-methylguanine, 3-methyladenine (3-meA), and *O*6-methylguanine adducts.

Peer review

This is a good descriptive study in which authors analyzed the antidiabetic effects of chitooligosaccharide (COS) on proliferation of pancreatic islet cells in streptozotocin-induced diabetic rats. The results are interesting and suggest that COS is a potential therapeutic substance that could be used in the treatment of diabetes mellitus. The authors also studies the interesting questions about the molecular mechanism involved in COS biological activity.

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CLINICAL RESEARCH

A randomized, double-blind, placebo-controlled trial assessing the efficacy and safety of tegaserod in patients from China with chronic constipation

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Abstract

AIM: To evaluate the efficacy and safety of tegaserod, 6 mg twice daily (b.i.d.), in men and women with chronic constipation (CC) from China.

METHODS: This was a multicenter, double-blind, placebo-controlled study. Following a 2-wk treatment-free baseline period, patients were randomized to receive either tegaserod (6 mg b.i.d.) or placebo (b.i.d.) for 4 wk. An analysis of covariance with repeated measures was used to determine the overall effect of treatment for the primary efficacy variable; the change from baseline in the number of complete spontaneous bowel movements (CSBMs) during the 4-wk treatment period. Secondary efficacy endpoints included other measures of response in terms of CSBMs, and patients' daily and weekly assessment of bowel habits. Safety was also assessed, based on the incidence and severity of adverse events (AEs).

RESULTS: A total of 607 patients were randomized to receive either tegaserod (n = 304) or placebo (n = 303). Tegaserod treatment resulted in a rapid and significant increase from baseline in the adjusted mean number of CSBMs per week over wk 1-4 compared with placebo (1.39 *vs* 0.91, P = 0.0002). A statistically significant difference in favor of tegaserod was also observed for a mean increase $\geqslant$ 1 CSBM/wk over wk 1-4 (47.7% *vs* 35.0%, tegaserod *vs* placebo, respectively, P = 0.0018) and for the absolute number of $\geqslant$ 3 CSBMs/wk over wk 1-4 (25.0% *vs* 14.5%, tegaserod *vs* placebo, respectively, P = 0.0021). Improvements in other symptoms of CC were also seen in the tegaserod group, including improved stool form and reduced straining. In addition, more patients in the tegaserod group reported satisfactory relief from their constipation symptoms. The frequency and severity of AEs was comparable between tegaserod and placebo groups, with the exception of a greater incidence of diarrhea in patients receiving tegaserod (3.6%) compared with placebo (1.7%).

CONCLUSION: Tegaserod treatment improved multiple symptoms of CC and was associated with a favorable safety profile.

Key words: Chronic constipation; tegaserod; China; Complete spontaneous bowel movement; Placebo-controlled; Stool

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INTRODUCTION

Chronic constipation/chronic idiopathic constipation (CC) is a gastrointestinal (GI) motility and sensory disorder that is commonly reported in many regions of the world, including Asia[1], North America[2], and Europe[3]. An epidemiological study conducted in Beijing concluded that 6.1% of the adult population were suffering from the symptoms of CC[4]. This compares with a survey of 3282 people in Hong Kong, of whom 14% were deemed to be suffering from CC[1]. The disorder is more common in women and elderly people[5]. The symptoms of CC impact on patients' quality of life (QoL) and result in frequent visits to physicians, particularly by older patients[1,6]. In

Hong Kong, approximately 25% (n = 820) of those surveyed were reported to visit a physician as a result of their symptoms[1].

The diagnosis of CC has primarily centered on the infrequency of the patients' bowel movements (BMs). However, CC is also associated with other symptoms that include straining, hard stools, feelings of incomplete evacuation, abdominal bloating, and abdominal discomfort/pain. While the pathophysiology of CC is still unclear, a proportion of patients with CC are assumed to have impaired GI motility[7]. This prolongs the length of time that stools remain in the bowel, allowing increased absorption of water from the stools which become hard and difficult to pass. Rome Ⅱ criteria refined the diagnosis of CC by providing a consensus definition that is frequently used in clinical research, and can serve as a useful guide for physicians[8]. The Rome Ⅱ criteria combine symptoms of straining, stool form and feelings of incomplete evacuation with measures of bowel frequency (less than three BMs per week).

A systematic review concluded that there were too few well-designed, randomized, placebo-controlled trials to support the efficacy of many of the available treatments for CC such as bulking laxatives (e.g., psyllium), osmotic laxatives [lactulose or polyethylene glycol (PEG)], and stimulant laxatives (senna or bisacodyl)[2]. Furthermore, other symptoms can be aggravated by laxative treatment (e.g., bloating)[9]. A further review found good evidence for the efficacy of PEG and tegaserod (Grade A recommendation) and moderate evidence to support the efficacy of lactulose and psyllium (Grade B recommendation)[10]. Other treatments for CC include the modification of patients' eating habits (increasing the consumption of dietary fiber/bulking agents), biofeedback training (where patients are taught relaxation and defecation techniques), and in severe cases, surgery. Evidence for the efficacy of these agents, however, is limited[7,11].

Targeting the pathophysiological basis of CC by stimulating intestinal motility and secretion may be a more appropriate approach for the treatment of the disorder, rather than using conventional treatments. Tegaserod is a selective agonist at the serotonin receptor, 5-HT$_4$, and has been shown to augment the release of neurotransmitters from the enteric nerves, hence stimulating intestinal peristalsis and secretion[12,13]. Two pivotal, randomized, placebo-controlled trials have demonstrated that tegaserod [2 mg or 6 mg twice daily (b.i.d.)] effectively treats the multiple symptoms of CC[14,15]. Tegaserod also effectively relieves the multiple symptoms of patients with irritable bowel syndrome (IBS) who suffer from constipation[16-18]. The majority of patients in the pivotal CC studies were Caucasian. Given that CC is a common disorder in China, the aim of the current study was to evaluate the efficacy and safety of tegaserod in men and women with CC from China.

MATERIALS AND METHODS

Study design

This was a randomized, multicenter, placebo-controlled, double-blind, parallel-group study, designed to evaluate the efficacy and safety of tegaserod in men and women with CC in China.

After completing an initial screening phase of up to 28 d, patients entered a 2-wk baseline period without study medication. At the end of the baseline period, eligible patients were randomized to receive either tegaserod 6 mg b.i.d. or placebo for 4 wk using a randomization list generated by Novartis Drug Supply Management, using Almedica Drug Label System, version 5.3 a, Almedica Technology Group Inc. All Novartis staff, other than the Drug Supply Management and the Biostatistics Quality Assurance Group, remained blind to the allocation of treatment until database lock. Randomization was performed in blocks using a 1:1 ratio. The randomization list was reviewed and locked by the Biostatistics Quality Assurance group. The identity of the treatments was concealed by using study tablets that were identical in appearance. The study was conducted in accordance with the Declaration of Helsinki and was reviewed by the Independent Ethics Committee or Institutional Review Board for each center.

Patient selection

Men and women 18 years of age or older with at least a 6-mo history of CC were eligible to participate in the study. CC was defined, according to a modification of Rome Ⅱ criteria, as less than three complete spontaneous bowel movements (CSBMs) per week accompanied by one or more of the following symptoms on more than 25% of occasions: very hard and/or hard stools (type 1 and/or 2 on the Bristol Stool Form Scale[19]), sensation of incomplete evacuation following at least 25% of BMs, and straining on at least 25% of days. All CC-related symptoms were confirmed by patient electronic diary (eDiary) data recorded during the baseline period.

Patients were excluded from entering the study if they had inflammatory bowel disease or other structural bowel disease, CC resulting from bowel surgery (with mechanical outlet obstruction, congenital anorectal malformation or clinically significant rectocele), abdominal pain/discomfort as the most bothersome symptom in the past 6 mo, past or current history or diagnosis of IBS, significant disorders or diseases that may interfere with completion of the study, or if they failed to complete the daily or weekly eDiary assessments during baseline. Patients who planned to use concomitant medications affecting bowel habits (including natural/homeopathic products) 1 wk prior to entry into baseline and during the study were also excluded, as were patients who used laxatives on more than two separate occasions during baseline. However, laxative use (bisacodyl, 15 mg/d) as a rescue medication was allowed for patients who did not experience a BM for at least 72 h.

Patients were excluded from the treatment phase if CC was not confirmed by the baseline eDiary data, if loose or watery stools were reported for 3 or more days during the baseline period, or for lack of compliance with the study protocol.

Assessments

The primary efficacy variable was the change from

baseline in the number of CSBMs per week during the 4-wk treatment period. Secondary efficacy variables included two response rates in terms of CSBMs: patients with a mean increase of one or more CSBM relative to baseline, and patients with an absolute number of three or more CSBMs per week during the 4-wk treatment period. Additional secondary efficacy variables included assessment of patients' bowel habits [i.e., stool form, frequency, and straining (which was recorded daily regardless of BM)], and patients' satisfaction with bowel habits, bothersomeness of constipation, distension/bloating, and abdominal discomfort/pain. Following each BM, patients were asked to record the time of the BM, whether the BM was accompanied by a feeling of complete evacuation (yes/no), and stool form (using the Bristol Stool Form Scale[19]). Patients evaluated their satisfaction with bowel habit on a 5-point scale ranging from 'not at all satisfied' to 'a very great deal satisfied', and bothersomeness of constipation, abdominal distension/bloating and abdominal discomfort/pain on a 5-point scale, which ranged from 'not at all bothersome' to 'a very great deal bothersome'. During the treatment phase of the study, patients also recorded whether they experienced satisfactory relief of constipation symptoms (yes/no) on a weekly basis.

The safety of tegaserod 6 mg b.i.d. *vs* placebo was evaluated by recording the frequency and severity of all adverse events (AEs) and serious adverse events (SAEs), by monitoring hematology, blood chemistry, urine and vital signs, and by performing electrocardiogram (ECG) evaluations. Other outcomes assessed but not presented in this paper included: patients' assessment of constipation on quality of life (PAC-QoL questionnaire) and patients' perception of study medication (PPSM questionnaire).

Statistical analysis

Planned enrollment was for 600 (n = 300 per arm) randomized patients with CC recruited from 15 centers across China. An assumption was made that the population distribution would be similar to that observed in the two pivotal CC trials[14,15]. Based on this assumption, the study was powered to detect a difference (tegaserod-placebo) in a change from baseline of 0.6 CSBMs/wk at a two-sided significance level of 5%. All statistical analyses were carried out with SAS® software (version 8.2).

As stated earlier, the primary efficacy variable was the change from baseline in the number of CSBMs per week during the 4-wk treatment period. Secondary efficacy variables included two response rates in terms of CSBMs: patients with a mean increase of one or more CSBMs relative to baseline, and patients with an absolute number of three or more CSBMs per week during the 4-wk treatment period. Additional secondary efficacy variables included assessment of patients' bowel habits [i.e., stool form, frequency, and straining (which was recorded daily regardless of BM)], and patients' satisfaction with bowel habits, bothersomeness of constipation, distension/bloating, abdominal discomfort/pain, and satisfaction with symptom relief (recorded weekly).

All efficacy analyses were performed on the intent-to-treat (ITT) population. Safety analyses included all patients who received at least one dose of study medication. An analysis of covariance (ANCOVA) model with repeated measures was used to analyze the overall effect of treatment for the primary efficacy variable (change from baseline in number of CSBM per week). The model included terms for treatment, week, study center, and baseline data as well as baseline data by week, and treatment by week interactions. To be defined as 'complete', BMs had to be associated with a sensation of complete evacuation. The BM was defined as spontaneous if no laxatives were taken during the 24 h prior to the BM.

Statistical analyses of the secondary efficacy variables [response rate relative to baseline (one or more CSBM per week) and response rate in terms of the absolute number of CSBMs (three or more CSBMs per week)] were carried out using a logistic regression model, with treatment and study center as factors, and the number of CSBMs per week at baseline as covariate.

Based on the daily eDiary assessments, the change from baseline was determined for the following variables: 1) the number of SBMs per week, 2) the number of days per week with no stools, hard or very hard stools, 3) the weekly mean straining score, and 4) the number of days with (too much) straining. The same ANCOVA model that was used for the primary endpoint was repeated for these variables. The number of patients with or without satisfactory relief [determined using a weekly eDiary assessment (patients were asked to consider whether they had satisfactory relief from their symptoms of CC in the past week)] were analyzed using Cochran Mantel Haenszel (CMH) tests, with center as a stratification factor.

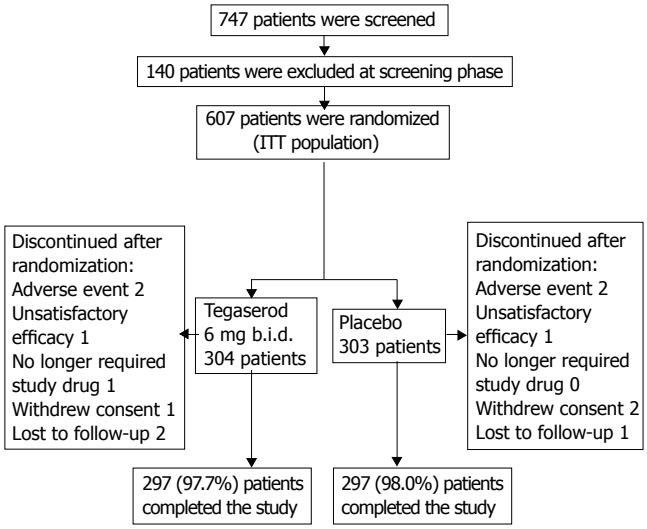

Figure 1 Participant flow.

RESULTS

Baseline

A total of 747 patients were screened for participation in this study, and 607 patients (81.3%) were randomized from a total of 15 centers to receive treatment with tegaserod 6 mg b.i.d. (n = 304) or placebo (n = 303) (Figure 1). The main reasons for screening failure were unacceptable laboratory values (5.0%), withdrawal of consent (4.3%)

Table 1 Patient demographic and baseline characteristics (ITT population)

Demographic variable	Tegaserod 6 mg b.i.d. (*n* = 304)	Placebo (*n* = 303)
Age (yr)		
Median	34.5	35
Range	18-80	18-78
Age group, years (*n*, %)		
< 35	152 (50.0)	151 (49.8)
35-64	132 (43.4)	135 (44.6)
⩾ 65	20 (6.6)	17 (5.6)
Sex (*n*, %)		
Male	70 (23.0)	61 (20.1)
Female	234 (77.0)	242 (79.9)
Race (*n*, %)		
Oriental	303 (99.7)	303 (100.0)
Other	1 (0.3)	0
Mean duration of constipation symptoms, months (SD)	100.3 (90.7)	94.9 (93.1)

and 'other' (4.3%). Most patients completed the double-blind treatment period (97.7% in the tegaserod group and 98.0% in the placebo group). One patient randomized to receive tegaserod did not receive study medication and was therefore excluded from the safety population.

Demographic and baseline variables were comparable between the tegaserod and placebo groups and most patients were Oriental [99.7% (tegaserod) *vs* 100.0% (placebo)] (Table 1).

Prior to randomization, the duration of patients' constipation symptoms was approximately 8 years in the tegaserod and placebo groups (Table 1). The mean number of CSBMs per week during the 2-wk baseline period was 0.36 in the tegaserod group and 0.31 in the placebo group. The most bothersome symptoms reported by patients subsequently randomized to tegaserod or placebo was straining (53.0% *vs* 56.1%), followed by feeling of incomplete evacuation (15.1% *vs* 15.2%), hard stools (13.5% *vs* 13.9%) and infrequent defecation (14.5% *vs* 11.9%). Laxative use in both treatment groups was comparable during the baseline period (35.5% and 36.0% of patients, randomized to tegaserod and placebo, respectively).

Primary efficacy variable

An increase from baseline in the overall number of CSBMs per week during the 4-wk treatment period was observed in patients receiving tegaserod (adjusted mean 1.39) and placebo (adjusted mean 0.91) (treatment difference; 0.48, 95% confidence interval; 0.23-0.73), yielding a statistically significant difference in favor of tegaserod ($P = 0.0002$, Figure 2 and Table 2). Tegaserod treatment significantly increased the number of CSBMs per week from baseline during each week of treatment, compared with placebo ($P < 0.05$) (Figure 3 and Table 2). Subgroup analysis revealed that men treated with tegaserod showed a greater increase from baseline in the overall number of CSBMs per week (wk 1-4) compared with women treated with tegaserod (the mean increase in the number of CSBMs per week was 1.67 in men and 1.29 in women).

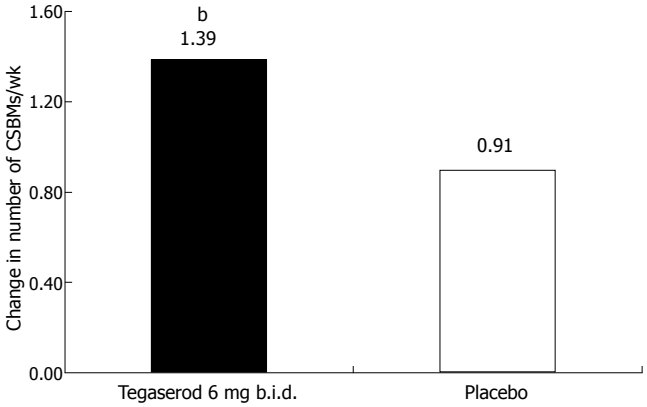

Figure 2 Change from baseline in the number of CSBMs per week (wk 1-4) by treatment (ITT population). Footnote: [b]*P* = 0.0002 *vs* placebo; Mean number of CSBMs per week at baseline: tegaserod 0.36; placebo 0.31.

Table 2 Treatment differences in change from baseline in number of CSBMs/wk for wk 1-4 (ITT population)

Time period		Tegaserod 6 mg b.i.d. *n* = 304	Placebo *n* = 303
wk 1-4	*n*	303	303
	Mean (SD)	1.38 (1.759)	0.89 (1.444)
	Adjusted mean[1]	1.39	0.91
	Median	0.75	0.25
	Min, max	-2.0, 9.0	-1.5, 7.5
	Tegaserod-placebo	0.48	
	(95% CI)[2]	(0.23, 0.73)	
	P value[3]	0.0002	
wk 1	Tegaserod–placebo	0.34	
	(95% CI)[2]	(0.05, 0.64)	
	P value[3]	0.0226	
wk 2	Tegaserod-placebo	0.54	
	(95% CI)[2]	(0.24, 0.84)	
	P value[3]	0.0004	
wk 3	Tegaserod–placebo	0.57	
	(95% CI)[2]	(0.27, 0.86)	
	P value[3]	0.0002	
wk 4	Tegaserod–placebo	0.47	
	(95% CI)[2]	(0.17, 0.77)	
	P value[3]	0.002	

CSBM: complete spontaneous bowel movement; SD: standard deviation; CI: confidence interval. Change from baseline (cfb) = post-baseline–baseline. A positive cfb indicates an increase in the number of CSBMs/wk. [1]Adjusted mean cfb. Calculated from least square mean estimate of repeated measures analysis. [2]Treatment difference (> 0 favors tegaserod). [3]Repeated measures model: cfb in number of CSBMs/wk = treatment (patient) + week + center + baseline + baseline *week + treatment *week.

Secondary efficacy variables

Analysis of response, defined as a mean increase of one or more CSBMs per week relative to baseline during the 4-wk treatment period, showed that treatment with tegaserod was significantly more effective than treatment with placebo [overall, tegaserod (47.7%) *vs* placebo (35.0%), $P = 0.0018$). While overall response was statistically significant for all 4 wk combined, statistical significance for individual weeks was reached at wk 2, 3 and 4 of treatment (Figure 4A).

Overall, treatment with tegaserod was superior to

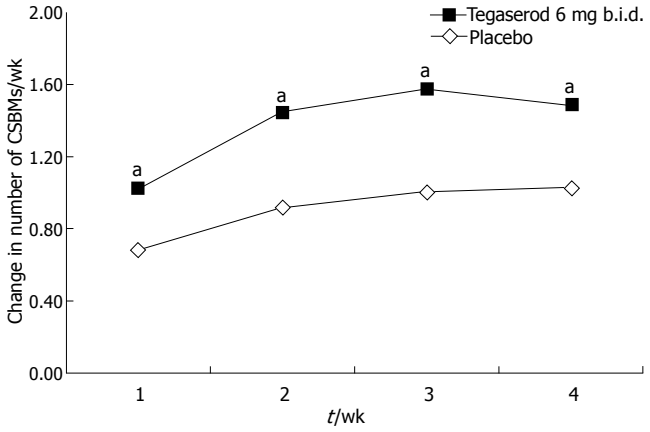

Figure 3 Change from baseline in the number of CSBMs per week by study week and treatment (ITT population). Footnote: $^{a}P < 0.05$ *vs* placebo; Mean number of CSBMs per week at baseline: tegaserod 0.36; placebo 0.31.

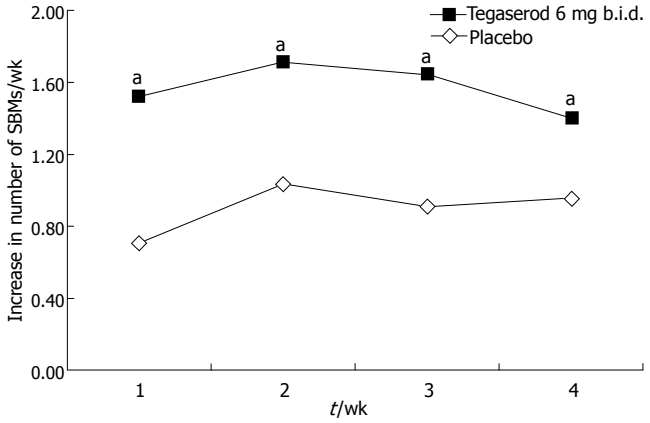

Figure 5 Change from baseline in number of SBMs per week by study week and treatment (ITT population). $^{a}P < 0.05$ *vs* placebo; Mean number of SBMs per week at baseline: tegaserod 2.33; placebo 2.26.

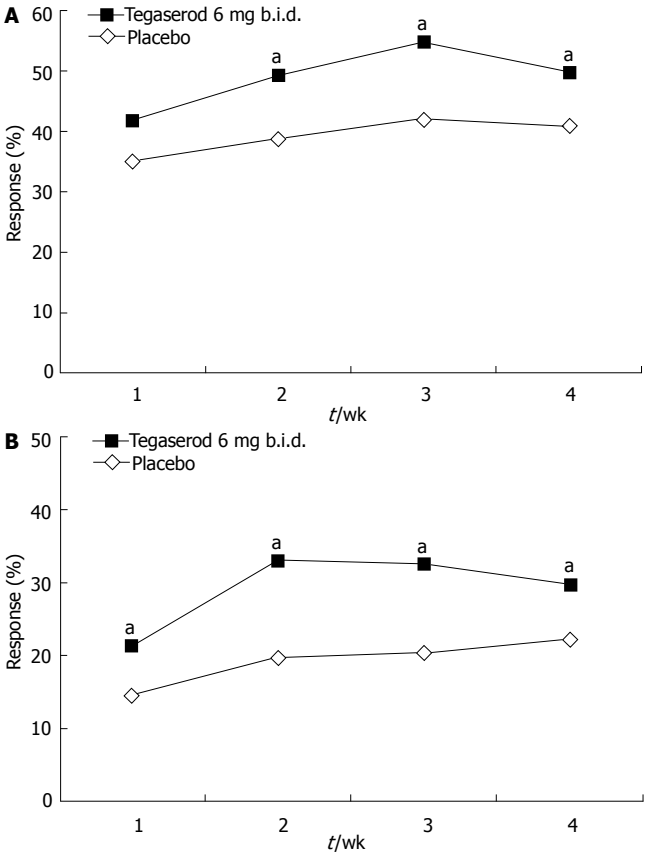

Figure 4 **A:** Response rate relative to baseline (increase of ≥ 1 CSBM per week) by week (ITT population); **B:** Absolute response rate (increase of ≥ 3 CSBM per week) (ITT population). $^{a}P < 0.05$ *vs* placebo; Mean number of CSBMs per week at baseline: tegaserod 0.36; placebo 0.31.

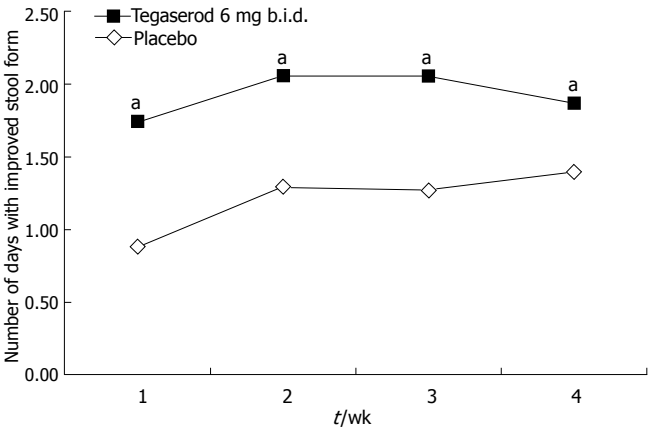

Figure 6 Change from baseline in number of days per week with no stool, hard, or very hard stools by treatment (ITT population). $^{a}P < 0.05$ *vs* placebo; An improvement was defined as a decrease in the number of days with no stool, hard, or very hard stools (Bristol stool score of 1 or 2[19]) relative to baseline.

treatment with placebo in terms of the absolute response rate (three or more CSBMs per week) [tegaserod (25.0%) *vs* placebo (14.5%), $P = 0.0021$]. This difference was statistically significant at each week of treatment (Figure 4B).

Assessment of constipation symptoms

Improvements in constipation symptoms were observed in patients receiving tegaserod over wk 1-4. Tegaserod significantly increased the number of SBMs per week compared with placebo (adjusted mean 1.57 *vs* 0.89, $P < 0.0001$), and this was statistically significant for each of the 4 wk of treatment ($P < 0.05$) (Figure 5). Compared with placebo, treatment with tegaserod also decreased the overall number of days per week (wk 1-4) with no stools, hard or very hard stools (adjusted mean -1.94 *vs* -1.19, $P < 0.0001$) and this was statistically significant for each of the 4 wk of treatment ($P < 0.05$) (Figure 6). Treatment with tegaserod also resulted in a decrease in the weekly mean straining score (adjusted mean -0.41 *vs* -0.33, $P = 0.0282$) and a decrease in the number of days per week with straining (adjusted mean -1.65 *vs* -1.24, $P = 0.0085$).

Significantly more patients receiving tegaserod than placebo responded positively to the question of whether they had 'satisfactory relief from their constipation symptoms over the past week of treatment' at all 4 wk of treatment (wk 1: 52.5% *vs* 35.0%; wk 2: 54.9% *vs* 40.5%; wk 3: 56.5% *vs* 41.3%; wk 4: 57.8% *vs* 44.2%; all $P < 0.05$) and at end of treatment (57.8% *vs* 43.9, $P < 0.05$) (Figure 7).

Trends in favor of tegaserod were observed for

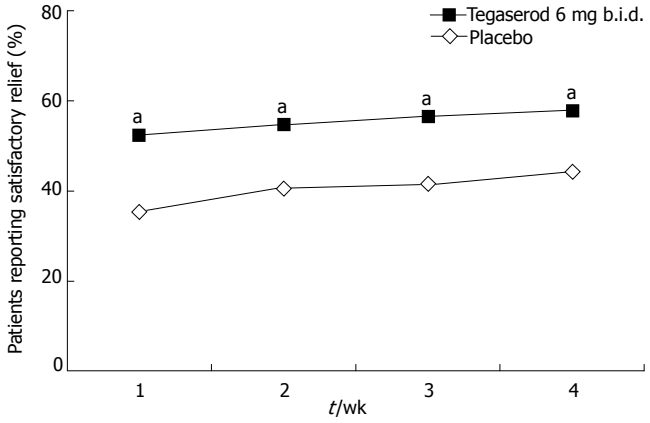

Figure 7 Satisfactory relief of constipation symptoms by week and treatment period (ITT population). $^{a}P < 0.05$ *vs* placebo.

the following secondary variables: bothersomeness of constipation, distension/bloating and abdominal discomfort/pain. Statistical significance was observed in wk 3 (bothersomeness of distension/bloating and abdominal pain/discomfort) and in wk 1-3 (bothersomeness of constipation). The number of patients using laxatives during the double-blind treatment period was higher in the placebo group (31.4%) compared with the tegaserod group (27.0%), although this difference was not statistically significant.

Safety assessments

The AEs reported in this study were mostly mild and transient. The overall frequency of AEs was similar in both the tegaserod and placebo groups (9.9% *vs* 11.2%) (Table 3). Diarrhea was the most common AE, reported by 3.6% of patients in the tegaserod group and 1.7% of patients in the placebo group. The study investigators considered the majority of cases of diarrhea to be mild (no cases were reported to be severe), and transient [median duration of first episode of diarrhea: 2 d (tegaserod group) *vs* 3 d (placebo group)]. All cases of abdominal pain were reported to be mild or moderate in severity and were reported with equal frequency in both groups (1.7% in each group).

The number of discontinuations due to AEs was the same in both treatment groups (0.7% each) (AEs resulting in discontinuation included diarrhea, dizziness, hypertension, rash, tinnitus, venous thrombosis in the limb, and vertigo). Other reasons for discontinuation included unsatisfactory therapeutic effect (0.3% in each group), patients no longer requiring study drug due to symptom improvement (0.3% in the tegaserod group and 0.0% in the placebo group), withdrawal of consent (0.3% in the tegaserod group and 0.7% in the placebo group) and patients lost to follow-up (0.7% in the tegaserod group and 0.3% in the placebo group). Five SAEs were reported, none of which were considered to be related to the study drug [one case of ureteric cancer in the tegaserod group (0.3%) and one case each of ankle fracture, pregnancy, hemorrhoid surgery, and venous thrombosis in the limb in the placebo group (1.3% in total)]. No cases of ischemic colitis were reported during this study, and no deaths occurred. Laboratory and ECG evaluations, and vital signs were comparable between treatment groups.

Table 3 Incidence of most frequent AEs, regardless of relationship to study drug (safety population)

Adverse event	Tegaserod 6 mg b.i.d. (n = 303) (%)	Placebo (n = 303) (%)
Diarrhea	11 (3.6)	5 (1.7)
Abdominal pain	5 (1.7)	5 (1.7)
Nasopharyngitis	1 (0.3)	7 (2.3)
Transaminases increased	3 (1.0)	1 (0.3)
Nausea	1 (0.3)	3 (1.0)
Abdominal distension	3 (1.0)	0
Dizziness	2 (0.7)	0
Leukopenia	1 (0.3)	1 (0.3)
Urinary tract inflammation	1 (0.3)	1 (0.3)
Abdominal pain upper	0	2 (0.7)

DISCUSSION

This is the first randomized, double-blind, placebo-controlled trial designed to evaluate the efficacy and safety of tegaserod in an adult population of men and women from China, who met the Rome Ⅱ diagnostic criteria for CC. The recent Rome Ⅲ criteria, published after this study was conducted, have further refined diagnostic criteria for CC[20].

The key efficacy analyses demonstrated that tegaserod improves multiple symptoms of CC. The results revealed that treatment with tegaserod was associated with a rapid and significant increase from baseline in the number of CSBMs at wk 1, which was sustained over each of the 4 wk of treatment. Secondary efficacy analyses also showed statistically significant improvements for tegaserod over placebo with regard to evaluation of bowel habits and satisfaction with constipation relief.

The responder rate for mean increase of one or more CSBM per week during the 4-wk treatment period [tegaserod (47.7%) *vs* placebo (35.0%)] was similar to the results obtained with Caucasian patients[14,15] suggesting that patients from China with CC respond in a similar fashion to those from Western countries.

Subgroup analysis confirmed that tegaserod relieved the multiple symptoms of CC in both men and women. This observation has clinical relevance, as fewer data are available in men, and further confirms the results from other pivotal studies[14,15].

Treatment with tegaserod was associated with a safety profile similar to that seen with placebo, although slightly more patients receiving tegaserod than placebo reported mild and transient diarrhea. Diarrhea is a predictable pharmacological event, and is likely due to tegaserod's promotile effect that stimulates peristalsis, reduces stool hardness and accelerates orocecal transit, promoting stool expulsion[12,13]. The increased incidence of diarrhea following treatment with tegaserod was similar to that reported in all other clinical studies of patients with CC from different ethnic groups[14,15,17,18].

The primary efficacy variable used in this study was the change from baseline in the number of CSBMs per week.

The assessment of SBMs discounts laxative-induced BMs, while further characterizing SBMs as 'complete' ensures that measurements are not simply based on an increase in the number of small hard pellets that are passed, which would leave symptoms unimproved for the patient. Hence, the definition of CSBM used in this study provides a subjective measure of BMs that are associated with a sense of complete evacuation, while in addition, providing an objective measure of the number of BMs. This assessment of CSBMs is considered to be able to detect changes that are meaningful to the patient.

Medications, such as laxatives, which are traditionally used to treat CC, may improve the frequency of BMs, but they do not treat the underlying causes of CC and have no proven effect on the multiple symptoms including straining, incomplete evacuation, abdominal bloating and abdominal discomfort/pain[9,21]. Therefore, in order to control their symptoms, patients often rely on multiple treatments, which are often ineffective. These include the increased intake of fiber, modifications in lifestyle and diet, and the use of prescription/non-prescription laxatives. This has led to high patient dissatisfaction and frustration with current treatments for CC[1], and hence there is a need for simple, safe, and effective first-line therapies to treat the multiple symptoms of patients with this disorder.

In conclusion, this was the first rigorously designed study to evaluate the efficacy and safety of tegaserod in an adult population of men and women from China with CC. The results of key efficacy analyses demonstrated that compared with placebo, tegaserod 6 mg b.i.d. increased the frequency of CSBMs, improved multiple symptoms of constipation, and was associated with a safety profile that is similar to that of placebo. Therefore, tegaserod offers an effective treatment option for patients from China with CC.

ACKNOWLEDGMENTS

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COMMENTS

Background

Chronic constipation/idiopathic constipation (CC) affects 11% to 14% of the Chinese population; however, currently prescribed first-line therapies for CC are suboptimal. Tegaserod is a selective partial agonist at the serotonin receptor, 5-HT$_4$, which is a well tolerated and effective treatment for the multiple symptoms of CC in Caucasian patients. However, its efficacy in patients from Asia-Pacific countries is unknown.

Research frontiers

There has been increasing interest in the use of serotonergic agents, such as tegaserod, for the treatment of gastrointestinal motility disorders. This study is the first randomized, double-blind, placebo-controlled trial designed to evaluate the efficacy and safety of tegaserod in men and women from China with CC.

Innovations and breakthroughs

The placebo-controlled trials demonstrating the promotile action of tegaserod on the gut (Degen *et al*, 2000 and Prather *et al*, 2000) led to the design of phase Ⅲ trials of tegaserod in patients with irritable bowel syndrome whose predominant symptom was constipation (IBS-C) and patients with CC. These studies demonstrated that treatment with tegaserod relieved multiple symptoms of these burdensome conditions (Johanson *et al*, 2004; Kamm *et al*, 2005; Müller-Lissner *et al*, 2001; Novick *et al*, 2002; Tack *et al*, 2005). As these studies were performed predominantly in patients from Western countries, this study aimed to evaluate the efficacy of tegaserod for the treatment of CC in patients from China.

Applications

The results of this study demonstrated that compared with placebo, tegaserod 6 mg b.i.d. increased the frequency of CSBMs, improved multiple symptoms of constipation, and was well tolerated in both men and women with CC from China.

Terminology

Complete spontaneous bowel movement (CSBM): The term 'CSBM' refers to the spontaneous occurrence of a bowel movement associated with a feeling of complete evacuation. Assessing bowel movements as 'spontaneous' discounts laxative-induced bowel movements, but further characterizing spontaneous bowel movements as 'complete' (CSBMs) ensures that measurements are not simply based on an increase in the number of small hard pellets that are passed, which would leave symptoms unimproved for the patient. The definition of CSBM used in this study therefore provides a subjective measure of bowel movements that are associated with a sense of complete evacuation, while in addition, providing an objective measure of the number of bowel movements. Tegaserod: A selective partial agonist of the 5-HT$_4$ receptor.

Peer review

This is an excellently designed, performed and presented study examining the efficacy of tegaserod in Chinese patients with chronic idiopathic constipation.

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CLINICAL RESEARCH

Influence of age on outcome of total laparoscopic fundoplication for gastroesophageal reflux disease

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Abstract

AIM: To demonstrate that age does not influence the choice of treatment for gastroesophageal reflux disease (GERD). We hypothesized that the outcome of total fundoplication in patients > 65 years is similar to that of patients aged ⩽ 65 years.

METHODS: Four hundred and twenty consecutive patients underwent total laparoscopic fundoplication for GERD. Three hundred and fifty-five patients were younger than 65 years (group Y), and 65 patients were 65 years or older (group E). The following elements were considered: presence, duration, and severity of GERD symptoms; presence of a hiatal hernia; manometric evaluation, 24 h pH-monitoring data, duration of operation; incidence of complications; and length of hospital stay.

RESULTS: Elderly patients more often had atypical symptoms of GERD and at manometric evaluation had a higher rate of impaired esophageal peristalsis in comparison with younger patients. A mild intensity of heartburn often leads physicians to underestimate the severity of erosive esophagitis. The duration of the operation was similar between the two groups. The incidence of intraoperative and postoperative complications was low and the difference was not statistically significant between the two groups. An excellent outcome was observed in 92.9% young patients and 91.9% elderly patients.

CONCLUSION: Laparoscopic antireflux surgery is a safe and effective treatment for GERD even in elderly patients, warranting low morbidity and mortality rates and a significant improvement of symptoms comparable to younger patients.

Key words: Gastroesophageal reflux disease; Esophagitis; Impaired peristalsis; Hiatal hernia; Laparoscopic total fundoplication

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INTRODUCTION

The population of elderly is rapidly growing globally, e.g. in the USA nearly 20 million of people will be more than 85 years old in the next fifty years[1]. Digestive diseases are common causes of morbidity and mortality in the elderly[2]. Among them gastroesophageal reflux disease (GERD) is usually more severe than in younger patients, which is frequently under-diagnosed and less treated[2]. This results in an increase of esophageal mucosal injuries and subsequent complications. Therefore, a more aggressive treatment has been advocated in these patients[3]. However, a higher morbidity and mortality of open surgery in the elderly, limited the number of these patients referred for surgical treatment. Moreover, their shorter life expectancy made surgery to be deemed a cost-ineffective strategy.

The advent of laparoscopic fundoplication has greatly reduced the morbidity of antireflux surgery and by now, it should be considered the surgical treatment of choice for GERD[4]. The aim of the current study is to review the outcome of young and elderly patients undergoing laparoscopic antireflux surgery for the treatment of GERD.

MATERIALS AND METHODS

From September 1992 to December 2005, 420 consecutive patients, 171 male and 249 female, mean age 42.8 years (range 12-80) with GERD underwent laparoscopic Nissen-Rossetti fundoplication. The preoperative and postoperative data were prospectively collected. Demographic data were obtained at the time of first visit. Sixty-five patients older than 65 years of age were defined as the elderly group (EG) whereas the remaining 355 younger than 65

years of age were defined as the young group (YG). Ethics board approval for collecting and using these data was obtained.

