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EDITORIAL

Multimodal pain stimulation of the gastrointestinal tract

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Abstract

Understanding and characterization of pain and other sensory symptoms are among the most important issues in the diagnosis and assessment of patient with gastrointestinal disorders. Methods to evoke and assess experimental pain have recently developed into a new area with the possibility for multimodal stimulation (e.g., electrical, mechanical, thermal and chemical stimulation) of different nerves and pain pathways in the human gut. Such methods mimic to a high degree the pain experienced in the clinic. Multimodal pain methods have increased our basic understanding of different peripheral receptors in the gut in health and disease. Together with advanced muscle analysis, the methods have increased our understanding of receptors sensitive to mechanical, chemical and temperature stimuli in diseases, such as systemic sclerosis and diabetes. The methods can also be used to unravel central pain mechanisms, such as those involved in allodynia, hyperalgesia and referred pain. Abnormalities in central pain mechanisms are often seen in patients with chronic gut pain and hence methods relying on multimodal pain stimulation may help to understand the symptoms in these patients. Sex differences have been observed in several diseases of the gut, and differences in central pain processing between males and females have been hypothesized using multimodal pain stimulations. Finally, multimodal methods have recently been used to gain more insight into the effect of drugs against pain in the GI tract. Hence, the multimodal methods undoubtedly represents a major step forward in the future characterization and treatment of patients with various diseases of the gut.

Key words: Pain; Gut; Experimental; Allodynia; Hyperalgesia; Neurophysiology

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INTRODUCTION

Abdominal pain is very common in the general population[1], and pain is the most prevalent symptom in the gastroenterological clinic[2]. Consequently, characterization of gut pain is one of the most important issues in the diagnosis and assessment of organ dysfunction. However, in clinical practice, the different symptoms of the underlying diseases confound the characterization of pain. These confounders may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions, such as fever and general malaise[3]. Furthermore, treatment with analgesics often causes sedation and other side effects. This will invariably bias the clinical evaluation of the pain-related symptoms. Hence, the patients tend to interpret other effects of the medication, e.g. an effect on the anxiety and depression relating to the disease, as a relief of pain[4]. Because of these confounding factors experimental pain models are often advantageous. Using these models, the investigator can control the experimentally induced pain (including the nature, localization, intensity, frequency and duration of the stimulus), and provide quantitative measures of the psychophysical, behavioral or the neurophysiological responses[3,5,6].

Experimental models have been used in different animal species. Here the investigators can study the neuronal activity in anesthetized or spinalized animals directly with invasive techniques or with assessment of behavior[7]. However, the neurobiology of the pain system differs between the animal species. This limits to a high degree the interpolation of findings from animal studies to man. Pain is the net effect of complex multidimensional mechanisms including intensity coding, affective, behavioral and cognitive components that involve most parts of the central nervous system. Furthermore, in humans, pain is closely related to linguistic terms and expressions. Thus, it is a complex sensory experience which is difficult to quantify with simple neurophysiological and/or behavioral methods. Therefore, animal experiments can only to some degree reflect the experience of clinical pain in humans and the interest in human experimental pain studies has increased rapidly during the last decade[3,8].

The primary advantages of experimental pain

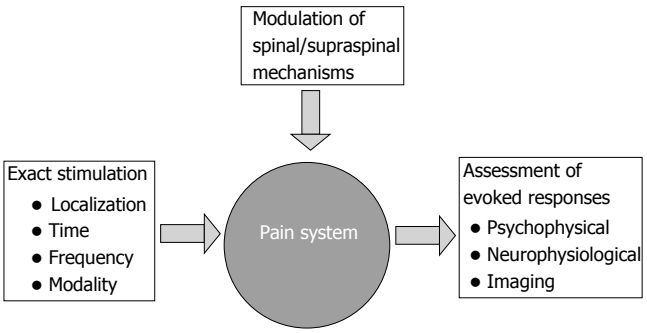

Figure 1 The concept for experimental induction, assessment and modulation of experimental gut pain in man.

approaches are that the stimulus can be controlled, delivered repeatedly and modulated, and that the responses can be assessed quantitatively with psychophysical and/or neurophysiological methods (Figure 1). Depending on the experimental model, different central mechanisms and conditions mimicking pathological pain can be studied. These are increased sensation to normal physiologic/non-painful and painful stimuli (allodynia and hyperalgesia, respectively). Experimental approaches can be applied in the laboratory for basic studies in healthy subjects and in patient groups, or used for preliminary screening of drug efficacy[9]. The methods can also be used in the clinic to characterize patients with sensory dysfunction and pain in organic and functional diseases[5,10-12]. The methods have been widely used in the skin and muscle[6]. However, due to the difficulties with access to the organs in the gastrointestinal (GI) tract, experimental pain testing is much more difficult than stimulation of the skin. The risk of perforation and other complications also limit the possibilities. Thus, most previous studies have relied on relative simple mechanical or electrical stimuli. These methods are easy to apply, but unless advanced modeling is used they have several limitations[3]. Most importantly, as pain is a multidimensional perception, it is obvious that the reaction to a single stimulus of a given modality can represent only a limited fraction of the entire pain experience. The possibility for combining different methods to stimulate the gut and evoke hyperalgesia will approximate the clinical situation, and give more comprehensive and differentiated information about the nociceptive system[3]. Multimodal models have clearly shown their value in testing of analgesics where a single stimulus has been inadequate assessing effects of specific drugs. Hence, Enggaard *et al*[13] showed that tricyclic antidepressants, which are valuable in treatment of functional pain disorders of the gut, increased the pain threshold to electrical stimuli, but did not reduce cold pressor pain. More sophisticated methods using a vide battery of tests will therefore be able to select the best test procedures to explore different basic aspects of pain as well as pharmacological modulations[9].

In this review, we outline the recent developments into test systems allowing standardized multimodal stimulations of the GI tract and their applications.

The rationale for multimodal stimulations of the gastrointestinal tract

The ideal experimental stimulus to elicit gut pain in man should be natural, minimally invasive, reliable in test-retest experiments and quantifiable[14]. The response to the stimulus should increase with increasing stimulus intensity and preferably the pain should mimic the observations in diseased organs by evoking phenomena, such as allodynia and hyperalgesia[8]. The different methods for pain stimulation of the human GI tract are electrical, mechanical, chemical, thermal and ischemic stimulation; further detailed informations regarding the advantages and shortcomings of these methods are explained elsewhere[3]. The ischemic stimuli are difficult to quantify in man and is normally not used as a direct stimulus. One of the major limitations of the different models is that they may not mimic clinical pain. Hence, they are relative short-lasting without the inflammation and subsequent activation of the many peripheral and central nervous mechanisms that are typically activated during diseases. Therefore, the basic neurobiological mechanisms in clinical pain may be different from those relating to an experimental stimulus[5,10]. For comprehensive experimental studies mimicking the clinical situation, a multimodal testing approach must therefore be used. A test battery where multimodal stimuli are used will increase the probability for activation of a range of relevant nervous mechanisms. Especially, if the stimulation is relatively long-lasting and includes modalities known to evoke peripheral as well as central sensitization, the likelihood that the model will mimic clinical pain is high despite the non-harmful nature of the stimulation.

In the GI tract, technical limitations of the currently available models have until now made a multimodal stimulation approach difficult. Some authors have combined mechanical and electrical stimuli[15,16] or used electrical stimuli combined with sensitization to acid[17]. The Center for Visceral Biomechanics and Pain in our department has recently introduced a multimodal pain model where mechanical, electrical, cold and warmth stimuli were combined. A summary of the findings in healthy subjects and patients is shown in Tables 1 and 2. Table 2 should be interpreted with caution as many data are still unpublished. In the multimodal model, mechanical stimulation is achieved with bag distension. Quantifications of the bag pressure and cross-sectional area are typically done by means of impedance planimetry or ultrasonography. Thermal stimulation is achieved by re-circulating fluid inside the bag with concomitant measurement of temperature. Chemical and electrical stimulation is done using side-holes placed proximal to the bag and by electrodes on the outside of the bag[18]. Sensitization with acid was added to the protocol to evoke allodynia and hyperalgesia together with increased referred pain areas (Figure 2)[19]. The multimodal approach gives the possibility for a differentiated stimulation of receptors in the superficial and deep layers of the gut. The possibility for induction of hyperalgesia and evoking central phenomena such as summation, allodynia and referred

Table 1 Multimodal comparison of clinical experimental data obtained in the esophagus from healthy volunteers

Group	Mechanical stimuli	Heat stimuli	Cold stimuli	Electrical stimuli	Sensitization with acid
Basic data	Differences between the sensations and referred pain areas was evoked by the stimulus modalities[18]. Reliability demonstrated[19,60] The sensation to mechanical stimulations was unaffected by relaxation of the smooth muscle[40,61]. Evidence for low and high threshold mechanoreceptors[22].	Reliability demonstrated. Stimulus-response functions obtained[18,19,60].	Reliability demonstrated. Stimulus-response functions obtained[18,19,60].	Reliability demonstrated. Stimulus-response functions obtained[18,19,60].	Allodynia and hyperalgesia evoked[19,26], although not consistent for mechanical stimuli (see text)[26,41]. Increased referred pain and amplitude of the nociceptive reflex indicating central hyperexcitability[19,26,46]. Acid perfusion sensitizes the oesophagus to heat but not cold, indicating sensitization of peripheral TRPV1 receptors[28,46]. Remote hyperalgesia was seen in the rectum after acid perfusion of the esophagus[20]. Hyperreactivity of contractions in esophagus, but tone was unaffected[26,46].
Gender differences	Males were more sensitive to stimulations, but an increased referred pain area was seen in females, reflecting sex differences in central pain processing[27,46].	No differences in sensation, but the referred area was larger in females[27,46].	As heat stimuli	Males less sensitive to single and repeated stimuli (Staahl et al., unpublished).	In females, the referred pain area increased to heat after acid sensitization, but no changes were seen to mechanical and cold stimulations[46].
Pharmacologic modulation	Oxycodone was better than morphine (and placebo) in attenuating mechanical pain[59].	Oxycodone was better than morphine (and placebo) in attenuating heat pain[59].	Not done	Both morphine and oxycodone attenuated the electrically evoked pain, but there were no differences between the opioids[59].	Not done

pain makes the models clinically relevant with respect to increasing the knowledge about peripheral and central pain mechanisms. The model have mostly been used in the esophagus, but recently also in the duodenum[20]. The pain assessment should ideally also be multimodal and, for example, include quantitative and qualitative sensations, assessment of referred pain and neurophysiological measurements[19].

Multimodal stimulation and peripheral receptors

The multimodal approach has given valuable information about the receptors in the gut wall. Theoretically, the thermal stimulation activates preferentially the receptors in the mucosa, the electrical stimulation penetrates into deeper layers of the gut and the mechanical stimulations affect predominantly receptors in the muscle layers (Figure 3).

It is generally believed that most visceral afferents are polymodal and respond to a wide range of stimuli including thermal stimuli[21]. However, sub-populations of these receptors exist and recently, we combined controlled distension with statistical modeling to demonstrate the existence of low and high threshold mechano-receptors in the human esophagus[22]. Most data on the sensation to thermal stimuli of the human viscera relate to few and relatively old studies[23-25], although some new studies have recently been published[18,19,26-29]. These studies point towards the existence of sensory pathways for thermal stimuli in the human GI tract. The thermal energy spreads from the superficial layers into the deeper layers of the gut depending on the temperature and conductance of the tissue[4]. However, as thermal stimuli are rather short-lasting in man mainly receptors in the mucosa are thought to be activated. Chemical stimulation with acid or capsaicin also activates receptors in the mucosa. Pedersen *et al*[28] used a multimodal approach to combine acid and heat stimuli of the esophagus. In this study, sensitization with acid resulted in a significant increase in the sensation to heat stimuli. The TRPV1 receptor is a polymodal detector of potential harmful stimuli, including noxious heat and protons[30]. Hence, it was suggested that TRPV1 receptors (or receptors with the same characteristics) were sensitized with acid and

Table 2 Multimodal comparison of clinical experimental data obtained in the esophagus from patient with different GI diseases

Patient group	Mechanical stimuli	Heat stimuli	Cold stimuli	Electrical stimuli	Sensitization with acid
Non-cardiac chest pain[41]	No differences to single stimuli, but increased pain to repeated stimuli and increased referred pain area, reflecting central hyperexcitability.	Not done	Not done	Not done	Increased sensation to mechanical stimulations after acid in patients only.
Esophagitis[31]	Patients were hyposensitive but with larger and more widespread referred pain. The distension induced more reactive contraction.	Patients were hypersensitive probably via increased activation of TRPV1 receptors.	No differences	Not done	Not done
Non-erosive reflux disease (Reddy *et al* unpublished data)	Patients were hyposensitive to mechanical stimuli. The distensions induced more reactive contractions in the esophagus in the patients and they had larger referred pain areas. Patients with pathological 24-h pH-measurement were more hyposensitive than the patients with normal pH profile.	The patients were hypersensitive to heat with increased referred pain areas to this modality.	No differences between patients and controls	Not done	Patients had a higher sensitivity score to acid perfusion.
Diabetes (Frøkjær *et al*, unpublished data)	Patients had hyposensitivity to distension, but increased referred pain areas, reflecting peripheral neuropathy and central hyperexcitability. Increased stiffness of the gut wall in diabetes.	As mechanical stimulations	Not done	As mechanical stimulations	Not done
Chronic pancreatitis (Dinmcevski *et al*, unpublished data)	No differences in sensation. No differentiated effect on morphine and oxycodone in attenuation of mechanical pain.	No differences in sensation Oxycodone attenuated heat pain better than morphine.	Not done	Larger referred pain area in the patients. Opioids were not better than placebo in attenuating electrical pain	Not done

subsequently resulted in increased firing of the afferents to heat stimulation. The role of this receptorsystem in the clinic was addressed in another study where selective hyperalgesia to heat but not cold stimuli was found in patients with reflux and grade B esophagitis[31]. The evidence for receptor-specific activation pattern in the experimental studies was supported in a recent study where the TRPV1 receptor was demonstrated in the human esophagus, and especially the receptor was up-regulated in esophagitis[32]. Thus, the multimodal approach gave valuable quantitative *in vivo* information about the receptor characteristics and pain mechanisms in healthy subjects as well as in patients with acid-evoked inflammation.

Multimodal stimulation and primary afferents

Electrical stimuli bypass the receptors and although all fiber populations (nociceptive as well as fibers mediating physiologic/non-nociceptive sensations) are excited by electrical stimuli, the relative proportion of activation depends on the stimulus intensity. With normal bipolar electrical stimulation, the current is believed first to activate receptors in the mucosa and submucosa, whereas the deeper layers are activated with increasing current[3]. The depth of activation also depends on the stimulation method and frequency[4]. In the gut, electrical stimulation is thought preferentially to activate thinly myelinated (Aδ) fibers[33]. Chemical and thermal stimuli, on the other hand, activate mainly non-myelinated (C) fibers and the terminals of mechanosensitive fibers are mainly localized in the muscle layers or have intraganglionic nerve endings[34,35]. Roughly speaking, the different modalities may therefore activate different fiber populations and, for example, may thermal and mechanical stimulation activate fibers in the mucosa and muscle layers, respectively. However, the difference between the stimulation paradigms may be of minor importance as Hobson *et al*[36] showed that the evoked brain potentials to mechanical and electrical stimulation of the gut were similar, reflecting that the same pathways were activated. The mechanical stimulation protocol may also be important. Hence, in the human

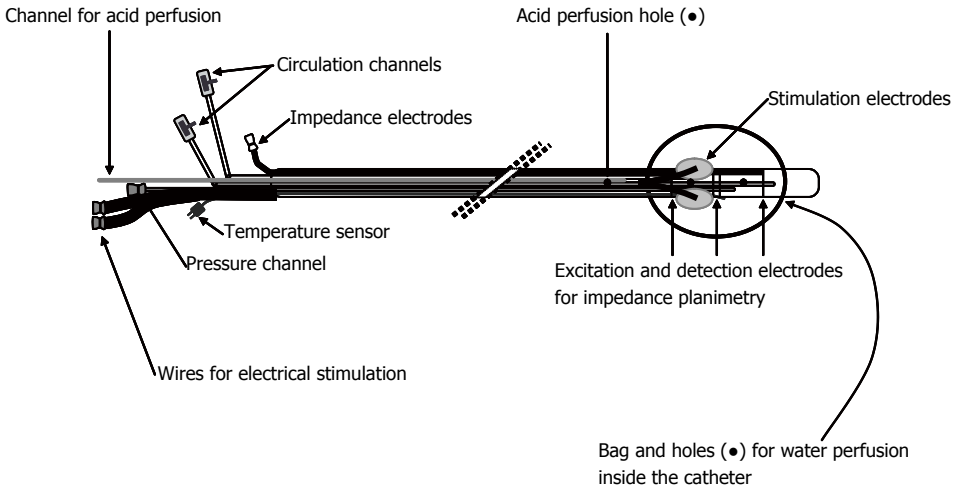

Figure 2 Schematic illustration of the probe used for multi-modal (electrical, mechanical, cold and warmth stimuli) of the esophagus.

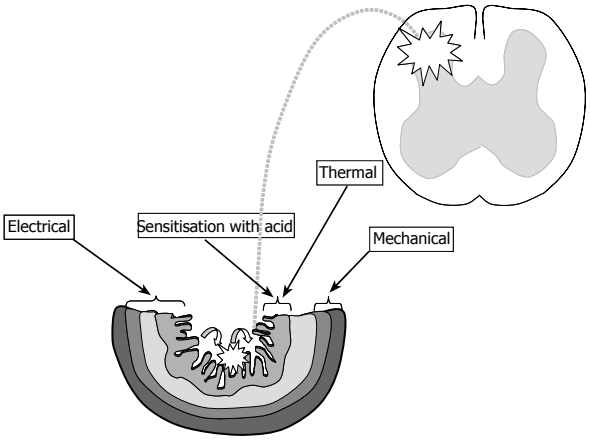

Figure 3 Schematic illustration of the proposed gut layers which are preferentially affected with: (1) Thermal stimuli (mucosa - dark grey and submucosa - light grey); (2) Mechanical stimuli (circular muscle layer - hatched grey and longitudinal muscle layer - prickled grey); (3) Electrical stimuli (all layers depending on stimulus intensity). Perfusion of the esophagus with acid (curved arrows) gives the possibility to evoke peripheral and (mainly) central sensitization (illustrated with stars).

rectum phasic distensions were shown preferentially to stimulate spinal pathways thought to mediate pain, whereas slow tonic stimuli mainly affect parasympathetic nerves[37]. We recommend a slowly ramp distension as it is more physiological and allows the subjects to assess the pain continuously. We also strongly recommend to precondition the tissue by two-three distensions until the stress-strain relationship becomes reproducible[38]. For advanced muscle analysis, we normally use butylscopolamine to relax the smooth muscle, and this does not seem to modify the sensation *per se* [20,39,40].

The multimodal approach has recently been used to compare the response to mechanical stimuli before and after chemical stimulation with acid in patients with non-cardiac chest pain[41]. In these patients, there is a normal sensory response to mechanical stimuli at baseline. However, after acid the evoked hyperalgesia resulted in a marked increase of the sensory response in the patients (Figure 4). Although peripheral sensitization may be important, the findings gave evidence for an amplification of central pain mechanisms manifested as allodynia, hyperalgesia, and increased and widespread referred pain areas to the mechanical stimulations. Mechanical stimulation together with advanced muscle analysis has also been used to explain the symptoms in patients with systemic sclerosis. In these patients, the contraction amplitude was smaller and there was an evidence for a stiffer gut wall in the small intestine[42]. The pain evoked by a controlled strain of the gut was increased and this may explain many of the symptoms reported in the clinic. In patients with diabetes, we also found evidence for increased stiffness in the duodenum using the multimodal approach (Frøkjær *et al*, unpublished data). This may reflect the increase in collagen deposition seen in these patients and may (together with autonomic neuropathy) explain the motor abnormalities seen in these patients.