Preoperative evaluation

Preoperatively all patients underwent Upper Gastrointestinal Endoscopy (UE), X-ray of barium swallow, esophageal manometry and 24-h pH monitoring. They were off peptic medications for thirty days. The medical evaluation included a structured questionnaire based on modified DeMeester symptom scoring system (Table 1). Measurement of hiatal hernia size was performed at the end of endoscopic examination after deflation of the stomach or by X-ray of barium swallow with video-fluoroscopy. The hernia size was measured as the distance between the centre of the diaphragmatic hiatus and the superior aspect of gastric folds. A hiatal hernia was deemed to be present if either gastric folds or a hernia pouch was present above the diaphragm between swallows. Esophagitis severity was assessed by means of Savary-Miller grading system. The location of Barrett's esophagus was noted; and the esophageal strictures, paraesophageal hernias and reinterventions were excluded from the study. Stationary esophageal manometry was carried out using 8-channel perfusion catheters, 4 disposed radially and oriented at 90° to each other and 4 positioned longitudinally at intervals of 5 cm. The catheter was perfused with distilled water using a low-compliance capillary pump at a constant infusion rate of 0.8 mL/min at 1.2 kg/cm^2. A system of pressure transducers transmitted data to an acquisition device (ACQ1^{TM}-Menfis bioMedica-Bologna, Italy) and from there to a personal computer. A specific software package (Dyno 2000^{TM}-Menfis bioMedica-Bologna, Italy) was used for data acquisition and processing. The following variables were assessed: (1) pressure of the lower esophageal sphincter; (2) relaxation of the lower esophageal sphincter (LES) in response to swallowing; (3) amplitude and propagation of peristalsis (esophageal peristalsis was considered impaired when < 30 mmHg). The LES was studied by both the stationary and the rapid pull-through methods. Esophagogastric pH monitoring was carried out using two glass probes which were connected to a portable, solid-state recorder (Digitrapper Proxima^{TM}-Synetics Medical, Sweden): the electrodes were placed, respectively, 5 cm above the proximal margin and 5 cm below the distal margin of the LES, identified by means of stationary manometry. For statistical analysis, results were expressed as a mean value ± SD; correlations among the various parameters were analysed using Fischer's exact test. The Wilcoxon signed rank test was used to compare the preoperative and postoperative modified DeMeester symptom score. American Society of Anaesthesiologists (ASA) grade was recorded at the time of surgery.

Postoperative evaluation

On an outpatient basis, the patients came to our department each six months for the first postoperative year and, after, each year and were invited to fulfil a standardized questionnaire dealing with presence of typical or atypical symptoms and based on the modified DeMeester score (Table 1). Satisfaction of the procedure and the will of undergoing the same operation after knowing its effects were defined as excellent outcome

Instrumental follow-up after surgery included: X-ray of barium swallow (performed at 1 year after surgery), esophageal manometry (performed at 6 mo, 1 year, and 2 years after surgery) and 24 h pH monitoring (performed at 1 year after surgery).

Statistical analysis was carried out using SPSS for Windows (version 12.0, SPSS Inc. Chicago, IL). Results were expressed as mean ± SD unless otherwise indicated. Student's *t* test, the Chi-square test, the Fischer's exact test and the Wilcoxon signed rank test were used as appropriate. *P* value < 0.05 was considered statistically significant.

Table 1 Modified DeMeester scoring system

Symptoms	Score	Description
Dysphagia	0	None
	1	Occasional transient episodes
	2	Require liquids to clear
	3	Impaction requiring medical attention
Heartburn	0	None
	1	Occasional brief episodes
	2	Frequent episodes requiring medical treatment
	3	Interference with daily activities
Regurgitation	0	None
	1	Occasional episodes
	2	Predictable by posture
	3	Interference with daily activities

Table 2 Preoperative evaluation: data and ASA score in EG and YG

Demographics	EG (> 65 yr)	YG (< 65 yr)	*P*
Age (mean yr ± SD)	72.6 ± 2.1	48.2 ± 3.2	< 0.05
Male:Female	1:1.5	1:1.7	NS
ASA score	2.2 ± 0.43	1.82 ± 0.51	< 0.05
Weight (mean ± SD)	64.4 ± 5.1	65.3 ± 6.4	NS

Table 3 Incidence of pre-operative symptoms in EG and YG

Symptoms	EG (%)	YG (%)	*P*
Heartburn	44/65 (67.7)	298/355 (83.9)	< 0.05
Acid regurgitation	39/65 (60.0)	277/355 (78.0)	< 0.05
Solid food dysphagia	22/65 (33.8)	27/355 (7.6)	< 0.05
Chest pain	18/65 (27.7)	51/355 (14.4)	< 0.05
Respiratory complication (chronic cough, sleep apnoea, asthma, laryngitis)	27/65 (41.5)	19/355 (5.4)	< 0.05

RESULTS

Preoperative data

Demographics data and ASA score of the two groups are listed in Table 2. In the YG, the mean duration of preoperative symptoms was 4.6 ± 2.3 years (range 1-11) whereas in the EG it was 8.3 ± 2.5 years (range 5-22). Tables 3 and 4 depict the incidence and severity of typical and atypical

Table 4 Severity of preoperative symptoms in EG and YG (mean ± SD)

Symptoms	EG	YG	*P*
Heartburn	1.7 ± 0.87	2.7 ± 0.74	< 0.05
Acid regurgitation	1.5 ± 0.96	2.3 ± 0.89	< 0.05
Solid food dysphagia	1.6 ± 0.76	0.5 ± 0.2	< 0.05
Chest pain	1.6 ± 0.82	1.5 ± 0.87	> 0.05
Respiratory complication (chronic cough, sleep apnoea, asthma, laryngitis)	1.8 ± 1.04	1.0 ± 0.45	< 0.05

Table 5 Preoperative manometric evaluation in EG and YG

Manometry	EG	YG	*P*
LES pressure (mmHg)	11.2 ± 1.5	11.0 ± 1.2	> 0.05
Impaired esophageal peristalsis (< 30 mmHg) N° of patients	43/65 (66.2%)	114/355 (32.1%)	< 0.05

Table 6 Preoperative evaluation: incidence, size of HH and pH metric data in NERD, ERD and Barrett patients in EG and YG

	EG NERD	YG NERD	*P*	EG ERD	YG ERD	*P*	EG Barrett	YG Barrett	*P*
Patients *n* (%)	15/65 (23.1)	220/355 (62)	< 0.05	45/65 (69.2)	125/355 (35.2)	< 0.05	5/65 (7.7)	10/355 (2.8)	< 0.05
Hiatal Hernia *n* (%)	13/15 (86.7)	148/220 (67.3)	< 0.05	39/45 (86.7)	97/125 (77.6)	< 0.05	4/5 (80)	8/10 (80)	-
Hiatal Hernia size (cm)	1.2 ± 0.18	0.3 ± 0.1	< 0.05	4.1 ± 1.9	2.3 ± 0.2	< 0.05			
De Meester score	13.1 ± 1.2	12.4 ± 1.2	> 0.05	17.5 ± 1.4	14.3 ± 1.2	> 0.05	18.2 ± 1.3	16.2 ± 1.4	> 0.05
(%) time pH < 4 (total)	11 ± 3	6 ± 2	< 0.05	26 ± 3	11 ± 5	< 0.05	27 ± 6	27 ± 5	> 0.05
(%) time pH < 4 (supine)	12 ± 4	7 ± 2	< 0.05	28 ± 4	13 ± 4	< 0.05	29 ± 5	30 ± 8	> 0.05
(%) time pH < 4 (upright)	9 ± 4	5 ± 2	< 0.05	15 ± 5	5 ± 3	< 0.05	25 ± 2	22 ± 7	> 0.05

symptoms in both groups. At manometric evaluation, no statistically significant differences in the mean LES pressure were found when the two groups were compared (*P* = NS) but the EG had a higher rate of impaired esophageal peristalsis (defined as peristaltic waves with a pressure value lower than 30 mmHg) in comparison with their younger counterparts (*P* < 0.05) (Table 5). Incidence of Hiatal Hernia (HH) was 89.2% (58/65) in elderly patients and 71.3% (253/355) in young patients (*P* < 0.05).

Table 6 shows the prevalence of HH and esophagitis and pH metric values either in Non-erosive reflux disease (NERD) and in Erosive reflux disease (ERD) patients. In the EG, 45/65 (69.2%) patients presented with esophagitis (ERD group): 11 of 45 (24.4%) had a grade I esophagitis while 34 out of 45 (75.6%) had a grade Ⅱ-Ⅲ esophagitis. In the YG, 125/355 (35.2%) patients presented with esophagitis (ERD group): 76 out of 125 (60.8 %) had a grade I esophagitis while 49 of 125 (39.2%) had a grade Ⅱ-Ⅲ esophagitis.

Therefore, in the EG, a significant higher grade of esophagitis has been found along with a higher incidence of Barrett esophagus (Table 6).

A pathologic DeMeester score was found at pH-monitoring in all patients of both subgroups: in the YG, it was 12.4 ± 1.2 and 14.3 ± 1.2, whereas in the EG it was 13.1 ± 11.2 and 17.5 ± 1.4 respectively for NERD and ERD subgroups. The mean percentage of total time < 4 at 24-h pH monitoring in NERD and ERD subgroups, is shown in Table 6.

Perioperative results

All the interventions were completed *via* laparoscopic approach. Mean operative time was 45 ± 14 min in YG and 61 ± 15 min in EG. No mortality was observed in both groups. A major complication occurred in 4/420 patients (1.0%), all among the YG. Mean postoperative hospital stay was 2.4 ± 0.9 d in YG (range 1-5) and 3.8 ± 1.0 d in EG (range 1-7) (*P* < 0.05). Normal activity resumed in 8.3 ± 3.4 d in YG and 12.5 ± 9.0 d in EG (*P* < 0.05) (Table 7).

Table 7 Perioperative results in EG and YG

Intraoperative results	EG	YG	*P*
Operative time (m)	61 ± 15	45 ± 15	< 0.05
Operative blood loss (mL)	50 (0-120)	30 (0-100)	< 0.05
Major complications	0	4/355 (1.1%)[1]	-
Mortality	0	0	-
Postoperative recovery			
Post operative hospital stay (d)	3.8 ± 1.0	2.4 ± 0.9	< 0.05
Resumption of normal activity (d)	12.5 ± 9.0	8.3 ± 3.4	< 0.05

[1]1/335 intraoperative mucosal tear, 3/335 postoperative bleeding (1 splenectomy).

Postoperative results

We followed up clinically 408 (97.1%) of 420 patients, 62 (95.3%) patients in the EG and 338 (95.2%) patients in YG. Two patients in the EG died four years after surgery for no surgery correlated event. In the YG, the mean follow-up was 83.2 ± 7 mo (range 6-141) whereas in EG it was 60 ± 8 mo (range 6-95).

An excellent outcome was observed in 314/338 (92.9%) younger patients and in 57/62 (91.9%) elderly patients (*P* > 0.05). Both groups showed significant improvement

Table 8 Postoperative symptoms score in EG and YG (mean symptom score ± SD)

Symptoms	EG		*P*	YG		*P*
	Preop.	Postop.		Preop.	Postop.	
Heartburn	1.7 ± 0.87	0.2 ± 0.12	< 0.05	2.7 ± 0.74	0.3 ± 0.11	< 0.05
Acid regurgitation	1.5 ± 0.96	0.3 ± 0.13	< 0.05	2.3 ± 0.89	0.2 ± 0.12	< 0.05
Solid food dysphagia	1.6 ± 0.76	0.4 ± 0.12	< 0.05	0.5 ± 0.2	0.2 ± 0.15	< 0.05
Chest pain	1.6 ± 0.82	0.3 ± 0.21	< 0.05	1.5 ± 0.87	0.2 ± 0.13	< 0.05
Respiratory complication (chronic cough, sleep apnoea, asthma, laryngitis)	1.8 ± 1.04	0.3 ± 0.11	< 0.05	1.0 ± 0.45	0.2 ± 0.12	< 0.05

Preop: preoperative; Postop: postoperative.

Table 9 Postoperative side effects in EG and YG

	EG	YG	*P*
Postoperative side effects: number patients (%)			
Dysphagia	2/62 (3.2%)[1]	11/338 (3.3%)[2]	> 0.05
Heartburn	2/62 (3.2%)[3]	12/338 (3.6%)[4]	> 0.05
Hyperflautolence	1/62 (1.6%)	6/338 (1.8%)	> 0.05
Early satiety	2/62 (3.2%)	9/338 (2.7%)	> 0.05
Bloating	1/62 (1.6%)	3/338 (0.9%)	> 0.05
Chest pain	0	2/338 (0.6%)	> 0.05

[1]2 dilation; [2]5 dilation, 6 laparoscopic re-fundoplication; [3]2 reassumed peptic medications; [4]8 reassumed peptic medications, 4 laparoscopic re-fundoplication.

Table 10 Postoperative manometric evaluation at 24 mo after surgery in EG and YG

Manometry	EG (36 Pts)		*P*	YG (230 Pts)		*P*
	Preop.	Postop.		Preop.	Postop.	
N-HPZ pressure (mmHg)	11.2 ± 1.5	28.2 ± 1.5	< 0.05	11.0 ± 1.2	28.1 ± 1.2	< 0.05
Increase of mean peristalsis waves patients *n* (%)		28/36 (77.8%)			100/230 (43.5%)	

Preop: preoperative; Postop: postoperative.

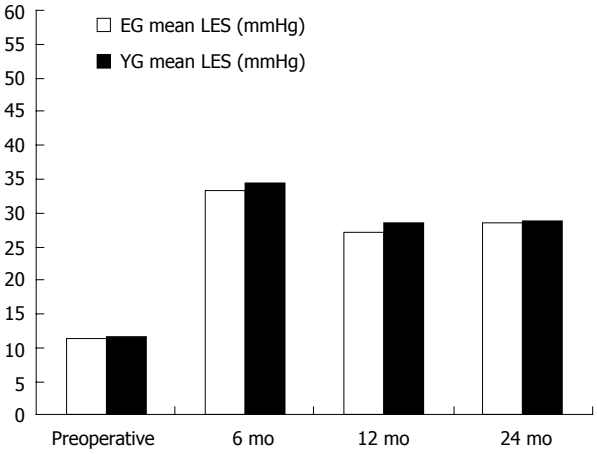

Figure 1 Modification in LES (mean in mmHg) in EG and YG.

in clinical symptom score (Table 8). At 6 mo, persisting postoperative dysphagia (DeMeester score 2-3) leading to > 15% of weight loss was observed in 11 (3.3%) of 338 patients in YG, 2 patients in the group with preoperative impaired peristalsis and 9 in the group with normal esophageal motility (Table 9). In EG, persisting postoperative dysphagia was relieved in 2 (3.2%) of 62 patients, both in group with normal preoperative esophageal peristalsis (Table 9).

No statistically significant difference was observed between patients with normal and impaired peristalsis. Five patients in YG and both 2 patients in EG were treated with endoscopic dilatation, whereas 6 patients in YG underwent a laparoscopic redo-funduplication with partial resolution of dysphagia. Recurrent heartburn was observed and confirmed with 24 h pH monitoring follow-up in 14/408 patients (3.4%), which was due to a disrupted wrap, an herniated wrap, and a slipped Nissen detected at X-ray barium in 7, 4, and 3 cases, respectively.

Ten patients reassumed their peptic medications; the remaining 4 patients, all in YG, underwent redofunduplication with partial resolution of symptoms. Respiratory symptoms showed a significant improvement in both groups (Table 9). Other data regarding hyper-flatulence, early satiety and bloating are depicted in Table 9.

Esophageal manometric follow-up (performed at 6, 12, and 24 mo after surgery) was made in 331 (81.1%) of 408 patients at 6 mo (48/62, 77.4% in EG and 283/338, 83.7% in YG), 275/408 (67.4%) at 12 mo (38/62, 61.3% in EG and 237/338, 70.1% in YG), and 266/408 (65.2%) at 24 mo (36/62, 58.1% in EG and 230/338, 68.0% in YG). Stationary esophageal manometry showed a significant improvement in the mean new high pressure zone (N-HPZ) value in comparison with preoperative values in the two groups ($P < 0.05$) (Table 10 and Figure 1); Manometric evaluation at 24 mo after surgery showed an increase of mean peristalsis waves in 28/36 (77.8%) patients of the EG and 100/230 (43.5%) patients of the YG.

Twenty-four hour pH monitoring at 1 year after surgery was performed in 205/408 (50.2%) patients. There was a significant postoperative decrease in DeMeester score and percentage of time pH < 4 during 24 h (Table 11).

DISCUSSION

Gastroesophageal reflux disease (GERD) is a common disorder in the western population; periodically symptoms

Table 11 De Meester score and percentage of reflux time during 24 h in EG and YG, preoperative and 1 yr after surgery

	Preoperative	1 yr after surgery
DeMeester score		
EG	15.1 ± 1.1	1.2 ± 0.7
YG	13.4 ± 1.5	1.1 ± 0.2
(%) time pH < 4		
EG	9.1 ± 0.6	1.4 ± 0.3
YG	8.2 ± 0.7	0.9 ± 0.8

occur in approximately 20% of adults in USA[5]. Its cost has been estimated to be $24.1 billion annually[2].

By 2020 more than 16% of population in USA are expected to be more than 65 years old while nearly 20 million ought to be more than 85 years old[6]. In the elderly, the prevalence of GERD is nearly the same among the general population, but complicated GERD appears to be more common than in young people[6].

Several authors have reported a higher incidence of esophagitis as well as Barrett esophagus in older patients[7-11]. Collen[7] found that esophagitis and Barrett esophagus were almost twice in patients aged 60 years than in young people (81% *vs* 47%, $P < 0.002$). Zhu[8] observed that the percentage of time with pH < 4 was 32.5% in older patients with GERD *vs* 12.9% in younger ones ($P < 0.05$). Furthermore, among elderly patients with esophagitis, nearly 21% had grade Ⅲ-Ⅳ disease compared with only 3.4% of younger patients ($P = 0.002$). Cameron[9] demonstrated that the prevalence of Barrett's esophagus increased with age to reach a plateau by the seventh decade. Fass[11] reported that the mean incidence rate of erosive esophagitis was 74% in the elderly and 64% in the younger patients and the frequency of symptoms was lower in the elderly group. David[12] demonstrated that the prevalence of severe esophagitis increased with age: only 12% in GERD patients < 21 years old in comparison with 37% in patients > 70 years old had severe esophagitis.

Also in our study, the elderly group (EG) had a higher rate of erosive esophagitis (69.2% *vs* 35.2.%) and a lower rate of Grade I esophagitis (22.2% *vs* 60.8%). Moreover, incidence of Barrett's esophagus as well as mean percentage of total time < 4 at pH-monitoring were significantly higher in the EG (Table 7). The frequency of reflux episodes has been reported to be similar either in the elderly or in young people whereas the duration of individual reflux episodes seems to be longer in the elderly[13]. However, it is not clear which factors lead to a more severe GERD in the elderly.

The etiopathogenesis of GERD seems to be multifactorial. The alteration may include a defective antireflux barrier, abnormal esophageal-clearance, altered esophageal mucosal resistance, and delayed gastric emptying[14].

Hiatal hernia (HH) as a structural defect of the antireflux barrier is a determining factor of GERD, by impairing both the diaphragmatic component and the clearance of acid refluxate from the distal esophagus[15]. HH has been identified in 60% of patients > 60 years old[16]. Furthermore, several studies found a higher frequency of esophagitis in patients with HH compared with patients without HH, and the severity was proportional to the size of HH. In our study, we noted a significant higher rate of HH in the EG (Table 6). Previous studies excluded any adverse effect of aging on the lower esophageal sphincter (LES) of healthy subjects[17,18]. Similarly, we did not find any significant difference in LES pressure between the EG and the YG. However, an impaired esophageal peristalsis (waves pressure < 30 mmHg) has been found in 66.7% of the EG and 33.4% of the YG (Table 5). It is not clear whether impaired peristalsis is a cause of or affects a more severe GERD, since we noted an increased amplitude of peristaltic waves both in the EG and in the YG at the postoperative manometric follow-up evaluation (Figure 1).

Changes in motility seen in older patients is related to long- term esophageal acid exposure rather than to effects of aging on esophageal smooth muscle and on collagen production that is increased in chronic inflammation[18,19]. In our study, the mean duration of preoperative symptoms was significantly longer in the EG. Probably a vicious circle begins in these subjects between cardia incontinence, increasing reflux and impaired peristalsis determining a reduction of esophageal clearing[20]. The realization of antireflux procedure seems to break this circle. Some authors described the increased amplitude of peristalsis in patients undergoing total fundoplication[21,22].

Besides, Sonnemberg showed an age dependent fall in salivary bicarbonate production while physiologic levels of gastric acid secretion remained stable in advanced age. These factors may increase esophageal acid exposure because of delayed acid clearance[23]. We found elderly patients having less frequency and severity of symptoms like heartburn and acid regurgitation than younger patients[24] (Tables 3 and 4). Raiha[24] hypothesized that typical symptoms should not be considered as expression of acid reflux in older patients. However, it is not clear which factors reduce frequency and severity of these symptoms although these patients have a higher rate of acid exposure and develop a more severe esophagitis.

Several studies have shown that altered esophageal pain perception to acid in the elderly is the result of an ageing process that may be responsible for an increased severity of GERD[11]. On the other hand, frequency and severity of atypical symptoms have been reported to be higher in elderly people with GERD[24]. Also in our study, we found a statistically significant higher rate of atypical symptoms such as dysphagia for solids, chest pain and respiratory symptoms in the EG (Tables 3 and 4).

Therefore, a mild intensity of heartburn often leads physicians to underestimate the severity of erosive esophagitis and its complications.

Surgical correction of GERD has been shown to be a cost-effective treatment by reducing long-term complications such as Barrett esophagus and stricture and by eliminating the need of a life-long medical therapy especially for young patients. However, a high morbidity and mortality rate of open surgery performed in the elderly, limited the number of these patients referred to surgical units[25]. Since a laparoscopic Nissen fundoplication

has been reported for the first time, a growing number of antireflux procedures have been performed in the USA[26]. Several studies showed laparoscopic surgery to be a safe and effective treatment for GERD being able to improve quality of life and warranting an early return to daily activities[27].

In elderly population with GERD, laparoscopic surgery has proven to be effective with low morbidity and mortality rates. Richter[2] observed that Laparoscopic Nissen Fundoplication did not increase the mortality, morbility and hospital stay in the elderly patients compared to younger surgical patients. Kalmoz[28] showed that age should not be considered a contraindication to laparoscopic surgical treatment of GERD as 97% of elderly patients would choose surgical treatment again if necessary. Bammer[29] reported that laparoscopic surgery is a good option for the treatment of severe GERD in octo- and nonagenarians, with an excellent follow-up in 93% of elderly patients. Except for preoperative disease severity, we did not find any significant difference in perioperative and postoperative results as well as in subjective and objective outcome between the two groups. The only observed differences in the operative time and blood loss seem to be related to the high ASA scores and the higher incidence and size of hiatal hernia in the EG.

Statistically significant improvement in heartburn, acid regurgitation, chest pain and respiratory complications of GERD was observed in both EG and YG (Table 9). An excellent outcome was observed in 314/338 (92.9%) younger patients and in 57/62 (91.9%) elderly patients.

A poor outcome was observed in 27 patients, 23/338 (7.1%) in YG and 4/62 (6.5%) in EG; persisting dysphagia occurred in 11/338 (3.3%) in YG and 2/62 (3.2%) in EG; and 12/338 (3.6%) in YG and 2/62 (3.2%) in EG had recurrent of heartburn. Differences between the two groups were not statistically significant also regarding the incidence of other side effects (flatulence, early satiety, etc) (Table 10). Outcome was not dependent on the presence of disordered esophageal motility.

There have been debates in literature regarding the realization of partial fundoplication in patients with defective esophageal peristalsis, and it seemed reasonable therefore, to choose this kind of wrap in elderly patients. Many authors supported the realization of a partial fundoplication in patients with impaired esophageal peristalsis to lower the incidence of persistent postoperative dysphagia[30-32]; moreover, partial wrap was considered as effective as total wrap to control gastroesophageal reflux, and short-term follow-up seemed to validate the choice of partial fundoplication[33,34]. Later on, partial antireflux procedure showed its inadequacy to assure a good protection from reflux at a long-term follow-up[35-37]. Livingston[38] reported a 1.4% recurrence rate of reflux in patients with total fundoplication versus 6.7% in those with partial fundoplication. At a long-term follow-up, Fernando[39] observed that 38% of Toupet patients used PPI versus 20% with Nissen. Jobe[40], in a ten years follow-up, noted a recurrence rate for reflux until 51% in patients treated with partial fundoplication (Toupet and Dor). Moreover, total fundoplication seems not to determinate a higher incidence of postoperative dysphagia compared with the partial wraps, even in patients with impaired peristalsis[41,42]. Patti[43] analysed the long-term results of patients treated with partial versus total antireflux procedures: efficacy was higher for total fundoplication (recurrence of reflux in 4% of patients with total fundoplication versus 19% in patients with partial fundoplication), while the incidence of postoperative dysphagia was similar in both groups, even in patients with impaired esophageal peristalsis (8% Toupet versus 9% Nissen). Pessaux[44], at a three-month follow-up, noted a dysphagia rate of 4.2% in patients treated with Nissen fundoplication versus 5.9% with Nissen-Rossetti wrap and 6.9% in those treated with Toupet. In a prospective randomized trial, Bessell[45] concluded that calibrating the antireflux wrap according to esophageal motility was not necessary, because the postoperative persistent dysphagia rate was similar between patients with total or partial wrap. Velanovich[46] did not find any statistically significant difference in postoperative dysphagia rate related to esophageal motility disorders (MD) (15.8% MD+ versus 16.4% MD-) in a group of patients undergoing total fundoplication.

Besides, total wrap seems to bring about an improvement of esophageal peristalsis. Heider[47] observed an increase of 47% of mean peristaltic waves in distal esophagus compared with preoperative time ($P < 0.01$), with the normalization of the esophageal motility in 74% of patients. Diaz de Liano[48], at 1 year follow-up, noted an augment of esophageal peristalsis in 43% of patients with impaired peristalsis undergoing total fundoplication. Scheffer[49] showed an increase of mean amplitude of peristalsis from a preoperative value of 57 mmHg to 86 mmHg at 3 mo follow-up and 92 mmHg at 2 years after surgery in a group of 34 patients. Oleynikov[50] in a trial comparing total and partial fundoplication noticed that in patients undergoing partial wrap, the mean amplitude of peristaltic waves increased from 27.8 mmHg before surgery to 35.6 mmHg postoperatively ($P > 0.05$), while in patients treated with total fundoplication, these values were respectively 28.2 mmHg versus 49.0 mmHg ($P < 0.05$). These evidences strongly support the choice of performing a total fundoplication also in elderly patients, which is often affected by severe impairment of esophageal peristalsis.

Our choice since 1972, has always been favorable to the total fundoplication, without section of short gastric vessel. We usually perform intraoperative endoscopy and manometry in order to calibrate antireflux wrap[51]. Usually, we calibrate the n-HPZ at values ranging from 20 to 45 mmHg ('hypercalibrated Nissen'), building the wrap around the gastroscope (with a diameter of 9 mm). This hypercalibration, in contrast with the 'floppy Nissen' of Donahue and DeMeester[52], resulted from the retrospective evaluation of a former series in which we used to calibrate the fundoplication to pressure values similar to those of a normal sphincter ('normocalibrated Nissen': 10-20 mmHg). This experience was followed by a high rate of gastroesophageal reflux recurrence (28.5%) in the first 12 mo after surgery[51], demonstrating that high pressure zone (HPZ) values of the Nissen-Rossetti wrap decrease after surgery with time (Figure 1). It is effective to

protect from GERD while avoiding a persistent dysphagia because a routine intraoperative manometric control of the wrap is always performed at the end of the procedure. Our preference for total calibrated wrap led us to consider it also in the treatment of patients affected with severe motility disorders such as achalasia and epiphrenic diverticula with excellent results[52,53].

In conclusion, laparoscopic antireflux surgery, is a safe and effective treatment for GERD even in elderly patients warranting low morbidity and mortality rates and a significant improvement of symptoms comparable to younger patients. Preoperative defective esophageal peristalsis is not a contraindication to total laparoscopic fundoplication.

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CLINICAL RESEARCH

Chios mastic treatment of patients with active Crohn's disease

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Abstract

AIM: To evaluate the effectiveness of mastic administration on the clinical course and plasma inflammatory mediators of patients with active Crohn's disease (CD).

METHODS: This pilot study was conducted in patients with established mild to moderately active CD, attending the outpatient clinics of the hospital, and in healthy controls. Ten patients and 8 controls were recruited for a 4-wk treatment with mastic caps (6 caps/d, 0.37 g/cap). All patients successfully completed the protocol. CD Activity Index (CDAI), Nutritional Risk Index (NRI), C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), and total antioxidant potential (TAP) were evaluated in the plasma at baseline and at the end of the treatment period. Results were expressed as mean values ± SE and $P < 0.05$ was considered to indicate statistical significance.

RESULTS: Patients exhibited significant reduction of CDAI (222.9 ± 18.7 *vs* 136.3 ± 12.3, $P = 0.05$) as compared to pretreament values. Plasma IL-6 was significantly decreased (21.2 ± 9.3 pg/mL *vs* 7.2 ± 2.8 pg/ mL, $P = 0.027$), and so did CRP (40.3 ± 13.1 mg/mL *vs* 19.7 ± 5.5, $P = 0.028$). TAP was significantly increased (0.15 ± 0.09 *vs* 0.57 ± 0.15 mmol/L uric acid, $P = 0.036$). No patient or control exhibited any kind of side effects.

CONCLUSION: The results suggest that mastic significantly decreased the activity index and the plasma levels of IL-6 and CRP in patients with mildly to moderately active CD. Further double-blind, placebo-controlled studies in a larger number of patients are required to clarify the role of this natural product in the treatment of patients with CD.

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disease of unknown etiology that may affect any level of the gastrointestinal tract[1-3]. It is well established that immunological mechanisms are involved in the pathogenesis of the disease. Inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), have a pivotal role in induction and amplification of the inflammatory cascade. Particularly, IL-6 stimulates T-cell and B-cell proliferation and differentiation[4], while it mediates the hepatic expression of acute phase proteins[5]. Increased concentration of TNF-α and monocyte chemoattractant protein-1 (MCP-1) have been reported in patients with CD[6]. Additionally, during chronic inflammation, when sustained production of reactive oxygen and nitrogen species occurs, antioxidant defenses may weaken, resulting in a situation termed oxidative stress[7]. Thus, in patients with CD, elevated oxidized low-density lipoprotein levels have been reported compared to healthy controls[6].

Despite the large number of therapeutic agents available today, none can be considered as completely satisfactory either due to resistant cases or because of significant side effects. To our knowledge, there are only scattered reports of natural compounds that potentially reverse relapse in CD. Trebble and co-workers[8] demonstrated an anti-inflammatory activity of fish oil and antioxidant supplementation evaluated in mononuclear cells of CD patients, while Lavy *et al*[9] demonstrated the effectiveness of the antioxidant β-carotene in a rat model as a prophylactic dietary measure in reducing the effects of acid induced enteritis, thus raising the possibility that patients with CD may benefit from the consumption of natural β-carotene. Also the flavonoid rutin, a well-established antioxidant compound, has been suggested as a therapeutic agent in CD. Rutin has been shown to attenuate pro-inflammatory cytokine production in both colonic

mucosa and peritoneal macrophages of experimental animals[10]. Treatment with food phytochemicals has been shown to be safe, sustainable and practical and changes of dietary habits have been advocated in the therapy of CD[11].

Pistacia lentiscus var. Chia (Anacardiaceae), well known as Chios mastic gum, is an evergreen shrub widely distributed in the Mediterranean region. Many ancient Greek authors, including Dioscurides and Theophrastus, mentioned Chios mastic for its healing properties in intestines, stomach and liver. Mastic has also been reported to possess antioxidant[12] and antibacterial[13] activity. With reference to gastrointestinal disorders, the effectiveness of the resin against peptic ulcers is evident[14] in most studies, while only in two reports there is no effect on *H pylori* eradication *in vivo*[15,16]. Furthermore, regarding gastric mucosa, the plant has been shown to be hepatoprotective in tetrachloride-intoxicated rats[17] and to suppress the extent of iron-induced lipid peroxidation in rat liver homogenates[18], without any toxic effect. A major constituent of mastic, namely oleanolic acid, is among the best-known triterpenes with biological properties against chemically induced liver injury in laboratory animals, exerting anti-inflammatory and antitumor-promotion effects[19]. This background information led us to examine the effects of supplementation with mastic in patients with active CD. This study is the first ever reported to evaluate mastic for possible clinical effectiveness in patients with CD.

MATERIALS AND METHODS

Study population

Ten consecutive patients with established CD and eight healthy controls were recruited to participate in the trial. All patients were attending the outpatient clinic of the Department of Gastroenterology, Saint Panteleimon General State Hospital in Nicea, Athens. Clinical evidence of mild to moderate Crohn's disease exacerbation was defined by a score of CD Activity Index (CDAI) higher than 150. Patients with clinical evidence of recurrence and CDAI higher than 400 were excluded from the study. Patients receiving mesalazine or antibiotics during the time of relapse were asked to continue treatment. None was receiving elemental diet or parenteral nutrition or antioxidant/mineral supplements and none was under treatment with immunosuppressives, immunomodulators and/or corticosteroids. Eight healthy volunteers with normal serum concentrations of C-reactive protein (CRP) (< 5 mg/L) and albumin (> 40 g/L) served as controls. Assessed by Medical History questionnaires, controls included in the study were healthy persons without chronic inflammatory disorder. Exclusion criteria for control recruitment were a body mass index (BMI) higher than 30 and anti-inflammatory drug treatment or antioxidant vitamin/mineral supplementation prior to trial. All volunteers gave a written consent after having received thourough information about the aims and procedure of the study. The Ethical Committees of both Harokopio University and Saint Panteleimon General State Hospital approved the protocol. Table 1 shows some demographic characteristics of patients and controls.

Table 1 Demographic characteristics and medications of patients with CD and controls

Characteristic	Patients	Controls
Age (yr)		
Mean	36.9	31.5
Range	18-73	25-45
Sex		
Female	5	4
Male	5	4
Duration of disease (yr)	6.4 (± 3.9)	-
Concomitant medication		-
None	3	
Mesalazine	3	-
Metronidazole	2	
Azathioprine	2	
Location of Crohn's disease		-
Small bowel	4	-
Small and large bowel	6	-
Fistulizing disease	3	-

Preparation of mastic caps

A UV source device (Jost/Ba-ro, Type FDLT 250/-80 × 2500) was used for sterilization of the Chios Mastic resin. Then, the sterilized mastic granules were milled to fine powder (particle size < 400 μm) by using a Hosokawa Alpine Mill (Fine Impact Mill 100 UP2). The encapsulation of powder was performed using the Profill Capsule filling System (Torpac Inc.). Capsule cells (capsugel, V caps, size 0) were made of Hpromellose (hydroxypropyl methylcellulose) and each contained 0.37 (± 0.02) g of mastic powder.

Intervention trial protocol

Dissolution time was measured according to standard methods[20] and was found to last approximately 7 min. Patients and healthy controls were subjected to a 4-wk supplementation with mastic caps (6 caps/d, 2.2 g in total) over a period from June 2005 to January 2006. Dietary assessment was accomplished applying Food Frequency Questionnaire (FFQ) and 24 h recalls. Dietary instructions were given to both healthy controls and patients as to maintain consumption of food rich in anti-inflammatory and antioxidant ingredients as poor as initially assessed by FFQ and 24 h recall interviews. Assessment of compliance during the trial was tested applying 24 h recalls twice a week. Mastic, either in the form of gum or as a sweet or bread ingredient, and fish oil, either crude or in the form of supplement, was not allowed in either group. The daily energy intake was evaluated by means of 24 h recalls. Blood samples were obtained for plasma isolation and subjected to CRP and albumin measurements prior and after the trial. At the same time points, plasma cytokine and antioxidant potential measurements were performed. Body weight was measured using electronic scales initially and at the end of the trial.

Disease activity index evaluation

The Crohn's Disease activity was evaluated by means

of the CDAI[21]. The CDAI incorporates eight related variables: the number of liquid or very soft stools per day, the severity of abdominal pain or cramping, general well being, the presence or absence of extraintestinal manifestations of CD, the presence or absence of an abdominal mass, the use of antidiarrheal drugs, hematocrit, and body weight. Scores range from 0 to 600 with higher scores indicating more severe disease activity. A score of 151 to 200 corresponds with mild disease activity; moderate disease has a score of 201 to 400, and scores of 401 or greater represent severe disease activity.

Biochemical measurements

CRP concentrations were analyzed immunoturbidimetrically on a Beckman Synchron CX5 fully automated chemistry analyzer. Albumin was measured by means of the bromocresol green method on the same analyzer.

Cytokine assays

Plasma cytokines from patients with CD and controls were assessed by quantitative enzyme-linked immunosorbent assays (ELISA) (R & D Systems Abingdon, UK) according to the manufacturer's instructions. Sensitivity limits of TNF-α, IL-6, and MCP-1 ELISAs are, respectively, 1.6 pg/mL, 0.70 pg/mL and 5.0 pg/mL. Plasma cytokines from patients with CD and controls were assessed in duplicate.

Plasma total antioxidant potential assay

Total antioxidant potential (TAP) in plasma was assessed by a colorimetric, quantitative assay for TAP in aqueous samples (OxisResearch Portland, USA) according to the manufacturer's instructions. The results of the assay were expressed as mmol/L of uric acid equivalents. The sensitivity of the assay is 30 μmol/L uric acid equivalents.

Statistical analysis

Results were expressed as mean ± SE. The Mann-Whitney Test was used for comparing differences between patients and controls prior the intervention. Differences reported primarily and at the end of the study within individual groups, were tested for significance by the Wilcoxon signed ranks test. Calculated $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Alterations of CDAI and induction of remission

The CDAI score was assessed at baseline and after the 4 wk treatment with mastic. All patients receiving mastic showed a reduction of the CDAI as compared to pretreatment values. The reduction of the mean CDAI value was statistically significant (from 222.9 ± 18.7 to 136.3 ± 12.3, $P = 0.05$) (Figure 1). The two main elements of CDAI showing the most striking improvement were the number of liquid stools per day and the score of general well being.

Nutritional risk index

One of the clinically useful measures of nutritional status in CD is the Nutritional Risk Index (NRI), which is calculated based on serum albumin levels and body weight using the following equation: NRI = [1.519 × albumin (g/L)] + [0.417 × (current weight/usual weight) × 100]. A NRI > 100 denotes absence of nutritional risk. NRI values between 97.5 and 99.9 correspond to a mild nutritional risk, NRI values from 83.5 to 97.5 to moderate nutritional risk, and NRI values lower than 83.5 to severe nutritional risk.

The patients' "usual weight" was the body weight at the time of remission, as reported in medical records at the hospital and confirmed by each single patient. NRI of healthy controls was normal at the start of the study and remained unchanged after the mastic supplementation (data not shown). The mean NRI value of CD patients increased from 87.5 ± 3.7 before treatment to 91.5 ± 3.2 at the end of treatment ($P = 0.059$). This increase was evident at the end of the second week of mastic supplementation and remained constant thereafter until the end of the trial.

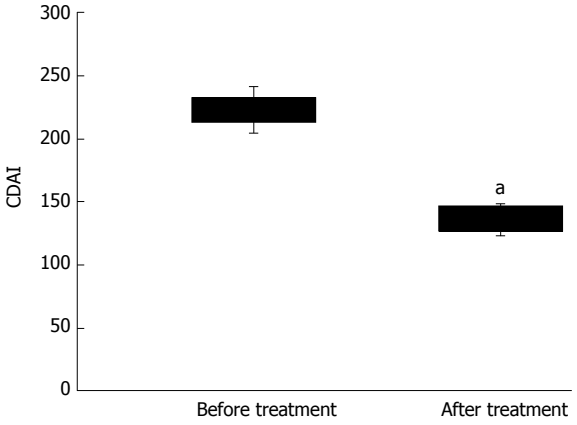

Figure 1 Crohn's disease activity index (CDAI) was decreased in patients with active Crohn's disease ($n = 10$) after 4-wk treatment with mastic caps ($^aP < 0.05$). Horizontal bars represent the mean value (± SE).

CRP

Prior to mastic treatment, CRP levels were significantly higher in CD patients (40.3 ± 13.1 mg/mL) than in healthy controls (2.4 ± 0.7 mg/L) ($P = 0.002$). Treatment with mastic caps of healthy controls resulted in no modifications in CRP values (2.3 ± 0.6 mg/L), which remained at concentrations ⩽ 5.0 mg/mL in all individuals. In CD patients, mean CRP levels were significantly decreased after treatment (from 40.3 ± 13.1 mg/mL to 19.7 ± 5.5, $P = 0.028$) (Figure 2).

IL-6 plasma concentration

IL-6 was below detection in healthy controls prior to therapy, while in patients it was significantly elevated compared to controls ($P = 0.034$). As with CRP, IL-6 in controls remained unaltered, while in patients it decreased significantly (from 21.2 ± 9.3 pg/mL to 7.2 ± 2.8 pg/mL, $P = 0.027$) (Figure 3).

TNF-α plasma concentration

Patients with active CD had TNF-α plasma concentrations 10-fold higher compared to controls before therapy (27.1 ± 9.7 pg/mL *vs* 2.6 ± 1.5 pg/mL, $P = 0.009$). After

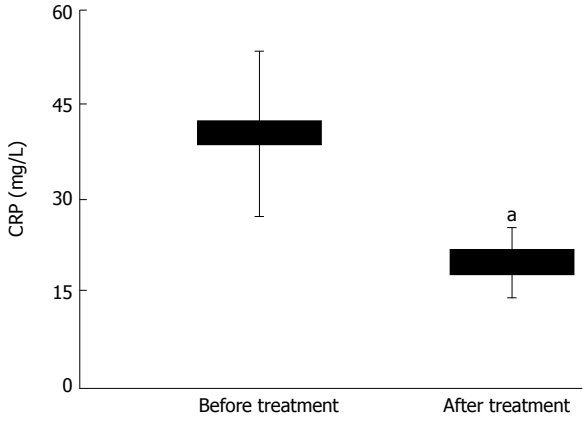

Figure 2 C-reactive protein (CRP) concentrations in patients with active Crohn's disease (n = 10) before and after 4-wk treatment with mastic caps ($^aP < 0.05$). Horizontal bars represent the mean value (± SE).

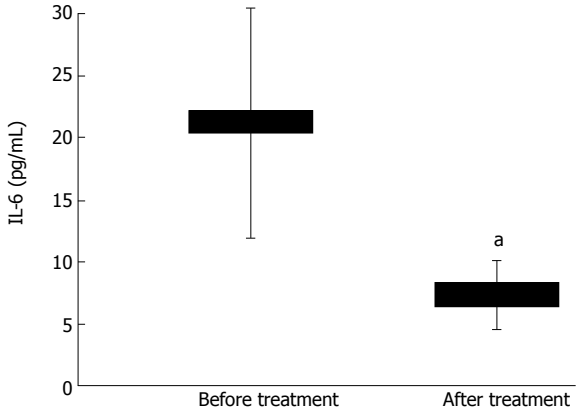

Figure 3 Plasma concentrations of interleukin-6 (IL-6) were suppressed in patients with active Crohn's disease (n = 10) after 4-wk treatment with mastic caps ($^aP < 0.05$). Horizontal bars represent the mean value (± SE).

treatment, plasma TNF-α decreased in patients, although this decrease did not reach statistical significance (27.1 ± 9.7 pg/mL to 16.4 ± 4.7 pg/mL, $P = 0.114$).

MCP-1 plasma concentration

In the case of MCP-1, patients with active CD had MCP-1 plasma concentrations 2.5-fold higher compared to controls (140.7 ± 43.9 pg/mL *vs* 57.5 ± 11.8 pg/mL, $P = 0.368$). Although not statistically significant, a decrease was observed in MCP-1 in CD patients at the end of the trial (76.6 ± 20.9 pg/ mL, $P = 0.074$).

Plasma TAP

TAP was significantly different between the two groups before mastic treatment (healthy controls, 0.4 ± 0.06 *vs* CD patients, 0.15 ± 0.09 mmol/L uric acid, $P = 0.003$). As shown in Figure 4, TAP was significantly increased in individual groups after mastic treatment (controls, 0.4 ± 0.06 *vs* 0.5 ± 0.05 mmol/L uric acid, $P = 0.025$; CD patients, 0.15 ± 0.09 *vs* 0.57 ± 0.15 mmol/L uric acid, $P = 0.036$).

Side-effects

No patient exhibited any side effects. However, during the third day of treatment, one female patient with CD of the small and large bowel reported an abrupt onset of constipation. She was advised to reduce the dose for two days. After that, she continued treatment without further complaints. No other untoward effect was reported.

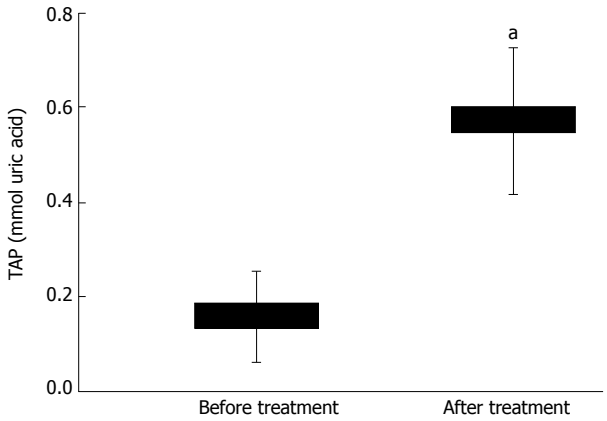

Figure 4 Plasma total antioxidant potential (TAP) was upregulated in patients with active Crohn's disease (n = 10) after 4-wk treatment with mastic caps ($^aP < 0.05$), indicating absorption of antioxidants and an improved *in vivo* antioxidant status. Horizontal bars represent the mean value (± SE).

DISCUSSION

Chios mastic has been previously shown to exert various biological properties *in vitro*[12], in experimental animal models[18] and in humans[14]. In the current study, we demonstrated that mastic was effective in the regulation of inflammation, evaluated by CRP, IL-6, TNF-α and MCP-1 in plasma, as well as in the regulation of oxidative stress, evaluated by TAP. In more details, mastic treatment significantly decreased the CDAI, which probably occurred through decrease of the pro-inflammatory IL-6, inducing remission in seven out of ten patients. Another important observation was that mastic resulted in improvement of the nutritional status, as shown by NRI.

Nutritional support in patients with CD has a primary role in inducing remission and malnutrition is very common in CD. While several factors, such as malabsorption and increased resting energy expenditure in underweight patients, may contribute to malnutrition[22], decreased oral intake is the primary cause. The methods used to support patients with CD are enteral and parenteral nutrition, in terms of protein-calorie intake. NRI is one of the most useful measures of nutritional status and points out severely malnourished patients when less than 83.5[23]. Hereby we show that NRI in patients supplemented with mastic was increased, however not significantly, perhaps due to the limited number of subjects. Particularly, NRI was increased in nine out of ten patients supplemented with mastic, two of whom experienced no nutritional risk (data not shown). The main element of NRI showing improvement was body weight gain. Based upon the fact that daily energy intake was unchanged during the trial (data not shown), increase in body weight and in NRI is due to the fact that mastic treatment resulted in decrease of liquid stools and therefore improvement in nutrient absorption.

The observed decrease in NRI in one of the patients was due to body weight loss, despite the fact that the number of liquid stools decreased. The daily energy intake of this young patient was gradually reduced and, according to her statement long after the end of the protocol, she was on a diet for weight loss.

The importance of IL-6 in patients with CD has been well documented. In patients with active CD, mRNA for IL-6 is overexpressed in the inflamed mucosa[24] and IL-6 is thought to play a crucial role in the pathogenesis of CD. Elevated IL-6 in plasma of patients with CD has been previously described[25]. Accordingly, we report that in patients with CD plasma concentration of IL-6 was significantly higher versus the control group. Significant decrease in IL-6 with mastic treatment was observed in patients following a decrease in plasma CRP (Figure 2). Because IL-6 is the main cytokine factor responsible for hepatic induction of acute phase proteins in CD, respective decrement in CRP is reasonable. In view of the fact that (1) oleoresins consist of triterpenes[26] with established anti-inflammatory and antioxidant effects[19,27] and (2) mastic contains antioxidant phenolic compounds[28], it is more likely that the plasma IL-6 decrease observed in CD patients was due to these compounds.

TNF-α showed an unsignificant (P = 0.114) 1.6-fold decrease in CD patients. On the other hand, the difference in TNF-α concentrations between patients and controls at baseline was significant. The data reported about TNF-α in CD are somewhat contradictory. Whereas some groups were able to demonstrate increased concentrations of TNF-α in CD compared to healthy controls[29], others were not[30]. Because TNF-α induces MCP-1 secretion via the activation of nuclear factor-kappa B[31], it is likely that the slight decrease in MCP-1 was due to the lower activation of the nuclear factor-kappa B pathway secondary to the decrease in TNF-α.

Oxidative stress has been proven to upregulate IL-6 gene expression[32]. We show that mastic treatment resulted in increase of plasma TAP in CD patients (Figure 4) as well as in controls. Plasma is a heterogenous solution of diverse antioxidants and an increase in the antioxidant capacity indicates absorption of antioxidants and an improved *in vivo* antioxidant status[33]. Whether the antioxidant triterpenes and phenolics contained in mastic[12] are absorbed or act on the exposed gastrointestinal mucosa, remains uncertain. Generally, our knowledge on the absorption and bioavailability of polyphenols is still limited, and the few studies in humans show that some are well absorbed and others hardly absorbed[34]. The unabsorbed may remain in the lumen and become available for fermentation in the colon. A substantial proportion of the gastrointestinal mucosa is therefore exposed to these compounds, or to their bacterial and systemic metabolites[35]. However, phenolic compounds do not seem to be absorbed as well as vitamins C and E, and hence their concentrations can be much higher in the lumen of the gastrointestinal tract than are ever achieved in plasma or other body tissues, making the action in the gastrointestinal tract more likely. Even less are the data on the absorption of triterpenes. Glycyrrhetinic acid, the triterpene derivative of glycyrrhizin, has been shown to be bioactive in experimental gastric lesion models[36] and has also been detected in the serum of experimental animals[37].

In conclusion, subjecting CD patients with mild to moderate activity to mastic treatment seems to improve the clinical features of the disease and to regulate inflammation and antioxidant status. The use of natural products as primary treatment in CD should attract wider support and research, with increasing awareness of the harm of the long-term use of corticosteroids. Whether it is time for gastroenterologists to embrace the concept that natural products, such as mastic, may be beneficial to CD needs further research in larger cohorts.

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CLINICAL RESEARCH

Cytomegalovirus infection in severe ulcerative colitis patients undergoing continuous intravenous cyclosporine treatment in Japan

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Abstract

AIM: To investigate active cytomegalovirus (CMV) infection following the cyclosporine A (CyA) treatment of steroid-refractory ulcerative colitis (UC).

METHODS: Twenty-three patients with severe UC not responding to steroid therapy (male 14, and female 9) enrolled at Nagoya University Hospital from 1999 to 2005. They received continuous intravenous infusion of CyA (average 4 mg/kg per day) for 1 mo. Serum and colonic biopsy samples were collected before CyA treatment and 4 d, 10 d, 20 d, and 30 d after treatment. Patients were evaluated for CMV by using serology (IgM antibody by ELISA), quantitative real-time PCR for CMV DNA, and histopathological assessment of hematoxylin and eosin (HE)-stained colonic biopsies. CMV infection was indicated by positive results in any test.

RESULTS: No patients had active CMV infection before CyA treatment. Eighteen of 23 UC patients treated with CyA were infected with active CMV (IgM antibody in 16/23 patients, 69.6%; CMV DNA in 18/23 patients, 78.2%; and inclusion bodies in 4/23 patients, 17.3%). There was no difference in the active CMV-infection rate between males and females. Active CMV infection was observed after approximately 8 d of CyA treatment, leading to an exacerbation of colitis. Fifteen of these 18 patients with active CMV infection (83.3%) required surgical treatment because of severe deteriorating colitis. Treatment with ganciclovir rendered surgery avoidable in three patients.

CONCLUSION: Our results suggest that active CMV infection in severe UC patients treated with CyA is associated with poor outcome. Further, ganciclovir is useful for treatment of CMV-associated UC after immunosuppressive therapy.

Key words: Cytomegalovirus; Cyclosporine; Ulcerative colitis; Ganciclovir

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INTRODUCTION

Cytomegalovirus (CMV) infection is one of the most common infectious complications after immunosuppressive therapy. It occurs mainly as a secondary infection in CMV-seropositive patients. CMV infection is a common viral infection in humans, occurring in 40%-100% of adults[1]. CMV infections are generally asymptomatic or are manifested as a mild mononucleosis-like syndrome[2]. Significant CMV disease may occur in various organs such as the retina, lung, and gastrointestinal tract, and the target organ is related to the etiology of immunosuppression[1]. Gastrointestinal (GI) CMV infection is rare in immunocompetent individuals. Clinically significant GI CMV infection generally occurs in immunecompromised patients[3]. In the gastrointestinal tract, CMV disease can occur in all locations, from the mouth to the rectum, and generally involves the formation of ulcers in the mucosa, often accompanied by hemorrhage[1].

Ulcerative colitis (UC) is common all over the world and is generally more frequent than Crohn's disease (CD)[4]. UC is thought to result from the inappropriate and progressive activation of the mucosal immune system driven by the presence of normal luminal flora. The aberrant response is most likely facilitated by defects in both the barrier function of the intestinal epithelium and the mucosal immune system[4].

Cyclosporine A (CyA) selectively inhibits immune

responses mediated by T lymphocytes by modulating the interaction of calcineurin-calmodulin[5]. This recognition has led to its use in patients with severe UC, with variable results. In an uncontrolled study, approximately 60%-80% of patients suffering from severe corticosteroid-refractory ulcerative colitis responded to cyclosporine therapy[6,7]. Since an intravenous infusion of CyA has clinical benefits in patients with steroid-resistant UC, it has been generally accepted that CyA selectively blocks the activation of helper and cytotoxic T cells and acts by inhibiting the nuclear factor of activated T cells and cytokine gene expression[8].

In Japan, patients with severe, corticosteroid-refractory or corticosteroid-dependent UC are frequently treated with strong immunosuppressive agents, including CyA. Therefore, patients with inflammatory bowel disease (IBD) are expected to be at an increased risk of infection with CMV. Despite the frequent use of immunosuppressive drugs in patients with UC, data on the frequency of CMV infection and its clinical significance in patients with UC are limited. The aim of this study was to describe our experience with active CMV infection following treatment with CyA for UC.

MATERIALS AND METHODS

Patients

Twenty-three patients with severe UC enrolled at Nagoya University Hospital from 1999 to 2005. They did not respond to a minimum of 7 d of intravenous systemic steroid therapy (prednisolone, more than 30 mg/d). The diagnosis of UC was based on clinical, endoscopic, radiological, and histological parameters. The study was approved by the Ethical Committee of the Graduate School of Medicine, Nagoya University, and all samples were obtained with informed consent in accordance with the Helsinki Declaration.

CyA treatment

Twenty-three patients received a continuous intravenous infusion of CyA in the form of Sandimmun solution (Novartis Pharma KK, Tokyo, Japan) at an average daily dose of 4 mg/kg for 1 mo. The CyA dose was adjusted to maintain a whole-blood CyA concentration of less than 500 pg/L. Complete blood cell counts, C-reactive protein (CRP), liver function tests (aspartate aminotransferase and alanine aminotransferase), renal function tests (creatinine and blood urea nitrogen), and clinical evaluation were performed before CyA treatment and then 4 d, 10 d, 20 d, and 30 d after treatment.

Collection of specimens and blood serum samples

Serum samples and colonic biopsy specimens were obtained from all patients before CyA treatment and then 4 d, 10 d, 20 d, and 30 d after treatment. Multiple biopsy samples were obtained from the inflamed area during colonoscopy for histopathological examination of inflammatory activity and CMV inclusion bodies; these samples were fixed in buffered neutral formalin. EDTA-treated venous blood (5 mL) was obtained from each patient under aseptic conditions for serological studies. The plasma was separated by centrifugation.

Histopathology

The colonic biopsy samples were paraffinized, sectioned, and stained with hematoxylin and eosin (HE). These sections were microscopically evaluated for the presence of characteristic cytomegalic cells and "owl's eye" nuclear inclusion bodies. Histologically, the activity of IBD was classified according to a standard system described previously [9].

Sample preparation

For the PCR assays, DNA was extracted from 200 μL of plasma by using the QIAamp Blood Kit (QIAGEN Ltd, Tokyo, Japan), eluted in 100 μL of distilled water, and stored at -30℃ until analysis.

Serological tests

The presence of anti-CMV IgM antibodies in all sera were tested by using the CMV IgM ELISA kit (Genesis Diagnosis Ltd., UK), a commercially available kit, by using the positive and negative controls provided with the kit. Virus-specific IgM antibodies were measured in the plasma regardless of the PCR results.

Real-time semi-quantitative PCR assay

The PCR primers used were from the immediate early (IE) gene[10]. The upstream primer was 5'-GACTAGTGT GTGATGATGCTGGCCAAG-3', and the downstream primer was 5'-GCTACAATAGCCTCTTCCTCATC TG-3'. A fluorogenic probe (5'-carboxyfluorescein-AGCCTGAGGTTATCAGTGTAATGAAGCGCC-3') was located between the PCR primers[11]. PCR was carried out by using a TaqMan PCR kit (PE Applied Biosystems, Foster City, CA), as described previously[11]. Briefly, 10 μL of the DNA extraction solution from the samples was added to a PCR mixture containing 10 mmol/L of Tris (pH 8.3); 50 mmol/L of KCl; 10 mmol/L of EDTA; 5 mmol/L of $MgCl_2$; 100 mmol/L of dATP, dCTP, dGTP, and dTTP; 0.2 mmol/L of each primer; 0.1 mmol/L of fluorogenic probe; and 1.25 U of AmpliTaq Gold (PE Applied Biosystems). After activation of the AmpliTaq Gold for 10 min at 95℃, 50 cycles each of 15 s at 95℃ and 1 min at 62℃ were carried out in a Model 7700 Sequence Detector (PE Applied Biosystems). Real-time fluorescent measurements were taken and a threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 × SD of the base line). For a positive control, a plasmid that contained the IE gene was constructed using the pGEM-T vector (Promega, Madison, WI) and was termed pGEM-IE. A standard graph was constructed using the C_T values obtained from the serially diluted pGME-IE. The C_T values from the clinical samples were plotted on the standard curve, and the copy number was calculated automatically by using Sequence Detector v1.6 (PE Applied Biosystems), a software package for data analysis. Samples were defined as negative when the C_T value exceeded 50 cycles[12]. The DNA copy numbers in

Table 1 Clinical and epidemiological characteristics of patients

	Male	Female	Total
Age, yr (median, range)	30 (18-56)	29 (8-56)	27 (8-56)
Gender	14	9	23
CMV infection diagnosed	11	7	18
Recent corticosteroid therapy	14	9	23
Outcome: Surgery	9	6	15
Ganciclovir therapy	2	1	3

Table 2 Utility of various tests for detection of infection with CMV in CyA treated patient with UC

Parameter	Real-time PCR (%)	Serum IgM antibodies (%)	Inclusion bodies in HE stained biopsy (%)
Number	18	16	4
Sensitivity	100	88.88	22.22
Specificity	100	100	100
PPV	100	100	100
NPV	100	71.4	26.3

the plasma were expressed per milliliters. The minimum detection level was 100 copies/mL of plasma.

Criteria for diagnosis of CMV infection

Positive results in any tests (IgM antibody, CMV DNA, or inclusion bodies in HE stained sections) were considered as evidence of CMV infection.

Statistical analysis

The results were expressed as mean ± SD. The *P* values below 0.05 were considered significant. The accuracy of each test was calculated considering a positive result in any test for CMV as evidence of infection.

RESULTS

Twenty-three patients were treated with intravenous CyA and they evidenced no severe side effects of the drug, such as renal failure or liver failure. The patient characteristics are listed in Table 1. All patients had received corticosteroids before the initiation of treatment with CyA. The degree of the severity of UC in all patients was pan colitis. Prior to treatment with CyA, active CMV infection was not observed in any of the patients. Eighteen of the 23 UC patients treated with CyA were infected with CMV (IgM antibodies in 16/23 patients, 69.6%; CMV DNA in 18/23 patients, 78.2%; and inclusion bodies in 4/23 patients, 17.3%). The rate of CMV detection by real-time PCR was significantly higher than other methods ($P < 0.05$). There was no difference in the CMV infection rate between males and females (males, 11/14; 78.57% and females, 7/9; 77.7%) (Table 2). With the exception of four of the tissue sections, all the sections from our UC patients who were treated with CyA were negative for CMV as demonstrated by histochemistry. After CyA treatment, none of the CMV-positive patients had pneumonia, hepatitis, retinitis, nephritis, or pancreatitis. Their only clinical symptoms were those of gastrointestinal disorder caused by colitis.

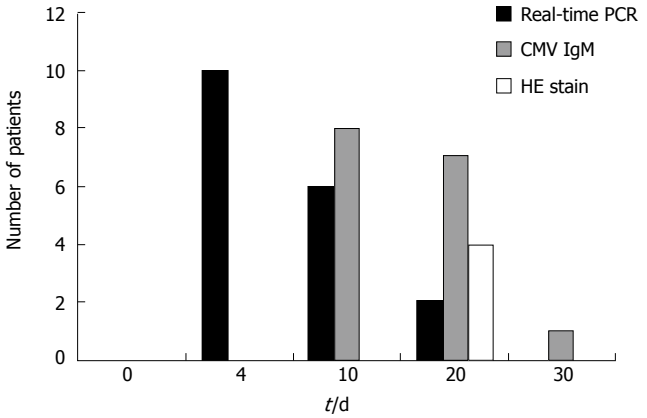

Figure 1 Comparison of initial CMV detection by day among the three methods (Real-time PCR, CMV IgM, and HE stain).

The median time of onset of the CMV infection after CyA treatment was 8.5 d (range: 4-20 d).

Real-time PCR detection is the most rapid of the three methods used (CMV IgM, HE biopsy, and real-time PCR results) (Figure 1). In particular, the HE biopsy method is slower and less sensitive than the other methods.

All patients showed an improvement in their symptoms after the initiation of the CyA treatment. Four CMV-negative patients treated with CyA showed a drastic improvement in their symptoms, and one CMV-negative patient who received CyA showed slight improvement. The degree of hematochezia progressively decreased after CyA treatment. CMV-negative patients continued to show an improvement in their symptoms during CyA treatment; further, even though CyA treatment was terminated, no deterioration in the degree of bowel symptoms was observed. On the other hand, all CMV-infected patients demonstrated an aggravation of the symptoms of colitis, i.e., an increase in the body temperature and the degree of hematochezia; after CMV infection ($P < 0.05$) (Figure 2). Further, the inflammatory values, i.e., the white blood cell (WBC) count and the CRP values worsened after CMV infection ($P < 0.05$) (Figure 3). However, an assessment of liver and renal functions after infection did not reveal any deterioration (Figure 4). After 10 d of CyA administration, CMV DNA was detected in 16 of the 18 UC patients, however, the clinical symptoms were improving. On the other hand, after 20 d, the clinical symptoms worsened in the CMV infected patients and CMV IgM and the histopathology results were positive. The values of the CMV IgM titer and the number of CMV DNA copies both increased after infection (Figure 5). The increased CMV load was followed by an increase in the severity of colitis.

Fifteen of these 18 patients with CMV infection (83.3%) required surgical treatment because of uncontrolled, severe deteriorating colitis. Perforation of the colon was observed in one of the patients after CMV infection; he underwent an emergency total colectomy. Nevertheless, in our study,

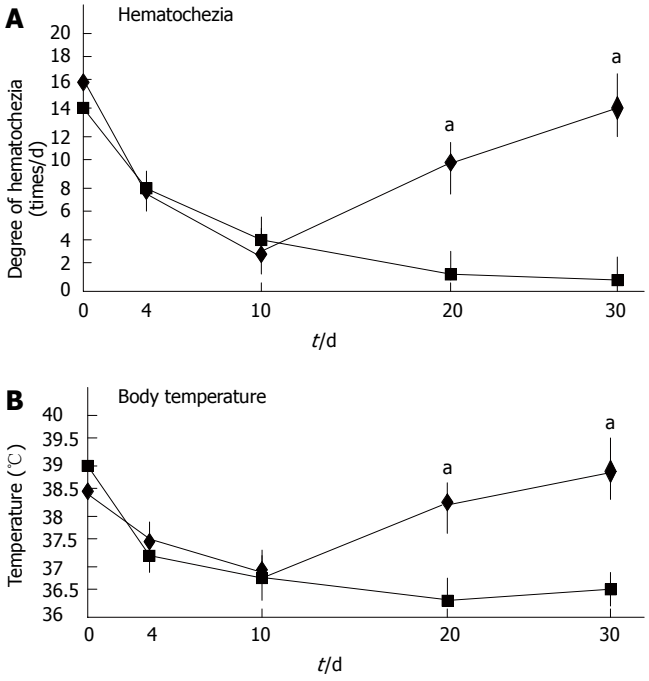

Figure 2 Change in clinical symptoms (hematochezia and body temperature) between CMV-positive and CMV-negative UC patients during intravenous CyA treatment. Panel **A** showed the change in the degree of hematochezia. Panel **B** showed the change in body temperature. The mean values of each group were plotted. Symbols: ◆, CMV positive; ■, CMV negative. $^{a}P < 0.05$.

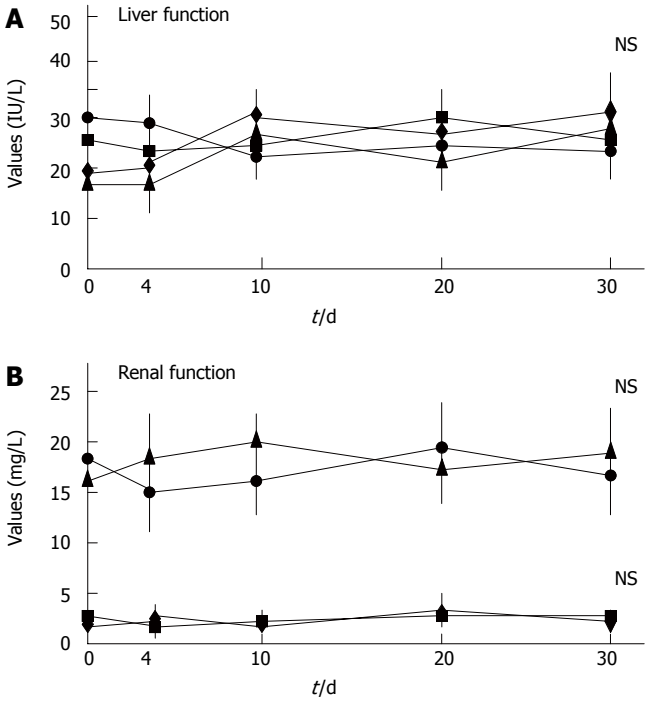

Figure 4 Change in liver function and renal function between CMV-positive and CMV-negative UC patients during intravenous CyA treatment. Panel A showed the change in liver function. The mean values of each group were plotted. Symbols: ◆, aspartate aminotransferase in CMV positive; ■, aspartate aminotransferase in CMV negative; ▲, alanine aminotransferase in CMV positive; ●, alanine aminotransferase in CMV negative. Panel B showed the change in renal function. The mean values of each group were plotted. Symbols: ◆, creatinine in CMV positive; ■, creatinine in CMV negative; ▲, blood urea nitrogen in CMV positive; ●, blood urea nitrogen in CMV negative, respectively. NS represented not significant.

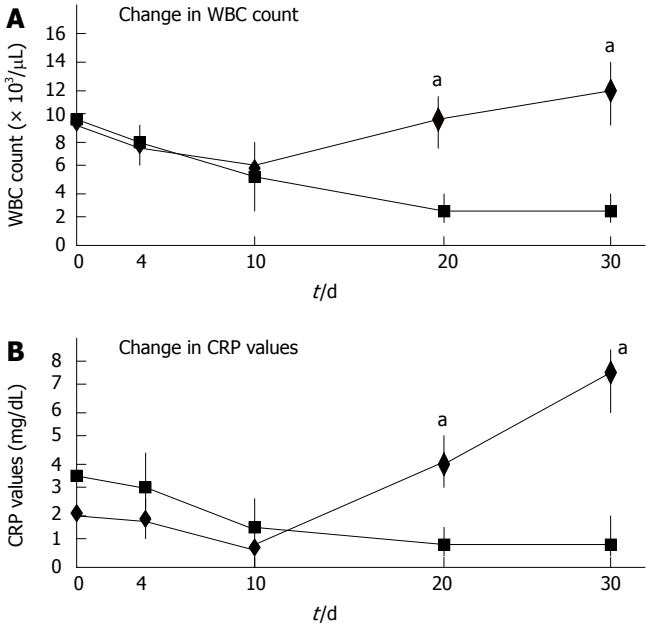

Figure 3 Change in inflammation markers between CMV-positive and CMV-negative UC patients during intravenous CyA treatment. Panel **A** showed the change in WBC count. Panel **B** showed the change in CRP value. The mean values of each group were plotted. Symbols: ◆, CMV positive; ■, CMV negative. $^{a}P < 0.05$.

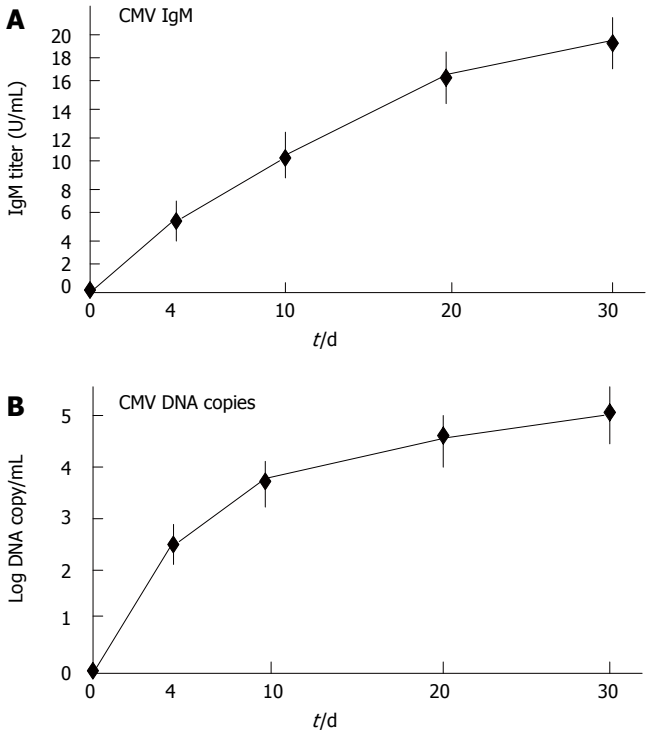

Figure 5 Change in mean CMV IgM value and CMV viral DNA load after CMV infection. Panel **A** showed the change in mean CMV IgM value after infection. Panel **B** showed the change in mean CMV viral load after infection.

there were no deaths among the UC patients after CMV infection.

Only three patients (males 2, and female 1) showed an improvement in the severity of colitis and did not require

a colorectomy since they were treated with ganciclovir following the identification of the CMV infection. The CMV IgM values did not decrease after ganciclovir treatment. However, in these three patients, the CMV DNA copy number decreased and did not show any further increase. Further, CMV infection was detected in one of the patients by an HE histopathological study. After ganciclovir administration, the owl's eye sign in the colon tissue was not observed. Thus, the three patients who were treated with ganciclovir were not reinfected with CMV although CyA treatment was continued.

DISCUSSION

We clarified a possible relationship between CMV infection and UC treated with CyA. We showed a significant deterioration in the clinical symptoms and the inflammatory response after active CMV infection. However, no impairment in hepatic or renal function was observed since we could not detect either CMV hepatitis or acute renal failure. Our results suggest that CMV infection in patients with severe UC treated with CyA is associated with a poor outcome because of severe deterioration in UC.

In general, a primary CMV infection, which is usually acquired early in life, is overcome by the humoral and cellular immunological response. Thereafter, the virus remains latent, particularly in endothelial cells and monocytes. Reactivation occurs during the use of immunosuppressive drugs. The viral load is the highest during active infection and the lowest during the latency stage in immunocompetent persons. We observed that CyA treatment may cause the reactivation of CMV and a subsequent deterioration in colitis. During a CMV infection, an increase in intestinal permeability has been shown to occur in kidney transplant recipients[14]. This defect in the barrier function may facilitate the exposure of the mucosal immune system to antigens from the luminal flora. CMV may also spread from the mucosa to the bloodstream because of such a defect in the barrier function, leading to CMV viremia in the bloodstream.

Steroid-resistant UC is defined as persistent active disease despite high-dose systemic corticosteroid therapy. A history of steroid resistance increases the possibility of complications such as CMV infection. CMV infections were detected in 4.6% to 13% of the patients diagnosed with UC[15,16]. Up to 33% of the patients with severe steroid-refractory UC were found to harbor the CMV virus. CMV was detected in 25% of the patients with steroid-refractory UC as compared to only 2.5% of patients with medically non-refractory UC. All the cases of UC with CMV infection in other investigations were clinically severe and steroid-resistant, and approximately 70% of the cases suffered from an acute exacerbation of symptoms and required immediate emergency surgeries. These results may suggest that CMV is not a coincidental occurrence but an exacerbating factor[17].

Although it is important to prevent the development of CMV disease[18], the availability of rapid, sensitive, and reliable methods for the early diagnosis of CMV infection is desirable[15]. As mentioned above, CMV disease can take many forms, depending upon the type of patient group under consideration. A consensus is available from the international CMV workshop for the definition of CMV disease[19]. The guidelines are purposefully rigorous and have aided the interpretation of clinical trial data and population based studies. However, they are likely to lead to an under appreciation of the contribution of CMV to patient morbidity. This is particularly true in the case of the histopathological diagnoses of CMV. Gastrointestinal symptoms along with histopathologically detected CMV are widely acceptable diagnostic criteria for CMV infections. In particular, the histopathological study of colon tissue is considered acceptable for CMV detection. Many CMV inclusion bodies were found in the surgical specimens of the UC cases in which CMV infection was detected, whereas CMV infection had not been detected by biopsy prior to surgery. CMV infection was observed in only 1 of the 55 cases investigated by endoscopic biopsy; however, it was detected in 8 of the 39 surgical cases[17]. Thus, it was rather difficult to detect CMV infection by biopsy, possibly because of sampling limitations. The characteristic inclusion bodies are not readily visible in routinely performed HE staining and CMV-infected cells are not always cytomegalic; therefore, the histopathological examination of the colonic biopsies had the lowest sensitivity. It has been suggested that CMV inclusion bodies are found more frequently in the right colon than in the left[16]; hence, multiple biopsies were taken from the colon, particularly from the inflamed and ulcerated areas, considering the fact that CMV exhibits tropism for the inflamed sites[20]. Therefore, it is likely that the false-negative results in the present study were related to sampling errors. Although our study did not confirm this, an immunohistochemical study of biopsy specimens may be more useful in diagnosis; however, immunohistochemistry is not a convenient tool for routine diagnosis.

The investigation of plasma CMV DNA is a simple, noninvasive, and nontraumatic method for evaluating and monitoring the CMV infection in patients. Procedures such as endoscopy and biopsies are traumatic and uncomfortable for a UC patient. The CMV-associated disease is generally the result of the reactivation of latent viruses rather than reinfection with the virus, and the measurement of CMV antibodies is often of no diagnostic use.

The real-time PCR assay was found to be as useful as the pp65 antigenemia assay because they were highly correlated[12]. Unlike the conventional qualitative PCR assay, which is not beneficial for determining the termination of antiviral therapy[21], this assay showed that the copy numbers of CMV DNA decreased and disappeared in response to anti-CMV therapy. Additionally, with this PCR method there is almost no room for bias due to subjective assessments by technicians. One investigator reported that real-time PCR is more sensitive (92%) than the pp65 test (88%) with regard to positive findings. Further, the correlations between real-time PCR and the pp65 antigenemia assay were statistically

significant[22]. The advantage of quantitative techniques is that by defining a "threshold value" of CMV load, they may allow a distinction between the commonly occurring, clinically irrelevant CMV infection and the levels of active CMV replication that are likely to lead to clinical disease[23].

However, there is still disagreement with regard to the optimal type of sample material, for example, leukocyte fractions versus plasma versus whole blood, and to the desirable sensitivity, which depends on the initial sample volume.

We used a quantitative CMV PCR assay that was able to quantify CMV DNA in plasma over the 100 copies/mL. The assay is fully controlled for maximal efficiency ~~in~~ at all steps. This is achieved by employing the widely used principle of an "internal control" and taking it one step further as a control for extraction efficacy.

Although the quantitative results obtained with different assays are difficult to compare because of the absence of an international standard, the results obtained with quantitative PCR correlate well with those of a number of other assays, and its sensitivity is generally higher[24]. The high sensitivity of the quantitative PCR assay is partly due to the use of a relatively large sample volume and the concentration of the DNA contained therein. Using plasma appears to avoid the loss of sensitivity as reported previously as compared to using whole blood or leukocytes[25]. Using plasma may also avoid the possibility of amplifying "latent" CMV from leukocytes. The exclusion of CMV DNA of intracellular origin may increase the clinical relevance of latent CMV detection; this in turn may decrease the predictive value of the test for active CMV infection[26]. Plasma is also easier to handle than cellular fractions such as leukocyte preparations and is better standardized, particularly in leukopenic patients. In HIV-positive individuals, the CMV load in whole blood and plasma has also been shown to be an important indicator of pathogenesis[27]. The presence of CMV DNA in the blood of HIV-positive patients identifies a group of patients who are almost 20 times more likely to progress to CMV disease than those who remain negative for CMV DNA in their blood[28]. In addition, an increasing CMV load in the blood was associated with an increased risk of disease progression[28]. CMV load was correlated with tissue samples obtained at the postmortem examinations of the HIV patients with histological evidence of CMV inclusions, and it was found that a viral load of > 5 000 000 genomes/μg DNA is required before CMV inclusions are observed[29]. It should be noted that some of these manifestations do not satisfy the criteria outlined for the diagnosis of CMV disease. Thus, it appears likely that cases of CMV colitis may also be dismissed because they do not meet the definition of CMV infection, despite the presence of clinical symptoms and CMV DNA in the blood. It is important to appreciate that with the advent of more sensitive molecular-based assays, a reappraisal of many of these definitions may be required.

In conclusion, a CMV infection in patients with steroid-resistant UC should be ruled out prior to initiating aggressive immunosuppressive therapy such as CyA for steroid-resistant UC. Further, ganciclovir as an anti-viral agent is useful for the treatment of CMV-associated UC after immunosuppressive therapy.

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COMMENTS

Background

Cytomegalovirus (CMV) infection is one of the most common infectious complications after immunosuppressive therapy. Ulcerative colitis (UC) is thought to result from the inappropriate and progressive activation of the mucosal immune system driven by the presence of normal luminal flora. Cyclosporine A (CyA) selectively inhibits immune responses mediated by T lymphocytes by modulating the interaction of calcineurin-calmodulin. This recognition has led to its use in patients with severe UC. In Japan, patients with severe UC are frequently treated with CyA and are expected to be at an increased risk of infection with CMV. Despite the frequent use of immunosuppressive drugs in patients with UC, data on the frequency of CMV infection and its clinical significance in patients with UC are limited.

Research frontiers

The aim of this study was to describe our experience with active CMV infection following treatment with CyA for UC in Japan.

Innovations and breakthroughs

Twenty-three patients with severe UC not responding to steroid therapy at Nagoya University Hospital received continuous intravenous infusion of CyA (average 4 mg/kg per day) for 1 mo. Serum and colonic biopsy samples were collected from all patients before CyA treatment and 4 d, 10 d, 20 d, and 30 d after treatment and were evaluated for CMV by using serology (IgM antibody by ELISA), quantitative real-time PCR for CMV DNA, and histopathological assessment of hematoxylin and eosin -stained colonic biopsies.

Applications

No patients had active CMV infection before CyA treatment. Eighteen of 23 UC patients treated with CyA were infected with active CMV (IgM antibody in 16/23 patients, 69.6%; CMV DNA in 18/23 patients, 78.2%; and inclusion bodies in 4/23 patients, 17.3%). Fifteen of these 18 patients with active CMV infection (83.3%) required surgical treatment because of severe deteriorating colitis. Treatment with ganciclovir rendered surgery avoidable in three patients. Active CMV infection in severe UC patients treated with CyA is associated with poor outcome. Further, ganciclovir is useful for treatment of CMV-associated UC after immunosuppressive therapy.