Multimodal stimulations and central pain mechanisms

In diseases of the GI tract, central sensitization and neuroplastic changes are probably of major importance to understand the sensory response as manifested by pain and hyperalgesia. Central pain mechanisms may be evoked by multiple stimuli (either temporal or spatial summation), resulting in central amplification of the response. The response is comparable to early phase of the frequency-dependent "wind-up" seen in animal experiments. In practice the central integration can be evoked by repeated electrical stimulation above 0.5 Hz[43,44], resulting in increased local and referred pain. Recently, the multimodal probe was used to give repeated mechanical stimulation in patients with non-cardiac chest pain. In this study, the number of stimuli tolerated was significantly lower in the patients compared with healthy controls, reflecting central hyperexcitability as a key to understanding the symptoms in these patients[41].

Sensitization of the esophagus with acid is an another possibility to evoke central (and peripheral) sensitization. Previously, it was shown that acid perfusion of the distal esophagus resulted in an amplified response to electrical, mechanical and thermal stimuli[19,26]. The central changes were documented in an experiment where there was an amplification of the nociceptive reflex[19]. The reflex was evoked by stimulation of the sural nerve, resulting in

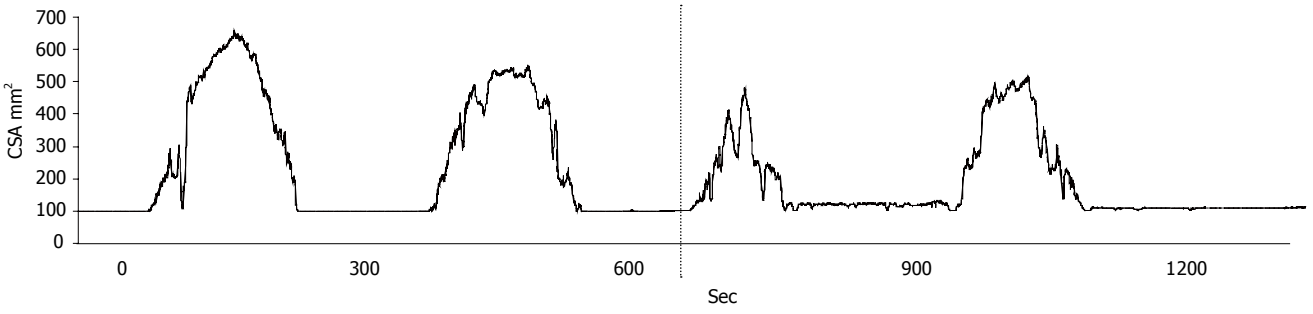

Figure 4 Cross-sectional area (CSA) at moderate pain during two distensions of the esophagus in a patient with non-cardiac chest pain. The distensions were performed before and after perfusion of the distal esophagus with acid (illustrated with the stippled line). A clear reduction in the tolerated mechanical stimulus was seen after acid perfusion.

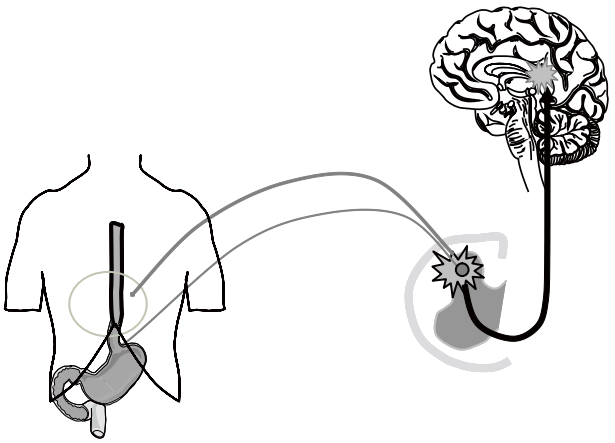

Figure 5 Referred pain in the somatic tissues is believed to be generated by central mechanisms, where visceral and somatic nerves converge on nerves in the same area of the spinal cord or at supraspinal centers. The phenomena also includes unmasking of latent connections and focal central hyperexcitability of the neurons.

activity of the biceps muscle of the thigh. The connection from the primary afferents to the motor neurons is a polysynaptic spinal pathway, which can be modulated by other afferent input, spinal neuronal excitability, and activity in descending control systems[5]. The reflex was evoked together with a painful mechanical stimulus of the esophagus. In the experiment, an amplification of the reflex was seen after sensitization of the esophagus with acid, reflecting central changes at the spinal cord level. Evidence for central changes to acid perfusion was also demonstrated by Sarker *et al*[17] who demonstrated a decreased pain threshold to electrical stimulation of the proximal esophagus after acid perfusion of the distal part. As the proximal esophagus was not affected by the acid, only central changes would explain the findings.

Referred somatic pain to visceral stimuli is regarded as a phenomenon generated by central mechanisms due to visceral nerves terminating in the same area of the spinal cord as somatic afferents[45] (Figure 5). Assessment of the referred pain area to electrical, mechanical and thermal stimulation can be used to determine the central response to these differentiated modalities[19]. The referred pain area to electrical, mechanical, cold and heat pain differs in size and localization reflecting the different peripheral (and hence central) nerves that are activated[18]. The increase in referred pain areas after acid perfusion is also an evidence for central sensitization caused by the chemical stimulation[26,46]. Recently, Pedersen *et al*[28] showed that the referred pain to heat but not cold stimulation of the esophagus increased after acid perfusion of the organ. This has also been shown in a more recent study[46]. As discussed previously, selective sensitization of the TRPV1 receptors by acid could result in an increased afferent barrage after a heat stimulus, which again was manifested as an increase in the referred pain area. The changes in local and referred pain to mechanical stimulations may, however, be difficult to determine as the acid also evokes secondary contractions that may squeeze the bag and influence the stimulus parameters[26]. On the other hand, an increase in the referred pain after acid perfusion is typically seen to mechanical stimulation of the esophagus in healthy subjects[19,26]. Increased referred pain to mechanical stimulations was also seen in patients with esophagitis and in non-erosive reflux disease, reflecting that facilitation of central pain mechanisms is important in the understanding of these diseases[31]. In patients with diabetes and chronic pancreatitis, we found hypoalgesia to peripheral stimulation, whereas there was a significant increase in the referred pain area. These data were interpreted as a descending inhibition of the afferent input counterbalancing central hyperexcitability (Frøkjær *et al*, unpublished data). Hence, the multimodal approach may be used explaining the symptoms in these patients, and may be used to evaluate the stage of disease in a more mechanism-based manner. Central changes may also result in allodynia and hyperalgesia to stimulation of other viscera[47]. Such changes are regarded important in the understanding of functional gut disorders where abnormal sensation to physiologic stimuli, such as feces or air in the gut, may contribute to the symptoms (allodynia). Recently, a multimodal approach was used to assess the sensation of the proximal esophagus, duodenum and rectum after sensitization of the distal esophagus with acid[20]. In this study, an increased sensitivity to mechanical stretch in the three gut segments was seen after acid perfusion. This was mainly due to increased sensitivity in the rectum being very remote from the experimentally inflamed esophagus.

Neuroplastic changes at the cortical level may also be shown by multimodal stimulations of the gut. Thus, Sarker *et al*[48] showed changes in the evoked brain potentials to

electrical stimulation of the proximal esophagus after acid perfusion of the distal segment. Recently, we showed that acid perfusion resulted in neuroplastic changes at the cortical level reflected. Reduction in latency and a backward shift of the electrical dipole in the anterior cingulate dipole were observed to electrically evoked pain in the esophagus after acid perfusion of the organ (Sami *et al*, unpublished data). Such changes were also found when the gut was electrically stimulated in patients with irritable bowel syndrome[49]. The backward shift in the cingulate activation after sensitization with acid in healthy subjects may, therefore, represent the central nervous system change corresponding to the allodynia and hyperalgesia to gut stimuli found in patients with functional disorders of the gut.

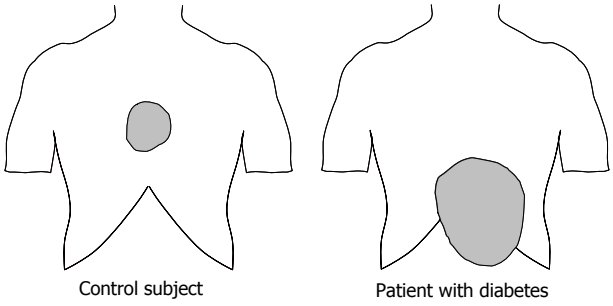

Figure 6 Referred pain area to mechanical distension of the esophagus in a typical healthy subject, and in a patient with diabetes mellitus and autonomic neuropathy. The patient complained of severe nausea and pain in the epigastrium. The referred pain area in the diabetic patient was larger and abnormally localized.

Gender differences to multimodal pain stimulations

Women are diagnosed more frequently with chronic visceral pain disorders than men[50]. Extensive evidence indicates that females and males differ in their nociceptive processing, although it seems modality- and tissue-specific. The reason for the female predominance is not known, but sex differences are found in basic GI functions, such as gallbladder emptying and colon transit[51,52]. For most studies using experimentally delivered somatic pain stimuli, females have lower thresholds, less tolerance, and higher pain ratings than males[53]. However, few studies have focused on sex-related differences in visceral pain in man, and these have been contradictory with respect to sex differences[51]. The multimodal probe was recently used to investigate any differences to mechanical and thermal stimuli of the esophagus. The results were somewhat ambiguous, but in general males seemed to be more sensitive to the stimuli[27,46]. However, a greater size of the referred pain areas to the different stimuli was seen in women. After acid perfusion, the males were also more sensitive than females to distensions, but no differences were found in response to the thermal stimuli[46]. In the females, only the referred pain area was increased to heat stimulations after sensitization with acid. The bigger referred pain areas may thus reflect that the central processing of pain to visceral stimuli differs between males and females as previously shown by our group and by Kern *et al* [54]. Thus, the multimodal stimulations revealed a differentiated response to peripheral and central pain mechanisms, which may explain the sex-related differences seen in several gastrointestinal disorders.

Multimodal stimulations outside the esophagus

Accarino *et al*[15] used multimodal stimulation (mechanical and electrical) of the jejunum. The verbal response to electrical stimuli and distension was compared, and no differences in the evoked response were found. This led the authors to conclude that the practical differences between the two modalities may probably be of minor importance. Recently, we used thermal and mechanical stimuli of the duodenum in healthy subjects[20] and in patients with diabetes and autonomic neuropathy (Frøkjær *et al*, unpublished data). The diabetes patients showed hypoalgesia to mechanical and electrical stimuli, whereas no changes were found to heat stimulation compared with controls. Furthermore, the referred pain area in the abdomen was enlarged in the patients (Figure 6). Such data may enhance our knowledge about peripheral and central pain mechanisms in these patients with implications for the treatment.

Mechanical stimulation of the rectum is one of the most used methods in experimental visceral research. Electrical and thermal stimulations have also been used in the rectum[3], but combinations of the methods were not done. However, combinations of mechanical and electrical stimulations have been used in assessment of evoked brain potentials[55] and to assess the effect of viscero-visceral hyperalgesia[20]. The stomach and other parts of the digestive system have not yet been studied with multimodal stimulations. Although the complicated anatomy, nervous innervation and function of these organs should be taken into account, multimodal models are obviously highly warranted.

Multimodal stimulations in drug research

Experimental models are widely used in research of the effect of analgesics. The differentiated information of the drug effect can be used as "proof-of concept", dose-efficacy analysis, and for designing further clinical trials. An approach to mimic the clinical situation is the use of multimodal tests, where different receptor types and mechanisms are activated. The multimodal model has clearly shown its value in somatic pain testing, where a single stimulus has been inadequate to test, for example, pathophysiological changes and effects of specific drugs[9]. Hence, differentiated effects could reflect how the drugs can modify different disease mechanisms. In the esophagus, Sarkar *et al*[56] recently used a model where the upper esophagus was stimulated following sensitization of the distal segment with acid. The secondary hyperalgesia in the proximal part was reduced with a prostaglandin inhibitor, demonstrating the preferentially central action of prostaglandins in this model. Recently, we used a multimodal (and multi-tissue) approach to test the effect of opioids. Opioids are widely used in treatment of visceral pain despite the many side effects. Opioids preferentially attenuate nociceptive responses produced by central integration (spinally amplified signals) to tonic activation

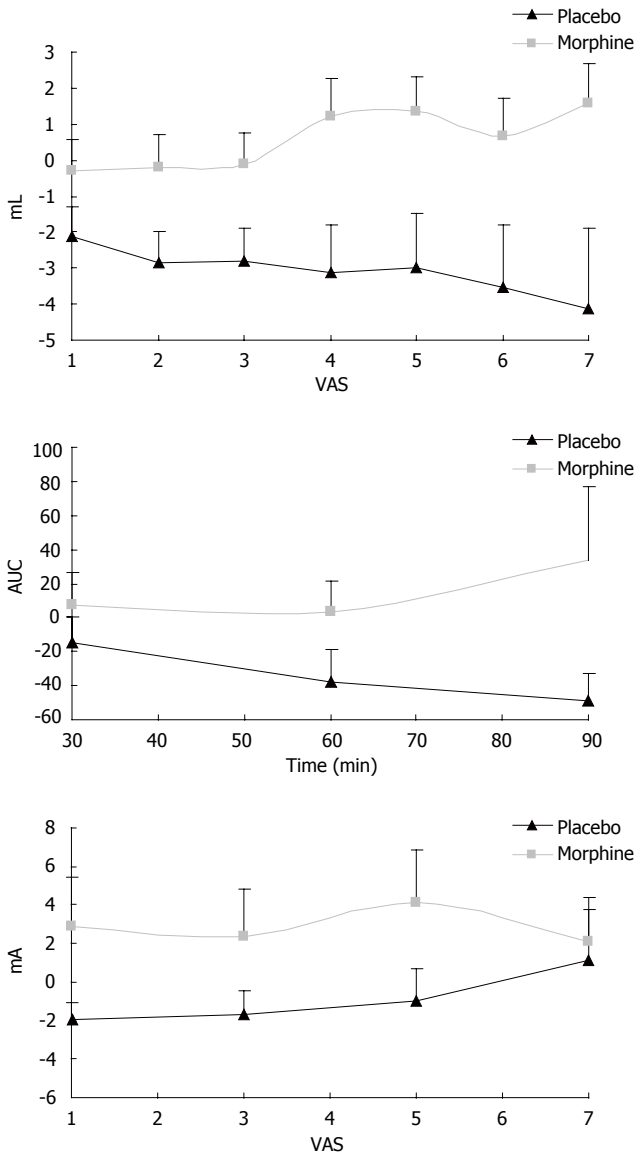

Figure 7 Change in sensory rating compared with baseline recordings for oral morphine and placebo using multimodal stimulation of the esophagus. Top picture: Morphine attenuates mechanically evoked pain better than placebo 60 min after drug intake ($P < 0.01$). VAS on the X-axis denotes the sensory rating at a visual analogue scale with 5 as the pain threshold. ML at the Y-axis denotes the bag volume. Middle picture: Morphine attenuates heat pain better than placebo ($P < 0.05$). Here the X-axis illustrates time after drug intake (min) and AUC on the Y-axis denotes the area under the temperature curve used as a proxy for the thermal energy. Bottom picture: Morphine also worked better than placebo ($P < 0.05$) on electrical stimulation 60 min after drug intake.

of unmyelinated fibers[4,5,7]. Therefore, evaluation of the anti-nociceptive effects of opioids may be clearer using slow rates of temperature or tonic pressure. In the viscera typically only one modality (pressure) has been used in the testing of analgesics[58]. However, Staahl *et al* (unpublished data) recently compared the effects of morphine and placebo on the pain thresholds to multimodal stimulation of the esophagus. A clear effect of morphine in attenuating heat, electrical and slow-ramp pressure stimulations was found (Figure 7). Morphine can definitely attenuate GI pain in the clinical situation and the model, therefore, proved its validity. The model was also used to differentiate between morphine and oxycodone, the latter was believed also to affect κ-opioid receptors thought to be predominant on visceral afferents. In equipotent doses, oxycodone was better than morphine in attenuating visceral pain, whereas there were no differences between the drugs on pain evoked in the muscle and skin[59]. The study thus demonstrated a different pharmacological profile of oxycodone compared to morphine, and therefore oxycodone may be a useful alternative to morphine in the treatment of visceral pain syndromes. We recommend that future studies evaluating analgesics in the GI tract should use a multimodal approach to get the necessary insight into visceral pain mechanisms and the effect of drugs in the gut. This will facilitate the design of subsequent clinical (phase III) studies. Hence, a substitution of the current "trial and error design" with a more mechanisms-based approach will reduce the economic and human burden in the development of new drugs targeted against pain in the GI tract.

CONCLUSION

Multimodal pain stimulation in the human GI tract is a newly developed experimental approach that mimics the clinical pain to a higher degree than previous models. The method has been used to gain more insight into basic peripheral and central pain mechanisms as well as characterizing patients with different diseases of the GI tract. Together with the possibility for pharmacological testing, the models represent a major step forward in the experimental characterization and treatment of patients with gastroenterological diseases.

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REVIEW

Diagnostic criteria for autoimmune chronic pancreatitis revisited

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Abstract

Autoimmune chronic pancreatitis (AIP) is increasingly being recognized worldwidely, as knowledge of this entity builds up. Above all, AIP is a very attractive disease to clinicians in terms of its dramatic response to the oral steroid therapy in contrast to ordinary chronic pancreatitis. Although many characteristic findings of AIP have been described, definite diagnostic criteria have not been fully established. In the year 2002, the Japan Pancreas Society published the diagnostic criteria of AIP and many clinicians around the world use these criteria for the diagnosis of AIP. The diagnostic criteria proposed by the Japan Pancreas Society, however, are not completely satisfactory and some groups use their own criteria in reporting AIP. This review discusses several potential limitations of current diagnostic criteria for this increasingly recognized condition. The manuscript is organized to emphasize the need for convening a consensus to develop improved diagnostic criteria.

Key words: Autoimmune chronic pancreatitis; Diagnostic criteria

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INTRODUCTION

Autoimmune chronic pancreatitis (AIP) can be defined as a chronic inflammation of the pancreas due to an autoimmune mechanism; autoimmunity is responsible for producing the pancreatic lesion[1,2]. AIP is a distinctive type of chronic pancreatitis that shows reversible improvement of pancreatic morphology and function with oral steroid therapy, in comparison to other types of chronic pancreatitis which hardly respond to various treatments[1-5].

AIP is increasingly being recognized to be a worldwide entity[6-8]. The sudden increment in cases reported probably reflects the growing awareness of the entity, rather than a rise in the true incidence. The diagnosis of AIP is, however, still challenging. Many groups have cited the diagnostic criteria proposed by the Japan Pancreas Society (Table 1)[9], whereas some groups have used their own criteria in reporting AIP[1,2,4,5,10]. This makes it difficult to compare studies from different centers, judge the relevance of comparisons and establish evidence on AIP. Above all, the largest problem was that a substantial portion of patients revealed clinical findings compatible to AIP and even responded to the steroid, yet failed to fulfill the Japanese diagnostic criteria[11,12]. This review revisits currently used diagnostic criteria of AIP focusing on the Japanese ones, discusses the potential limitation of diagnostic criteria and raises some important issues related to the diagnosis of AIP. In view of our experiences of a relatively small cohort of 28 patients, we propose a new revision that may help physicians diagnose AIP. By revising the diagnostic criteria of AIP, more patients may benefit from this diagnosis and be spared from burdensome surgery.