Terminology

CMV infection is one of the most common infectious complications in immune-compromised patients. UC is chronic inflammation colitis thought to result from the inappropriate and progressive activation of the mucosal immune system driven by the presence of normal luminal flora. CyA is a drug to inhibit immune responses selectively mediated by T lymphocytes by modulating the interaction of calcineurin-calmodulin.

Peer review

Severe ulcerative colitis is a potentially life-threatening condition. Traditional treatment for such patients is high-dose intravenous corticosteroids but up to 40% of patients become refractory to this treatment. In these patients CyA therapy has been shown to have an initial positive clinical response in many patients. However, i.v. cyclosporine is recommended only as short term "bridging" therapy to induce remission followed by azathioprine or 6-mercaptopurine maintenance therapy. In the present study Minami and coworkers investigated the frequency of the development of a CMV infection during a continuous intravenous cyclosporine treatment in ulcerative colitis and the clinical outcome in CMV infected vs. non infected patients. The study is of interest.

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CLINICAL RESEARCH

Patients without hepatocellular carcinoma progression after transarterial chemoembolization benefit from liver transplantation

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Abstract

AIM: To assess the outcome of patients, who underwent transarterial chemoembolization (TACE) for hepatocellular carcinoma (HCC) and subsequently liver transplantation (OLT) irrespective of tumor size when no tumor progression was observed.

METHODS: Records, imaging studies and pathology of 84 patients with HCC were reviewed. Ten patients were not treated at all, 67 patients had TACE and 35 of them were listed for OLT. Tumor progression was monitored by ultrasound and AFP level every 6 wk. Fifteen patients showed signs of tumor progression without transplantation. The remaining 20 patients underwent OLT. Further records of 7 patients with HCC seen in histological examination after OLT were included.

RESULTS: The patients after TACE without tumor progression underwent transplantation and had a median survival of 92.3 mo. Patients, who did not qualify for liver transplantation or had signs of tumor progression had a median survival of 8.4 mo. The patients without treatment had a median survival of 3.8 mo. Independent of International Union Against Cancer (UICC) stages, the patients without tumor progression and subsequent OLT had longer median survival. No significant difference was seen in the OLT treated patients if they did not fulfill the Milan criteria.

CONCLUSION: Selection of patients for OLT based on tumor progression results in good survival. The evaluation of HCC patients should not only be based on tumor size and number of foci but also on tumor progression and growth behavior under therapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer related mortality and the fifth most common cancer[1] worldwide. Nearly 500 000 cases are diagnosed each year in the United States and the incidence increased from 1.4 cases per 100 000 between 1976 and 1980 to 2.4 cases per 100 000 from 1990 to 1995. Furthermore, the associated mortality rate and hospitalization showed an increase of 41% and 46%, which demands a significant challenge in the management of HCC patients[2].

The stage of disease divides HCC therapy into curative versus palliative approaches. Curative treatments are reserved for patients without portal vein invasion or distant metastases and include percutaneous ablation, surgical resection and liver transplantation[3-5]. Palliative attempts include liver-directed therapies, rarely systemic chemotherapy, and are offered when local extra-hepatic spread or distant metastases are present. Palliative therapy via transarterial chemoembolization (TACE) may be offered for unresectable HCC. TACE involves the injection of chemotherapeutic agents into the hepatic artery[6,7]. Multiple large randomized and controlled trials have failed to demonstrate a beneficial effect in survival of patients treated with TACE, but meta-analysis of these trials show a slight beneficial effect in survival in comparison with conservative treatment[8].

For localized HCC in curative situations, which is defined by the absence of macro-vascular invasion and metastatic disease with well-preserved hepatic function, the initial treatment of choice is hepatic resection. In case of cirrhosis, optimal candidates for surgical resection show a single lesion less than 5 cm in size, with no complications of end-stage liver disease and no significant portal hypertension (portal pressure gradient less than 10 mmHg). Nevertheless, after 5 years there are significant

recurrence rates (70%) in HCC patients after surgical resection, and the 5-year survival rate is 30%. Taken together only about 5% of patients are ideal candidates for hepatic resection[9-11]. Therefore liver transplantation (OLT) is the only curative approach that addresses the HCC lesion as well as the underlying liver cirrhosis[12-15].

Initial reports of liver transplantation in patients with HCC showed poor outcomes with a recurrence rate up to 50% and a 5-year survival of less than 40%. In these reports many patients underwent OLT in the setting of advanced HCC. As a consequence the Milan criteria have been put forward to provide guidelines that help select HCC patients for curative OLT. The goal of this effort was to achieve comparable survival rates in liver transplanted patients with HCC and patients without concomitant neoplasias[12].

Patients fulfilling the criteria (single nodule < 5 cm or up to three nodules each < 3 cm) have a favorable prognosis with 3-year survival rates of 75% up to 85% and a recurrence rate of less than 15%. However, a retrospective cohort analysis showed comparable survival rates in patients who had solitary nodules less than 6.5 cm or 3 nodules with a combined diameter of less than 8 cm[13], demonstrating that OLT is a potentially curative approach for patients with HCC extending the Milan criteria.

However, the general scarcity of donor livers hampers timely liver transplantation. In the interim specific therapy such as TACE can be initiated to stabilize the patient's health condition. Because the Milan criteria do not take into account tumor progression following non-surgical intervention strategies, patients treated with TACE cannot be necessarily evaluated on the basis of these criteria. We therefore selected patients for liver transplantation based on the lack of tumor progression during the waiting time and determined the clinical outcome in patients who were treated with TACE and subsequently underwent liver transplantation.

MATERIALS AND METHODS

Study design and characteristics of patients

From January 1995 to March 2002, 77 patients with HCC were seen at the Department of Surgery, University of Goettingen. The diagnosis of HCC was confirmed in all patients either by biopsy of the tumor or by a serum alpha-fetoprotein (AFP) measurement. In addition, in 7 patients who underwent OLT, HCC was diagnosed in the histological examination of the explanted organ.

Patient demographics showed a male: female ratio of 70:14 and a mean age of 59 ± 11.4 years (range 31-84). The main underlying disease of HCC was liver cirrhosis (n = 63, 75%), which could be assigned to the diagnoses of alcohol (n = 23), hepatits C (n = 22) and hepatitis B (n = 18) (Table 1).

Age, gender, Child-Pugh score and tumor stage of all patients are shown in Tables 2 and 3. At the time of HCC diagnosis, 10 (12%) patients had no evidence of impaired liver function, 28 (33%) patients presented with class A, 29 (35%) with class B, and 17 (20%) with class C impaired liver function according to the Child-Pugh classification.

Table 1 Diagnostic chart of patients with HCC

Diagnoses	*n*	Age (yr, mean ± SD)
No liver disease	10	61 ± 12.2
Fibrosis	3	66.7 ± 0.6
Alcohol induced cirrhosis	23	60 ± 8.3
Hepatitis (C) cirrhosis	22	55.9 ± 9.9
Hepatitis (B) cirrhosis	18	55 ± 14.1
Others[1]	8	56 ± 16.9
Total	84	59 ± 11.4

[1] Haemochromatosis 2, Caroli-syndrome 1, primary biliary cirrhosis 1, primary sclerosing cholangitis 1, hepatitis C 1, acute liver faliure 1, cryptogene cirrhosis 1. The majority of patients had liver cirrhosis secondary to viral hepatitis and alcohol-related liver disease.

Table 2 Cirrhosis scoring of study patients according to Child-Pugh Score

Child-Pugh Score	*n*	Age	Gender	
		(yr, mean ± SD)	Male	Female
A	28	56 ± 13.2	26	2
B	29	57 ± 9.7	25	4
C	17	56 ± 9.1	15	2
Total	74	56.3 ± 11.3	66	8

HCC was classified at hospital admission.

Table 3 Staging of the patients according to UICC

UICC stage	*n*	No cirrhosis	Cirrhosis	Age (yr, mean ± SD)
Ⅰ	5	1	4	52 ± 11.7
Ⅱ	13	1	12	60 ± 13.5
Ⅲ	11	3	8	60.8 ± 8.0
Ⅳ	55	5	50	58 ± 11.6
Total	84	10	74	59 ± 11.4

Table 4 Treatment modalities of patients with HCC

Treatment	*n*	No cirrhosis	Cirrhosis
No treatment	10	3	7
TACE, not listed for OLT	32	6	41
TACE, died waiting for OLT	15		
TACE and liver transplantation	20	0	20
Liver transplantation	7	1	6
Total	84	10	74

The tumor staging according to the UICC criteria of patients used in this study is shown in Table 3.

Ten (12%) out of 84 patients were not treated because they died before the treatment was started (n = 4), they refused treatment (n = 1) or TACE could not be done due to their cardio-pulmonary risk (n = 5). The remaining 74 patients were treated as seen in Table 4. In 7 transplanted patients, the HCC was diagnosed after liver transplantation and therefore they were not treated before OLT. In the other 67 patients TACE was done and 35 of them were listed for OLT. The reasons for not listing a patient for

liver transplantation were no additional liver cirrhosis (n = 10), age older than 65 (n = 11) and persistent alcohol disease (n = 11). From the initial 35 patients who were listed for OLT, 15 showed tumor progression after TACE and were therefore subsequently removed from the transplantation list. These patients showed a median time of tumor progression of 3.1 mo. The remaining 20 patients underwent OLT with a median time on waiting list of 7.6 mo.

Chest X-ray, computed tomography (CT) and staging by the TNM scoring system of the UICC was performed in all the patients. Tumors that were first identified by histopathology of the explanted liver were classified as incidental tumors.

Selection criteria for OLT

Patients were selected for OLT based on the guidelines of Transplantions Gesellschaft (DTG). In addition, patients with extrahepatic tumor manifestation did not qualify for OLT. Tumor size or number of tumors were not taken into account for listing the patient. The patients received TACE and were restaged every 6 wk during waiting time. Evidence of tumor progression resulted in removement of the patients from the waiting list.

Transaterial chemoembolization protocol

Patients were listed for OLT and immediately obtain TACE. TACE was performed in cases of advanced HCC stage or when tumors progressed during the staging work-up every 6 wk. Patients in advanced tumor stage (downstaging group) were listed when they responded to the first TACE treatment cycle. Sixty-seven patients were subjected to selective TACE before transplantation. The chemoembolization solution contained 50 mg epirubicin, 10 mL lipiodol and 3 mL water-soluble contrast material. Embolization was performed until blood flow to the tumor stopped.

The following day CT scanning was performed to determine the lipiodol uptake by the tumor tissue. Each TACE cycle was repeated every 6 wk and ultrasound, CT scan and AFP levels were assessed. Response to TACE is defined as constant size of the tumor and/stable AFP levels. Patients showing a positive response to TACE remained on the waiting list and were monitored by a CT scan (every 3 mo) and determination of AFP level (monthly). Patients with tumor progression under TACE treatment were discharged from the waiting list (non-responder).

Post-transplantation management and follow-up

Immunosuppressive therapy following OLT consisted of a drug regimen of Prograf in combination with corticosteroids. Corticosteroids were gradually tapered and discontinued within 3 mo. Prograf was continued for one year after OLT unless side-effects were seen. The frequency of the outpatient visits thereafter varied according to the patient conditions and types of complications. No anti-cancer treatment was given after transplantation. All patients were followed up weekly in the outpatient clinic for the first month after discharged from the hospital. Screening for tumor recurrence was assessed by determination of a-fetoprotein (AFP) and ultrasonography every 3 mo. A routine CT scan of the abdomen and chest was performed every year, and additional imaging techniques (bone scan, magnetic resonance imaging) were done if HCC recurrence was suspected. The medical records and pathologic reports were analyzed retrospectively.

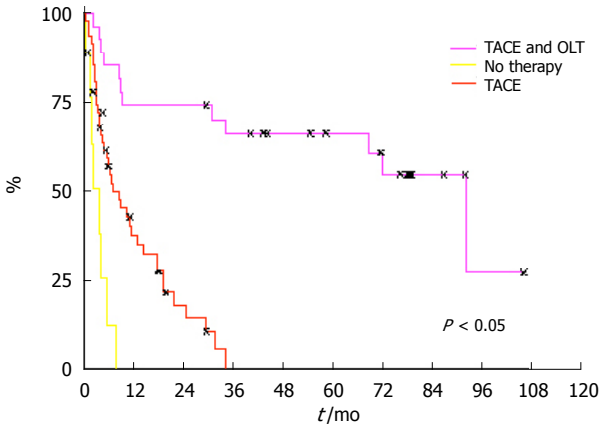

Figure 1 Survival probabilities for the first 5 yr after treatment. Overall HCC patient survival who had received TACE and OLT, only TACE and patients who received no treatment.

Statistical analysis

We analyzed the statistical significance on recurrence and survival of tumor-related risk factors, tumor size, number of nodules, and Milan criteria. We used the Kaplan-Meier method to measure survival and the log-rank test to analyze statistical differences. Results are expressed as mean ± SD. One-way ANOVA was used for comparisons between the groups when one measurement per experiment was available. Non-normally distributed data was calculated after rank transformation. Data were calculated by univariate ANOVA. Tukey's-HSD test was used for post hoc comparisons. $P < 0.05$ was considered significant in difference. Survival was estimated using Kaplan-Meier/log-rank analysis. All calculations were performed using SPSS 10.0®, standard version.

RESULTS

Tumor progression after TACE

Chemoembolization was well tolerated in the majority of patients. The most common complaints after TACE were pain, transient fever, and nausea. No patient developed major complications, which required surgical intervention. After TACE, 20 (29.2%) showed response and 47 (70.1%) showed tumor progression.

Tumor progression defines a group with good survival after OLT

As expected, the patients without treatment had the worst outcome with a median survival of 3.8 mo as seen in Figure 1. The Patients who were treated with TACE but did not qualify for liver transplantation due missing sighs of liver cirrhosis (n = 10), age older than 65 (n = 11), persistent alcohol disease (n = 11) or tumor progression (n = 15) had a median survival of 8.4 mo (Figure 1). The

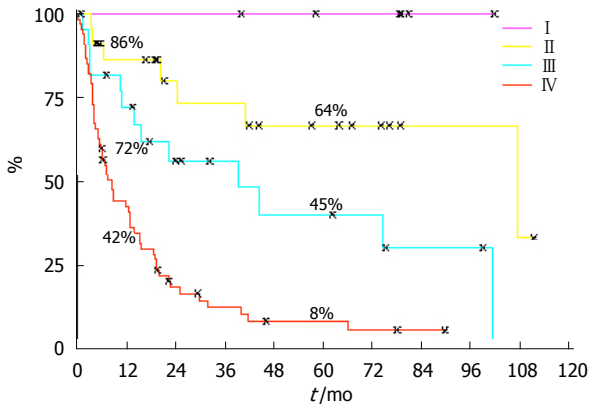

Figure 2 Kaplan-Meier patient survival curves showing survival dependent on UICC stage.

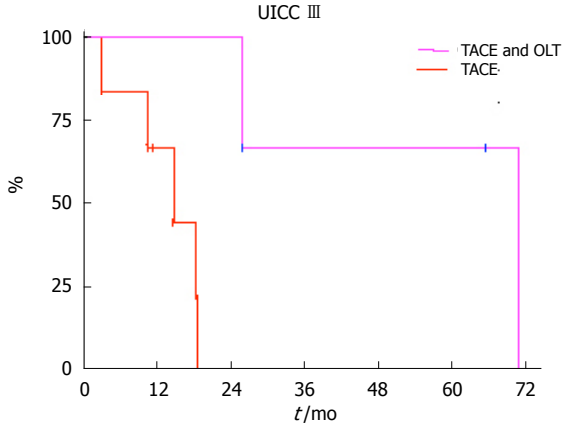

Figure 4 Kaplan-Meier patient survival curves. OLT improved survival UICC stage Ⅲ.

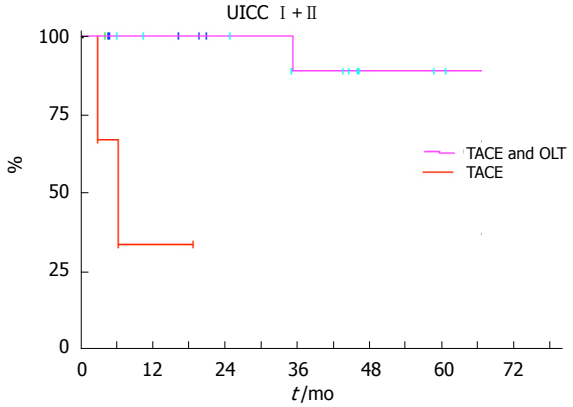

Figure 3 Kaplan-Meier patient survival curves demonstrating that OLT improved survival UICC stage Ⅰ and Ⅱ.

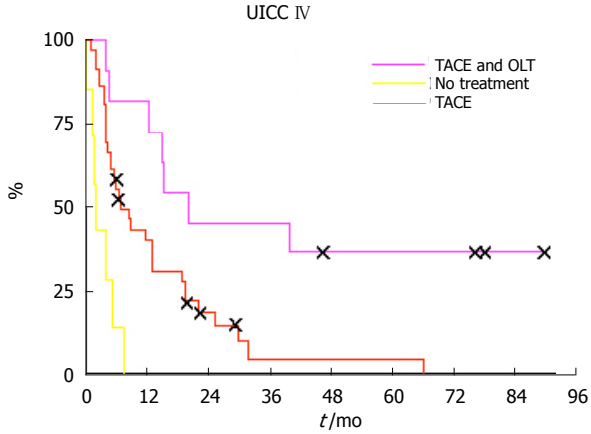

Figure 5 Kaplan-Meier patient survival curves. OLT improved survival UICC stage Ⅳ.

patients who showed no signs of tumor progression during waiting time had an average of 3 sessions of TACE (range, 1 to 10) before OLT. The TACE treated and liver transplanted patients (n = 20) had a significant better outcome compared with TACE treated patients with a median survival of 92.3 mo (Figure 1). The time of median survival in the transplanted group is comparable to OLT patients with non-malignant diseases (median survival of 101.6 mo) and confirms the selection criteria for OLT are suitable to select HCC patients for liver transplantation.

Survival dependent on UICC stage

Survival analysis revealed that the patient overall survival was dependent on the UICC stage. Patients with UICC stage Ⅰ (n = 5) had an overall survival of 100%, stage Ⅱ (n = 13) 64%, stage Ⅲ (n = 11) 45% and stage Ⅳ only 8% (n = 55) (Figure 2).

Lack of tumor progression and subsequent OLT improves survival irrespective of UICC stage

Patients, who fulfilled the selection criteria for liver transplantation and had no signs of tumor progression, underwent subsequently OLT. Independent of UICC stage, the patients with TACE and OLT had a better survival (Figures 3, 4 and 5). None of the patients with UICC stage Ⅰ on the waiting list showed signs of tumor progression. In UICC stage Ⅱ and Ⅲ, 4 patients had signs of tumor progression during waiting time. Seven of the UICC stage Ⅳ patients displayed signs of tumor progression. Independent of the UICC stage the patients without tumor progression and subsequent OLT had a significant better median survival. Patients who did not fulfill the criteria for liver transplantation or showed signs of tumor progression in UICC stage Ⅲ had a median survival of 14.2 mo compared to 39.5 mo in the liver transplanted group (Figure 4). In UICC stage Ⅳ patients the median survival was 8.6 versus 15.6 mo, respectively. The patients who were not treated at all had the worst survival with a median survival time of 3.8 mo (Figure 5). Taken together, independent of the UICC stage, the survival was significantly better in patients who qualified for liver transplantation and without any evidence of tumor progression while waiting for OLT (P > 0.05).

Patients without tumor progression have a comparable outcome irrespective of the Milan criteria

Patients, who fullfilled the Milan criteria, are considered to have an excellent outcome. Interestingly, in our study there was no significant difference (P = 0.19) in survival

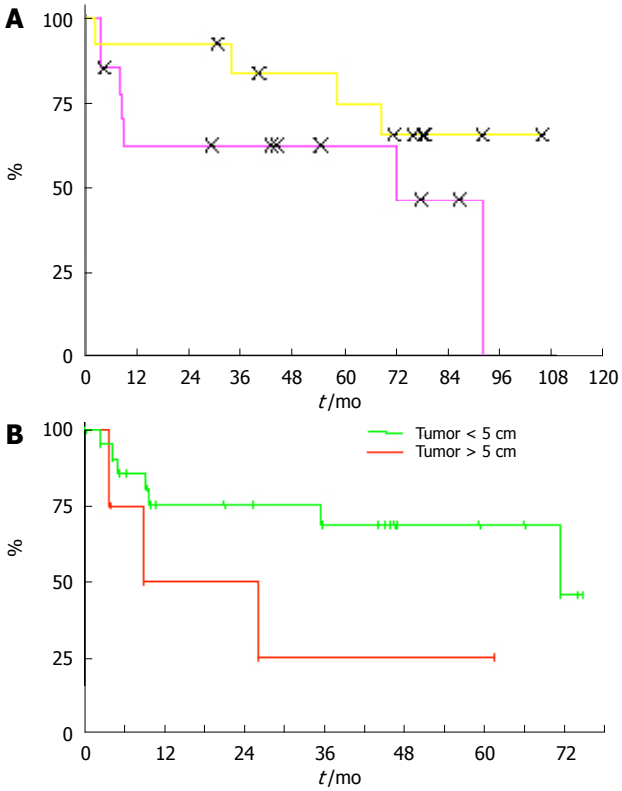

Figure 6 Survival probabilities for the first 5 years after liver transplantation according to the Milan criteria (**A**) and tumor size (**B**).

of patients, who fullfilled the Milan criteria compared to the more advanced patients (Figure 6A). However, the patients with tumors less than 5cm had a significantly better median survival ($P = 0.03$) compared to patients with tumors larger than 5 cm (Figure 6B).

DISCUSSION

Liver transplantation is the only approach that addresses the multifocal hepatocellular carcinoma and also treats the underlying cirrhosis but the limited availability of donor organs and the subsequent immunosuppression urge criteria of patient selection. Our data suggest that patients, who do not show signs of tumor progression after TACE treatment benefit from OLT irrespective of UICC stage. Without tumor progression, even patients outside of the Milan criteria do have a good outcome. Therefore we suggest that patient selection for OLT should be based on tumor size and additionally on assessment of tumor progression.

The current UNOS policy for organ allocation among patients with HCC favors those with tumors confined within the limits of diameter and number of nodules defined by the Milan criteria[12]. These were derived from a prospective study showing significantly better recurrence-free survival for 35 patients meeting the proposed criteria than for 13 patients with HCC exceeding criteria because of preoperative tumor stage underestimation based on pathologic analysis of the liver explants (92% *vs* 59% at 4 years). Other study groups proposed to "expand" the limits concerning OLT for HCC[16], because studies using the extended UCSF criteria (solitary tumor < 6.5 cm, three or fewer nodules with the largest lesion < 4.5 cm, total tumor diameter < 8 cm or without gross vascular invasion) do not worse the outcome of patients after OLT[13]. Therefore, concerns arise whether the Milan criteria may be too restrictive, thus excluding patients who would otherwise benefit from OLT. The decision for OLT is based on tumor size and number of tumor within the liver but not on growth behavior of the tumor or response of the tumor after bridging therapy. In our present study, 12 patients were found to have tumor characteristics exceeding the Milan criteria and underwent OLT. These patients were selected on the tumor progression after TACE and the difference in survival among those patients compared to patients meeting the Milan criteria did not reach statistical significance ($P = 0.19$). Thus patients without tumor progression not fulfilling the Milan criteria might benefit from OLT.

Vitale *et al*[17] described a very low occurrence of drop-outs from waiting list, though they had a quite long median waiting time of 11 mo[18]. In their study they used the following criteria to exclude patients from OLT: short-listing, general contraindication to transplant, extrahepatic spread, vascular invasion or poorly differentiated HCC (G3) at pre-OLT percutaneous biopsy. Size and number were not considered absolute selection criteria. They suggest that adoption of different selection criteria accounting more for tumor biology (grading) rather than tumor size and number and a use of a pre-OLT multimodal strategy probably may guarantee a lower number of drop-outs. Our results demonstrate that patients who show no further tumor progression under TACE treatment significantly ($P < 0.05$) benefit from OLT. It seems to be an easier biological grading method than the routine percutaneous biopsy for liver lesions because of the given potential risk of tumor seeding along the biopsy tract.

Furthermore, current imaging techniques have a high incidence of false-negative and false-positive results when evaluating HCC in cirrhosis. Only in 14.3% of OLT patients, tumor diameter was correctly identified by pretransplant radiological examinations shown by Sotiropoulos *et al*[19,20]. Sensitivity of radiological imaging was especially poor for tumors between 1 and 2 cm and less than 1 cm (21% and 0%, respectively), indicating that detection of small HCCs, especially in end-stage cirrhotic livers, remains problematic. A critical appraisal of patient characteristics together with great caution when interpreting imaging studies is recommended to determine candidacy for transplantation. Otherwise many patients are not given the opportunity to undergo OLT.

TACE involves the injection into the hepatic artery of chemotherapeutic agents such as doxorubicin, mitomycin, or cisplatin with lipiodol, to promote intra-tumor retention of the medications. TACE has the advantage of treating larger tumor-areas, being repeatable and perhaps downstaging patients. Disadvantages of TACE include significant toxicity and acute liver failure, especially when treating large areas[17]. Even if downstaging by TACE has been reported, TACE has not been shown to improve survival or recurrence of HCC after transplantion. Even in patients who were not transplanted, multiple large randomized controlled trials have failed to demonstrate

a survival benefit. Only a small survival benefit in comparison with conservative treatment was seen in meta-analysis of these trials. In addition, none of the current studies have examined the ability of TACE to sustain patients on the transplantation waiting list. Our data demonstrate that the patients, who have no tumor progression after TACE benefit substantially from OLT. Whether these patients would progress without TACE remains speculative[19,21-26].

In contrast to other malignant diseases, assessment of HCC patients on the waiting list should not only account for the current stage, it although should evaluate the tumor progression. Multiple genetic lesions within the HCC cells, which modulate growth, cell cycle, apoptosis and invasion, define tumor progression[27-31]. These genetic lesions are not defined in number and location and therefore prospective evaluation based on molecular pattern is not possible. On the other hand it is practicable to assess tumor growth by size and AFP levels.

Therefore, we conclude that the growth behavior of the tumor (defined as progression under anti-cancer therapy) could provide simple but helpful information about the recurrence rate and the outcome of HCC patients after OLT. We propose that growth behavior (P0 for no progression and P1 for progression) should be added to staging systems for classification of HCC to select patients for OLT[32-34].

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CLINICAL RESEARCH

Esophageal mesenchymal tumors: Endoscopy, pathology and immunohistochemistry

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Abstract

AIM: To study the endoscopic, pathological and immunohistochemical features of esophageal mesenchymal tumors.

METHODS: Twenty-nine patients diagnosed as esophageal mysenchymal tumors by electronic endoscopy and endoscopic ultrasound (EUS) were observed under light microscopes, and all tissues were stained by the immunohistochemical method. The expression of CD117, CD34, SMA and desmin were measured by staining intensity of cells and positive cell ratios.

RESULTS: Endoscopically, esophageal gastrointestinal stromal tumors (GISTs) and leiomyomas (LMs) had similar appearances, showing submucosal protuberant lesions. They all showed low echo images originated from the muscularis propria or muscularis mucosa on EUS. Endoscopy and EUS could not exactly differentiate esophageal GISTs from LMs. Microscopically, there were two kinds of cells: spindle cell type and epitheloid cell type in esophageal GISTs. Leiomyomas and leiomyosarcomas were only of spindle cell type. One malignancy was found in five cases of esophageal GISTs, and one malignancy in 24 cases of leiomyomas and leiomyosarcomas. Using Fisher's exact method, the differences of malignant lesion proportion were not significant between esophageal LMs and GISTs, 1/5 *vs* 1/24 ($P > 0.05$). All cases of esophageal GISTs were positive for CD117, and 3 cases were also positive for CD34. The 24 cases of leiomyomas and leiomyosarcomas were all negative for CD117 and CD34. The differences of positive rates of CD117 and CD34 were significant between esophageal GISTs and LMs, 5/5 *vs* 0/24, 3/5 *vs* 0/24 ($P < 0.005$). All leiomyomas and leiomyosarcomas were positive for SMA, and desmin. Among 5 cases of esophageal GISTs, 2 cases were SMA positive, and 1 case was desmin positive. The differences in positive rates and expression intensity of SMA and desmin were significant between esophageal LMs and GISTs, 24/24 *vs* 2/5, 24/24 *vs* 1/5 ($P < 0.005$).

CONCLUSION: The most common esophageal mesenchymal tumors are leiomyomas, and esophageal GISTs are less common. Most of esophageal LMs and GISTs are benign. Endoscopy and EUS are the effective methods to diagnose esophageal mesenchymal tumors and they can provide useful information for the treatment of these tumors. However, they cannot exactly differentiate esophageal GISTs from LMs. Pathological, especially immunohistochemical features are useful to differentiate GISTs from leiomyomas.

Key words: Esophageal mesenchymal tumors; Gastrointestinal stromal tumors; Leiomyomas; Endoscopy; Pathology; Immunohistochemistry

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INTRODUCTION

Traditionally, the gastrointestinal mesenchymal tumors (GIMTs) have been almost uniformly classified as gastrointestinal leiomyomas (LMs). However, recent evidence indicates that most mesenchymal tumors of the gastrointestinal tract are gastrointestinal stromal tumors (GISTs)[1]. It is difficult to differentiate esophageal GISTs from LMs because of their similar appearance. GISTs frequently have malignant potential, therefore, it is important to differentiate GISTs from LMs. GISTs arising in the gastrointestinal tract have been known quite well. Whether there are the same stromal tumors in the esophagus, and whether stromal tumors are the most

frequent mesenchymal tumors of the esophagus, are big concern of clinical doctors[2,3].

MATERIALS AND METHODS

Specimen collection

All the patients were in- and outpatients from the First Affiliated Hospital of Nanchang University during June 2004 to November 2005 and they all met with the following two criteria: (1) Endoscopically, the tumors showed submucosal protuberant lesions, and they showed low echo images originated from the muscularis propria or muscularis mucosa on EUS. (2) Microscopically, they were diagnosed as esophageal mesenchymal tumors. All tissue specimens were obtained by the following 3 methods: biopsy, endoscopic mucosal resection (EMR) or surgical operation.

Methods

All tissue specimens were fixed in 10% formalin and processed routinely for paraffin embedding. Sections of 4-mm thick were stained with hematoxylin and eosin, and observed by light microscopy. Then all cases were stained for CD117, CD34, SMA, and desmin. All antibodies were purchased from Beijing Zhongshan Corporation. The detailed procedures were carried out according to instructions of the kits.

Criteria for histopathology

First, esophageal mesenchymal tumors: According to the criteria of 2005 WHO Oncopathology and Genetics[4], if spindle cells and epitheloid cells were shown microscopically, esophageal mesenchymal tumors can be diagnosed. Sencond, criteria for assessing malignancy of gastrointestinal stromal tumors: according to the criteria of 2005 WHO Oncopathology and Genetics and the advice of Singer and Miettinen[4-6], GISTs were diagnosed as malignant when the following criteria were met: tumor size $\geqslant$ 5 cm, nuclear mitotic figure > 5/50 HPF. GISTs were diagnosed as benign: tumor size < 5 cm, nuclear mitotic figure < 5/50 HPF. GISTs were diagnosed as potentially malignant: tumor size $\geqslant$ 5 cm, nuclear mitotic figure < 5/50 HPF or tumor size < 5 cm, nuclear mitotic figure > 5/50 HPF. At the same time, tumor hemorrhage/necrosis, peripheral invasive growth, lymph node metastasis and metastasis to another organ are all considered also. Third, criteria for leiomyosarcomas: according to the criteria of 2005 WHO Oncopathology and Genetics and internal reports[4,7,8], tumor size $\geqslant$ 5 cm, nuclear mitotic figure > 5/50 HPF, tumor hemorrhage/necrosis, peripheral invasive growth and metastasis.

Assessment for immunohistochemical results

Positive results were indicated if the cytoplasm was stained brown, and cell membrane was stained positive for CD34 and CD117. The categories were (+): more than 10% of cells stained; (-): less than 10% of cells stained. Positive control: CD117, an indicator of the known GISTs; CD34, an indicator of vascular endothelial cells in tumors; SMA, an indicator of normal smooth muscles in vascular walls or esophageal walls; Desmin, an indicator of normal smooth muscles in esophageal walls. Negative control: the primary antibody was replaced by PBS for negative control.

Table 1 Clinical findings of 29 cases of esophageal mesenchymal tumors

Tumor type	*n*	Dysphagia	Heart burn/ retrosternal pain	Hemorrhage	Stomachache	Asymptomatic
LMs	24	4	7	1	2	10
GISTs	5	1	1	1	0	2
Total	29	5	8	2	2	12

Tested by Fisher's exact method, the differences of symptoms are not significant between esophageal LMs and GISTs ($P > 0.05$).

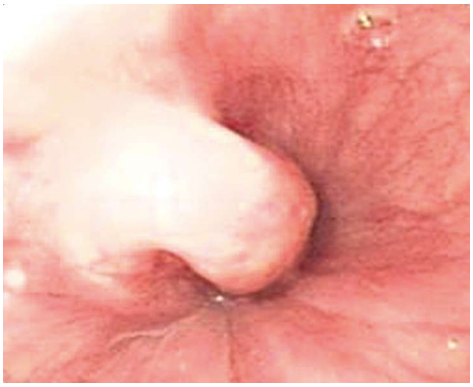

Figure 1 Endoscopic image of esophageal stromal tumors.

Statistical analysis

Data was tested using Fisher's exact method. A P value less than 0.05 was considered statistically significant.

RESULTS

Clinical data

Among 29 cases of esophageal mesenchymal tumors diagnosed by endoscopy, pathology and immunohistochemistry, 5 cases were esophageal GISTs, 23 cases were leiomyomas and 1 case was leiomyosarcoma. In the group of esophageal GISTs, 3 cases were male, and 2 cases were female. Their age ranged from 44-63 years (mean 52 ± 7.8 years). In the group of leiomyomas and leiomyosarcomas, 13 cases were male, and 11 cases were female. Their age was between 24-68 years (mean 55 ± 10.2 years). The symptoms of esophageal mesenchymal tumors are summarized in Table 1.

Endoscopic and EUS characteristics

Endoscopically, GISTs showed submucosal protuberant lesions such as hemisphere, nodosity, strip or irregular shape, and had smooth surface, wide bases and the same color as its adjacent mucosa (Figures 1 and 2). The malignant lesions showed ulceration or hemorrhage, and had no clear boundaries with the normal tissue. Benign tumors varied from 0.5-3 cm in size. One malignant tumor

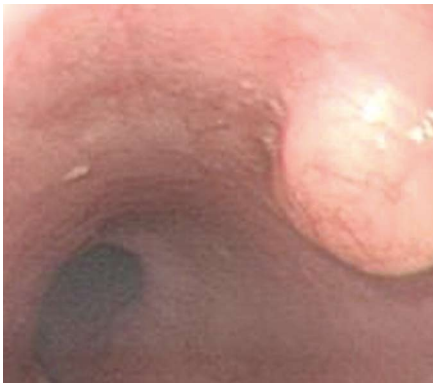

Figure 2 Endoscopic image of leiomyomas.

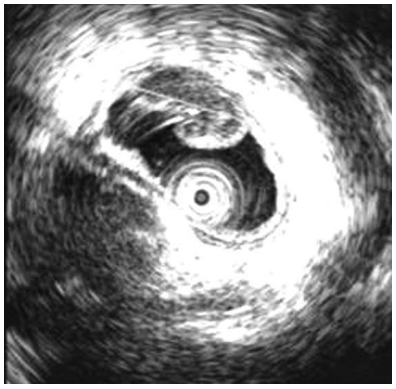

Figure 3 EUS image of esophageal stromal tumors: originated from muscularis propria.

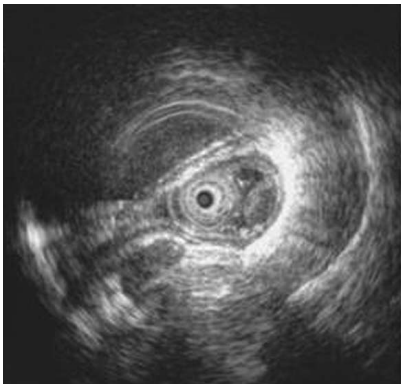

Figure 4 EUS image of leiomyomas: originated from muscularis mucosa.

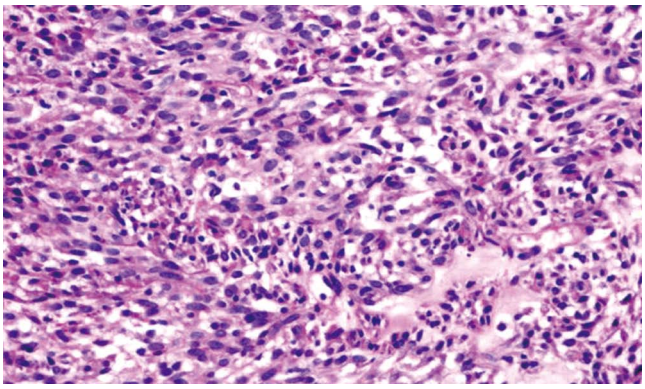

Figure 5 Malignant esophageal stromal tumor: tumor cells were intensely stained, but there was no visible mitoschisis. (HE × 200).

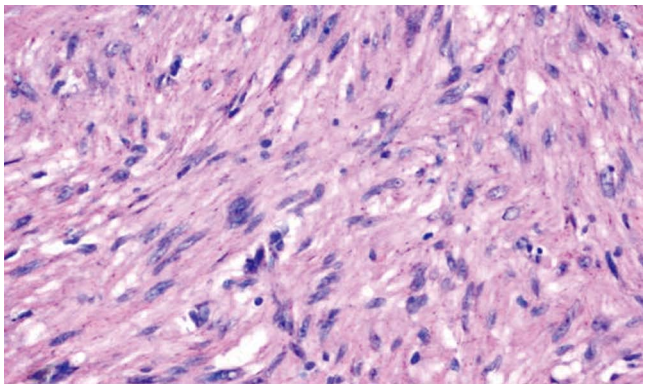

Figure 6 Esophageal leiomyoma: cells were all spindle, with abundant eosinophilic cytoplasm (HE × 200).

was especially large, extending to the cardia and body of the stomach. LMs showed submucosal protuberant lesions such as polyps, hemisphere, pillar, dumb bell, with smooth surfaces. Tumors varied between 0.5-3 cm in size, and had clear borders. One leiomyosarcoma showed irregular nodosity. Its size was 3 cm × 4 cm with anabrotic surfaces.

On EUS, GISTs showed round, spindle-shaped or irregular low echo images originated from the muscularis propria or muscularis mucosa, from which internal echoes were homogeneous or heterogeneous. Two cases were originated from the muscularis mucosa and 3 cases from the muscularis propria (Figures 3 and 4). LMs also showed low echo images originated from the muscularis propria or muscularis mucosa and their internal echoes were homogeneous or heterogeneous. Eleven cases were originated from the muscularis mucosa and 12 cases were originated from the muscularis propria. Endoscopy and EUS could not differentiate esophageal GISTs from LMs because of their similar appearances.

Pathological characteristics

Among 5 cases of GISTs, 4 cases were of spindle cell type, and one case was of epitheloid cell type. There was no mixture of cell types. The cells of GISTs were more intense than leiomyomas, and had a less eosinophilic cytoplasm. The spindle cells were arranged in braid, sarciniform, or cord-like and their nuclei were rod -like. The epitheloid cells were round, orbicular-ovate or polygon and their nuclei were conspicuous. According to the above mentioned criteria, there was 1 case of malignancy at the inferior segment of the esophagus, which size was uncertain, with hemorrhage and necrosis on its surface. Microscopically, the tumor cells were intense, but there was no visible mitoschisis (Figure 5).