OUR EXPERIENCES

We reviewed the clinical, radiologic, laboratory and histologic features of 28 patients with AIP who responded to the oral corticosteroid. The response to the steroid was defined as improvement in clinical symptoms, negative conversion of detected autoantibodies, normalization of elevated level of IgG or IgG4, and reversion of abnormal pancreatic imaging including CT and endoscopic retrograde pancreatogram.

The diagnosis of AIP in our hospital was made by the criteria as shown in Table 2. For the diagnosis of AIP, imaging criterion (CT and ERCP findings) was an essential component. If the patient fulfilled the imaging criterion (diffuse enlargement of pancreas and diffuse or segmental irregular narrowing of main pancreatic duct) together with laboratory and/or histopathologic criteria, the patient was diagnosed of AIP. Even though a patient fulfilled imaging criterion only, in other words, the laboratory criterion and histopathologic criterion were incompatible

Table 1 Diagnostic criteria for autoimmune pancreatitis by the Japan Pancreas Society

	Diagnostic criteria
I	Imaging criterion: Diffuse narrowing of the main pancreatic duct with irregular wall (more than 1/3 length of the entire pancreas) and enlargement of the pancreas
II	Laboratory criterion: Abnormally elevated levels of serum gamma-globulin and/or IgG, or the presence of autoantibodies
III	Histopathologic criterion: Marked lymphoplasmacytic infiltration and dense fibrosis
For diagnosis, criterion I must be present, together with criterion II and/or III	

Table 2 Diagnostic criteria for autoimmune pancreatitis in Asan Medical Center

Inclusion Criteria
Criterion I. Pancreatic imaging (essential)
(1) CT: Diffuse enlargement (swelling) of pancreas
and (2) ERCP: Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion II. Laboratory findings
(1) elevated levels of IgG and/or IgG4
or (2) detected autoantibodies
Criterion III. Histopathologic findings
Fibrosis and lymphoplasmacytic infiltration
Criterion IV. Response to the steroid
Definite diagnosis: Criterion Ⅰ and any of criterion Ⅱ-Ⅳ

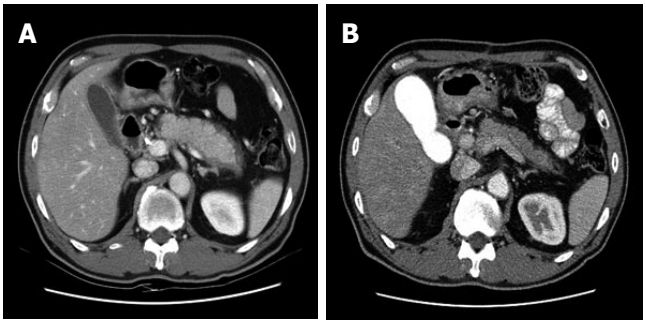

Figure 1 Abdominal CT findings. **A:** Diffusely enlarged pancreas without any calcification or stones in a 59 year-old man. A capsule-like low-density rim can also be seen around the pancreas; **B:** After steroid therapy, the pancreas returned to its normal size and the rim disappeared.

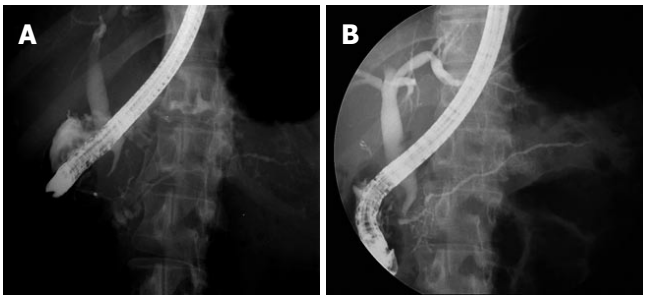

Figure 2 ERCP findings. **A:** Diffuse irregular narrowing of more than 2/3 of the entire length of main pancreatic duct noted in the 49 year-old woman; **B:** After steroid therapy, evidently widening of the main pancreatic duct.

or not available, steroid was administered and the patient was diagnosed as AIP if the response to the steroid was shown.

The patients' mean age was 55.3 years (range, 32-78 years) and they were comprised of 22 males and 6 females. None of the patients had a history of alcohol abuse or other predisposing factors for chronic pancreatitis.

On CT, all the patients revealed a diffusely enlarged pancreas with no or mild peripancreatic fat infiltration. All the patients had no any typical findings of ordinary chronic pancreatitis, such as multiple parenchymal calcifications or pancreatic ductal stones. Capsule-like low-density rim surrounding the pancreas was shown in five (18%) patients (Figure 1). On direct pancreatogram, 8 (29%) patients showed diffuse irregular narrowing of main pancreatic duct which involved entire main pancreatic duct or at least more than 2/3 of entire length of main pancreatic duct (Figure 2). In seven patients with segmental irregular narrowing, total length of ductal involvement was less than 2/3 of the entire length of main pancreatic duct and it was between 1/4 and 1/3 (Figure 3).

The IgG level was increased (>1 800 mg/dL) in 14 (50%) patients (Table 3). The IgG4 level was measured in 23 patients and it was increased in 15 (65%) patients. In 4 patients, the IgG4 level was increased without elevation of IgG level. Autoantibody was detected in 15 (68%) patients.

Pancreatic tissue specimens were obtained from 19 patients. Percutaneous ultrasound-guided core biopsy with an 18-gauge needle was performed in 17 patients and open biopsy in 2 patients. The biopsy specimen of 14 (74%) patients showed marked inflammatory infiltrates mostly of lymphocytes and plasma cells and dense fibrosis of the pancreatic tissue. However, one patient showed predominant eosinophilic granulocytes infiltrating into the pancreatic parenchyma rather than lymphoplasma cell infiltration. Overall, the biopsy findings were non-diagnostic, that is, either lymphoplasma cell infiltration or fibrosis was minimal or absent, in 5 of 19 (26%) cases.

Seven of 28 (25%) patients who responded to the steroid did not satisfy the Japanese imaging criterion because the extent of ductal narrowing was less than one third of entire length of main pancreatic duct. Another two patients showed normal IgG level, negative results of autoantibody measurements and non-diagnostic pancreatic histopathology. Taken together, 9 of 28 (32%) patients did not meet the Japanese diagnostic criteria for AIP, yet responded to the steroid.

DIAGNOSTIC CRITERIA

The diagnosis of an autoimmune disease is always an impetus to the clinician. Autoimmune conditions often lack pathognomonic findings on histopathology. The sensitivity and specificity of serologic markers also leave controversies in the diagnosis. To overcome these problems, combinations of common clinicopathologic findings are often used to guide physicians in diagnosing autoimmune diseases.

During the past decade, Japanese investigators have described many common clinicopathologic findings of AIP by reporting over three hundred cases domestically.

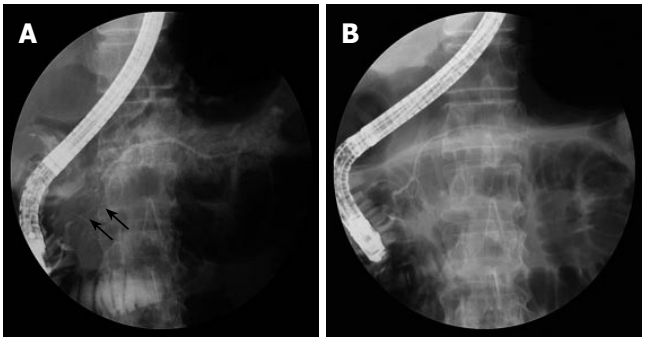

Figure 3 ERCP findings. **A:** Segmental narrowing (arrows) of the main pancreatic duct noted at the pancreatic head. The extent of irregular narrowing is less than 1/3 of the entire length of main pancreatic duct; **B:** Direct pancreatogram revealing a normal-appearing main pancreatic duct following steroid therapy.

Based on this experience, the Japan Pancreas Society published the "Diagnostic Criteria for Autoimmune Pancreatitis" in the year 2002[9]. The criteria are constituted of 3 components: (1) Pancreatic imaging: diffuse irregular narrowing of the main pancreatic duct with irregular wall (more than 1/3 length of the entire pancreas) and diffuse enlargement of the pancreas; (2) Laboratory data: elevated levels of serum gammaglobulin and/or IgG, or the presence of autoantibodies; and (3) Histopathologic findings: fibrotic changes with lymphocyte and plasma cell infiltration. For the diagnosis of AIP, criterion 1 must be present, together with criterion 2 and/or 3 (Table 1). Interestingly, the criteria does not include symptoms or common laboratory findings, as they are not specific to AIP[13,14]. The condition commonly manifests as obstructive jaundice, weight loss, and recent-onset diabetes in elderly men. None of the patients diagnosed as AIP have a history of alcohol abuse or other predisposing factors for chronic pancreatitis. In contrast to other types of pancreatitis, severe abdominal pain is infrequently encountered. These features are similar to that of pancreaticobiliary malignancies, which are the most difficult and important entities to differentiate from AIP[15].

Imaging criterion

Radiologic imaging of the pancreas is an essential component of the Japanese criteria where it is mentioned as a "must" for diagnosing AIP. The criterion is stated as diffuse narrowing of the main pancreatic duct with irregular wall (more than 1/3 length of the entire pancreas) and diffuse enlargement of the pancreas which can be identified with ERCP (endoscopic retrograde cholangiopa ncreatography) and CT, respectively[9]. Although abdominal US is most frequently performed imaging method for the screening of pancreatic disease, overlying bowel gas or obesity often hinders the sonographic visualization of the pancreatic gland, rendering the examination limited in scope and quality.

On CT, most cases of AIP reveal a diffusely enlarged pancreas with no or mild peripancreatic fat infiltration. Phlegmonous changes of the pancreas, peripancreatic fluid collection or pseudocysts formation are rare. Diffuse enlargement of the pancreas correlates with the pathology of marked stromal edema, which manifests as diffuse swelling on gross examination[16,17]. Surprisingly, CT does not reveal any typical findings of chronic pancreatitis, such as multiple parenchymal calcifications or pancreatic ductal stones. Rather, AIP resembles mild form of acute pancreatitis according to Balthazar classification[18]. Some cases of AIP reveal peculiar CT findings, such as a capsule-like low-density rim surrounding the pancreas (Figure 1)[19]. This rim is thought to correspond to the histologic findings of an inflammatory process that contains fibrous changes involving the peripancreatic adipose tissue[20]. Delayed enhancement of the pancreatic parenchyma is another distinguishing feature of AIP. On arterial enhanced phase, the pancreas appears hypodense when compared to the spleen. On the delayed phase, attenuation increases when compared to early images. This reflects fibrosis with an associating inflammatory process. These characteristic patterns observed on CT are important in differentiating AIP from pancreatic cancer[15].

The term "enlargement" of the pancreas can be subjective and vague. The size of the pancreas may be affected by many factors, including body mass, ethnic group, gender and age. There are individual variations in the size of the gland, with smaller atrophic glands seen in older individuals[21,22]. While a pancreas may seem normal for a large young man, the same size can be described as "enlarged" for an elderly patient with small body mass. Nishino *et al*[23], therefore, describe that the pancreas is considered to be enlarged when the width of the pancreatic body or tail was more than two thirds of the transverse diameter of the vertebral body or the width of the pancreatic head is more than the full transverse diameter of the vertebral body.

The Japanese imaging criterion has described the ductal pathology as diffuse narrowing of the main pancreatic duct with irregular wall (more than one third of the length of the entire pancreas), which can be observed on direct pancreatogram[9]. However, we were confronted with confusion when applying this criterion. This may be because the terms used were imprecise and vague in the meaning. First, in the previous international symposium on chronic pancreatitis[24,25], pancreatic lesion was classified as focal, segmental, or diffuse according to the extent of involvement. In this international classification, "diffuse" is used when a process involves the entire pancreatic duct or at least more than two-thirds of the entire length of the main pancreatic duct, whereas lesions that are not continuous and involve the head, body or tail are defined as "segmental" (Figures 2 and 4)[24,25]. In Japanese criteria, the terms "diffuse" and "at least one third of the entire length" in the same sentence are contradictory to each other. Thus, it may be more appropriate that the term "diffuse" irregular narrowing of main pancreatic duct in Japanese imaging criterion (Table 1) is changed to "diffuse or segmental" irregular narrowing. In AIP cases of segmental involvement, the intervening normal-appearing duct upstream to the stricture shows no or minimal dilatation in spite of the long stricture (Figure 4). These findings differentiate AIP from pancreatic cancers, which reveal stricture associated with marked upstream ductal dilatation[26,27]. Second, "narrowing" of the main pancreatic duct is a term based on subjective assessment.

Table 3 Clinical characteristics of 28 patients with autoimmune chronic pancreatitis

Age/sex	Other autoimmune diseases	IgG (mg/dL)	IgG4 (mg/dL)	Auto antibody	ERCP	CT findings	Pancreas pathology	Response to steroid
54/M	+	3570	1764	-	S	DE	N-C	Y
60/M	+	4500	810	+	S	DE	N-C	Y
32/M	-	974	13	-	S	DE	ND	Y
68/M	-	2010	350	-	S	DE	Diagnostic[b]	Y
52/M	+	1440	32	+	S	DE	ND	Y
59/M	-	1880	324	-	S	DE	Diagnostic	Y
53/M	+	1990	150	+	S	DE	Diagnostic	Y
56/F	-	2730	1464	+	S[a]	DE	N-C	Y
74/M	-	2210	N-C	-	S	DE	N-C	Y
52/M	-	2100	N-C	-	D	DE	N-C	Y
53/F	-	1240	29	-	S[a]	DE	Diagnostic	Y
45/M	-	1470	N-C	+	D	DE	Diagnostic	Y
58/M	-	4100	780	+	S	DE	Diagnostic	Y
63/F	-	1200	10	-	D	DE	Diagnostic	Y
61/M	-	1500	190	-	S[a]	DE	Diagnostic	Y
63/M	-	1780	658	+	S[a]	DE	Diagnostic	Y
49/F	-	2000	445	+	D	DE	N-C	Y
59/M	-	1230	840	+	D	DE	Diagnostic	Y
51/M	-	1570	310	-	D	DE	Diagnostic	Y
68/M	+	3550	1360	+	S	DE	N-C	Y
33/M	-	1350	66	-	S[a]	DE	N-C	Y
36/M	-	1060	50	+	D	DE	ND	Y
60/M	-	1930	N-C	+	S[a]	DE	Diagnostic	Y
64/F	-	1470	N-C	+	S	DE	ND[c]	Y
70/M	+	1820	310	+	D	DE	Diagnostic	Y
44/M	-	1110	36	-	S	DE	Diagnostic	Y
34/F	+	1070	11	-	S	DE	ND	Y
78/M	-	4570	1580	+	S[a]	DE	N-C	Y

S: Segmental irregular narrowing of main pancreatic duct; D: Diffuse irregular narrowing of main pancreatic duct; DE: Diffuse enlargement of pancreas; N-C: Not checked; ND: Non-diagnostic; [a]: Extent of irregular narrowing was less than 1/3 of the entire length of main pancreatic duct; [b]: Lymphoplasma cell infiltration and fibrosis; [c]: Eosinophilic infiltration.

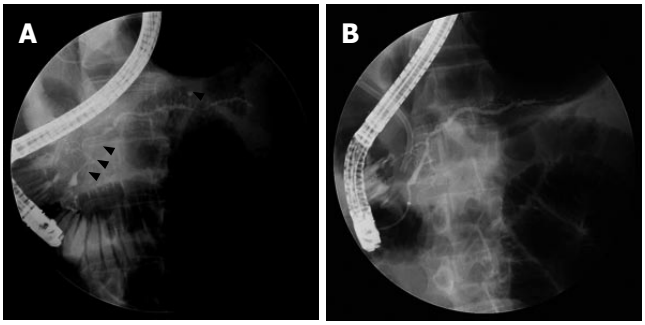

Figure 4 ERCP findings. **A:** Segmental irregular narrowing (arrow heads) of the main pancreatic duct that involves the pancreatic head and tail portion is noted in a 53-year-old man; **B:** After steroid therapy, the narrowing sites on preceding ERCP were resolved. Biliary stenting for narrowing of intrapancreatic common bile duct is noted.

At the present time, there are no references for how thin "narrowing" is. Like the size of pancreatic gland, duct diameter also varies by age, gender, and size of patient, *etc*. In the cases of AIP, most patients are elderly, which complicates matters because the diameter of the main pancreatic duct physiologically increases with age[28,29]. In patients with AIP, the ductal diameter widens to normal caliber after steroid therapy (Figures 2 and 4).

Some authors suggested that as a Japanese length criterion of direct pancreatogram, "more than 1/3" of ductal narrowing (Table 1) should be changed to "more than 1/4" because the extent of ductal narrowing was between 1/4 and 1/3 of the entire length of main pancreatic duct in some cases of AIP[30]. Actually, in our series, the extent of irregular narrowing was less than 1/3 of the entire length of main pancreatic duct in 7 of 28 (25%) patients (Figure 3, Table 3). Moreover, a variant form of AIP that is characterized by focal parenchymal swelling with a localized short stenosis of the main pancreatic duct and evident upstream dilatation has been reported[31]. Because this focal type is a rare form of AIP and mainly diagnosed after laparotomy, further discussion is beyond the scope of this review. However, studies reported that segmental narrowing progressed to diffuse narrowing on serial ERCPs without steroid treatment[32,33]. In other words, "focal" and "segmental" types can evolve into "diffuse" with time, which implies that the total length of the stricture can be less than one third at an earlier stage of the disease[32,33]. Last, "irregular wall" is another confusing term. While Japanese criteria use the term of "irregular" to portray the marginal irregularity of the narrowed ductal wall[30], we have experienced AIP patients with direct pancreatograms that reveal smooth margins not uncommonly. This may be related to the fact that pathologic specimens often reveal inflammation confined to the subepithelial space with an intact ductal epithelial lining[34,35]. Therefore, the term "irregular wall" may not always be appropriate for AIP. On the other hand, the Cambridge classification for chronic pancreatitis uses

"irregular" (*i.e.*, 'irregular dilatation' of main pancreatic duct) to describe the overall contour of the main pancreatic duct[24]. These pancreatographic findings may be explained by the heterogenous pattern of inflammatory infiltrates and fibrosis noted in pathology[34].

The intriguing point of imaging is that CT findings resembling mild acute pancreatitis are quite unusual when associating ERCP findings, which reveal diffuse or segmental irregular narrowing of the main pancreatic duct, are considered. Narrowing of the main pancreatic duct with or without intrapancreatic common bile duct stenosis on ERCP is a finding that is rarely seen in acute pancreatitis, but often in chronic pancreatitis[36]. While diffuse enlargement without calcification or stone is common in acute pancreatitis, this finding is rare in chronic pancreatitis and parenchymal atrophy is more frequently observed in ordinary chronic pancreatitis. CT findings have no evident chronic parenchymal changes in contrast to the ductal pathology detected on ERCP[37]. Coexistence of such contradicting radiologic findings (CT and ERCP) in the same patient was a rare yet peculiar association that was consistently observed in patients with AIP[38].