Twenty three cases of LMs were composed of well-differentiated smooth muscle cells of spindle cell type and the cells were arranged as braid, sarciniform. The tumors were moderately cellular with abundant eosinophilic cytoplasm (Figure 6). According to the criteria, there was one leiomyosarcoma, which size was 3 cm × 4 cm, with ulceration and bleeding on its surface and without clear borders. Microscopically, there were abundant spindle cells with a few mitoschisis.

Among 29 cases of esophageal mesenchymal tumors, one case with malignancy (20%) was found in 5 cases of esophageal GISTs, and one case with malignancy (4.2%) was found in 24 cases of leiomyomas and leiomyosarcomas. Using Fisher's exact method, the differences of malignant lesions proportion were not significant between esophageal LMs and GISTs, 1/5 *vs* 1/24 ($P > 0.05$).

Immunohistochemical results

Twenty nine cases of esophageal mesenchymal tumors

Table 2 Expression of CD117 in 29 cases of esophageal mesenchymal tumors

Tumor type	*n*	Positive rate (%)
GISTs	5	5
LMs	24	0
	$P < 0.003$	

Table 3 Expression of CD34 in 29 cases of esophageal mesenchymal tumors

Tumor type	*n*	Positive rate (%)
GISTs	5	3 (60)
LMs	24	0
	$P < 0.003$	

Table 4 Expression of SMA in 29 cases of esophageal mesenchymal tumors

Tumor type	*n*	Positive rate (%)
LMs	24	24 (100)
GISTs	5	2 (40)
	$P < 0.003$	

Table 5 Expression of desmin in 29 cases of esophageal mesenchymal tumors

Tumor type	*n*	Positive rate (%)
LMs	24	24 (100)
GISTs	5	1 (20)
	$P < 0.003$	

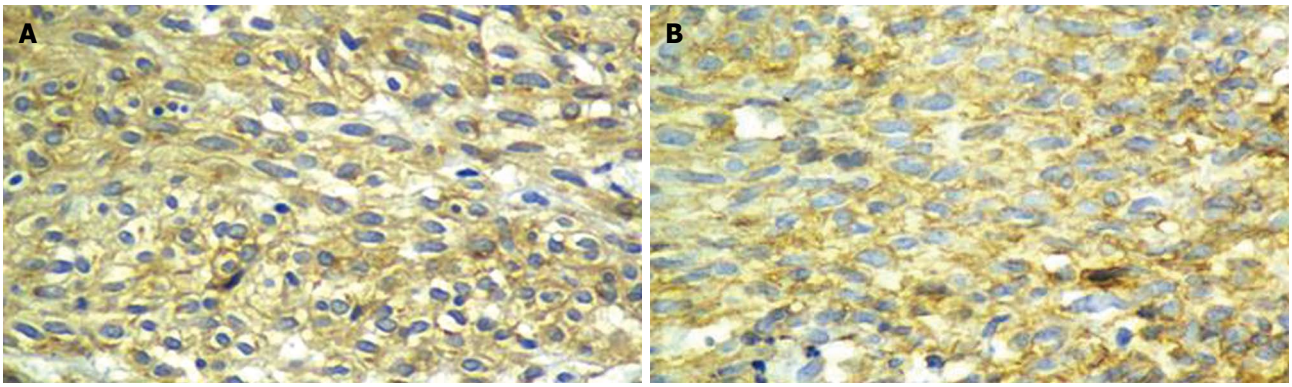

Figure 7 Expression of CD117(× 200) and CD 34 (× 400) in malignant esophageal stromal tumor: showing yellow or brown granules in cell cytoplasm and (or) membrane. **A**: CD117; **B**: CD34.

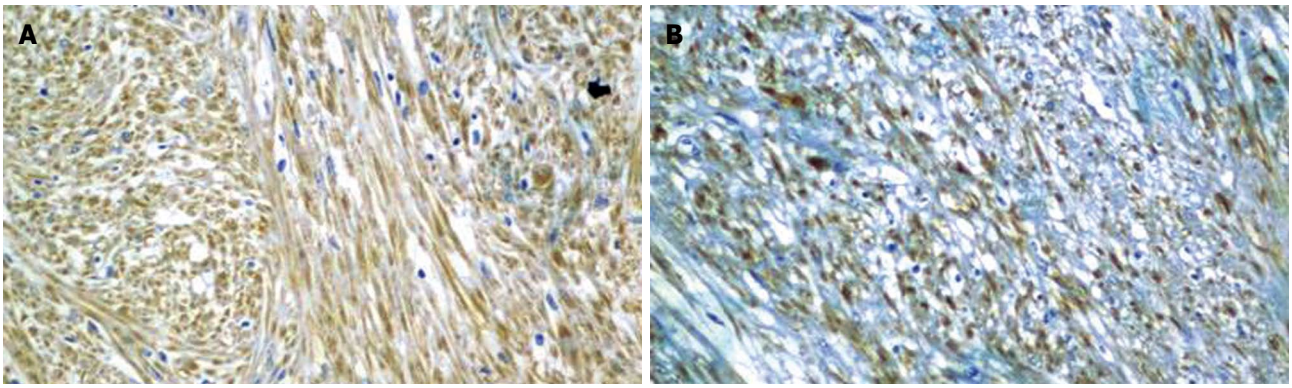

Figure 8 Expression of SMA (**A**) and Desmin (**B**) in esophageal leiomyomas (× 200): showing yellow or brown granules in cell cytoplasm.

were all stained positive for CD117, CD34, SMA and desmin. The results are summarized in Table 2, Table 3, Table 4 and Table 5, Figures 7 and 8.

DISCUSSION

Gastrointestinal mesenchymal tumors have long been classified as LMs, including leiomyoma and leiomyosarcoma. In 1983 Mazur and Clark introduced the term of gastrointestinal stromal tumors (GISTs). GISTs are a kind of potentially malignant tumor. Most scholars believe that the stomach is the predilective site of GISTs, next are the intestines, the colon and the rectum. And GISTs seldom occur in the esophagus[9,10].

Gouveia[11] reported that esophageal GISTs, though less in number, were also made up of fusiform cells, and their mitotic index was low. CD117 and CD34 were expressed, and the malignant degree was low. Among 57 cases of esophageal mesenchymal tumors, studied by Madalie[12], 14 cases of esophageal GISTs were found, and only 2 cases were malignant. Wang[13] reported that 9 cases of GISTs were found among 44 cases of esophageal mesenchymal tumors, and 3 cases were malignant. In our study, among 29 cases of esophageal mesenchymal tumors, 5 cases were demonstrated to be GISTs by endoscopy, pathology, and immunochemistry, and the other 24 cases were LMs. This result further proved that esophageal GISTs exist in the gastrointestinal tract, though they are fewer in number than esophageal LMs, accounting for only 20%[12-14].

Esophageal mesenchymal tumors mostly occur in people of middle and old age, especially over 50 years old, occasionally in children. In the current study, all the 5 cases were over 40 years old, and 3 of them were over 50 years old (the youngest one was 40 years old). The mean age was 52 years. Esophageal leiomyomas may occur at any age. In our group, age of patients with LMs was from 20 to 70 years, and males were slightly more than females. The clinical manifestations of esophageal mesenchymal tumors are closely correlated with the size, nature, and growth pattern of the tumor. In the earlier period, clinical symptoms are nonspecific. When the tumor volume becomes bigger grossly and grows intracavitarily, symptoms such as dysphagia, heart burn, and retrosternal pain may become obvious. Some patients may have upper gastrointestinal hemorrhage.

The appearance of esophageal GISTs and leiomyomas are similar under the endoscope. They are generally globular, hemispheroid, polypoid, with tubercular eminences. The surface of benign GIMTs is smooth, while there is ulceration or hemorrhage on the surface of malignant GIMTs. EUS can not only examine the wall of the esophagus, but can also estimate the topography of the extent, location of lesions, and their relation to the surrounding organs[15,16]. EUS can discriminate GIMTs from other protrusion lesions of the esophagus. Usually esophageal mesenchymal tumors show a low echo image. Though EUS can help doctors to make therapeutic decisions by surgery or by endoscopy, it cannot help doctors to judge the type of the GIMTs.

Esophageal GISTs originate from between the walls of the esophagus. They are a kind of proliferation of spindle cells or epithelioid cells. No matter where the tumor is originated, the cells of GISTs are more abundant than that of leiomyomas and have less eosinophilic cytoplasm. The tumor cells are interlaced，dispersed, or paliformly arranged. In our study, cell nests made up of spindle cells were found in three cases of esophageal GISTs. These cell nests can only be found in GISTs, but not in LMs. This result is coincident with that of Franquemont[17].

There is no definite criterium for differentiation between benign and malignant esophageal mesenchymal tumors. We determined the nature of tumors according to their infiltration, metastasis, volume, and nuclear mitotic figure. In the present study, one case of interstitialoma occurred at the inferior segment of the esophagus. Its diameter was more than 5 cm. There was ulceration and hemorrhage on the surface, but no obvious mitoschisis. This tumor can still be judged as malignant. Kimiyoshi[18] suggested a criterium for differentiation between benign and malignant GISTs: hemorrhage or necrosis, the diameter of the tumor > 5 cm, Ki-67 labeling index (LI) > 3%. If the tumor has any one of the items above, it is malignant. If none of the items above can be found, then it is benign. Kimiyoshi also found that cellulosity, nuclear atypia, and mitoschisis were not related to the nature of the tumor. This is different from the traditional diagnostic criteria. And further studies are needed. Leiomyosarcomas are not common, and its diagnostic criteria are not well studied. According to the criteria of WHO, there was only 1 case of leiomyosarcoma in the present study. The tumor occurred at the inferior segment of the esophagus. Intensive fusiform cells could be seen under the microscope. Mitoschisis could be seen accidently. The diagnosis was low potential malignant leiomyosarcoma. The incidence of esophageal leiomyosarcoma is low.

In our study, the difference between the ratio of malignant GISTs (1/5) and that of leiomyosarcoma (1/24) was insignificant. It showed that the biological behaviour of GISTs was related to the site of the tumor. Esophageal GISTs are not as malignant as those in the gastrointestine. It was also noted that 1 case of esophageal leiomyosarcoma and 1 case of esophageal interstitialoma both occurred in the inferior segment of the esophagus adjacent to the cardia. Whether the predilection site for malignant lobus intermedius tumor is the inferior segment of the esophagus is still to be studied.

In 1998, Kindblom *et al*[19] found that GISTs expressed CD117, which provided an effective means to study GISTs. CD117 is sensitive and specific. Studies reported that the sensitivity was 90%-100%[20,21]. In this study, all 5 cases of esophageal interstitialoma expressed CD117, while no case of leiomyoma expressed CD117. Both the sensitivity and specificity were 100%. This result is coincident with most overseas studies. However, not all GISTs expressed CD117. Debiec-Rychter found that in some of the malignant or recurrent cases of GISTs, CD117 was not expressed[22]. CD34 is a sensitive immunochemistry marker of GISTs. CD34 was expressed in 60%-70% cases of GISTs[23,24], but barely expressed in leiomyomas and myoschwannomas[25,26]. In our group the sensitivity of CD34 was 60%, and the specificity was 100%. Smooth muscle actin(SMA) is widespread and strong positive in smooth muscles, and also positive in GISTs. This shows that some GIST cells could differentiate into smooth muscles. Desmin is a key index for diagnosis and differential diagnosis of GIMTs, which is strongly expressed in smooth muscles, while barely expressed in GISTs. In this group, desmin was positive in all 24 cases of leiomyoma (leiomyosarcoma); only positive in 1 GIST. This is consistent with previous reports[27,28].

Our study provided evidence that all esophageal mesenchymal tumors had the same immunohistochemical features, and combined detection of CD117, CD34, SMA and desmin can help differentiate esophageal GISTs from LMs.

In summary, the present study demonstrates that

the traditional classification is not precise by combined analysis of the clinical, endoscopic, pathological and immunohistochemical features of esophageal mesenchymal tumors. Clinical findings, endoscopy and EUS can be helpful for diagnosis of esophageal mesenchymal tumors, but cannot determine the nature of tumors. Pathology and immunohistochemistry play an important role in their diagnosis and differential diagnosis.

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CLINICAL RESEARCH

High-altitude gastrointestinal bleeding: An observation in Qinghai-Tibetan railroad construction workers on Mountain Tanggula

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Abstract

AIM: To investigate the gastrointestinal bleeding (GIB) in people from lowland to high altitude and in workers on Mountain Tanggula and its causes as well as treatment and prophylaxis.

METHODS: From 2001 to October 2003, we studied GIB in 13502 workers constructing the railroad on Mountain Tanggula which is 4905 m above the sea level. The incidence of GIB in workers at different altitudes was recorded. Endoscopy was performed when the workers evacuated to Golmud (2808 m) and Xining (2261 m). The available data on altitude GIB were analyzed.

RESULTS: The overall incidence of GIB was 0.49% in 13502 workers. The incidence increased with increasing altitude. The onset of symptoms in most patients was within three weeks after arrival at high altitude. Bleeding manifested as hematemesis, melaena or hematochezia, and might be occult. Endoscopic examination showed that the causes of altitude GIB included hemorrhage gastritis, gastric ulcer, duodenal ulcer, and gastric erosion. Experimental studies suggested that acute gastric mucosal lesion (AGML) could be induced by hypoxic and cold stress, which might be the pathogenesis of altitude GIB. Those who consumed large amount of alcohol, aspirin or dexamethasone were at a higher risk of developing GIB. Persons who previously suffered from peptic ulcer or high-altitude polycythemia were also at risk of developing GIB. Early diagnosis, evacuation, and treatment led to early recovery.

CONCLUSION: GIB is a potentially life threatening disease, if it is not treated promptly and effectively. Early diagnosis, treatment and evacuation lead to an early recovery. Death due to altitude GIB can be avoided if early symptoms and signs are recognized.

Key words: High altitude; Gastrointestinal bleeding; Hypoxic stress; Acute gastric mucosal lesion; Risk factors

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INTRODUCTION

Although cardiac, respiratory and neurological symptoms are more common among mountaineers and persons from lowland going to the high altitude, little work has been done on the effect of hypoxia on digestive system in either patients with altitude illness or in healthy individuals at high altitude. In fact, symptoms of the digestive system such as anorexia, epigastric discomfort, epigastralgia, heart burn, dyspepsia, nausea, vomiting, diarrhea, haematemesis, piles and peptic ulcers are frequently found in mountaineers and altitude sojourners[1,2]. Moreover, epidemiological and clinical studies suggest that gastrointestinal bleeding (GIB) is not uncommon at high altitude, and is often life-threatening[3,4]. There are two diagnostic criteria for acute mountain sickness (AMS), one is the ESQ Ⅲ[5] and the other is the Lake Louise consensus scoring system[6]. Gastrointestinal symptoms include nausea/vomiting, loss of appetite, stomach/abdominal pain, constipation,

however, the GIB is not included in the diagnostic criteria. Furthermore, data on GIB are available from Japanese, Chinese, and Peruvians, but rarely reported in western people[7].

Altitude problems are of great importance for the railroad construction workers at high altitude. The construction of railroad on Mountain Tanggula offered a unique opportunity for investigation and study of AMS, high altitude pulmonary edema (HAPE), and high altitude cerebral edema (HACE), as well as high altitude GIB. This paper describes the incidence, clinical features, and risk factors for GIB in the railroad construction workers on Mountain Tanggula.

MATERIALS AND METHODS

Areas and subjects

Between June 29, 2001 and October 31, 2003, altitude gastrointestinal problems including altitude GIB were studied at two hospitals near the construction site. One hospital is located on the Fenghoushan at an altitude of 4779 m and the other hospital is situated in the Kekexili area at an altitude of 4505 m. These two hospitals received patients from the construction sites, working at altitudes between 3486 m and 4905 m. A total of 8014 workers worked at Fenghoushan and 5488 in Kekexili over the past three years. The weather conditions in the two surveying regions and the evacuated areas in Golmud and Xining are summarized in Table 1.

The weather and climatologic data were provided by the Tanggula Meteorological Observatory Station and the Qinghai Weather Bureau. Workers were not only exposed to a hypoxic environment, but also to severe cold. The temperature in winter ranged between -27℃ and -36℃, with an annual average temperature of -3℃ to -7℃.

A questionnaire was delivered to the persons to be investigated, including age, gender, ethnicity, occupation, place of birth, length of time at low altitude, length of time after ascending to high altitude, history of smoking and consumption of alcoholic beverages, current and past medical history, and family history.

All subjects underwent a careful medical evaluation before and after ascending to high altitude. Physical examination, routine blood tests, chest roentgenograms, electrocardiograms, *etc* were performed. The protocol was approved by the Qinghai High Altitude Medical Research Institute Committee on Human Research. Informed consent was obtained verbally for each subject.

Diagnosis

The diagnosis of AMS was made using the Lake Louise scoring system (LLSS) in which two or more of symptoms monitored were defined as AMS[6]. The clinical diagnosis of GIB was based on the following criteria: (1) occurrence in unacclimatized individuals who were rapidly exposed to altitudes exceeding 3000 m; (2) onset of typical symptoms including epigastric discomfort, epigastric pain, haematemesis, melaena, or hematochezia; (3) significant decrease in hemoglobin concentration (Hb) or hematocrit value (Hct); (4) endoscopic diagnosis of GIB after descending to the Golmud (2808 m) or Xining (2261 m); (5) disappearance of symptoms and signs after treatment with blood transfusion and oxygen. Outcome was assessed according to the clinical and laboratory results.

Table 1 Weather conditions in the two surveying regions and the two evacuated areas-Golmud and Xining

Area	Fenghoushan	Kekexili	Golmud	Xining
Altitude (m)	4905	4505	2808	2261
PB (mmHg)	417	440	538	585
Average annual temperature (℃)	-7.0	-2.6	3.6	6.7
Annual precipitation (mm)	317	291	42	371
Annual sunshine time (h)	2712	2764	3101	2793
Relative humility (%)	57	58	34	57

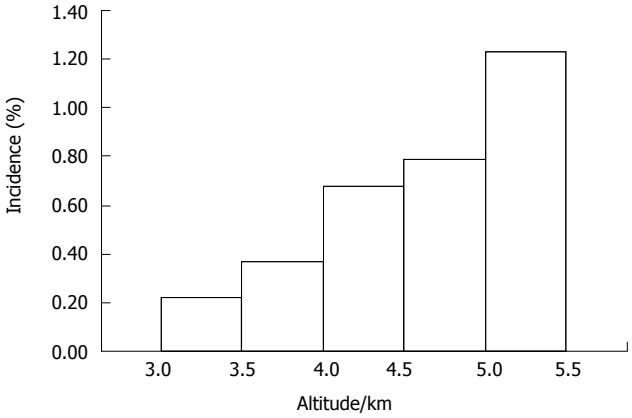

Figure 1 Frequency distribution of altitudes in patients with gastrointestinal bleeding diagnosed at two hospitals on Mountain Tanggula. The incidence of GIB increases with increasing altitude.

Statistical analysis

Statistical analysis was made by the IBM 3990 system. The incidence (cumulative case rate over a defined period in a defined population) of GIB in these populations was calculated. The frequency of risk factors for GIB was examined, using two by two tables, odds ratios, and chi square test. Comparison of mean numerical values was made by t test. $P < 0.05$ was considered statistically significant.

RESULTS

Overall incidence

We selected two typical areas where the local hospitals are located to investigate the altitude illness. From 2001 to 2003, GIB was found in 66 cases of 13502 workers on Mountain Tanggula during the period with an incidence of 0.49%.

Predisposing factors

The incidence of GIB was also found to be dependent upon many variables, including altitude, length of stay at high altitude, age, sex, labouring conditions, and ethnicity.

Altitude: The incidence of GIB at various altitudes is shown in Figure 1. Occurrence of GIB was rarely found below 3500 m. The highest frequency of GIB

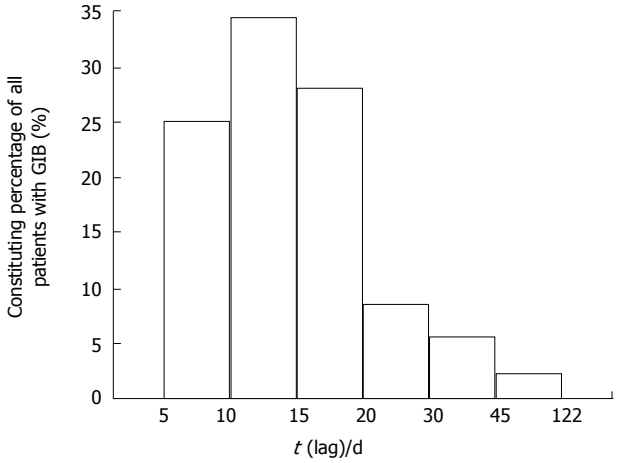

Figure 2 Time lag between ascent and onset of illness.

was observed between 4500 m and 5000 m, its incidence increased gradually with altitudes being 0.2% at 3500 m and 1.1% at 4905 m ($P < 0.01$).

Onset time of symptoms: Symptoms suggestive of GIB occurred in 17 out of 66 cases (25.8%) within 5-10 d after arrival at high altitude, in 24 cases (36.3%) within 11-15 d, 14 cases (21.2%) within 16-20 d, the remaining 11 cases(16.7%) within 21-122 d (16.7%) (Figure 2). The onset time of most symptoms was within three weeks after arrival at high altitude.

Age and gender: Many of the patients were young or middle-age adults, and the oldest was 46 years of age. The mean age of all cases was 34 ± 7.8 years.

GIB occurred frequently in men. Since the total number of females was small (21 persons), and could be neglected.

Occupation: The incidence of GIB in workers and carders was 0.51% and 0.46%, respectively. There was no significant difference between the two groups ($P > 0.05$).

Ethnicity: Interestingly, all the GIB patients were Han Chinese lowlanders. Over one-third of the workers were native highlanders (most of them were Tibetans who permanently lived at altitudes from 2500 m to 3200 m) and no GIB was found in them.

Endoscopic findings

All patients with altitude GIB were evacuated to the hospitals in Golmud or Xining. Among them, 28 had an endoscopic examination either at admission or within 24 h after admission and others were not able or refused to undergo an endoscopic examination. Endoscopic examination showed gastric ulcer in 11 cases, duodenal ulcer in 4, combined ulcers in 4, hemorrhage erosion in 5, bleeding gastritis in 4 and chronic atrophic gastritis in 1, respectively.

Risk factors

GIB was obviously related to drinking alcohol and use of aspirin and/or dexamethasone in 3 patients. Case one was a 37-year old railroad worker from lowland to a construction site at 4505 m. During the ascent, he was given 8 mg dexamethasone followed by 4 mg every eight hours for AMS prophylaxis. In the morning after arrival at 4505 m, he complained of severe headache. He was given 0.5 g of aspirin and dexamethasone, which did not relieve his headache. He drank approximately 300 mL of liquor (50% alcohol). After eight hours he suddenly became incapacitated with symptoms of upset stomach, abdominal pain, and severe fatigue. At about 6.0 AM on the third day, he had melaena and tarry black stool, thus aspirin and dexamethasone were immediately stopped. Within a few hours his condition became progressively worsened, and he was promptly evacuated to the Golmud Hospital (2808 m). At admission, his BP was 90/50 mmHg and Hb level was below 65 g/L. Gastro-fiberscopic examination was performed within six hours after admission and revealed diffuse gastric bleeding and erosion, and linear gastric ulcer combined with duodenal ulcer. Supplementary oxygen was given and an intravenous drip was put up. After 600 mL whole blood transfusion over about five hours, and administration of H_2 blocker (ranitidine), his BP gradually increased (110/80 mmHg), and malaena was stopped. He slowly improved over two weeks, his Hb increased to 105 g/L. He was discharged 21 d after admission and returned to his lowland home.

In addition to this case, we encountered 2 similar cases at the Kekexili construction site. Case two was a healthy young man who took aspirin and dexamethasone to prevent AMS and drank alcohol in the evening, he had brisk melaena in the next morning. Case three was a middle age worker, with a previous history of gastric peptic ulcer. He took aspirin for headache, after drinking 100 mL of hard liquor, he developed acute melaena.

Polycythemia (Hb > 210 g/L) was found in 5 patients with GIB having lived at altitude above 4000 m for more than 45 d.

Moreover six patients with GIB had a previous history of potential peptic ulcer and 5 had chronic gastritis.

Outcomes

Once the diagnosis was confirmed, the patients were either evacuated to the Golmud Hospital (2808 m), or treated as outpatients or sent to Xining (2261 m). Their conditions gradually improved after effective treatments.

DISCUSSION

Nomenclature and relation between AMS and altitude GIB

High-altitude GIB is a distinct clinical entity. However, the different terms of this syndrome have been used by different authors. The term "hypoxic gastric bleeding" is used loosely in some Peruvian scientists[8], "altitude digestive hemorrhage" is also used for this illness[9]. Since hemorrhage could occur in buccal mucosa, digestive mucosa, finger nails, as well as in gastrointestinal tract, urinary tract, retina and gums[10,11], it is named as "high altitude hemorrhage syndrome". GIB is one of the clinical manifestations of this syndrome[11]. However, GIB is almost an independent digestive mucosal lesion, causing gastrointestinal bleeding at high altitude. We hold that the term "high altitude gastrointestinal bleeding" (altitude GIB) is more suitable for this condition.

We also noted that approximately 58% of GIB patients are accompanied with AMS, and about 42% have altitude GIB alone. However, GIB has not been found to be associated with HAPE or HACE. Thus whether altitude GIB is a gastrointestinal type of AMS should be further studied.

Incidence of altitude GIB

The incidence of altitude GIB among workers constructing Qinghai-Tibetan highway on Mountain Tanggula from 1978 to 1984 was 1.1%[4]. The incidence of altitude GIB in workers constructing the Qinghai-Tibetan railroad at the same mountainous areas was 0.49% in 2001-2003, which is much lower than the reported incidence in mountaineers on Everest. During 1988 the China-Japan-Nepal Friendship Expedition to Mountain Qomolungma (Everest), a total of 52 Japanese mountaineers climbed from Tibetan (north) side (5154 m) to the summit (7790 m), five on the north side suffered from upper alimentary bleeding with an incidence of 9.6%[12], suggesting that the incidence of GIB increases with the increase of altitude.

Liu[13] reported that the occurrence of GIB in soldiers stationed between 3700 m and 5380 m for one year is 0.8% of the total patients and 1.5% of AMS cases at a Chinese Army Hospital located at the foot of Mountain Karakoram (3550 m) during the same period. However, endoscopy examination showed that the incidence of acute gastric mucosal lesion (AGML) in mountaineers is as high as 16%-49%[14-16], suggesting that some subclinical GIB exists.

Clinical features of altitude GIB

Of the 66 cases, 17 had altitude GIB within 10 d after arrival at above 3500 m. Most patients (55) had altitude GIB within 20 d, and 11 had altitude GIB after 21 d. These observations were incomparable with the onset of HAPE or HACE. In general, the mean time of HAPE or HACE occurrence after arrival at above 3500 m was three days with a range of 1-5 d.

The victims of GIB include both mountaineers and native highlanders[8,17]. Macedo[8] reported that two adult male native Quechua Indians presented a sudden and severe gastric bleeding of unknown etiology in the Andes. However, in our GIB patients, there were no Tibetan natives. It is possible that these individuals have greater genetic resistance to hypoxia at high altitude.

At high altitude, GIB may be manifested as haematemesis (vomiting of blood, either bright red or brown in color), melaena (tarry black, sticky stool) or hematochezia (fresh red blood per rectum, ruling out the diagnosis of piles). However, hemorrhage was manifested as melaena in almost all 64 patients except for two with haematemesis. Bleeding, however, might be occult, with normal appearance of stools. Occult blood in stools was detected in 10 mountaineers by Naito *et al*[18] using "OC-Hemodia Kit" which can detect only human hemoglobin without cross-reaction in any other animals during their Iwate Karakoram expedition in 1989, a total of 8 persons presented a positive reaction, suggesting that silent bleeding from gastrointestinal mucosa commonly exists in mountaineers.

GIB gives rise to symptoms and signs depending upon the rate and extent of bleeding. In our experiences, acute moderate bleeding (blood loss greater than 500 to 1000 mL) results in drowsiness, dizziness, oliguria, sweating and pallor. BP changes occur first in the form of orthostatic hypotension. Pulse rate seems to be a far less accurate parameter, particularly at high altitude, as tachycardia is common amongst such mountaineers. Acute massive bleeding is characterized by loss of greater than 1500 mL or 25% of the circulating blood volume within a period of minutes to hours, decreased systolic and diastolic BP, increased pulse rate, decreased hemoglobin concentration or Hct values. It is a critical indicator for the desperate situation. Therefore any patient experiencing malaena with or without hematemesis or associated symptoms or hypotension, should be evacuated promptly and hospitalized immediately for further evaluation and treatment[4].

Endoscopic findings and causes of altitude GIB

Endoscopy is highly advisable. Sugie *et al*[19] found gastro-duodenal mucosa lesions in 13 out of 22 mountaineers at Mountain Xixapangma (5020 m). The results of arterial blood gas analysis reported by Sugie *et al*[20] are as follows: PaO2 = 43 mmHg, PaCO2 = 23.5 mmHg, pH = 7.51, BE = 1.0. Zhao and Li[15,16] reported that the incidence of gastro-duodenal mucosal lesions is 49% in 51 young healthy male subjects at the altitude between 3658 m and 4200 m. Occasionally, upper digestive hemorrhage may occur in gastric cancer patients at altitude[21,22].

Saito[12] reported a male mountaineer on Mt. Everest expedition who developed massive GIB and serious anemia at the altitude of 7028 m. Endoscopy after his arrival at sea level, showed no abnormality[23]. Zhou[24] reported a case of a healthy lowland Han subject who developed GIB during a sojourn at Lhasa (3658 m) and died of recurrent massive GIB. Postmortem revealed diffuse superficial erosion in the stomach but no peptic ulcers.

Mechanism of altitude GIB

The mechanism of altitude GIB remains unclear. Pathological studies in patients died of altitude GIB revealed significant dilatation of arterioles and venules, formation of extensive blood capillaries in gastric mucosa[25] and hemorrhage into the gastric mucosa[26]. Different kinds of stress can cause AGML. It was reported that cold or hypoxic stress can induce AGML in rats[27,28]. However, on high mountains, cold stress usually accompanies the effects of hypoxic stress in mountaineers, suggesting that hypoxemia is probably the main factor for AGML in mountaineers who are healthy at sea level[19]. Kamiyama *et al*[29] have attributed the potential difference (PD) to the indicator of gastric integrity or gastric mucosa defensive mechanism that may play an important role in the pathogenesis of hypoxia-induced AGML. The PD is closely related to the transport of electrolytes by gastric mucosa cells depending on aerobic metabolism. Hypoxemia may cause changes

in tissue levels of oxygen resulting in decrease of PD. Strenuous exertion and hard work increase hypoxia stress and induce injury of gastric mucosa capillaries[14,17]. Naito *et al*[30] noted that peroxidation of phosohatidylcholine in gastric mucosa is another possible mechanism underlying AGML at high altitude. It was reported that ulcer index (UI) and phosphatidylcholine hydroperoxide (PCOOH) are higher in hypoxia group than in control group, though thiobarbituric acid (TBA) substance does not differ between the two groups, suggesting that lipid peroxidation in gastric mucosa may play a role in the pathogenesis of hypoxia-induced AGML. Acute diffuse lesions induced by stresses are pathologically and clinically distinct from gastro-duodenal mucosa lesions and present as an upper alimentary bleeding.

Fisk factors for altitude GIB

Alcohol: It is historically believed that alcohol could relieve and protect against AMS[4]. More recently, Houston[31] has recommended less alcohol use on mountains. It appears that alcohol may also be a risk factor for GIB at altitude. Ravenhill[32] has observed that alcohol increases the severity of symptoms of AMS. Those who drink a little alcohol under a simulated hypoxic condition (7620 m), may impair their mental and physical function[33]. Steele[34] has described three Sherpas who had severe epigastric pain, nausea and vomiting after heavy drinking on Mountain Everest. Zhou[24] reported three young men in Lhasa who developed massive gastric hemorrhage due to acute hemorrhagic gastritis after a hard drinking. High altitude native populations, such as Quechua Indians, like to drink inferior rough alcohol, which may be the aetiology of the increased prevalence of gastric ulceration[26]. From 1978 to 1984 when the Qinghai-Tibetan Highway was under construction, the incidence of GIB was higher (1.93%) in workers who drank more of it than in those (0.95%) who drank it less[4], suggesting that drinking alcohol increases the risk of developing GIB at altitude and alcohol should be avoided.

Aspirin or other NSAID agents: Aspirin has been shown to be effective in treating headache due to AMS[35]. Aspirin alone is not very effective in preventing headache due to AMS, whereas aspirin in combination with dexamethasone can achieve rather good results[36]. There is strong but not conclusive evidence that major bleeding episodes could result from acute, diffuse hemorrhagic lesions in the upper GI tract after ingestion of aspirin. Thus, relief of headache due to AMS is a difficult matter because aspirin and other non-steroidal anti-inflammatory drugs (NSAID) lead to GIB[7,37]. It was reported that drugs such as paracetamol (acetaminophen) or ibuprofen, have a strong anti-inflammatory action on headache or other pains and do not cause gastrointestinal bleeding[38].

Dexamethasone: Dexamethasone has been used in the prevention and treatment of AMS[39,40] because administration of it for a short time does not result in any problems at high altitude[31]. However, dexamethasone is one of the drugs that are strongly suspected of being ulcerogenic, especially in patients with a prior history of peptic ulcer disease, and may increase the risk of GIB at altitude. Concomitant use of dexamethasone, aspirin and alcohol has an additive or synergistic effect on the upper GI tract mucosa, and could lead to rapid and serious GIB as in our cases.

High altitude polycythemia: Polycythemia is a common feature seen in altitude residents and patients with hypoxemia[15,16,24]. Digestive symptoms such as epigastric pain, dyspepsia, anorexia, vomiting, and diarrhea can be frequently eliminated in 89%-100% of patients with high altitude polycythemia (HAPC)[24]. GIB is a common complication of HAPC. In our series five patients with GIB had HAPC. Zhou[24] reported polycythemia in 21 young male Chinese soldiers (mean age 26.6 years) stationed in Lhasa (3658 m) who had no previous history of peptic ulcer. GIB occurred following development of HAPC. They found that exposure to cold, strenuous exertion, and drinking alcohol are the main predisposing causes.

Zhao and Li[15,16] have reported their endoscopic examinations in 98 patients with HAPC (Hb > 210 g/L) in Lhasa, showing superficial gastritis in 29, gastric ulcer in 26, duodenal ulcer in 12, complex ulcers in 5, and atrophic gastritis in 3. Chu and Sun[41] examined 5 patients with HAPC (mean Hb = 225 g/L) in Madou (4300 m), diffuse bleeding and erosion as well as ulcerous necrosis were observed in their stomach, and electromicroscopic examination showed the ultrastructural characteristics of their gastric mucosal biopsies, such as irregularly arranged thick microvilli, microfilament enlargement of secretory canaliculus, and high density of enlarged mitochondria in parietal cells, increased zymogen granules and rough endoplasmic reticulum in principal cells, proliferation of vascular endothelial cells, microvilli-like appearance of their surface and basement membrance thickening. Such gastric mucosal lesions may be associated with gastric mucosal ischemia caused by microvascular thrombosis due to excessive polycythemia.

Peptic ulcerations at high altitude: Clinical observations on the Himalayas and Andes suggest that peptic ulcer occurs more frequently at high altitude than at sea level[21,24,42]. At high altitude, the ulceration rate is higher in gastric ulcer than in duodenal ulcer[43,44]. Berrio *et al*[42] reported that 100 cases of GIB have been diagnosed by endoscopic examinations in General Hospital at La Oroya (3800 m), gastric ulcer accounting for 33%, duodenal ulcer accounting for 23%, gastric erosion accounting for 23%, neoplasias accounting for 2%, respectively. The prevalence of gastric ulcer increases with increasing altitude, while the prevalence of duodenal ulcer does not[42]. Upper digestive hemorrhage is a common complication of peptic ulceration at high altitude[42,45], the hemorrhagic rate is 20%-66%[9,45].

It is well known that the incidence of gastric ulcers and bleeding is increasing in high altitude residents chronically exposed to a hypoxic environment[42,45-47]. In addition, hypoxic and cold stress-induced gastric mucosal lesion may explain in part, the high incidence of gastric ulcers in altitude populations.

In the present 66 cases of GIB, 4 had a previous history of peptic ulcer. Among them, GIB occurred

rapidly in 1 patient after using aspirin, suggesting that persons with known peptic ulceration should not go to high altitude mountains unless their symptoms have been well controlled before they go to high altitude mountains as complications in the field can be fatal[37].

Course and prognosis of altitude GIB

Altitude GIB can be life threatening and acute massive bleeding due to secondary effects of shock-increasing anoxia, cellular dysfunction and acidosis at high mountains may lead to death[4]. We have reported that the mortality of altitude GIB is as high as 6.8% in Qinghai-Tibetan Highway construction workers on Mountain Tanggula[4].

The emergency measures taken for acute altitude GID include early evacuation or just descent with oxygen inhalation and saline-infusion or blood transfusion. Drugs, such as H_2 blockers or proton pump inhibitors have been shown to be more effective in ameliorating GIB[4]. Mountaineers and highlanders should know well about the symptomatic self care (SSC) for altitude GIB[48], that is when symptoms such as epigastric pain or dyspepsia are present, a H2-receptor antagonist is effective for ulcers and GIB and the current therapy for altitude GIB.

In conclusion, early diagnosis, treatment and evacuation lead to an early recovery. Death due to altitude GIB can be avoided if early symptoms and signs are recognized.

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RAPID COMMUNICATION

Differences in characteristics of patients with and without known risk factors for hepatocellular carcinoma in the United States

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Abstract

AIM: To examine the clinical characteristics of a subgroup of patients with hepatocellular carcinoma (HCC) and compare them to those with known risk factors.

METHODS: We used the HCC database of 306 patients seen at our institution from January 1, 1995 to December 31, 2001. Of the 306 patients, 63 (20%, group 1) had no known risk factors (hepatitis C virus, hepatitis B virus, alcohol, hemochromatosis or cirrhosis from any cause) and 243 (group 2) had one or more risk factors.

RESULTS: The median age was similar in both groups, but there were disproportionate numbers of younger (< 30 years old), older (> 80 years) patients, women (33% *vs* 18%), and Caucasians (81% *vs* 52%) in group 1 as compared to group 2. There were fewer Asians (2% *vs* 11%) and African Americans (13% *vs* 27%) in group 1. Abdominal pain (70% *vs* 37%) was more common while gastrointestinal bleeding (0% *vs* 11%) and ascites (4% *vs* 17%) were less common in group 1 compared to group 2. Group 1 had larger tumor burden (median size 9.4 cm *vs* 5.7 cm) at the time of presentation, but there were no differences in the site (right, left or bilateral lesions), or number of tumors between the two groups.

CONCLUSION: HCC patients without identifiable risk factors have different characteristics and clinical presentation compared to those with known risk factors. Absence of cirrhosis and larger tumor burden may explain the differences in the presenting symptoms.

Key words: Hepatocellular carcinoma; Patient characteristics; Risk factors

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cancer in the world with more than 500 000 new cases reported per year[1,2]. The disease is unevenly distributed worldwide with a higher incidence in South-East Asia and Sub-Saharan Africa than in other regions of the world[1,2]. Although it is less common in the United States and Western Europe, there are data to suggest that the incidence may be increasing secondary to hepatitis C virus (HCV)[1-5]. The common risk factors that predispose to HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin, and cirrhosis in general[6-9]. In addition, hemochromatosis, alcoholism, and non-alcoholic fatty liver disease (NAFLD cirrhosis) increase the risk of developing HCC[10]. In the United States, alcoholism and hepatitis C are the leading predisposing causes of HCC[11]. However, a significant proportion of patients develop HCC despite the absence of any known risk factors including cirrhosis. There is only limited information on the differences in the characteristics and outcomes of patients with or without risk factors who develop liver cancer in the USA.

The purpose of this study was to define the clinical characteristics and presentation of patients without identifiable risk factors and compare them to those with known risk factors.

MATERIALS AND METHODS

For the purpose of this study, we studied patients who

Table 1 Patient characteristics *n* (%)

Category	Group 1 (*n* = 63)	Group 2 (*n* = 243)	*P*
Sex			
Male	42 (67)	198 (82)	< 0.05
Female	21 (33)	45 (18)	
Age (yr)			
Median (range)	66 (18–87)	61 (23–87)	
< 30	5 (8)	3 (1)	< 0.01
30-39.9	3 (5)	10 (4)	
40-49.9	6 (10)	35 (14)	
50-59.9	9 (14)	65 (27)	
60-69.9	10 (16)	76 (31)	
70-79.9	17 (27)	44 (18)	
> 80	8 (13)	7 (3)	
Unknown	5 (8)	3 (1)	
Race			
Asian	1 (2)	27 (11)	< 0.05
African American	8 (31)	66 (27)	
Caucasian	51 (81)	125 (51)	
Hispanic	1 (2)	8 (3)	
Other	1 (1)	12 (5)	
Unknown	1 (1)	5 (2)	
Country			
US born	58 (92)	201 (83)	
Immigrant	2 (3)	28 (11)	
Foreign visitor	3 (5)	14 (6)	

Table 2 Symptoms and signs at presentation *n* (%)

	Group 1 (*n* = 46)	Group 2 (*n* = 217)	*P*
Symptoms			
Abdominal pain	32 (70)	81 (37)	< 0.001
Fatigue	7 (15)	39 (18)	
Anorexia	7 (15)	22 (10)	
Nausea and vomiting	7 (15)	23 (11)	
Change in bowel habits	3 (7)	15 (7)	
Gastrointestinal bleed	0 (0)	24 (11)	< 0.05
None	8 (17)	85 (39)	< 0.01
Signs			
Weight loss	12 (26)	38 (18)	
Abdominal mass	4 (9)	14 (7)	
Jaundice	3 (7)	26 (12)	
Fever	3 (7)	14 (7)	
Ascites	2 (4)	37 (17)	< 0.05
Encephalopathy	1 (2)	19 (9)	
None	25 (54)	113 (52)	

presented to the Johns Hopkins Hospital with HCC from January 1, 1995 to December 31, 2001. A retrospective database was created with the approval of the Institutional Review Board. Patients with HCC were identified for inclusion in the database by searching the medical records using an ICD-9 code for liver cancer (155.0) and the Database of Pathology Departments using the term "hepatocellular carcinoma". The information was collected on all patients using the hospital's electronic patient record.

To be included in the study, a patient was 18 years or older, visited Johns Hopkins Hospital during the designated period, and had a confirmatory diagnosis of HCC. HCC was diagnosed based on histological confirmation or an elevated alpha fetoprotein (AFP) > 400 IU/mL with a liver image showing characteristic features of HCC. In the absence of elevated AFP or histological confirmation, characteristic liver image along with a clinical history compatible with HCC was necessary[12]. A compatible clinical history included known cirrhosis, HBV or HCV infection, hemochromatosis or history of alcoholism.

The risk factors for HCC were defined as HBV, HCV, cirrhosis from any cause (based on imaging and/or liver histology), aflatoxin, alcoholism, hemochromatosis, pre-malignant liver tumors and rare metabolic syndromes that are known to predispose to HCC. Patients without any known identifiable risk factors were included in group 1 and compared to those patients with one or more risk factors (group 2).

Statistical analysis was performed with SPSS version 10.0. Statistical tests included chi-square and Student-*t* tests. $P < 0.05$ was considered statistically significant.

RESULTS

Three hundred and six patients were seen with HCC at our institution from January 1, 1995 to December 31, 2001. Of the 306 patients, 63 (20%, group 1) had no known risk factors (HCV, HBV, alcohol, hemochromatosis or cirrhosis from any cause) and 243 (group 2) had one or more risk factors.

Of the 243 patients (group 2) with a known risk factor for HCC, hepatitis B was documented in 49 (20%), hepatitis C was present in 110 (45%) and 115 (47%) acknowledged moderate or abusive alcohol use. Rare disorders such as Wilson's disease, porphyria cutaneous tarda, autoimmune hepatitis, schistosomiasis, and sclerosing cholangitis were noted in one patient each. Cirrhosis was documented by histology in 164 (67%).

Demographic data of both groups are shown in Table 1. There was a male predominance in both groups but there was a higher proportion of females in group 1 (2:1 *vs* 9:2) compared to group 2. The median age was greater in group 1 with a disproportionate distribution of patients at the extremes of age.

Presenting signs and symptoms are shown in Table 2, with complete data available in 263 of the 306 patients. The most common presenting symptom in each group was abdominal pain, but it was more common in group 1. Other statistically significant differences noted were the frequency of gastrointestinal bleeding and the presence of ascites. Weight loss was comparable in both groups. As expected , HCC was not diagnosed during routine screening or surveillance in any patient of group 1 but in 46 (21%) of group 2 ($P < 0.001$).

Diagnostic imaging data revealed differences between groups 1 and 2 (Table 3). We excluded studies that were not done at our institution since films were not available for confirmation. Imaging studies showed a larger tumor diameter (median 9.3 cm, range 4-25 cm *vs* 5.7 cm, range 0.7-20 cm) in group 1 than 2. Approximately half of the patients (52% and 48%) in both groups had a solitary tumor, and the majority of tumors were located in the right liver (67% and 60%). A higher proportion of patients in group 2 had bilateral tumors (7% *vs* 24%, P = NS). Portal vein involvement was similar in both groups.

Histological examination demonstrated fibrolamellar

Table 3 Tumor imaging characteristics

	Group 1	Group 2
Size	*n* = 24	*n* = 159
Median (cm)	9.3	5.7
Minimum (cm)	4	0.7
Maximum (cm)	25	20
	n (%)	*n* (%)
< 2 (cm)	0 (0)	15 (9)
2.01-5.0	2 (8)	59 (37)
5.01-10.0	13(54)	68 (43)
> 10.0	9 (38)	7 (11)
Focality	*n* = 25	*n* = 149
Unifocal	13 (52)	72 (48)
Multifocal	12 (48)	77 (52)
Hemiliver	*n* = 30	*n* = 162
Right	20 (67)	97 (60)
Left	5 (17)	27 (17)
Bilateral	5 (7)	38 (24)

Please note that size could not be determined in 6 patients in group 1 and 3 in group 2. Similarly 'focality' could not be determined in 5 patients in group 1 and 13 in group 2.

variant HCC in 6/63 patients of group 1 and 0/243 patients of group 2.

DISCUSSION

In this study, we described the characteristics of patients who presented to a tertiary care center in the United States without known risk factors for HCC and compared them to those with one or more identifiable risk factors. The patients in group 1 without identifiable risk factors had a relatively higher proportion of women and Caucasians. The age distribution of this group was asymmetrical, with a disproportionate number of patients less than 30 years old and older than 80 years. The increased frequency of younger HCC patients in this group could be explained by the fibrolamellar variant of HCC that is known to affect younger patients without risk factors. This tumor was exclusively seen in group 1, 4 out of the 6 patients less than 30 years old had fibrolamellar variant. While fibrolamellar variant could explain the disproportionate number of younger patients in group 1, another explanation must be found for the increased number of patients over the age of 80 years in this group. It is certainly possible that these patients may have had occult viral hepatitis or alcohol use, and examination of liver tissue or peripheral blood monocytes may have detected occult HBV and HCV infections in some of them. The retrospective nature of this study also did not permit us to determine whether these patients had adequate tests to rule out viral hepatitis. Another demographic difference between the two groups of patients was the ratio of males to females. Group 1 had a relatively higher proportion of female patients, and it is possible that some of these patients may have progressed from adenoma.

The clinical presentation was also different in both groups. Group 2 was more likely to present without any symptoms (40% *vs* 17%) and this could be partly explained by the fact that many of these patients (19%) were diagnosed with HCC during surveillance or screening. Abdominal pain was the most common symptom in both groups, but it was more common in group 1 and this could be explained by the larger tumor burden. Despite the smaller (40% smaller) tumor size in group 2, portal vein involvement and metastases were similar in both groups, suggesting that there may be differences in tumor biology.

Our study suggested that there were differences in patient characteristics, symptoms, and tumor size in patients who presented with and without known risk factors for HCC. Absence of cirrhosis and tumor size may explain the differences in symptoms, and there is a suggestion that tumor biology may be different in these groups. The higher proportions of women and older patients without risk factors remain poorly explained. It is important to note that our study had all the inherent weaknesses of a retrospective study. It is more than likely that a more detailed diagnostic work-up may have revealed more risk factors in both groups. In addition, we could not independently confirm the laboratory test results in many patients. The prospective and complete collection of data on risk factors and tumor characteristics of patients diagnosed with HCC will further distinguish the differences between patients who present with and without risk factors.

Most patients with HCC have known risk factors such as HCV, HBV, or cirrhosis. Genetic changes that lead to HCC are complex and poorly understood, and most studies have focused on the genetic changes in the 'high risk' population[13,14]. Genetic changes that lead to HCC take place over 30-50 years, and this may partly explain the difficulty to define the sequential molecular changes that lead to HCC. There is increasing circumstantial evidence that the development of HCC, like most other cancers, is a multi-step process including inactivation or loss of tumor suppressor genes, activation or over expression of multiple oncogenes and heterozygosity of multiple chromosomes[13-18]. There is experimental evidence that p53, Rb1 and Wnt pathways are important molecular pathways involved in the development of HCC. The early genetic changes may vary depending on the etiology of liver disease and geographic location. Even in the same patient, there may be considerable genetic heterogeneity among different tumor nodules, suggesting that we may not find a common unifying pathway in the pathogenesis of HCC. However, accumulating evidence indicates that hepatocytes with multiple genetic changes may expand in a clonal fashion leading to dysplastic nodules and liver cancer. The molecular mechanisms of liver cancer in patients without known risk factors are difficult to explain. It is possible that many of these patients have been exposed to known or unknown carcinogens. Prospective studies should be designed to identify hitherto unidentified factors including the role of obesity or non-alcoholic fatty liver disease, occult HBV or HCV infections and genetic predisposition.

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RAPID COMMUNICATION

Clinical benefits of biochemical markers of bone turnover in Egyptian children with chronic liver diseases

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Abstract

AIM: To investigate the association between serum insulin-like growth factor 1 (IGF-1), osteocalcin, and parathyroid hormone (PTH) levels with the etiology and clinical condition of patients with chronic liver disease.

METHODS: Eighty children with hepatocellular damage were divided into 3 groups according to the etiology of disease infection: bilharziasis (9 patients), hepatitis B virus (HBV, 12 patients) and hepatitis C virus (HCV, 29 patients). The Child score index was found as A in 24 patients, B in 22 patients, C in 4 patients. Thirty healthy children served as control group. HBsAg, HBcAbIgM, HBcAbIgG, and anti-HCV were detected using ELISA technique. HCV-RNA was measured by reverse transcription polymerase chain reaction (RT-PCR). Anti-bilharzial antibodies were detected by indirect haem-agglutination test. Liver function tests were performed using autoanalyser. Serum IGF-1, osteocalcin and PTH levels were measured by ELISA technique. Abdominal ultrasonography was also conducted.

RESULTS: Serum IGF-1 level was significantly lower in all patient groups with liver diseases, while serum osteocalcin and PTH levels were significantly elevated in patients with HBV and HCV infections compared with the control group. Serum osteocalcin and PTH concentrations were measured with the severity of liver disease from Child A to C. Child A patients unexpectedly showed significantly reduced IGF-1 levels in comparison to patients staged as Child B or C. Serum osteocalcin level was negatively correlated with albumin (14.7 ± 0.54 *vs* 3.6 ± 0.10, $P < 0.05$), while that for PTH was positively correlated with total protein (70.1 ± 2.17 *vs* 6.7 ± 0.10, $P < 0.05$) in patients with HCV infection.

CONCLUSION: Low serum IGF-1 level seems to play a critical role in the bone loss in patients with chronic liver disease. Elevated biochemical markers of bone remodeling suggest high-turnover in patients with viral infection and reflect severity of the clinical stage.

Key words: Liver disease; Bone turnover; Insulin-like growth factor-1; Osteocalcin; Parathyroid hormone

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INTRODUCTION

Bone tissues are metabolically active and undergo continuous remodeling throughout their life. Skeletal remodeling is achieved by two counteracting processes: bone formation which is accomplished through osteoblasts and bone resorption that is attributed to osteoclast activity[1]. Bone manifestations are well-known as extrahepatic complications of chronic liver disease[2]. Patients with chronic liver disease are at increased risk of developing hepatic osteodystrophy manifested as osteomalacia or osteoporosis[3]. Osteoporosis can be described as a disturbance in the bone remodeling process. Bone loss is a result of an imbalance of the bone remodeling process, where bone resorption exceeds bone formation[4].

The prevalence of osteoporosis in patients with chronic liver diseases ranges from 10% to 60%[5,6]. In general, secondary factors such as malabsorption and nutritional deficiencies may cause bone changes in chronic liver disease[7]. Insulin like growth factor (IGF) family is considered as important anabolic hormones, which play a role in anabolic metabolism and stimulating DNA synthesis, cell proliferation and meiotic division throughout life[8]. Since most circulating IGFs are synthesized by hepatocytes, lower levels of these parameters could be found in patients with liver diseases[8].

Regulation of bone metabolism is achieved by various factors such as mechanical motion, minerals, and hormones, all influencing bone turnover[4]. Osteocalcin is a protein produced by osteoblasts. Its level is reduced in the presence of osteodystrophia and can be recommended as a very sensitive and specific marker for bone formation/turnover[9].

Parathyroid hormone has a dual effect on bone cells. It can stimulate osteoblast activity and lead to substantial increase in bone density. In contrast, when secreted continuously at relatively high rates, as in hyperparathyroidism patients it can stimulate oseoclast-mediated bone resorption and suppress osteoblast activity[1]. The anabolic effect of parathyroid hormone (PTH) on osteoblasts is probably both direct and indirect via growth factors such as insulin like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β)[1].

However, the role of hepatocellular dysfunction in hepatic osteodystrophy is not clear. Therefore, this study aimed to clarify the influence of hepatocellular dysfunction on bone loss, and to outline the clinical benefits of controlling serum IGF-1, osteocalcin and parathyroid hormone levels in children with chronic liver diseases.

MATERIALS AND METHODS

Eighty children were enrolled in this study, from Liver Institute, Menoufyia University Inpatient and Outpatient Clinic and divided into: (1) Bilharziasis group: 9 patients (males whose age ranged from 6 to 15 years). (2) HBV group: 12 patients (10 males and 2 females whose age ranged from 3 to 15 years). (3) HCV group: 29 patients (22 males and 7 females whose age ranged from 2 to 16 years). (4) Control group: 30 apparently healthy children (22 males and 8 females whose age ranged from 3 to 15 years) with no history or clinical evidence of liver disease or any other diseases. (5) History was obtained from all individuals and clinical examination was carried out.

Specimen collection

Under complete aseptic condition, 5 mL of blood was taken from all the patients and controls. Each blood sample was divided into two portions: a small portion (1.8 mL) was taken on 3.8% Na-citrate as anticoagulant (0.02 mL) to obtain plasma and a larger portion of blood sample was allowed to clot. Sera were separated and stored at -80℃ till being tested, while the plasma sample was used immediately to measure prothrombin time and concentration.

Serological test

Hepatitis surface antigen (HBsAg), hepatitis B core antibody IgM (HBcAb IgM) and hepatitis B core antibody IgG (HBcAb IgG) were measured by ELISA technique using kits from Dia-Sorin Biomedica Co., according to the methods of Boniolo *et al*[10], Tedder and Wilson[11] and Hoofangle *et al*[12] respectively.

Anti-HCV was detected by third generation of ELISA using kit from the Biochem ImmunoSystem Inc[13], HCV-RNA extraction was carried out by reverse transcription polymerase chain reaction (RT-PCR) according to the method described by Ravaggi *et al*[14]. Anti-bilharzial antibodies were detected by indirect haem-agglutination test (IHA) according to the method of Hoshino *et al*[15], using kits of Fumoze-France.

Table 1 Serum IGF-1, osteocalcin and PTH levels in patient groups and control group (mean ± SE)

	Control (n = 30)	Bilharziasis (n = 9)	HBV (n = 12)	HCV (n = 29)
IGF-1 (ng/mL)	277.1 ± 12.2	94.0 ± 4.30[b]	105.1± 10.3[b]	230.0 ± 10.5[b]
Osteocalcin (ng/mL)	5.7 ± 0.3	6.8 ± 0.66	16.5 ± 0.76[b]	14.7 ± 0.54[b]
PTH (ng/mL)	61.6 ± 2.7	63.3 ± 3.03	83.4 ± 4.30[b]	70.1 ± 2.17[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

Liver function tests

Serum AST, ALT, alkaline phosphatase (ALP), GGT , total and direct bilirubin, total protein, and albumin levels were measurd using EKTOCHEM 750XRC analyzer. Prothrombin time and concentration were also estimated in plasma according to the method of Poller[16].

Biochemical markers of bone turnover

Serum insulin like growth factor-1 (IGF-1), osteocalcin and PTH levels were measured by ELISA technique using kits of BioSource-Belgium[17-19].

Abdominal ultrasonography

To assess the size and echopattern of the liver, the size of the spleen and the presence of ascitis or any other abnormalities in the abdomen were detected using RT-X200 Prob 3.5 MHZ convex of General Electric Company -USA.

Statistical analysis

The obtained data are presented as mean ± SE. The difference between two groups was calculated using unpaired *t*-test, while the difference between more than two groups was calculated using one way analysis of variance ANOVA according to Snedecor and Cochran[20]. Least significant difference (LSD) test was used to compare the means of Child classification according to Walter and Duncan[21] at probability 0.05.

RESULTS

Patients with chronic liver diseases were divided into three groups according to the etiology: group 1 (9 patients with bilharziasis whose mean age was 10.8 ± 1 years), group 2 (12 patients with HBV whose mean age was 11.5 ± 0.6 years, and group 3 (29 patients with HCV infection whose mean age was 11.8 ± 1.5 years). Thirty children whose mean age was 10.5 ± 0.5 years and were sex matched with normal liver served as control group.

Table 1 depicts the results of serum IGF-1, osteocalcin and PTH levels in patients with either bilharziasis or HBV and HCV infections as well as in controls. The results revealed that patients with bilharziasis and those with either HBV or HCV infections showed a significant

Table 2 Liver function tests in different patient groups and control group (mean ± SE)

	Control (n = 30)	Bilharziasis (n = 9)	HBV (n = 12)	HCV (n = 29)
ALT (U/L)	20.5 ± 0.9	38.0 ± 6.8[a]	72.6 ± 12.4[a]	107.0 ± 28.1[a]
AST (U/L)	18.4 ± 0.8	46.0 ± 7.5[a]	71.2 ± 10.8[a]	120.0 ± 21.8[a]
ALP (U/L)	74.0 ± 9.3	82.0 ± 11.8	214.0 ± 41.2[a]	165.0 ± 28.9[a]
GGT (U/L)	26.0 ± 2.1	37.6 ± 9.9	54.0 ± 1.5[a]	59.9 ± 11.1[a]
T. bilirubin (mg/dL)	0.84 ± 0.03	0.9 ± 0.16	1.8 ± 0.20[a]	1.3 ± 0.13[a]
T. protein (mg/dL)	6.9 ± 0.10	7.1 ± 0.30	6.8 ± 0.20	6.7 ± 0.10[a]
Albumin (mg/dL)	4.0 ± 0.06	3.9 ± 0.10	3.5 ± 0.19[a]	3.6 ± 0.10[a]
Prothrombin (%)	93.0 ± 1.3	77.0 ± 4.0[a]	69.0 ± 6.0[a]	65.6 ± 2.50[a]

[a]$P < 0.05$ *vs* control group.

Table 3 Alterations in serum IGF-1, osteocalcin and PTH levels according to the severity of the disease from class A to C (mean ± SE)

Severity	A (n = 24)	B (n = 22)	C (n = 4)	LSD at 0.05
IGF-1 (ng/mL)	152.6[b] ± 7	186.8[a] ± 20.3	196.0[a] ± 36.4	32.67
Osteocalcin (ng/mL)	12.6[b] ± 0.9	14.6[a] ± 0.9	15.8[a] ± 1.2	1.73
PTH (ng/mL)	71.3[b] ± 3.0	70.3[b] ± 2.7	86.5[a] ± 4.3	5.36

Within each row the means followed by different letters are significantly different. LSD: Least significance difference. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

Table 4 Effect of age and sex on serum IGF-1, osteocalcin and PTH (mean ± SE)

	Age (yr)			Sex		
	3-9	10-16	P	Male	Female	P
IGF-1 (ng/mL)	153.2 ± 59.3	182.6 ± 84.5	0.268	175.2 ± 84.2	177.1 ± 58.4	0.948
Osteocalcin (ng/mL)	13.8 ± 4.7	13.7 ± 4.2	0.941	13.1 ± 4.1	16.4[a] ± 4.1	0.037
PTH (ng/mL)	71.1 ± 14.0	72.3 ± 13.8	0.786	73.1 ± 13.8	67.2 ± 13.1	0.249

[a]$P < 0.05$ *vs* males.

decrease ($P < 0.01$) in serum IGF-1 level compared to the controls. The most significant decrease in serum IGF-1 was recorded in patients with bilharziasis followed by HBV-infected and HCV-infected patients. Serum osteocalcin level showed a significant increase ($P < 0.01$) in patients with HBV and HCV infection compared to the controls. The most significant increase in serum osteocalcin level was observed in HBV-infected patients. Patients suffering from bilharziasis had no significant ($P > 0.05$) change in serum osteocalcin level compared to the controls. Serum PTH level was significantly increased in HBV-infected patients ($P < 0.01$) and in those with HCV infection ($P < 0.05$) compared to the controls. No significant change in serum PTH level was detected in patients with bilharziasis compared to the controls (Table 1).

Liver function tests in patients with bilharziasis or HBV and HCV infection as well as in the control are presented in Table 2. Serum ALT and AST activities showed a significant ($P < 0.05$) increase in all patient groups compared to the control group. The most significant increase in serum ALT and AST activities was recorded in the patients suffering from HCVinfection. The activities of ALP, GGT and total bilirubin in serum revealed a significant increase ($P < 0.05$) in patients with HBV and HCV infection compared to the control, while no significant change ($P > 0.05$) was recorded in patients with bilharziasis as compared to the control group. Serum total protein level showed significant decrease ($P < 0.05$) in patients with HCV infection, whereas patients with either bilharziasis or HBV had no significant ($P > 0.05$) change as compared to the control (Table 2). Serum albumin level revealed a significant ($P < 0.05$) decrease in patients suffering from either HBV or HCV infection compared to the control. No significant change in serum albumin level was observed in patients with bilharziasis compared to the control. Patients suffering from either bilharziasis or HBV and HCV infection had a significant ($P < 0.05$) decrease in prothrombin concentration compared to the control. The most significant decrease in prothrombin concentration was detected in HCV-infected patients (Table 2).

Alterations in serum IGF-1, osteocalcin and PTH level according to the severity of the disease from Child class A to C are depicted in Table 3. Significant ($P < 0.05$) change was detected in the three serum markers (IGF-1, osteocalcin and PTH) in each grade of the disease. The mean values of the three serum markers were markedly increased with the severity of the disease and the highest value was recorded in Child class C of the disease using LSD at probability 0.05.

Noteworthy, our results revealed that serum IGF-1, osteocalcin and PTH levels were not age or sex dependent except for osteocalcin level which was higher in females (Table 4).

On correlating the three bone markers with each other and liver function indices using Pearson correlation, there was only significant negative correlation between serum osteocalcin and albumin ($r = -0.409$, $P = 0.027$) and significant positive correlation between PTH and total protein ($r = 0.451$, $P = 0.014$) in patients with HCV infection (Figure 1A and B), while no significant correlation was recorded between IGF-1, PTH or osteocalcin in our patients.

DISCUSSION

Patients with chronic liver disease are prone to develop hepatic osteodystrophy. In the majority of cases it is

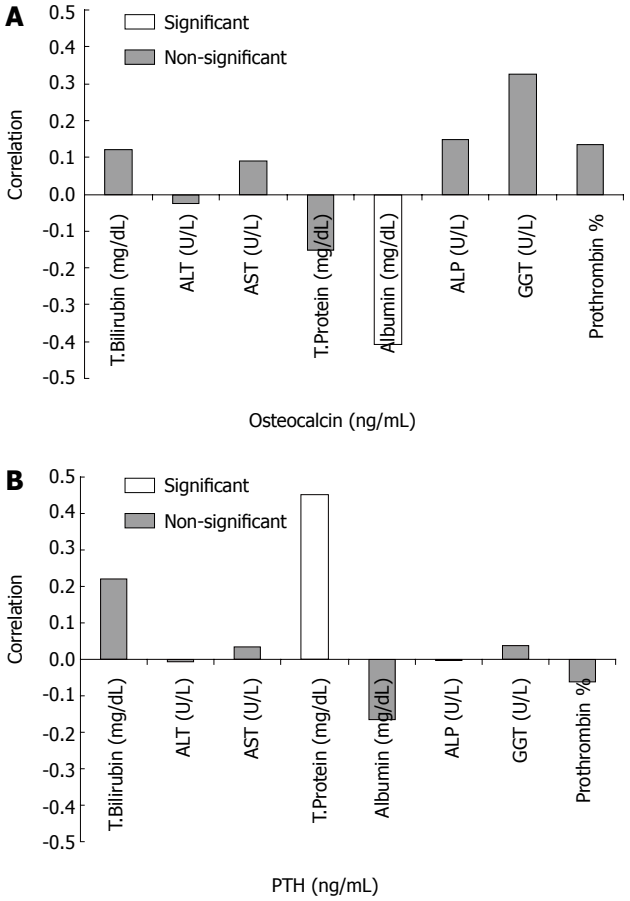

Figure 1 Correlation coefficient between serum osteocalcin and albumin levels (**A**) & between serum PTH and total protein levels (**B**) in patients with HCV infection.

characterized by development of osteoporosis and/or osteomalacia with possible persistence of minor or major disability[3]. Osteoporosis reflects loss of bone (both matrix and its mineral) and osteomalacia is due to defective mineralization of osteoid[22]. Osteoporosis is a frequent complication of end-stage liver disease irrespective of its etiology. The prevalence varies between 9% and 60%, the highest being observed in cholestatic disorders and alcoholic liver disease[23].

The present study aimed at elucidating the influence of bilharziasis, hepatitis B or C infections on the development of osteoporosis in children. Serum levels of IGF-1, osteocalcin and PTH were measured and correlated with the routine liver functions as well as the child-pough score. The results of the present work showed that serum IGF-1 level was significantly decreased in all children infected with bilharziasis, HBV and HCV, while unexpectedly it showed significant increase with the severity of liver diseases from A to B or C classification but its level was still lower than that in the control.

These results are greatly supported by the finding of Hassan *et al*[24] who showed a significant reduction in the level of IGF-1 in children with schistosomiasis with or without hepatic fibrosis. Also, our results are in agreement with the findings of Orsini *et al*[25] who found similar results in hepatosplenic schistosomiasis patients and the results of Mazziotti *et al*[26] in patients with hepatocellular carcinoma (HCC) and hepatitis C virus-related cirrhosis. The reduction in IGF-1 level also can precede the diagnosis of HCC[26]. Similarly, decreased serum IGF-1 level has been recorded both in patients with viral cirrhosis[9,27] and in patients with hepatitis B infection without cirrhosis[28].

In schistosomiasis patients with hepatic fibrosis, the depressed circulation of IGF-1, free T4 and growth hormone may be responsible for stunted stature[24]. Moreover, low serum IGF-1 level in hepatosplenic schistosomiasis patients has been found to be related to the clinical form of hepatosplenic schistosomiasis[25]. The level of IGF-1 is elevated in patients with schistosomiasis with or without liver fibrosis and relates to its clinical form[25], suggesting that the severity of liver dysfunction can affect the level of IGF-1. As liver function of patients with bilharziasis is usually better than those with cirrhosis due to HBV and HCV infection and usually scored as child A, this may partly explain the relatively unexpected low level of IGF-1 in our patients with less sever liver affection (child A group).

The present study recorded a significant increase in serum osteocalcin level in patients with viral hepatitis infection only. Also a negative correlation was obtained with the level of serum albumin in patients with HCV infection. Moreover, serum osteocalcin level was significantly increased in child B and C patients than in child A patients.

It has been reported that serum osteocalcin level is higher in cirrhotic patients than in control, suggesting that cirrhotic patients have high bone turnover. In addition, osteodystrophy associated with hepatic cirrhosis is due to a defect in alpha-hydroxylation (1, $25(OH)_2D$) by the kidney due to the decrease in its primary substrate, the liver $25(OH)_2D$. This could be attributed to the reduced availability of vitamin D, inadequate conversion of vitamin D to $25(OH)_2D$, accelerated metabolism of $25(OH)_2D$, and urinary loss of $25(OH)_2D$ with its transport protein[29]. However, some studies reported that serum osteocalcin level is low in cirrhotic patients and osteopenia in these patients is not due to a decrease in bone formation[30,31], but may be a consequence of hepatic osteodystrophy due to low plasma vitamin D and calcium levels[31].

Concerning the changes in serum PTH level, the present study recorded significant increase in patients with Hepatitis B and C infections. As regard a positive correlation was obtained with serum total protein level among the group of HCV infection. Moreover, serum PTH was significantly increased in class B and C patients than patients of class A.

In hepatocellular dysfunction some previous studies reported that serum PTH level is high in patients with primary biliary cirrhosis[32], liver cirrhosis[33], HBV infection[34], as well as in dogs with schistosomiasis[35] and in children with cholestatic and non-cholestatic liver disease[36]. On the other hand, other studies reported that PTH is unchanged in patients with chronic viral liver disease[37] and post hepatic cirrhosis[38]. Kirch and co-workers[33] have found a significant correlation between PTH and the parameters of liver functions such as prothrombin, albumin and bilirubin, suggesting that the increasing PTH level is related to liver dysfunction. This may explain why the elevation of PTH level is due

to the impaired liver function rather than secondary hyperparathyroidism[33]. Moreover, elevated PTH-related protein was observed with hyperglobulinemia and hypoalbuminemia in dogs with schistosomiasis[35], and this may explain the obtained positive correlation between PTH and serum total protein levels in patients with HCV infection due to hyperglobulinemia observed in our study.

Hepatic osteodystrophy (HOD) begins at the stage of chronic non-cirrhotic liver injury and bone loss is connected with liver damage, suggesting that the principal pathogenesis of HOD is attributed to intestinal Ca-malabsorption as a result of low serum albumin and villous atrophy[39]. Lower ionized calcium resulting from deficient intestinal absorption due to low $25(OH)_2D$, leads to increase in PTH level[36].

The significant rise in serum osteocalcin level in our female patients indicated that their bone turnover was accelerated. This result is in agreement with Steinberg *et al*[40] who found that female sex is a risk factor for bone turnover and low bone density.

In conclusion, serum IGF-1, osteocalcin and PTH appear to be markers of bone metabolism in children with hepatocellular damage. Low level of IGF-1 seems to play a role in the bone mass loss in patients with chronic liver disease. Elevated biochemical markers of bone remodeling suggest high bone turnover in children with viral infections. Fortification of diet by ergocalciferol is essential in patients with chronic liver disease and regular bone density measurements are necessary in these patients.

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RAPID COMMUNICATION

Per rectal portal scintigraphy as a useful tool for predicting esophageal variceal bleeding in cirrhotic patients

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Abstract

AIM: To investigate potential roles of per rectal portal scintigraphy in diagnosis of esophageal varices and predicting the risk of bleeding.

METHODS: Fifteen normal subjects and fifty cirrhotic patients with endoscopically confirmed esophageal varices were included. Patients were categorized into bleeder and non-bleeder groups according to history of variceal bleeding. All had completed per rectal portal scintigraphy using ^{99m}Technetium pertechnetate. The shunt index was calculated from the ratio of ^{99m}Technetium pertechnetate in the heart and the liver. Data were analyzed using Student's *t*-test and receiver operating characteristics.

RESULTS: Cirrhotic patients showed a higher shunt index than normal subjects (63.80 ± 25.21 *vs* 13.54 ± 6.46, $P < 0.01$). Patients with variceal bleeding showed a higher shunt index than those without bleeding (78.45 ± 9.40 *vs* 49.35 ± 27.72, $P < 0.01$). A shunt index of over 20% indicated the presence of varices and that of over 60% indicated the risk of variceal bleeding.

CONCLUSION: In cirrhotic patients, per rectal portal scintigraphy is a clinically useful test for identifying esophageal varices and risk of variceal bleeding.

Key words: Portal scintigraphy; Portal hypertension; Cirrhosis; Esophageal varices; Bleeding

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INTRODUCTION

Esophageal variceal bleeding is a serious and potentially life-threatening complication of liver cirrhosis[1-4]. No simple noninvasive method which accurately predicts esophageal variceal bleeding is available so far and endoscopists have had to perform endoscopy every 3 to 6 mo to evaluate patients with previously bleeding esophageal varices[5,6]. However, this approach is costly and not all patients with liver cirrhosis and esophageal varices are good candidates for such procedures[7].

Per rectal portal scintigraphy is a noninvasive method for evaluation of portosystemic shunting using portal shunt index (SI) calculated from radioactivity curves of the liver and the heart. In humans, such an SI shows a good correlation with portal pressure measured by percutaneous transhepatic portography or intraoperative method[8]. Portal scintigraphy is also clinically useful, especially in establishing prognosis of cirrhotic patients with varying shunt indices[9]. Evaluating portal pressure using per rectal portal scintigraphy may be easily performed and might provide information on risk of variceal bleeding.

The aim of this study was to determine the benefit of per rectal portal scintigraphy for evaluation of non-bleeding esophageal varices, bleeding esophageal varices and normal control subjects. We also compared different portal shunt indices, Child-Pugh grades and endoscopic appearances in various groups and examined the correlation between the portal SI and risk of variceal bleeding.

MATERIALS AND METHODS

Normal subjects and patients

Normal subjects: Normal adult volunteers of both sexes, with a minimum age of 18 years, were recruited. An attempt was made to obtain subjects in well distributed age groups, and equal in number for both sexes. All of the subjects were in good health, with no history of the liver and vascular diseases, or use of medication, alcohol or any substances that might affect the liver. The liver function test was normal, and tests for viral hepatitis (B, C) were negative or revealed immunized status. Hepatic ultrasonography was also performed to rule out structural liver disease or hepatocellular carcinoma.

Patients: Cirrhotic patients of either alcoholic or viral in etiology who had signs of portal hypertension, and a history or physical signs suggesting esophageal varices, or even esophageal variceal bleeding, were asked to participate in this study. Only patients with endoscopically confirmed esophageal varices were included. The esophageal varices were graded according to criteria developed by the Japanese Research Society for Portal Hypertension[10]. Thorough medical examination and interview for the history of gastrointestinal bleeding were performed. Only cirrhotic patients with esophageal varices were included in this study. The initial laboratory tests performed in this study included complete blood count, liver function test, viral hepatitis (B, C) profile, and renal function test. All patients were graded according to Child-Pugh classification. The etiology of bleeding was considered to be variceal if either actively bleeding varices were observed by endoscopy or if varices were endoscopically found without other sources of bleeding.

Informed consent were obtained from all patients and the protocol was in conformity with the ethical guidelines of the Declaration of Helsinki and the Clinical Research Committee at Chiang Mai University Hospital.

Measurement of the portal shunt index

The procedure used for per rectal portal scintigraphy was the same for both normal subjects and cirrhotic patients[9]. After fasting overnight for at least 6 h, the rectum was emptied by administration of laxatives (unison enema 100 mL). A polyethylene device (Terumo® feeding tube Fr.8) was inserted deep into the rectum with the tip of the tube being placed in the upper rectum, 20 cm above the anal verge, to avoid absorption into the systemic circulation via the inferior rectal vein in the lower rectum. To generate time-activity curves, a gammascinti camera with a large field of view (Apex SP4, El-Scint Co., Israel), equipped with a low-energy, multipurpose, parallel-hold collimator was used. The collimator was positioned over the patient's abdomen so that the field of view would always include the heart, the liver, and the spleen. Ten millicuries (2 mL) of ^{99m}Technetium pertechnetate, followed by 15 mL of air, was infused into the rectum through the tube. The sequential images were generated from counts in their areas of interest (AOI). For color display, the summed images were reconstructed by grouping of 20 s per image from the original 60 images. ^{99m}Technetium pertechnetate is commonly used in diagnostic scanning and imaging both in clinics and research. The procedure is safe and readily applicable because of the amount and short half life of radioactive substance used. Portal pressure increases shortly after an episode of variceal bleeding[11] and is stable over 72 h after bleeding[12]. Therefore, per rectal portal scintigraphy in patients of the bleeding group was performed 72 h after bleeding when the portal pressure returned to baseline level in order to determine the risk of that episode of bleeding.

Calculation of the portal shunt index

To calculate the amount of blood that entered the portal system and went to the liver and the heart, we used the portal SI[8]. This index was derived from the ratio of ^{99m}Technetium pertechnetate in the heart and the liver at the exact time as shown in the following equation.

$$SI = \frac{\sum_{0}^{(n+25)/5} x_i(H)}{\sum_{0}^{(n+25)/5} x_i(L) + \sum_{0}^{(n+25)/5} x_i(H)} \times 100\%$$

n = time at which radionuclides appeared in the area of the liver (s).

n' = time at which radionuclides appeared in the area of the heart (s).

$Xi\ (L)$ = the count per 5 s over the AOI of the liver.

$Xi\ (H)$ = the count per 5 s over the AOI of the heart.

In normal portal circulation, the transit time of blood circulation from the liver to the heart via hepatic veins and inferior vena cava was 18-26 s, and the scinti images were acquired in 5 s per image. The SI was calculated at the time of n + 25 s. The results were shown as the pattern of time-activity curves and summed-images.

Statistical analysis

Results were expressed as mean ± SD. The significance of difference between mean values was evaluated by Student's *t*-test. Differences with probability values of less than 0.05 were considered to be significant. The selection of an appropriate cutoff point of portosystemic shunt for the presence of esophageal varices and the risk of variceal bleeding was analyzed by the receiver operating characteristic (ROC)[13].

RESULTS

The normal control group consisted of eight male and seven female subjects (aged 28-59 years, mean 44.3 years). Fifty cirrhotic patients (45 males and 5 females), of various etiologies, aged between 30 and 70 years (mean 46.9 years) were recruited in this study. The baseline characteristics of all study patients are shown in Table 1. The patients were categorized into two groups according to their history of variceal bleeding, namely a non-bleeding group including 25 patients and a bleeding group with 25 patients. Bleeding was the first episode in all 25 patients in the bleeding group. All patients had received neither endoscopic variceal surveillance nor prophylactic treatment to prevent variceal bleeding. The two groups of patients showed similar baseline characteristics with the exception of alanine aminotransferase, total bilirubin, and prothrombin time. The severity of liver disease in the bleeding group was higher than that of the non-bleeding group. In the non-bleeding group, etiologies of liver disease were alcoholic in 9, hepatitis viral B in 6, hepatitis viral C in 5, and alcoholic and virus in 5 cases. In the bleeding group, etiologies of liver disease were alcoholic in 20, hepatitis virus B in 3, and alcoholic and virus in 2 cases. The mean SI in normal subjects was 13.54% ± 6.46%. For the cirrhotic patients, the mean SI was 63.80% ± 25.21%. Normal subjects were found to have significantly lower average SI than cirrhotic patients with esophageal varices ($P < 0.01$). If cirrhotic

Table 1 Baseline characteristics of the study patients

	Control (n = 15)	Non bleeder (n = 25)	Bleeder (n = 25)
Age (yr)	44.27 ± 8.48	49.32 ± 10.25	44.25 ± 8.86
Sex (M/F)	8/7	20/5	25/0
Presence of ascites (%)	0	10 (40)	7 (28)
Hepatic encephalopathy (%)	0	0	3 (12)
Serum albumin (g/dL)	4.55 ± 0.26	3.16 ± 0.54	2.76 ± 0.83
Alanine aminotransferase (U/L)	18.93 ± 4.03	51.88 ± 45.9	77.12 ± 61.41[b]
Serum bilirubin (mg/dL)	0.65 ± 0.19	2.49 ± 1.16	5.27 ± 6.55[b]
Prothrombin time (s)	-	2.67 ± 1.02	3.27 ± 2.43[b]
Etiology of cirrhosis			
Alcohol	-	9	20
Hepatitis B virus	-	6	3
Hepatitis C virus	-	5	0
Alcohol and virus	-	5	2
Child-Pugh A	-	13	8
B	-	9	9
C	-	3	8

[b]$P < 0.01$ *vs* bleeder and non bleeder group.