We believe that clinicians should vigorously obtain direct pancreatograms when patients show unusual clinical features or atypical clinical manifestation in patients suspected of pancreaticobiliary malignancies. Although direct pancreatogram holds a critical position in diagnosing AIP, hardships follow in obtaining it. Due to the possible risk related to the procedure, the visualization of pancreatic duct is not always carried out when the main pancreatic duct is not dilated on US or CT in patients with diffuse pancreatic swelling alone[39,40]. There are also pitfalls in interpreting pancreatograms. Because the main pancreatic duct is extrinsically compressed, it is technically difficult to attain whole ductal images and, sometimes, sufficient amount of contrast is not introduced into the main pancreatic duct. An "abrupt cutoff" of the main pancreatic duct may conceal a true "irregular narrowing" when ERCP is performed inadequately. Deep cannulation of catheter up to the tail portion guided by a thin guide wire can help circumvent this problem. While magnetic resonance cholangiopancreatography (MRCP) is replacing diagnostic ERCP in many pancreaticobiliary diseases, it seems to be limited in delineating the detailed pathology of main pancreatic duct associated with AIP. AIP is basically a "narrow-duct" disease and the resolution of MRCP is inferior to that of ERCP in this aspect[41,42].

Laboratory criterion

Abnormally elevated levels of serum gamma globulin and/or IgG or the presence of autoantibodies are described as the laboratory criterion of AIP by the Japan Pancreas Society[9]. In general, these features are known facets of autoimmune diseases, which provide laboratory evidence of an autoimmune pathogenesis[43]. By measuring various autoantibodies, a number of candidates have emerged for clinical usage in AIP. Detection rates for autoantibodies, however, have varied among reports[44,45]. This difference may be accounted by the number of measured autoantibodies, which can affect detection rates of autoantibodies; rates tend to increase as more autoantibodies are checked[46]. Among the conventional autoantibodies that are commonly investigated in autoimmune diseases, anti-nuclear antibody and rheumatoid factor are more frequently detected in AIP. Other markers, including anti-smooth muscle antibody and anti-mitochondrial antibody, have failed to show detection rates above 10%. On the other hand, anti-lactoferrin antibody (ALF) and anti-carbonic anhydrase II antibody (ACAII) are relatively organ-specific autoantibodies, which reveal the highest detection rates (over 50%) among autoantibodies in AIP[47]. Lactoferrin is normally present in the pancreatic acinus and carbonic anhydrase II in the ductal cells of the pancreas. ALF and ACA II, however, require a special laboratory for measurement that is unavailable to many clinicians. And, even if ALF and ACA II are measured, these markers are not 100% sensitive[48,49]. This implies that although carbonic anhydrase II and lactoferrin are considered to be the most likely target antigen for AIP, cases without ALF and ACAII, despite a good response to steroid therapy, also exist. ALF and ACAII are not specific to AIP as they may be elevated in other diseases[48,49]. Elevated levels of ACAII were also reported in patients with pancreatic cancer or ordinary chronic pancreatitis[10,50,51].

Elevated IgG and hypergammaglobulinemia are not always seen in patients with AIP. IgG4, a subtype of IgG, levels have been reported to be able to distinguish AIP from other pancreatic disorders with a high sensitivity (95%) and specificity (97%)[52]. Moreover, some cases of AIP show elevated serum levels of IgG4 in spite of normal IgG levels[53]. We, therefore, believe that both IgG and IgG4 levels should be measured in all patients suspected with AIP. In Japanese criteria, however, the increased IgG4 level was not included in the laboratory criteria and only mentioned in the appendix that there are cases with elevated levels of IgG4[9]. The Japanese criteria for laboratory data states that IgG levels should be higher than 1 800 mg/dL (normal range, 614-1295 mg/dL[54]). Although cutoff value of IgG was set at 1 800 mg/dL in Japanese criteria, another group uses different cutoff value (1 700 mg/dL) of IgG for the diagnosis of AIP[55]. If we set cutoff value of IgG at higher level, the specificity of IgG increases but the sensitivity decreases. More evidence on sensitivity and specificity according to each cutoff value should be provided for IgG before cutoff level is established.

While serologic markers have provided a base in understanding AIP, there are still controversies. As autoantibodies and IgG can rise non-specifically during the course of various injuries and diseases, the mere increment can not indicate a cause-and-effect relationship. Moreover, elevation of IgG4 level has been reported in patients suffering from pancreatic carcinoma and other types of chronic pancreatitis[50]. A number of groups have tried to find other laboratory indicators of AIP. HLA may identify patients susceptible to AIP. One report mentioned that frequencies of DRB1*0405 and DQB1*0401 were significantly higher in patients with AIP when compared with chronic calcifying pancreatitis[56]. At the present time, however, further studies are required to evaluate the value of each laboratory indicator and find a more reliable one.

Histopathologic criterion

The Japanese criteria described the histopathologic findings of AIP as marked inflammatory infiltrates mostly of lymphocytes and plasma cells and dense fibrosis of the pancreatic tissue[9]. In one report, however, lymphoplasma cell infiltration was minimal in one third of AIP cases. In general, marked infiltration of inflammatory cells is noted in early stage of an autoimmune disease, but fibrosis becomes predominant as the disease progresses. This suggests that degree of inflammatory cell infiltrates and fibrosis and predominance of either one may be dependent on the stage of AIP[57]. The characteristic lymphoplasmacytic infiltration and fibrosis may not be pathognomonic to AIP. Alcoholic chronic pancreatitis (ACP) also showed an abundant amount of lymphoplasma cell infiltration and fibrosis[58]. Although periductal inflammation and fibrosis were observed in both ACP and AIP, the detailed histologic patterns differed. While ACP mainly revealed dense interlobular fibrosis with a cirrhosis-like appearance, AIP showed loose fibrosis in both interlobular and intralobular region[58-60]. AIP showed more severe and diffuse acinar atrophy than ACP. As for extracellular matrix proteins, collagen type IV, a component of the intact basement membrane that ensures accurate regeneration of tissue, was preserved in cases associated with AIP, whereas ACP showed a significant loss of this collagen subtype[58]. Not only may this help differentiate AIP from ACP, but also aid our understanding of the regeneration of acinar structures and regression of fibrosis following steroid treatment. More specific histologic features, *i.e.*, the pattern of fibrosis and inflammation, should be supplemented for histopathologic criterion of AIP.

Periductal fibrosis and inflammatory infiltrates surrounding the duct like a cuff compress the ductal lumen into a star-like structure[61]. Intrapancreatic portion of common bile duct is often involved and the biliary involvement in AIP develops by the same mechanism as the pancreatitis[23]. Some reports of AIP show predominant neutrophilic or eosinophilic granulocytes infiltrating into the pancreatic parenchyma rather than lymphoplasma cell infiltration[62]. This acute inflammatory component of AIP is characterized by focal detachment, disruption and destruction of the duct epithelium due to invading granulocyte, which has been named "granulocytic-epithelial lesions" of the ducts. The extension and severity of chronic and acute changes in AIP vary from case to case, and even from area to area within the same pancreas.

Many clinicians come across difficulties in obtaining adequate pancreatic specimens for histologic evaluation[48]. Fine-needle aspiration biopsy often fails to gain sufficient amounts of pancreatic tissue. Large-bore needle biopsies may be used to yield adequate amounts for pathologic examinations. However, this is at the cost of increasing risks of procedure-related complications. Because of the patchy nature of inflammation seen in AIP, percutaneous biopsy may not be diagnostic due to sampling error problems[13]. Another problem is the potential risk of tumor seeding during biopsy in patients in whom cancer can not be omitted. Due to this reason, endoscopic ultrasonogram (EUS)-guided biopsy is recommended for patients in whom pancreatic cancer can not be excluded[63]. This approach is useful because the pancreatic head is the most frequently involved portion in AIP. One recent paper has reported the usage of EUS-guided trucut biopsy, which may help surmount the above problems and allow optimal histologic examination in AIP[64].

Although histologic evaluation offers a gold standard in diagnosing many disease entities, its role maybe a little different in autoimmune conditions, as seen in primary sclerosing cholangitis and autoimmune hepatitis[65,66]. In these conditions, the histopathologic findings are not disease-specific. Biopsy is often performed to exclude other entities that coexist or show resemblance rather than to make a diagnosis. Taken together, the role of histopathologic examination in patients suspected of AIP may be the exclusion of other diseases, such as malignancy, rather than the confirmation of diagnosis.

Response to the steroid

Although the dramatic response to the steroid is a well-known phenomenon in AIP, a detailed steroid schedule has not been fully established at the present time. Prednisolone is usually initiated at 30-40 mg per day and tapered after confirmation of the response to the steroid. The response to the steroid is defined as improvement in clinical symptoms, negative conversion of detected autoantibodies, normalization of elevated levels of IgG, and reversion of abnormal pancreatic imaging, including CT and endoscopic retrograde pancreatography[58]. In cases of obstructive jaundice associated with intrapancreatic common bile duct narrowing, however, biliary stenting is often needed additionally. The dose of oral corticosteroid may be tapered by 5 mg each 2-4 wk. Eventually, the steroid is completely discontinued or maintained at a dose of 2.5-10 mg/d according to the preference of the doctor[50,52,67].

The response to the oral steroid provides a circumstantial evidence of underlying autoimmune pathogenesis[68]. Among the responses to the oral steroid therapy, recovery of pancreatic ductal narrowing is top priority in the differential diagnosis of AIP. For the recovery of pancreatic ductal narrowing, histologic recovery including periductal fibrosis should be accompanied. We already reported histologic recovery, especially regression of pancreatic fibrosis in patients with AIP after steroid therapy[69]. Relief of pancreatic ductal narrowing by steroid administration is a unique and specific finding that can not be seen in any other type of chronic pancreatitis or pancreatic cancer (unpublished observation, Myung-Hwan Kim, MD). And, because marked improvement of pancreatic ductal narrowing can be observed at as early as 2 wk after steroid therapy[32,50], steroid trial may be a practical diagnostic tool that has clinical impact, especially when differentiation from cancer is an issue. This is analogous to autoimmune hepatitis where steroid treatment is justified when the entity is highly suspected, and the response to treatment is incorporated into the diagnostic scoring system[66]. If steroid therapy fails to show clinical improvement, imaging studies should be performed again to differentiate AIP from pancreatic cancer. There are opposes against

including the response to the steroid into the diagnostic criteria of AIP. They argue that steroid is not usually prescribed in any other type of chronic pancreatitis and one has to strongly suspect AIP in the first place to ever consider steroid therapy. To observe the response to the oral steroid in patients suspected of AIP, however, may be a diagnostic trial as well as therapeutic trial. Actually, in one Japanese university hospital and Italian group, they use the response to the steroid as one diagnostic criterion of AIP[50,70].