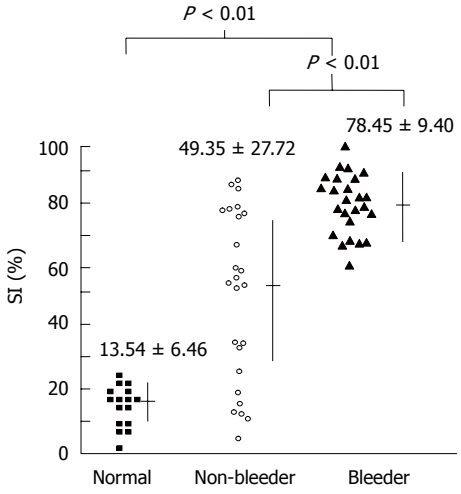

Figure 1 Comparison of portal shunt index in normal subjects and cirrhotic patients, with and without variceal bleeding. Data shown as mean (horizontal bar) ± SD (vertical bar).

patients were grouped as non-bleeding and bleeding, the mean SIs were 49.35% ± 27.72% and 78.45% ± 9.40%, respectively. The average SI in bleeding group was significantly higher than in non-bleeding group ($P < 0.01$), as shown in Figure 1.

Analysis was done to discover any difference in SIs when the patients were sub-grouped according to etiologies (i.e. alcoholic, viral, or both), presence of ascites, endoscopic appearances and Child-Pugh grade. Details are shown in Table 2. The mean SI was 78.87% ± 22.05% in cirrhosis from alcohol, 56.67% ± 24.08% in cirrhosis from virus, and 41.23% ± 23.47% in cirrhosis from alcohol plus viruses. The average SI in the etiology group of alcohol significantly differed from that of virus, and also that of alcohol plus virus ($P < 0.05$). The mean SI was 62.87% ± 28.62% in cirrhotic patients without ascites and 65.92% ± 23.64% in cirrhotic patients with ascites. The difference between both groups was not significant ($P = 0.35$). We used Child-Pugh classification to estimate the severity of liver disease in both bleeding and non-bleeding groups as a whole. The mean SI was 52.60% ± 25.31% in Child-Pugh grade A, 69.29% ± 22.34% in Child-Pugh grade B, and 76.64% ± 22.01% in Child-Pugh grade C. The SI in Child-Pugh grade A differed significantly ($P < 0.05$) from those in Child-Pugh grades B and C. Although the SI in Child-Pugh grade B seemed to be lower than that in Child-Pugh grade C, the difference was not statistically significant ($P = 0.20$).

Portal shunt index and endoscopic appearances of esophageal varices

The esophageal varices were endoscopically graded according to the Japanese Research Society for Portal Hypertension by color, forms, location, diameters, and the red color sign (RCS). Variceal forms were classified into three types: straight (F_1), enlarged tortuous (F_2), and largest sized (F_3). The mean SI was 57.03% ± 27.63% in F_1 group, 67.47% ± 24.99% in F_2 group, and 72.65% ± 8.77% in F_3 group (Table 2). Mean SI in F_3 group was higher than in F_2 and F_1 groups. The difference between the groups was statistically significant ($P < 0.05$). Mean SI in RCS-negative group was 52.94% ± 26.31% and in RCS-positive group was 68.16% ± 23.78%. The difference between both groups was not significant ($P = 0.054$).

Portal shunt index and risk of esophageal variceal bleeding

The receiver operating characteristic (ROC) showed the cutoff point of portal SI between normal subjects and cirrhotic patients at 20% with a 90.0% sensitivity and 53.3% specificity. The cutoff point of portal SI between the cirrhotic patients with and without variceal bleeding was 60% with a 96.0% sensitivity and 72.0% specificity (Figure 2). Basically, the cirrhotic patients with esophageal varices who had a portal SI of more than 60% might have high risk of variceal bleeding, and the patients who had a portal SI more than 20% were likely to develop esophageal varices.

Table 2 Portal shunt index in cirrhotic patients according to the etiologies of cirrhosis, presence of ascites, Child-Pugh grade and endoscopic appearances

			n	Portal shunt index (%)
Etiology of cirrhosis				
	Alcohol		29	78.87 ± 22.05
	Virus[1]		14	56.67 ± 24.08
	Alcohol with virus[1]		7	41.23 ± 23.47
Ascites	Absence		33	62.87 ± 28.62
	Presence		17	65.92 ± 23.64
Severity of liver diseases		Child A	21	52.60 ± 25.31
		Child B	18	69.29 ± 22.34
		Child C	11	76.64 ± 22.01
Endoscopic appearances		F_1	14	50.03 ± 27.63
		F_2	20	67.47 ± 24.99
		F_3	16	72.65 ± 8.77
		RCS-positive	27	68.16 ± 23.78
		RCS-negative	23	52.04 ± 26.31

[1] Including hepatitis B virus and hepatitis C virus.

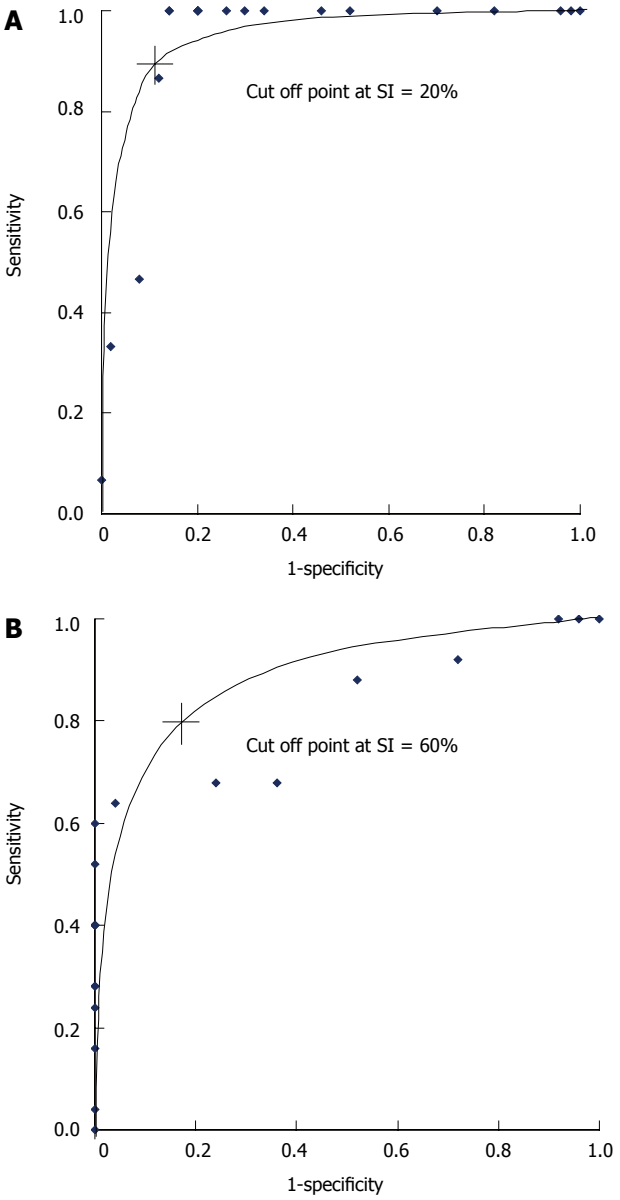

Figure 2 ROC curves of the portal shunt indices. **A**: Between normal subjects and cirrhotic patients with esophageal varices; **B**: Between cirrhotic patients with and without variceal bleeding (bleeder *vs* non-bleeder). + Indicates a cutoff point with maximum discrimination.

DISCUSSION

In this study, we used per rectal portal scintigraphy to detect and investigate portosystemic shunting in order to determine the difference among the bleeding, the non-bleeding cirrhotic and the normal control groups. We found that the SI was higher in the bleeders compared with other groups. The SI correlated with the severity of liver disease and the size of esophageal varices. Bleeding from the varices was a major complication of portal hypertension that may vary according to the severity of portal hypertension. The cumulative survival rate of patients with esophageal varices was significantly lower than that of patients without varices[14]. The cumulative survival rate of patients with portosystemic shunting seen by scintillation splenoportography was also significantly lower than that of patients without such a shunt [15]. Many factors could potentially be used to predict variceal bleeding in cirrhotic patients with esophageal varices. These predictors include the following: (1) local factors such as variceal size, vessel radius, or red color sign; (2) hemodynamic factors: portal pressure > 12 mmHg, blood volume, and collateral blood flow; and (3) severity of liver disease[10]. In general, the direct measurement of portal pressure is performed invasively and not readily available in many clinical settings. In those circumstances, indirect measurement of portal pressure, such as portosystemic shunt evaluation may be used instead. Recently, various methods of measuring portosystemic shunt have been developed, and the relationships between the risk of variceal bleeding and the extent of portosystemic shunt have been studied.

Per rectal portal approach in the measurement of portal circulation using various radionuclides has been described as a relatively non-invasive method by many researchers[9,16-19]. In our present study, we chose to perform non-invasive per rectal portal scintigraphy using ^{99m}Technetium pertechnetate because this radionuclide is well absorbed by the rectum, has a short half life and large doses could be used[20]; it also generates images useful for diagnosis and is economical. The SI from per rectal scintigraphy also correlated well with the degree of portal pressure[9]. In this study, the mean SI in cirrhotic patients with esophageal varices was significantly higher than that in normal subjects. The cutoff level of SI indicating development of esophageal varices derived from the ROC was 20%. All six cirrhotic patients with esophageal varices whose SIs were less than 20% belonged to Child-Pugh grade A and had less severe variceal forms without red color signs than patients with esophageal varices and an SI more than 20%. None of these six patients with small varices bled.

We also studied the association between Child-Pugh class, etiology of cirrhosis and portal scintigraphy in cirrhotic patients with esophageal varices. The mean SI

in Child-Pugh grade C was higher than that of Child-Pugh grade B and grade A, with a significant difference between grade C and A. The SI tended to be higher in patients with more severe liver disease. Because the Child-Pugh classification correlated well with the severity of liver disease, serial measurement of portal circulation by SI might be helpful in investigating the progress of cirrhosis. The SI in alcoholic cirrhosis was significantly higher than other etiologies because of more severe liver disease in alcoholic group. The SI in patients with alcohol and virus cirrhosis was lower compared with cirrhosis induced by alcohol or virus alone. This might possibly result from the small number of patients in alcohol with virus group. The SIs in cirrhotic patients with and without ascites were not significantly different. In comparison of the SI in different variceal forms, there were significant differences among the size of varices. Higher SIs were found in patients with higher variceal forms. There was a good relationship between SI and the size of esophageal varices.

The SI values in the present study were significantly different in cirrhotic patients with and without variceal bleeding. It was presumed that an increasing flow of blood through the collateral of inferior mesenteric veins measured by portal scintigraphy reflected the increasing pressure in the portal system, the development and the size of esophageal varices. This increasing pressure was at a certain level, adequately high for the varices to rupture. So the higher the SI, the higher was the risk of bleeding. In this study, the SI was clearly demonstrated to be a useful predictor of the risk of bleeding. By ROC, an SI of more than 60% correlated with esophageal variceal bleeding. We presumed that if cirrhotic patients had an SI of more than 20%, they would have adequate portal pressure to develop the esophageal varices, and if the SI was more than 60% they carried the risk of variceal bleeding. However, further prospective, longitudinal studies are needed to confirm these results.

In conclusion, this study suggests that portosystemic shunt, represented by the SI and evaluated by per rectal portal scintigraphy, is higher in cirrhotic patients with esophageal varices. The magnitude of blood shunting through these tributaries correlates well with the etiology and severity of cirrhosis and the risk of variceal bleeding. An SI of more than 60% in cirrhotic patients with esophageal varices reflects a high risk of variceal bleeding.

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RAPID COMMUNICATION

Increased plasma malondialdehyde and fructosamine in anemic *H pylori* infected patients: Effect of treatment

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Abstract

AIM: To unravel the possible association of malondialdehyde (MDA) and fructosamine in anemic *H pylori* infected patients and to observe the alteration in MDA and fructosamine levels in these patients after treatment for one month.

METHODS: Fructosamine, MDA and glucose were estimated in 22 anemic *H pylori* infected patients and 16 healthy controls. Hematological parameters were also evaluated in both the groups using Sysmex-K-100 automated cell counter. The *H pylori* infected patients were randomly divided into two groups. *H pylori* infected patients in Group Ⅰ received both iron supplementation and anti-*H pylori* therapy, while patients in Group Ⅱ received only iron supplementation. All the biochemical and hematological parameters were estimated after one month of treatment.

RESULTS: In anemic *H pylori* infected patients, while MDA (5.41 ± 2.16 *vs* 2.26 ± 0.50; $P < 0.05$) and fructosamine (2.64 ± 0.93 *vs* 1.60 ± 0.35; $P < 0.05$) were significantly increased, iron (32.72 ± 14.93 *vs* 110.25 ± 26.58; $P < 0.05$), hemoglobin (6.9 ± 2.6 *vs* 12.66 ± 0.74; $P < 0.05$) and ferritin (28.82 ± 16.27 *vs* 140.43 ± 30.72; $P < 0.05$) levels were significantly decreased compared with the controls. With partial correlation analysis, fructosamine was found to have a significant positive correlation with MDA. In Group Ⅰ, while MDA level decreased significantly (3.11 ± 1.73 *vs* 5.50 ± 2.46; $P < 0.05$), there was a significant increase in iron (84.09 ± 29.51 *vs* 36.09 ± 17.81; $P < 0.05$), hemoglobin (10.40 ± 1.11 *vs* 7.42 ± 1.90; $P < 0.05$) and ferritin (116.91 ± 63.34 *vs* 30.46 ± 17.81; $P < 0.05$) levels after one month. There was no significant change in the levels of fructosamine in group Ⅰ after treatment. Similarly, no significant alterations were noted in the levels of MDA, fructosamine, hemoglobin or ferritin in Group Ⅱ patients after one month of treatment.

CONCLUSION: An increased level of fructosamine and MDA was found in anemic *H pylori* infected patients. Present data supports the premise that lipid peroxides *per se* do play a role in the glycation of plasma proteins. Furthermore, the findings from this study indicate that treatment for both anemia and *H pylori* infections is required for lowering the levels of lipid peroxides in these patients.

Key words: *H pylori*; Anemia; Fructosamine; Malondialdehyde; Iron; Glycation

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INTRODUCTION

H pylori, a gram negative bacillus is the most common pathogenic bacteria in the world[1]. Even though approximately half of the population in the world has *H pylori* infection, the prevalence and severity vary greatly among countries and population groups within the same countries. The overall prevalence of *H pylori* is strongly correlated with socio-economic conditions[1]. The prevalence among middle aged adults is over 80% in many developing countries as compared with 20%-50% in industrialized countries[2].

Accumulating evidences suggest an association between gastric *H pylori* infection and low iron stores and anemia[3-6]. Epidemiological studies have demonstrated a close relationship between serum ferritin and presence of anti-*H pylori* IgG[3,4]. A fall in serum ferritin reflects declining body iron stores and is an accepted marker of iron deficiency. It has also been found that eradication of *H pylori* infection in iron-deficient anemic patients was found to reverse the iron deficiency status in both children and adults[5,6].

Spontaneous nonenzymatic modifications of protein are commonly reported in tissues with slow turnover and they are considered by several authors as a possible common mechanism involved in the progression of many pathological conditions[6]. Among the nonenzymatic processes, oxidative stress and glycation have aroused a particular interest in recent years[7,8].

Glycation is a non-enzymatic condensation reaction between reducing sugars and free amino groups at NH_2-terminus or susceptible ε-amino groups of lysine residues of proteins. The reaction is initiated by the reversible formation of a schiff base, which undergoes a rearrangement to form a relatively stable Amadori product[9]. The pathological consequences of these alterations very much depend on the nature of proteins involved as well as on their function and concentration in a particular organ[9]. The rate of formation of glycated protein is considered to depend on the ambient concentration of glucose and half life of the protein[9]. However, there is convincing evidence that concentrations of nonenzymatically glycated protein are increased in many non-diabetic pathological states[10-13]. Elevated concentrations of glycated hemoglobin have been found in myocardial infarction, chronic renal failure, and nephrotic syndrome patients with normal blood glucose concentrations[10-12]. Similarly high concentrations of fructosamine are reported in non-diabetic chronic renal failure and rheumatic arthritis patients[12,13]. Increased levels of glycated hemoglobin (HbA_{1C}) have also been documented in iron deficiency anemic patients without any history of diabetes[14-16].

We have recently demonstrated that lipid peroxides *per se* can enhance the process of protein glycation[17]. This result was in agreement with the findings of Jain *et al*[18]. We have also demonstrated that the process of lipid peroxidation and glycation are closely associated in patients with chronic renal failure, hyperthyroid, asthma, nephrotic syndrome and patients with rheumatoid arthritis[19-24].

Even though there are substantial reports demonstrating the presence of excess lipid peroxides in patients with *H pylori* infection, there is a dearth of information regarding the levels of glycated proteins in these patients with anemia. Given the importance of glycated protein in causing pathological complication in various diseases, it was deemed pertinent to investigate the levels of glycated plasma protein and the possible association with the levels of lipid peroxides in *H pylori* infected patients who were anemic. Furthermore, we explored the effect of treatment on the levels of these parameters. This study is the first to describe an association between lipid peroxides and glycated protein in anemic *H pylori* infected patients.

MATERIALS AND METHODS

Blood sample (3 mL) was obtained from 22 anemic patients with *H pylori* infection and 16 age matched healthy subjects. Anemic patients were recruited from the outpatient department of our institute, JIPMER, Pondicherry, India. Only patients of 13 years of age or older were enrolled for this study. Anemic patients were selected based on the hemoglobin levels (Hb < 11 g/dL) and peripheral blood smear suggesting iron deficiency anemia. Selected patients underwent detailed physical examination and laboratory evaluation.

One milliliter of the whole blood in EDTA was used for the analysis of hemoglobin and red cell indices using Sysmex-K-100 automated cell counter (Sysmex Singapore Pvt. Ltd, Singapore). The rest of the sample was centrifuged at 3000 r/min for 10 min. The plasma was separated and analyzed for lipid peroxides, fructosamine, iron, ferritin and glucose. Plasma ferritin level was determined by ELISA using human ferritin enzyme immunoassay test kit (IBL Immunobiological Laboratories, Hamburg, Germany). Fructosamine was measured by p-indonitrotetrazolium violet kinetic method using the Raichem Kits (Haemagen Diagnostics, San Diego, CA) adapted to 550 Express Plus Analyzer (Ciba Corning Diag., Oberlin, OH). The concentration of lipid peroxides in plasma was measured by thiobarbituric acid method[25]. Plasma iron and glucose were measured by fully automated ferrozine and glucose oxidase methods respectively in Ciba Corning 550 Express Plus. All patients who were found to have iron deficiency by the above parameters underwent stool examination on three consecutive days for the presence of hookworm ova on microscopy and for occult blood by benzidine test.

After informed consent, upper gastrointestinal endoscopy was done and multiple biopsy specimens were obtained from the antral mucosa for rapid urease test and histology. Tissue sections were stained for *H pylori* with Geimsa. *H pylori* infection was defined as a visible organism seen under microscopy and a positive rapid urease test. Patients with histories of consumption of nonsteroidal anti-inflammatory drugs (NSAIDs), anticoagulants or corticosteroids, those with hematological disorders or stool samples positive for occult blood or hookworm ova, and those with duodenal or gastric ulcers or carcinoma stomach at endoscopy were excluded from the study.

Patients positive for *H pylori* infection by rapid urease test and on histology were randomly assigned to two groups (Group I and Ⅱ) by creating block sizes of six or eight and linking to five-digit random numbers (Rand Corporation; New York: The Free Press, 1955). Patients in Group I received oral ferrous sulfate tablets 200 mg thrice a day for 1 mo, and a 14-d course of anti-*H pylori* therapy consisting of clarithromycin 250 mg bid, lansoprazole 30 mg bid and tinidazole 500 mg bid. Those in Group Ⅱ received only oral ferrous sulfate tablets as above. All the above-mentioned biochemical and hematological parameters were assayed after 1 mo therapy. This study was approved by the ethics committee of JIPMER. Informed consent was obtained from all subjects.

Statistical analysis

Student's *t* test was used to estimate the differences between the groups. Correlation was assessed by the partial correlation analysis. All results are presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

All the parameters tested in both the *H pylori* infected

Table 1 Comparison of biochemical and hematological parameters in *H pylori* infected anemic patients and controls

Parameters	Control group (n = 16)	Test group (n = 22)
Hemoglobin (g/dL)	12.66 ± 0.74	6.9 ± 2.6[a]
Iron (μg/dL)	110.25 ± 26.58	32.72 ± 14.93[a]
Ferritin (ng/mL)	140.43 ± 30.72	28.82 ± 16.27[a]
Fructosamine (nmol/L)	1.60 ± 0.35	2.64 ± 0.93[a]
MDA (mmol/L)	2.26 ± 0.50	5.41 ± 2.16[a]
Glucose (mg/dL)	81.19 ± 9.74	83.50 ± 8.27

[a]$P < 0.05$ *vs* control group.

group and healthy controls are given in Tables 1 and 2. Fructosamine levels were significantly higher in *H pylori* infected patients compared to controls. Levels of lipid peroxides were significantly increased in the test group than the healthy age-matched controls. In the test group, a significant correlation ($r = 0.50$, $P = 0.02$) was observed between fructosamine and MDA using partial correlation analysis controlling for blood glucose level. As previously reported, hemoglobin, serum iron and ferritin levels were significantly reduced in *H pylori* infected patients when compared with controls.

Response to therapy

Patients in group I had a greater increase in mean hemoglobin level (2.98 g/dL) after one mo than those in Group Ⅱ (1.07 g/dL). The increases in mean serum iron (48 μg /dL *vs* 15.91 μg/dL) and ferritin levels (86.45 ng/mL *vs* 25.28 ng/mL) after one mo of treatment were also more marked in Group I than in Group Ⅱ. There was also a significantly decreased MDA levels in Group I when compared with Group Ⅱ after one mo of treatment. However, there was no significant alteration in fructosamine levels in both the groups after one mo of treatment.

DISCUSSION

Free radicals and other reactive oxygen species (ROS) are generated by all aerobic cells and are known to participate in a great variety of deleterious reactions[26]. The oxidative damage caused by free radicals is believed to play a pivotal role in the pathogenesis of *H pylori* infection[27-29]. Lipid peroxidation is one of the reactions set into motion as a consequence of the formation of these radicals in cells and tissues[26]. The initiation of lipid peroxidation has been considered the proximal cause of cell membrane destruction and cell damage[26]. Increased amounts of malondialdehyde (MDA) have been found in patients infected with *H pylori*[30-32]. Our results also indicate an increased lipid peroxidation in *H pylori* infected patients.

Lipid peroxidation can damage proteins, lipids, carbohydrates and nucleic acids. Plasma membranes are the critical targets of lipid peroxides[26]. Apart from participating in these deleterious reactions, lipid peroxides *in vitro* have been found to enhance the glycation of proteins[18]. We have also recently reported that MDA can enhance the glycation of hemoglobin *per se*[17].

Table 2 Changes in hematological and biochemical parameters of groups after treatment

Parameters	Group Ⅰ (*H pylori* treatment + oral iron therapy) (n = 11)	Group Ⅱ (Oral iron therapy) (n = 11)
Hemoglobin (g/dL)		
Baseline	7.42 ± 1.90	6.38 ± 2.37
1 mo after therapy	10.40 ± 1.11[a]	7.45 ± 1.94
Iron (μg/dL)		
Baseline	36.09 ± 18.97	29.36 ± 9.10
1 mo after therapy	84.09 ± 29.51[a]	45.27 ± 21.36[a]
Ferritin (ng/mL)		
Baseline	30.46 ± 17.81	27.18 ± 15.26
1 mo after therapy	116.91 ± 63.34[a]	52.46 ± 39.21
Fructosamine (nmol/L)		
Baseline	2.28 ± 0.62	2.99 ± 1.06
1 mo after therapy	2.13 ± 0.63	2.27 ± 0.97
MDA (mmol/L)		
Baseline	5.50 ± 2.46	5.32 ± 1.94
1 mo after therapy	3.11 ± 1.73[a]	3.81 ± 1.61

[a]$P < 0.05$ *vs* the baseline value before treatment.

We have found that lipid peroxides are closely associated with glycated hemoglobin in hyperthyroid, chronic renal failure and asthma patients[20-22]. We observed a significant association between MDA and fructosamine in non-diabetic asthma, nephrotic syndrome, rheumatoid arthritis and chronic renal failure patients[19,22-24].

In the present study, the levels of fructosamine were increased significantly in anemic patients infected with *H pylori* when compared with healthy controls. Among the various methods proposed for the measurement of glycated serum proteins, fructosamine is the method of choice for the clinicians[9]. As albumin is the most abundant protein in serum and contains multiple lysine residues, measurement of fructosamine is mainly the determination of glycated albumin[9].

In anemic patients, the concentrations of HbA_{1C} have been reported to be increased[14-16]. Several hypotheses have been formulated to explain the increase in glycated hemoglobin concentrations in these patients. It has been proposed that in iron deficiency the quarternary structure of the hemoglobin molecule may be altered, thus glycation of the β-globin chains occur more readily[14]. According to some investigators, the increase in glycated hemoglobin in non-diabetic anemic patients is mainly attributed to the decrease in hemoglobin levels in these patients[16].

To our knowledge, no study to date has attempted to clarify whether there is any increase in glycated protein levels in anemic, *H pylori* infected patients and whether this increase is the consequence of an increased lipid peroxidation among these patients.

To verify this hypothesis, we tested a well-defined group of anemic, *H pylori* infected patients before and after one month of treatment. In our study, the increased MDA was found to be significantly associated with increased fructosamine concentrations ($r = 0.50$, $P = 0.02$) before treatment, even when the proposed effect of

glucose on the concentrations of fructosamine was refuted by partial correlation analysis. The mechanism by which MDA enhances the glycation process has not been clearly elucidated. MDA is thought to enhance the process of protein glycation by acting as an anchor between sugar and hemoglobin moieties[18]. It has also been suggested that oxidative stress can facilitate the autoxidation of glucose to dicarbonyl intermediates, an early step in the Maillard reaction[33].

There was a significant decrease in MDA levels in *H pylori* infected patients after one month of treatment with both ferrous sulfate and anti-*H pylori* therapy. Previous reports have indicated that the levels of lipid peroxides decrease significantly after treatment for *H pylori* infection[30,32]. Similarly, in iron deficiency anemia it has been found that supplementation with iron reduces the levels of glycated hemoglobin[14]. There was no significant decrease in MDA levels in the test group treated with only oral iron. In both the test groups, there was no significant reduction in fructosamine levels after one month of treatment. There also existed no significant association in the anemic *H pylori* infected group between fructosamine and MDA after one month of treatment. One reason for these observed results can be attributed to the half-life of glycated proteins.

Recent studies have uncovered a myriad of pathological events induced by glycated albumin. These include increasing the expression of extracellular matrix protein, activation of protein kinase C, and stimulating the expression of transforming growth factor β_1 and its primary signaling receptor, the TGF-β type Ⅱ receptor. Apart from these alterations, glycated albumin has been found to alter the levels of NF-κB. These data support the hypothesis that glycated albumin is a sufficient stimulus to set into motion pathogenic signaling pathways. Several investigators have also reported that *H pylori* can activate NF-κB in human gastric mucosa *in vivo* and cultured gastric epithelial cells *in vitro*, thus the pathogenesis may be mediated through NF-κB[34-36]. Studies have shown that these alterations in molecular pathways play an essential role in the *H pylori* induced inflammation and associated complications.

In conclusion, this study gives a snapshot of an increased glycated serum protein and lipid peroxide levels in *H pylori* infected patients who were anemic as well. These data from the present study also supports the notion that alteration in the levels of MDA in anemic *H pylori* infected patients may be the basis for enhanced levels of fructosamine. Furthermore, the findings from our study indicate that treatment for both anemia and *H pylori* are required for reducing the levels of lipid peroxides in these patients.

It would be interesting to investigate the levels of fructosamine in non-anemic *H pylori* infected patients and it is worthwhile to investigate if additional supplementation of antioxidants would potentiate any further attenuation of protein glycation in *H pylori* infected patients when compared with the regular therapy. A longer follow-up investigation after treatment would shed more light into the above-mentioned alterations in anemic *H pylori* infected subjects.

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CASE REPORT

Esophagotracheal fistula caused by gastroesophageal reflux 9 years after esophagectomy

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Abstract

Fistula between digestive tract and airway is one of the complications after esophagectomy with lymph node dissection. A case of esophagotracheal fistula secondary to esophagitis 9 years after esophagectomy and gastric pull-up for treatment of esophageal carcinoma is described. It was successfully treated with transposition of a pedicled pectoralis major muscle flap.

Key words: Esophagus; Fistula; Surgery; Complications; Esophagitis; Reflux

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INTRODUCTION

The denervated stomach as an esophageal substitute recovers intraluminal acidity with time. Increased risk for acid reflux after incurs prolonged esophageal exposure to gastric acid which may lead to esophageal mucosal injury. A patient with esophagotracheal fistula caused by reflux esophagitis 9 years after esophagectomy with postoperative radiation therapy was treated with transposition of a pedicled pectoralis major muscle flap. This complication caused by esophagitis has been described.

CASE REPORT

A 59-year old man presented with a sudden onset of dyspnea and increasing cough productive of gastric contents and saliva. Significant past medical history included an esophagectomy with a reconstruction of gastric tube pulled up through the posterior mediastinum 9 years earlier for esophageal squamous cell carcinoma (pT1, pN0, pM0, pStage I), followed by irradiation (58 Gy) due to positive for malignancy of proximal surgical margin of the esophagus with histological examination. After the operation he had relapses of gastric ulcer and reflux esophagitis. There was no evidence of cancer recurrence over the follow-up period.

He was symptom-free except for cough after swallowing and regurgitation. None of abnormal shadows was detected in chest radiographs. Upper gastrointestinal endoscopy showed a fistula with a diameter of 5 mm on the anterior wall of the esophagus, 20 mm proximal to the esophagogastric anastomosis (Figure 1A), and a gastric ulcer just below the anastomosis (Figure 1B). No malignant feature was detected around the fistula and pathological examination of biopsy specimens from the gastric ulcer showed chronic inflammation. Bronchoscopic examination demonstrated the fistula in the membranous portion of the trachea, 90 mm distal to the vocal cord and 50 mm proximal to the bifurcation (Figure 2). Computed tomography confirmed the fistula located on a level with the upper wedge of sternum and no image of cancer recurrence (Figure 3). Twenty-four pH monitoring in the cervical esophagus was failed because of his rejection.

The patient received proton pomp inhibitors before operation with a slight improvement in his clinical symptoms, but no apparent improvement in the bronchoscopic examination. Surgery was performed 20 d after administration. A cuff of spiral endotracheal tube (8 mm in diameter) was situated below the fistula in the trachea. With the patient in the supine position, a U-shaped skin incision in the anterior cervix and median sternotomy to the 3rd rib were made. Previous radiation therapy made the procedure difficult. After lysis of adhesions, cervical esophagus and the upper portion of gastric tube were freed. Then, the common wall between esophagus and trachea was carefully dissected free in a cranial to caudal direction until the fistula was identified. No malignant changes were found macroscopically. The wall of the fistula was cut sharply, taking care to avoid tearing of the membranous portion of trachea widely. The fistula orifice in the esophageal wall was closed primarily in layers and the orifice in the trachea was also sutured primarily. The

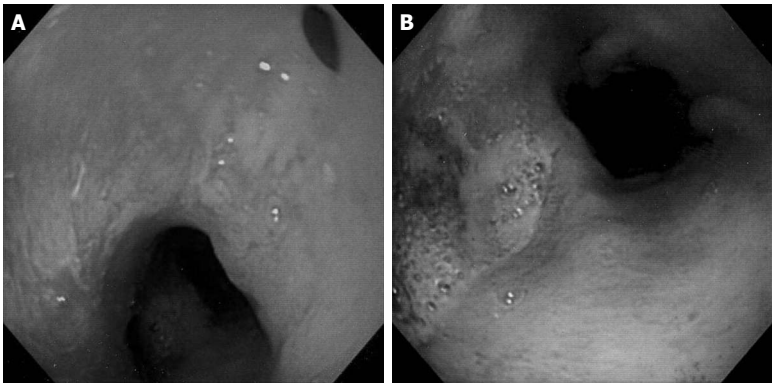

Figure 1 Upper gastrointestinal endoscopy showing a fistula in the esophagus, 20 mm proximal to the anastomosis (**A**), and a gastric ulcer just below the anastomosis (**B**).

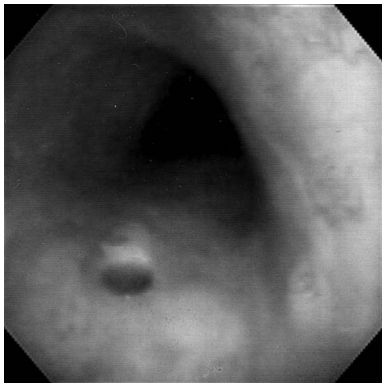

Figure 2 Bronchoscopic examination showing a fistula in the membranous portion of the trachea, 90 mm distal to the vocal cord.

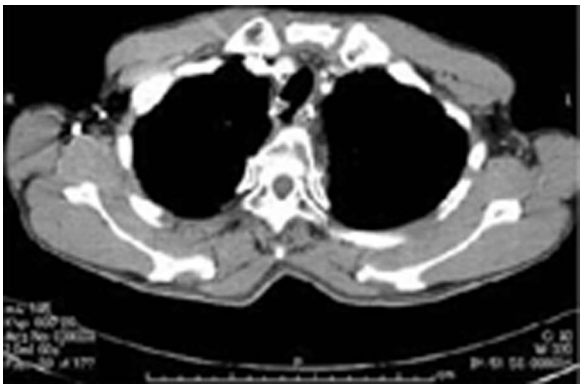

Figure 3 Computed tomography showing an esophagotracheal fistula located on a level with the upper wedge of sternum.

medial wedge of the left clavicle, 1st and 2nd ribs, and the left half of the sternum to the 2nd rib were excised. The left pectoralis major muscle flap was inserted and fixed between the trachea and the gastric tube. His endotracheal tube was extubated immediately after the operation. The patient's postoperative course was uneventful. On the 16th postoperative day, feeding was started when no leak was found after a gastrograffin swallow revealed no leak. The patient was discharged on the 28th postoperative day. He had no evidence of esophagitis, gastric ulcer and fistula 9 mo after the operation.

DISCUSSION

Fistulae between digestive tract and airway are recognized as complications after esophagectomy. The digestive tracts include residual esophagus, esophagogastric anastomosis and gastric tube. Leakage from esophagogastric anastomosis can lead to localized mediastinitis and represents a possible cause of tracheoesophagogastric fistula. Gastric tube-to-airway fistula develops due to peptic ulcer, ischemia of gastric tube or airway, radiation or abscess in the mediastinum. Fistula between cervical esophagus and trachea is the rarest case in these three types of fistulae. This fistula caused by gastroesophageal reflux after esophagectomy has not been reported previously.

Peptic ulcers of pulled-up gastric tubes often cause hemorrhage, perforation and penetration[1]. Gutschow e*t al*[2] reported that the denervated stomach as an esophageal substitute recovers a normal intraluminal acidity with time, so that more than 3 years after surgery the 24-hour pH profile in the gastric cavity of almost all patients is similar to that in healthy subjects. Shibuya[3] showed that sever esophagitis is found in 75.6% of remnant esophagus after esophagectomy for esophageal cancer. Gutschow *et al*[2] showed that 38.5% of patients have reflux esophagitis in the remnant esophagus for 3 years or more after esophagectomy. In our patient, endoscopic examination more than 4 years after esophagectomy revealed often severe esophagitis and gastric ulcer. Although he was treated with a proton pomp inhibitor every time, he discontinued himself taking drugs because of symptom-free. Therefore, it is strongly suggested that repetition of severe esophagitis and postoperative radiation therapy influenced formation of his fistula. In this patient, *H pylori*-IgG was positive. Although evaluation of his gastric emptying was not made, he often had reflux-symptom after esophagectomy.

Myocutaneous or muscle flap, because of their rich blood supply, can be used to fill the dead space after fistulectomy and add vital tissue to prevent recurrent fistulization[1,4-6]. In the part of cervical area, sternocleidomastoid muscle and pectoralis major have been used. If the esophagus and/or gastric tube cannot be preserved, continuity of the gastrointestinal tract may be reconstructed with, for example, colonic or small bowel interposition[7].

No significant relationship has been found between reflux symptoms, such as heart burn and regurgitation, and endoscopic findings after esophagectomy in the previous reports[3,8]. Patients with denervated stomach may be free from symptoms of gastric ulcer. Therefore, endoscopic examination is necessary at least once a year for early detection of ulceration and esophagitis. Fistulae between digestive tract and airway are grave complications of esophagectomy because they frequently result in respiratory failure and life-threatening conditions. If gastric ulcer or esophagitis is detected in endoscopic examination after esophagectomy, this therapy should be

started immediately regardless of the interval between the examination and esophagectomy.

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CASE REPORT

A case of biliary gastric fistula following percutaneous radiofrequency thermal ablation of hepatocellular carcinoma

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Abstract

Percutaneous radiofrequency thermal ablation (RFA) is an effective and safe therapeutic modality in the management of liver malignancies, performed with ultrasound guidance. Potential complications of RFA include liver abscess, ascites, pleural effusion, skin burn, hypoxemia, pneumothorax, subcapsular hematoma, hemoperitoneum, liver failure, tumour seeding, biliary lesions. Here we describe for the first time a case of biliary gastric fistula occurred in a 66-year old man with a Child's class A alcoholic liver cirrhosis as a complication of RFA of a large hepatocellular carcinoma lesion in the Ⅲ segment. In the light of this case, RFA with injection of saline between the liver and adjacent gastrointestinal tract, as well as laparoscopic RFA, ethanol injection (PEI), or other techniques such as chemoembolization, appear to be more indicated than percutaneous RFA for large lesions close to the gastrointestinal tract.

Key words: Radiofrequency thermal ablation; Hepatocellular carcinoma; Biliary gastric fistula; Complications

Falco A, Orlando D, Sciarra R, Sergiacomo L. A case of biliary gastric fistula following percutaneous radiofrequency thermal ablation of hepatocellular carcinoma. *World J Gastroenterol* 2007; 13(5): 804-805

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INTRODUCTION

Percutaneous radiofrequency thermal ablation (RFA) is an emerging therapeutic modality in the management of liver malignancies[1], particularly in patients who cannot undergo surgery[2]. The increasing attractiveness of this technique is due to its effectiveness, safety and low morbidity rate[1,2]. Compared with ethanol injection (PEI), the first percutaneous therapy to be introduced, RFA is characterized by the need of a reduced number of treatment sessions. Both therapies are generally performed with ultrasound (US) guidance which allows considerable ease of application, due to their low costs and no X-ray exposure. Nevertheless, a number of potential complications of RFA have been described, occurring with a rate of almost 9%[3], including liver abscess, ascites, pleural effusion, skin burn, hypoxemia, pneumothorax, subcapsular hematoma, hemoperitoneum, liver failure, and tumour seeding. In addition, biliary lesions have been described in 1% of a large number of patients included in a review of the literature in this field[3], and two case reports describing enterobiliary[4] and biliary pleural[5] fistulae have been published so far.

CASE REPORT

Here we describe a case of biliary gastric fistula occurred in a 66-year old man with a Child's class A alcoholic liver cirrhosis as a complication of RFA of a large (4.0 cm × 4.5 cm) hepatocellular carcinoma (HCC) lesion in the III segment. Written informed consent was obtained from the patient before treatment. The ablation was performed under US-guidance [Eidos (EUB-525), Esaote, Genova, Italy] using a 3.5 MHz sector probe, with a lateral guide for electrode placement. The treatment was made under general anesthesia using a 20-cm long, 18-gauge electrode to apply the RF current (MIRAS RC, Invatec Italia, Brescia, Italy). The procedure lasted 30 min without apparent complications. In the night following the intervention, the patient experienced transient retching, responsive to metoclopramide administration, and moderate abdominal pain. An enhanced computed axial tomography (CT) scan of the abdomen, performed 22 d after the procedure, showed the presence of aerobilia within the left portion of the liver attributed to a biliary gastric fistula (Figure 1A). A subsequent conventional contrast-enhanced study of the first gastrointestinal tract, showed a fistulous tract starting from the lesser curvature of the stomach with concomitant spasm of the greater curvature, causing a communication with the left portion of the liver (Figure 1B).

DISCUSSION

Biliary gastric fistulae can be diagnosed on the basis of both direct and indirect radiological signs. The diagnosis

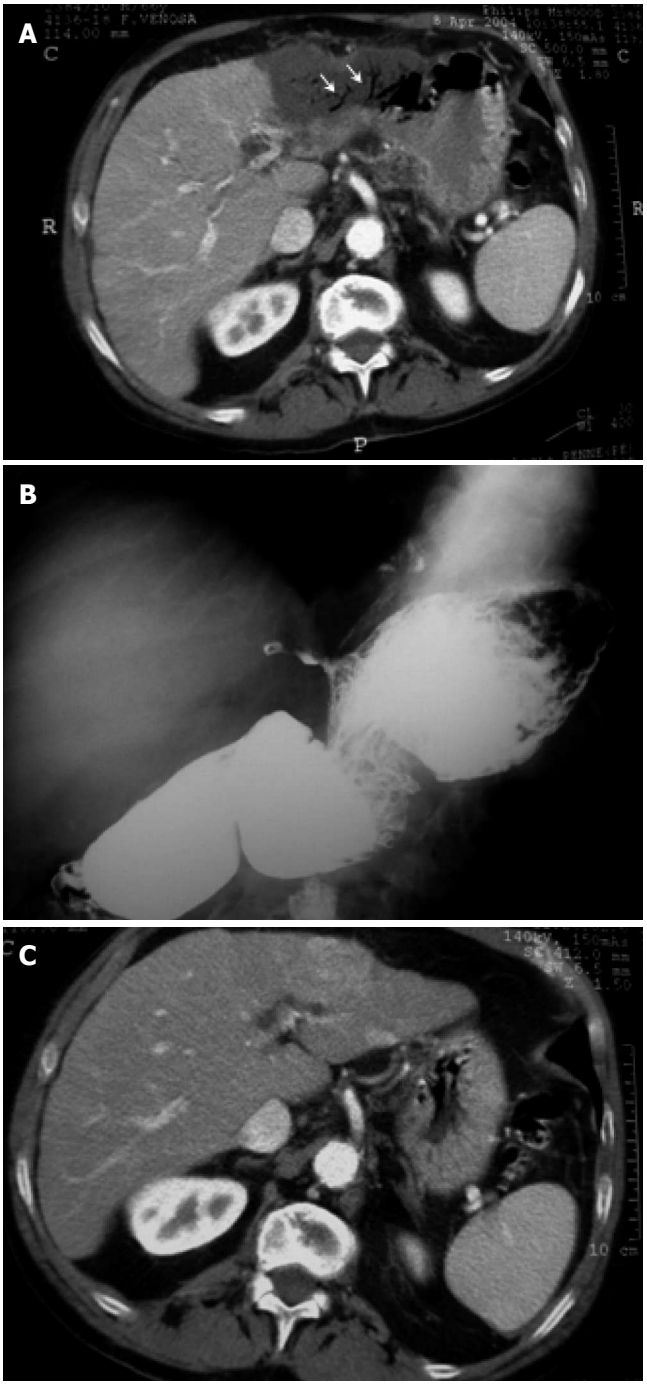

Figure 1 Enhanced CT abdominal scan performed after the percutaneous procedure showing air inclusions in the bile ducts within the left portion of the liver (**A**), radiograph of the first gastrointestinal tract showing a biliary gastric fistula starting from the lesser curvature of the stomach with concomitant spasm of the greater curvature (**B**), and enhanced CT scan of the abdomen performed before percutaneous thermal ablation of the HCC lesion in the III liver segment showing a left portion of the liver without aerobilia (**C**).

with indirect radiological criteria was made by enhanced CT. The presence of aerobilia confined to the left portion of the liver (Figure 1A), in the absence of surgery for biliary-digestive by-pass procedures and the fact that the thermoablative treatment was performed after a previous CT in which the fistula was not present (Figure 1C), led to the hypothesis of a iatrogenic alteration. This was confirmed by a conventional contrast-enhanced study of the first gastrointestinal tract, showing a fistulous tract starting from the lesser curvature of the stomach with concomitant spasm of the greater curvature, causing a communication with the left portion of the liver (Figure 1B).

Perforation of the gastrointestinal tract, a possible major complication of RFA of hepatic nodules, can be difficult to recognize as its clinical manifestations can be attributed to the normal course of the so called "post-ablation syndrome". Indeed, mild to moderate abdominal pain is not uncommon after RFA of large lesions, particularly when they abut the Glisson's capsule containing nerve endings.

We describe here for the first time in the literature a case of biliary gastric fistula as a possible complication of thermal ablation of HCC lesions. A recent study has shown the efficacy and safety of percutaneous RFA of HCC abutting the gastrointestinal tract[6]. Nevertheless, in the light of this case and previous descriptions of enterobiliary fistulae occurred following the same therapeutic modality, RFA with injection of saline between the liver and adjacent gastrointestinal tract, as well as laparoscopic RFA, PEI, or other techniques such as chemoembolization, appear to be more indicated than percutaneous RFA for large lesions close to the gastrointestinal tract.

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CASE REPORT

Spontaneous rupture of a hepatic hydatıd cyst into the peritoneum causing only mild abdominal pain: A case report

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Abstract

Hydatid disease is an endemic disease in certain areas of the world. It is located mostly in the liver. Spontaneous rupture of the hydatid cyst into the peritoneum is a rare condition, which is accompanied by serious morbidity and mortality generally. We present herein a case with a spontaneous rupture of a hepatic hidatid disease into the peritoneum without any serious symptoms. A 15-year-old boy was admitted to the emergency room with a mild abdominal pain lasting for a day. Physical examination revealed only mild abdominal tenderness. There was no history of trauma or complaints related to hydatid diseases. Ultrasonography showed a large amount of free fluid and a cystic lesion with irregular borders in the liver. He was operated on. Postoperative albendazol therapy was given for 2 mo. No recurrence or secondary hydatidosis was seen on CT investigation in the 3rd, 6th and 12th mo following surgery.

Key words: Hydatid disease; Spontaneous rupture; Liver

Karakaya K. Spontaneous rupture of a hepatic hydatıd cyst into the peritoneum causing only mild abdominal pain: A case report. *World J Gastroenterol* 2007; 13(5): 806-808

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INTRODUCTION

Hydatid disease is a parasitic infestation caused by Echinococcus granulosus[1-3]. Hydatid disease is an endemic problem in Turkey as well as in sheep-bearing regions of the world[1,2,4].

Although hepatic hydatid disease may be asymptomatic for many years, it can become symptomatic due to expansion, rupture or pyogenic infection[2,3,5]. Hydatid cyst rupture may cause mild to fatal complications[2,5]. Rupture of the hepatic hydatid disease generally occurs into the biliary tree. Rupture of the cyst in the peritoneal cavity is rare and generally followed by anaphylactic reactions.

In this study, we present a case of a spontaneous rupture of a hepatic hydatid cyst into the peritoneum, in which the patient was admitted to the emergency room for mild abdominal pain without any other symptom. Spontaneous rupture of the hepatic hydatid cyst into peritoneum without any serious symptom is unusual.

CASE REPORT

A 15-year-old boy perceived sudden abdominal pain in the epigastrium and right upper quadrant a day ago. After a short duration his pain was ameliorated spontaneously. The following day he perceived mild pain in the right lower quadrant. He had no pruritis, erythema or any other symptom except the abdominal pain. No history of blunt trauma was found.

Physical examination revealed a blood pressure of 100/70 mmHg, and a pulse rate of 78 beats/min. The patient was afebrile. Abdominal palpation revealed mild tenderness. He was comfortable on his movements. There was no history of nausea, vomiting or loss of appetite. Laboratory investigations were normal except mild leukocytosis (WBC: 11 000/mm^3).

Abdominal ultrasonography showed a large amount of free fluid and a cystic lesion measuring 10 cm × 12 cm with irregular borders in the right lobe of the liver. The anterior border of the cyst was incomplete. Floating membrane image was seen. The biliary ducts were normal in calibration.

The abdomen was exposed through a right paramedian incision. Approximately 3500 mL clear fluid was aspirated from the intraperitoneal space. There was a hydatid cyst in the right lobe of the liver. The cyst was 13 cm in size and had a ruptured area of 5 cm × 7 cm anteriorly (Figure 1). There were firm adhesions between the liver and right diaphragm. There was no daughter vesicle in the abdominal cavity. The germinative membrane was taken out completely (Figure 2). No biliary leakage was seen. We could not find any other pathological evidence on exploration. The cyst pouch was irrigated with hypertonic saline (20%) and then followed by isotonic saline. The peritoneal cavity was irrigated with isotonic saline. Introflexion procedure was performed. Latex drains were

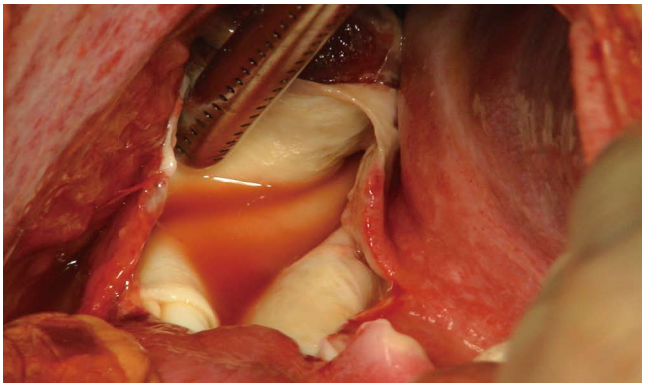

Figure 1 Ruptured hydatid cyst in the liver.

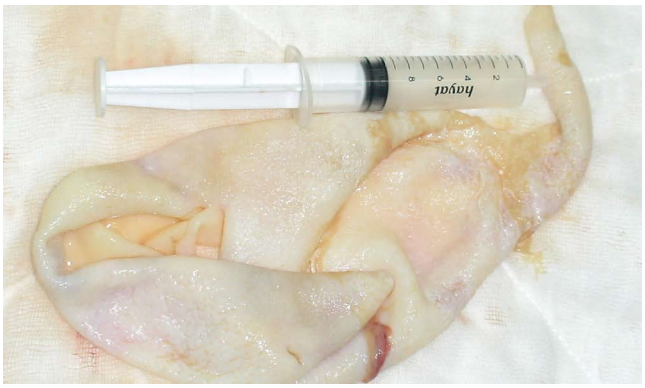

Figure 2 Germinative membrane extracted totally.

placed into the sub-hepatic and recto-vesicle areas.

The patient was discharged from the hospital on the 9th postoperative day without any complication. Postoperative albendazole therapy (10 mg/kg) was prescribed for 2 mo under the liver enzyme and blood count monitoring. The patient was controlled as an outpatient. Postoperative follow up was uneventful for 13 mo. No recurrence or secondary hydatidosis was seen on CT investigation in the 3rd, 6 th and 12th mo following surgery.

DISCUSSION

The hydatid cyst is typically filled with clear fluid (hydatid fluid). The cyst consists of an internal cellular layer (germinal layer) and an outer, acellular layer (laminated layer). As the cyst expands gradually, a granulomatous host reaction followed by a fibrous reaction forms a connective tissue layer, which is called a pericyst[1].

Hydatid disease is a serious health problem in endemic areas as well as in Turkey [6,7]. The diagnosis and appropriate surgical therapy is usually delayed because most of the hydatid cysts remain asymptomatic until it is getting complicated [1-3,7,8].

Precautions for the hydatid disease are mostly insufficient. Treatment of dogs with antihelmintic is the main procedure to control the parasite (Echinococcus spp.)[9]. In rural areas of Turkey this treatment is not applied routinely. In the rural area, sheep are home-slaughtered routinely. Dogs can access to the infected viscera. This is true in almost all of the developing or underdeveloped countries in the world[9].

Initially almost all of the hydatid cysts are asymptomatic. Later in time some symptoms depending on the involved organ, localisation of the cyst and pressure effect of the cyst on the surrounding tissues and structures develop[1,7,10]. Abdominal pain is the most commonly encountered symptom. The second one is the symptoms arising from the pressure effect of the cyst[7,8,11]. It is known that rupture of the cyst ameliorates the abdominal pain and the patient feels comfortable to some degree[2]. Later in time rupture of the hydatid cyst produces symptoms and signs of allergy or peritoneal irritation almost all the time[10]. No history of previous abdominal pain or any other symptom relating to the hydatid cyst was present in the case presented here.

Diagnosis of the hydatid cyst is mainly based on ultrasonography and computed tomography (CT)[1-3,7,8,10,11]. CT is especially valuable for the 3-dimensional localisation of the cyst preoperatively[8,11]. MRI, magnetic resonance cholangio pancreatography (MRCP), scintigraphy scans and laparoscopy may be useful for diagnosis of the rare, undiagnosed cases and for its complications. As we made the diagnosis of a ruptured hepatic hydatid cyst by US examination, the other investigational techniques were not used for preoperative evaluation.

Surgery is the preferred treatment for the cure of the hydatid disease[3,6,8]. Surgical procedures may differ from percutaneous aspiration instillation and reaspiration to complete excision of the cyst[1,6,7]. We performed introfleksion procedure on the patient. Rupture of the cyst is usually related to increased intracystic pressure. This may be related to trauma, or over enlargement of the cyst[2,10]. Perforation of the hydatid cyst may cause dissemination of the parasite and increased morbidity and mortality rate[1,2,9]. As cyst size increases, risk of rupture increases[5]. Cyst size measured 13 cm preoperatively in our case. Intraperitoneal rupture of the hydatid cyst can cause abdominal pain, allergy and anaphylaxis[1,2,5]. Mild abdominal tenderness was present in our patient on admission. The patient had no history of trauma or any event that increases intra-abdominal pressure such as coughing or constipation.

Medical treatment with albendazole is described elsewhere[1,6,7,10,11]. The patient was treated with albendazole 10 mg/kg for 2 mo under liver enzyme and blood count monitoring. Postoperative follow up was uneventful.

Intra-peritoneal hydatidosis is a major problem for the patients who have hydatid cyst rupture. The patient presented here had no recurrence or secondary hydatidosis.

In endemic regions, it is useful to consider hydatid cyst disease for patients with abdominal pain admitted to the emergency room. Rupture of the hydatid cyst may be fatal.

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CASE REPORT

Carcinosarcoma of the liver with mesenchymal differentiation

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Abstract

We report an extremely rare case where a mesenchymal differentiation, especially embryonal sarcoma, was demonstrated in cholangiocarcinoma. At autopsy, a yellowish-white tumor (15 cm x 12 cm) was found in the right hepatic lobe, and there were several daughter nodules in both hepatic lobes. Histologically, most of the main tumor and all of the daughter nodules examined showed sarcomatous changes (spindle cells, pleomorphic cells and hyalization). Histologic examination of a part of the main tumor disclosed a focus of adenocarcinoma within the tumor. The frequent transitions between the adenocarcinomatous areas and the sarcomatous areas suggested that sarcomatous transformation occurred in the cholangiocarcinoma and then spread rapidly. Immunohistochemically, the adenocarcinomatous elements were positive for cytokeratin, carcinoembryonic antigen (CEA) and epithelial membrane antigen, and negative in the sarcomatous cells. Vimentin was positive only in the sarcomatous elements. The findings of the present case support the view that carcinosarcomas represent carcinomas that develop sarcomatous elements via metaplasia of the epithelial element.

Key words: Cholangiocarcinoma; Carcinosarcoma; Mesenchymal differentiation

Sumiyoshi S, Kikuyama M, Matsubayashi Y, Kageyama F, Ide Y, Kobayashi Y, Nakamura H. Carcinosarcoma of the liver with mesenchymal differentiation. *World J Gastroenterol* 2007; 13(5): 809-812

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INTRODUCTION

Cholangiocarcinoma is usually a moderately to well differentiated adenocarcinoma with considerable demoplastic reactions. Nakajima *et al*[1] reported that 92 of 102 cases of cholangiocarcinoma were mucin-producing adenocarcinomas, and the remaining were adenosquamous (3 cases), squamous (3 cases), mucinous (1 case), or anaplastic carcinoma (3 cases). Only brief descriptions of sarcomatous changes and sarcomatous variants of cholangiocarcinoma can be found in a few studies or reviews dealing with a large series of cholangiocarcinoma cases[1,2] . However, detailed clinicopathological studies of sarcomatous changes in cholangiocarcinoma have not been reported in English and Japanese literature to the best of our knowledge.

Because we recently encountered an autopsy case of cholangiocarcinoma showing sarcomatous changes, we report this case to emphasize the autopsy findings, as well as the histogenesis of sarcomatous changes in cholangiocarcinoma.

CASE REPORT

A 74-year old Japanese woman was admitted to our hospital complaining of pain in the right hypochondrium and underwent a cholecystectomy. The main laboratory data were as follows: red blood cells 378 × 10^4/mm^3, white blood cells 5200/mm^3, serum total protein 8.0 g/dL, total bilirubin 0.8 mg/dL, serum glutamic-oxaloacetic transaminase 46 IU/L, serum glutamic-pyruvic transaminase 58 IU/L, lactic acid dehydrogenase 416 IU/L, alkaline phosphatase 476 IU/L, γ-glutamyl transpeptidase 119 IU/L, and C- reactive protein 2.9 mg/dL. Carcinoembryonic antigen (CEA) was 51.5 ng/mL, but serum α-fetoprotein (AFP), a protein induced by vitamin K absence or antagonists (PIVKA-Ⅱ) and carbohydrase antigen 19-9 (CA19-9) were within the normal range. Hepatitis B core (HBc) antibody, hepatitis C antibody (HCV-Ab), and human immunodeficiency virus (HIV) were all negative. A computed tomography scan of the abdomen revealed a low-density mass with renal invasion in all segments of the right hepatic lobe, without lymph node swelling or dilatation of the intrahepatic bile ducts. Magnetic resonance imaging revealed hypointensity on the T1-weighted images and heterogeneous hyperintensity on the T2-weighted images (Figures 1A and B). Angiography showed a malignant blush in the right lobe (not shown). A sonographically guided hepatic tumor biopsy showed the

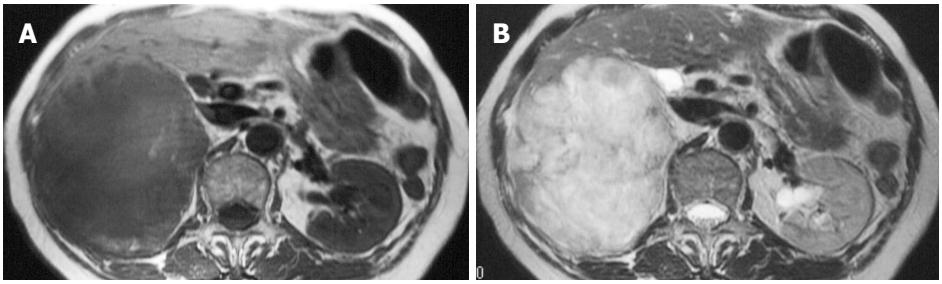

Figure 1 Magnetic resonance imaging showing hypointense areas in the right lobe of the liver on a T1-weighted image (**A**) and an inhomogeneously hyperintense mass on a T2-weighted image (**B**).

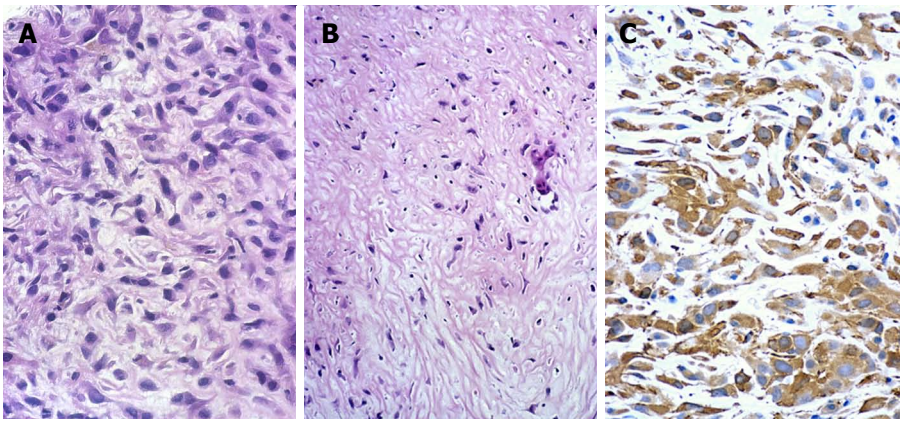

Figure 2 Needle biopsy showing a sarcomatous area consisting of interlacing bundles of atypical spindle cells [hematoxylin-eosin stain; magnification x 200 (**A**), x 100 (**B**)] and immunohistochemical staining showing positive α-SMA (**C**).

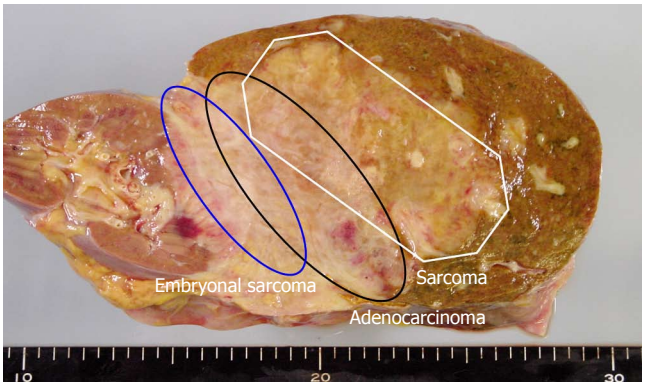

Figure 3 Gross appearance of hepatic tumor.

proliferation of spindle cells. An immunohistochemical study showed that α-smooth muscle actin (SMA) was positive, and keratin, vimentin, desmin, CEA, and S-100 protein were negative, thus leiomyosarcoma was suspected (Figure 2). Her general condition gradually worsened and she died of hepatic failure and disseminated intravascular coagulation (DIC) after two months.

Autopsy findings

The gross findings were a yellowish-white tumor (15 cm × 12 cm) with blurred borders in the right hepatic lobe (Figure 3) where the right branch of the portal vein and right kidney were occluded.

Microscopically, the majority of the main tumor and all daughter nodules examined showed a sarcomatous appearance. Elongated cells were arranged in bundles, occasionally interlacing. These histologic features were similar to those of fibrosarcoma or leiomyosarcoma. That is, these areas were composed of nonadhesive spindle-shaped or fusiform cells, and to a lesser degree, pleomorphic giant or multinuclear cells, the majority of the latter showing bizarre nuclei and prominent nucleoli (Figure 4). These sarcomatous areas looked like malignant leiomyosarcoma. There were many foci of coagulative necrosis within these sarcomatous areas. In addition, there was a well-differentiated tubular adenocarcinoma within the tumor (Figure 5). There were direct transitions between adenocarcinomatous elements and sarcomatous elements. However, the transitions were unclear. A hydropsy-like part was recognized at the side edge of the tumor, and tumor cells floating in mucinous cytoplasm were evidence of undifferentiated (embryonal) sarcoma (Figure 6).

These two elements on the representative section of tumor were mapped (Figure 3), showing adenocarcinomatous elements within the tumor and a considerable amount of sarcomatous elements surrounding this carcinoma. There were no histologic elements suggestive of hepatocellular carcinoma.

The nontumorous hepatic tissue showed nonspecific reactive changes with mild fibrous enlargement of the portal tracts, but not liver cirrhosis. There were no regenerative nodules throughout the liver, and HBsAg was not detected in the liver by orcein stain.

Primary antisera to keratin, vimentin, desmin, AFP, CEA, and S-100 protein were obtained from DAKO Corporation. Keratin was weakly positive in the adenocarcinoma cells. There was positive staining for CEA at the part of tubular adenocarcinoma. Vimentin was expressed only in the sarcomatous cells. There were no positive reactions for AFP, desmin, S-100 protein, or CA19-9 in the tumor cells.

It was diagnosed as cholangiocarcinoma and carcino-

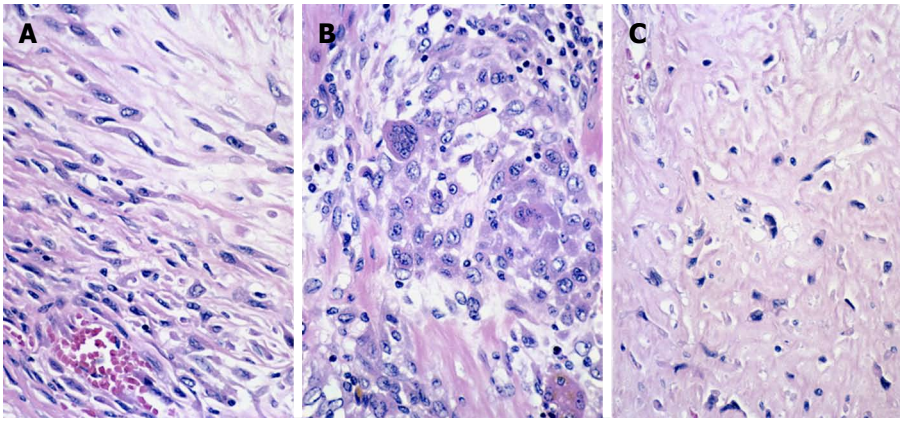

Figure 4 Spindle cells (**A**), pleomorphic areas (**B**), or hyalization (**C**) in leiomyosarcoma (hematoxylin-eosin stain; magnification x 160).

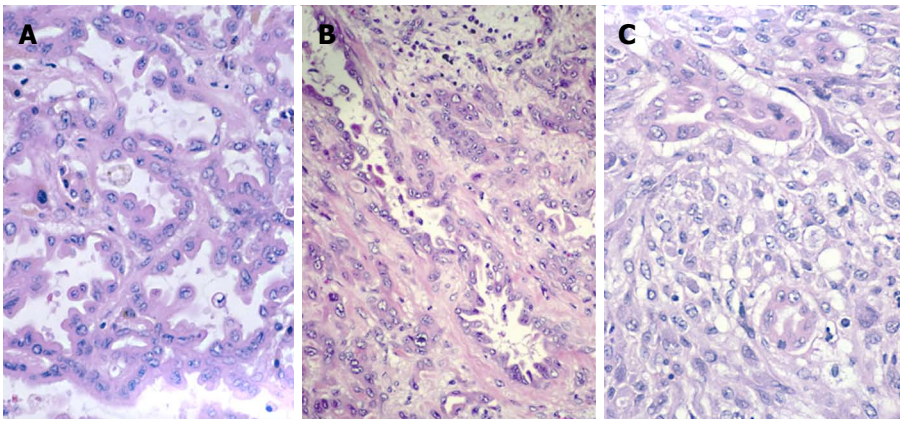

Figure 5 Cholangiocarcinoma focus (**A**) (hematoxylin-eosin stain; magnification x160), component surrounded by fibrosis (**B**) (hematoxylin-eosin stain; magnification x100), intimately mixed carcinomatous and sarcomatous components (**C**) (hematoxylin-eosin stain; magnification x 160).

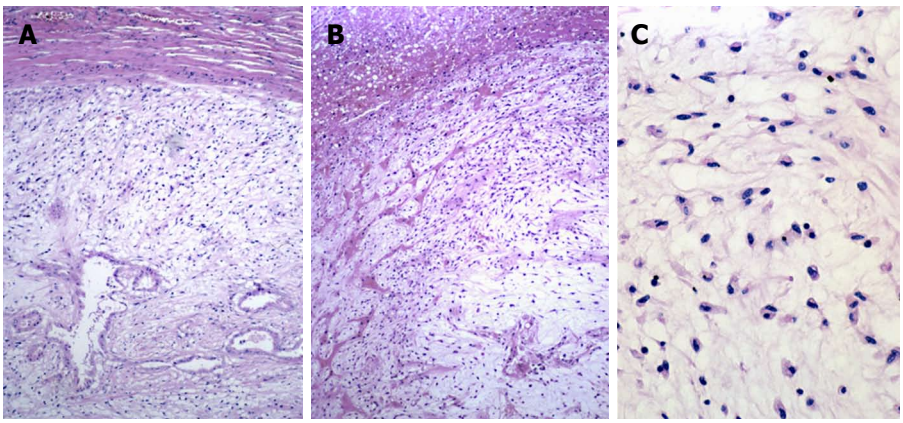

Figure 6 Sarcomatous portion of the tumor consisting of undifferentiated (embryonal) sarcoma [hematoxylin-eosin stain; magnification x 25 (**A**, **B**), x 160 (**C**)].

sarcoma with mesenchymal differentiation that specialized and coexisted in a lobe corollary tumor.

DISCUSSION

Sarcomatous transformation of primary cancer of the liver in adults is most common in hepatocellular carcinoma (HCC), especially after anticancer chemotherapy or transarterial embolization therapy[3]. In our patient, there were no lesions resembling HCC, and AFP was negative in the tumor, so that the derivation of sarcomatous cells from HCC was excluded.

Histologic mapping of a whole section of the main tumor showed adenocarcinomatous and sarcomatous elements within the main tumor. Considering the mapping, the sarcomatous elements would be interpreted as the result of the metaplastic transformation of pre-existing adenocarcinoma cells[4].

In our case, direct transitions between sarcoma and adenocarcinoma in the main tumor suggested that the sarcomatous changes might have developed secondarily from pre-existing cholangiocarcinoma. Most of the daughter nodules showed such sarcomatous changes, suggesting that sarcomatous elements grow and spread more rapidly than the adenocarcinomatous elements.

Thompson *et al*[5] stated that the carcinomatous and sarcomatous components may be monoclonal in origin and derived from single stem cells. Genetic analysis is one possible solution to this problem, as reported in esophageal carcinosarcoma[6].

What is more, various other hypotheses have been proposed to explain the biphasic appearance of sarcomatoid carcinomas[7,8]. Briefly, the explanations include the collision theory of independent neoplastic growth from multipotent stem cell origins, epithelial to mesenchymal conversion by epithelial-stromal interaction, and a combination of the two. The salient features in our case were the presence of dysplasia and adenocarcinoma *in situ*, morphological "transition" between carcinomatous and sarcomatous tissues in relation to expansion of invasion, and the detection of sarcomatous characteristics by immunohistochemistry in the epithelial component, which strongly support the histogenesis of epithelial to mesenchymal conversion. Gentile *et al*[9] reported that the presence of productive retroviral infection in the sarcomatous cells is related with tumor progression from the carcinomatous to the sarcomatous phase. The examination I performed confirmed that it was negative for viruses, but it was related with an unknown virus. However, why sarcomatous changes develop is a mystery.

In summary, sarcomatoid carcinoma derived from cholangiocarcinoma is an extremely rare tumor composed of mixed malignant epithelial and mesenchymal cells, with only 12 cases[10-12] reported to date who died of liver failure due to extensive metastatic growth of sarcomatoid carcinoma despite postoperative chemotherapy. The histologic features, stage, and outcome of the reported cases indicate that this neoplasm generally pursues a highly aggressive and malignant biological course with rapid growth and wide local infiltration, leading to a poor prognosis. Radical surgery with adjuvant chemotherapy, and close follow-up are necessary for the management of this disease.

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CASE REPORT

Metastatic melanoma to the common bile duct causing obstructive jaundice: A case report

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Abstract

Metastatic melanoma to the common bile duct is very rare with only 18 cases reported so far. We report a 46 year old women who, 18 mo after excision of a skin melanoma, developped a painless progressive obstructive jaundice. At operation a melanoma within the distal third of the common bile duct was found. There were no other secondaries within the abdomen. The common bile duct, including the tumor, was resected and anastomosed with Roux-en-Y jejunal limb. The patient survived 31 mo without any sign of local recurrence and was submitted to three other operations for axillar and brain secondaries, from which she finally died. Radical resection of metastatic melanoma to the common bile duct may result in lifelong relief of obstructive jaundice. It is safe and relatively easy to perform. In other cases, a less aggressive approach, stenting or bypass procedures, should be adopted.

Key words: Metastatic melanoma; Common bile duct; Jaundice

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INTRODUCTION

The great majority of malignant melanomas arise from the skin, squamous mucose membranes, retina, and leptomeninges. They can metastasize to almost every organ of the body. The intraabdominal viscera are involved in diffuse metastatic disease in approximately 60% of patients[1-3]. DasGupta and Brasfield in 1964 reported a 15% involvement of the gallbladder and 6% involvement of the remainder of the biliary tree[2].

CASE REPORT

A 46-year-old woman was admitted to our hospital with painless progressive obstructive jaundice. Previously she had been submitted to excision of a skin melanoma on the back in a local hospital. Our laboratory data showed an elevated bilirubin (235.9 μmol/L, direct bilirubin 138.4 μmol/L), alkaline phosphatase (1540 U/L), gama GT (638 U/L), SGOT (525 U/L), SGPT (870/U/L), and ESR (76/1 h). Ultrasonography showed a dilated gallbladder and common bile duct and suspected stone within the distal third of the common bile duct. In December 1996, a cholecystectomy was performed. An operative cholangiography through the cystic duct showed a tumorous mass within the distal common bile duct causing almost complete obstruction (Figure 1). The duodenum was mobilized, the common bile duct was opened and a piece of dark soft tumour tissue was removed. Frozen section biopsy showed a malignant melanoma. The common bile duct was then carefully dissected and transected 1 cm away from the lower edge of the tumor. The duct was proximally resected close to the convergence of the hepatic ducts. The distal end of the common bile duct was oversewn and the proximal end was anastomosed with Roux-en-Y jejunal limb. A careful search showed no other secondaries within the abdomen.

The recovery was uneventful. Histopathology revealed ill-defined epithelioid tumor cell aggregates infiltrating bile ducts and the surrounding fibro-adipose structures. There was strong brown granular intracytoplasmic pigmentation in some parts of the infiltrate (Figures 2 and 3). Fontana-Masson histochemical staining confirmed melanin pigment and positive strong anti-S100 protein and anti-HMB45 immunoreactivity with weak positivity to anti-melan A antibodies proved the diagnosis of a metastatic melanoma. The patient survived 31 mo without any signs of local recurrence. She had to be submitted to another three surgeries for the axillar and brain secondaries, which were a final cause of her death.

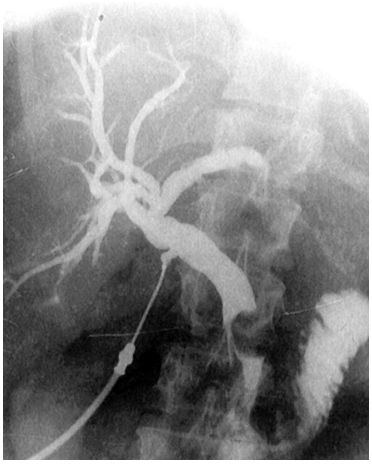

Figure 1 Showing filling defect within the distal common bile duct causing almost complete obstruction.

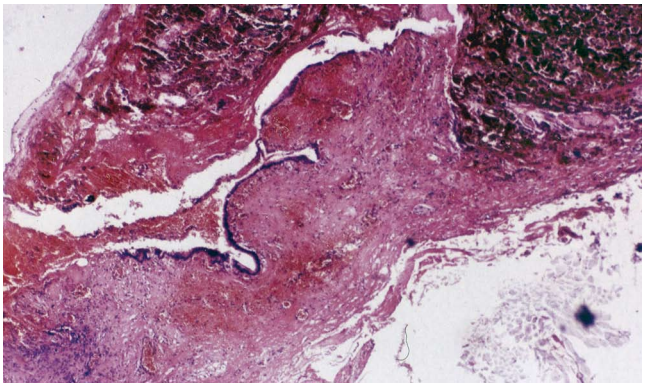

Figure 2 A pigmented malignant tumour evidently infiltrates extrahepatic bile duct. Later histochemical and immunohistochemical analyses showed melanoma cells (HE, × 13).

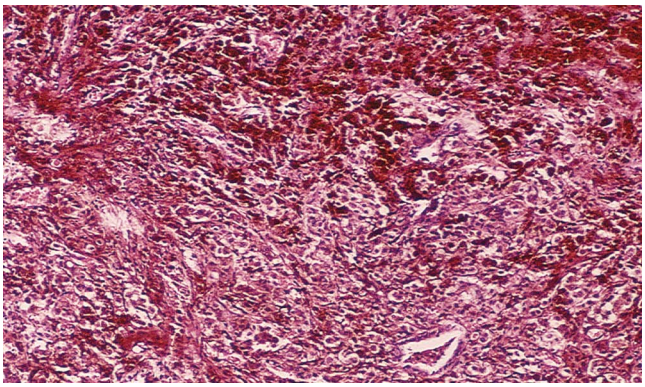

Figure 3 Higher magnification of the same tumour field showed insular and trabecular-to-solid histological organization of melanoma cells. Most of the cells were hyperpigmented with massive intracytoplasmic melanosome granules (HE, × 64).

DISCUSSION

The first case of a metastatic melanoma to the bile duct was described by Spigelberg in 1895, and the second by Duval in 1908[4]. A search throughout Medline showed 18 metastatic melanomas to the common bile duct reported so far[4-19]. Within this period, about 20 cases of primary malignant melanomas of the common bile duct have been described. However, in spite of confirmatory immunohistochemical stains, electron microscopic studies, the presence of junctional activity adjacent to the tumour, and tests to rule out other possible remote or concurrent primary sites, absolute exclusion of a metastatic melanoma from an unknown occult site or regressed site is not entirely possible[20]. The melanoma secondaries usually arise from the primary skin lesion but occasionally they may arise from primary or metastatic melanoma of the gallbladder[7,12,16].

Patients with metastatic melanoma to the common bile duct usually present with progressive painless obstructive jaundice[4,19]. Rarely the patients have hematobilia[10], pain[5,8], or cholangitis[19]. Laboratory data show a cholestatic jaundice. On ultrasonography this tumour is echogenic with little or no acoustic shadowing[9]. The exact preoperative diagnosis of the tumour is established, almost exclusively when localised at the Vater's papilla so that a biopsy can be taken[18], or on the basis of histologic examination of a small fragment of the tissue retrieved from the bile duct with a Dormia basket after papilotomy[19].

The current literature has few recommendations with regard to appropriate treatment of the melanoma metastases to the bile ducts[14,19]. It seems reasonable to perform radical surgical resection in patients with potentially curable disease and isolated deposits in the bile duct[14,19]. We believe it is paticularly indicated if resection is not too dangerous and if it is relatively easy to perform, as in the present case. A resection may be necessary in cases of serious hematobilia[10]. If there is a concurrent metastatic disease elsewhere, it is prudent to adopt a less aggressive approach in order to relieve obstructive jaundice, such as the bypass procedures[14], or stenting[18,19].

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Meetings

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

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
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Sevilla
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Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
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Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
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Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

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

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
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Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
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Lisbon
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Meeting ILTS 13th Annual International Congress
20-23 June 2007
Rio De Janeiro
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Meeting 9th World Congress on Gastrointestinal Cancer
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Books

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

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