There are also concerns of the possibility of cancer progression during a trial of steroid therapy in an operable patient[6]. Despite this risk, we believe that a trial with steroids can be used to guide diagnosis when used in a proper fashion. If a patient shows typical pancreatic images of AIP, a short course (about 2 wk) of steroid may differentiate AIP from pancreatic cancer due to the fact that pancreatic ductal images of malignancy do not change[32,50]. And if the results are equivocal or do not favor AIP, the diagnosis of AIP should undergo reevaluation and the possibility of laparotomy should be considered. By including the response to the steroid into the diagnostic criteria, we can overcome the fact that there are patients who do not satisfy laboratory data and histologic findings, yet reveal typical images and an excellent response to the steroid.

Association of other postulated autoimmune diseases

Autoimmune diseases tend to cluster, and one patient may have more than one autoimmune conditions[43]. This is also the case in AIP which is frequently associated with Sjögren's syndrome, retroperitoneal fibrosis, primary sclerosing cholangitis, primary biliary cirrhosis, and inflammatory bowel disease[71]. This association may be explained by the fact that carbonic anhydrase II and lactoferrin are present in the salivary gland and biliary duct as well as the pancreas. An autoimmune response against these common antigens may result in "autoimmune exocrinopathy" which describes an autoimmune disease that involves multiple exocrine organs[72]. On the basis of the absence or presence of a systemic autoimmune diseases, Okazaki *et al*[14] divided AIP into 2 major groups: Primary or secondary forms of the disease. In contrast to the Japanese criteria, Italian group has included the association of other postulated autoimmune disease into their diagnostic criteria of AIP (Table 4)[50].

The prevalence of other postulated autoimmune diseases, however, varies among papers with some reporting rates that exceed 50%[50]. Comorbidities are manifested at various time points on the natural course of AIP; some manifest before AIP and some simultaneously and others after remission[32]. Thus, primary form of AIP may need to be changed to secondary form in cases where other autoimmune diseases are not recognized at diagnosis of AIP but develop later. For patients with AIP, the presence of an associated autoimmune disease may help the diagnosis, but if not present at the onset of the disease, we must carefully search for it.

In addition, pancreatitis in patients suffering from other autoimmune diseases does not always indicate that the cause of pancreatitis is autoimmunity. For example, when pancreatitis occurs in patients with systemic lupus erythematosus, pancreatitis can be related to various causes, such as vasculitis or medications[73]. This also adds confusion in reporting AIP in patients with underlying autoimmune conditions. In these cases, the remarkable response to steroids can help identify autoimmunity as the cause of pancreatitis[74].

Table 4 Diagnostic criteria for autoimmune pancreatitis by Italian group

Diagnostic criteria
1 Histology and cytology
2 The association with other postulated autoimmune disease
3 Response to the steroid therapy

REVISED DIAGNOSTIC CRITERIA FOR AIP: OUR PROPOSAL

The Japan Pancreas Society proposed diagnostic criteria in 2002 (Table 1). In our experience, a considerable number of cases of AIP is diagnosed confidently when these criteria are applied. Clinicians, however, reported cases that benefited with steroid therapy that did not satisfy the criteria[11]. In our study, 9 of 28 (32%) patients with AIP did not fulfill Japanese criteria, yet responded to the steroid (Table 3). In other words, clinicians may miss a substantial portion of patients suffering from AIP when the diagnosis is confined to those who satisfy the criteria proposed by the Japan Pancreas Society.

On the basis of a single institute experience of 28 patients, we introduce the following system for the diagnosis of AIP (Table 5). We have designed a system where patients are stratified by evidence strength for AIP into "definite", "probable" and "possible". We respect the diagnostic criteria proposed by the Japan Pancreas Society and believe that it contains the strongest findings that support the diagnosis of AIP. Some of the original descriptions create confusion and the diagnostic criteria are not completely satisfactory. Descriptions have been, therefore, rephrased and the diagnostic criteria have been expanded to include more patients who can benefit from this diagnosis. While "definite" AIP is almost same as the original Japanese criteria, those who only reveal typical pancreatic images of AIP are diagnosed as "possible" AIP. This is because the combination of pancreatic imaging (CT and ERCP) seen in AIP is quite distinctive and rarely seen in any other disease entity. When other postulated autoimmune diseases are associated with typical pancreatic images of AIP, the suspicion index becomes higher and patients are designated as "probable" AIP. The diagnosis of AIP is confirmed by the unique response to steroid that can be observed by the dramatic resolution of pancreatic ductal narrowing, and then patients are reassigned to "definite".

We believe that patients who present with typical pancreatic images of AIP deserve a short course of steroids before undergoing surgical resection, despite the lack of any serologic markers of autoimmunity or

Table 5 A proposal of revised diagnostic criteria for autoimmune pancreatitis

Diagnostic Criteria
Criterion I. Pancreatic imaging (essential)
(1) CT: Diffuse enlargement (swelling) of pancreas
and (2) ERCP: Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion II. Laboratory findings
(1) elevated levels of IgG and/or IgG4
or (2) detected autoantibodies
Criterion III. Histopathologic findings
Fibrosis and lymphoplasmocytic infiltration
Criterion IV. Association of other postulated autoimmune disease
Definite
I+II+III+Ⅳ or I+II +III or I+II or I+III
Probable, I+IV; Possible, I only: Rediagnosed as "definite" if "response to the steroid" is present

Table 6 General features of autoimmune diseases

General features of autoimmune diseases
1 Elevated levels of serum gammaglobulin and/or IgG, or detected autoantibody
2 Lymphoplasma cell infiltration and fibrosis at lesional site
3 Association with other autoimmune diseases
4 Response to the steroid

pathognomonic histopathologic findings. The results of serologic markers and histologic examination in patients with AIP may be closely related to the disease activity (active *vs* inactive) or the stage (early *vs* late) of the disease. In our proposal, therefore, a diagnostic trial of steroid can be initiated even though serologic markers or pathologic findings do not fulfill the Japanese criteria or are not available, providing that pancreatic images are typical to AIP.

The Japan Pancreas Society emphasizes imaging (CT and ERCP) criterion and serologic abnormalities as important and relatively specific markers for this entity, while they do not use "the response to the steroid" and "association of other postulated autoimmune diseases" as diagnostic criteria (Table 1). However, Italian experience does not believe imaging findings and serologic abnormality are specific (Table 4). In our revised diagnostic criteria (Table 5), pancreatic imaging (CT and ERCP) is also essential and the findings are almost same as Japanese criteria. Instead, we abolished the condition "more than 1/3 of the entire length of main pancreatic duct" and added "segmental" irregular narrowing. In the laboratory criterion, we newly inserted elevated serum IgG4 level. Consequently, more patients may benefit from oral steroid therapy and can avoid unnecessary major operation. In addition to pancreatic imaging, we included the response to the steroid and association with other postulated autoimmune diseases in the diagnostic criteria, in order to reduce cases that might be occluded by the Japanese criteria. It is already well known that the general features of autoimmune diseases include detected serum autoantibodies or elevated IgG, lymphoplasmacytic infiltration and fibrosis at the lesional site, response to the steroid and association with other autoimmune diseases (Table 6)[68]. Among the general features of autoimmune diseases, one feature does not have more evidence of strength to be superior to the others in the diagnosis of AIP. We, therefore, used all aforementioned features of autoimmune disease for the diagnosis of AIP.

CONCLUSION

Autoimmune chronic pancreatitis (AIP) is a relatively new disease entity to many physicians, yet because of the clinical impact, they must vigilantly look for it. This review discusses several potential limitations of current diagnostic criteria for this increasingly recognized condition. The manuscript is organized to emphasize the need for convening a consensus to develop improved diagnostic criteria. These efforts will refine the diagnosis of AIP, which will lead to less inter-observer variation and provide a strong base for the research of this treatable condition.

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REVIEW

Anastomotic disruption after large bowel resection

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Abstract

Anastomotic disruption is a feared and serious complication of colon surgery. Decades of research have identified factors favoring successful healing of anastomoses as well as risk factors for anastomotic disruption. However, some factors, such as the role of mechanical bowel preparation, remain controversial. Despite proper caution and excellent surgical technique, some anastomotic leaks are inevitable. The rapid identification of anastomotic leaks and the timely treatment in these cases are paramount.

Key words: Colon; Colectomy; Anastomosis; Surgical complications

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INTRODUCTION

Anastomotic leakage following colorectal resection and primary anastomosis is a major clinical problem. The increased morbidity and mortality following anastomatic leakage are considerable, and lead to prolonged hospital stay. Leakage after partial colectomy with primary anastomosis may result in abscess formation, sepsis, multiple procedures and death. Despite vast improvements in surgical technique and devices, anastomotic leakage continues to be a clinical problem. The prevalence of intraperitoneal anastomotic leak varies in the literature between 0.5% and 30%, but is generally between 2% and 5%[1-3]. The double staple anastomotic technique does not appear to increase the risk for anastomotic leak, which has been reported to be 2.7%[4].

There are many factors that contribute to anastomotic leakage. Certainly, poor surgical technique can lead to an anastomotic leak. However, even when the operation is done technically well, anastomotic leaks are inevitable. Hence, a great deal of research has been done to elucidate the factors, which may decrease the rate of anastomotic leaks. Several factors have been identified that may impact on anastomotic leakage: adequacy of blood flow to the anastomoses, contamination, anastomotic technique, the presence of a pelvic drain, anastomotic tension, absence of active disease or distal obstruction, and the distance from the anal verge[5].

Numerous different techniques have been used to fashion a colorectal anastomosis. These techniques can be divided into 2 categories: hand sewn or stapled anastomosis. Hand sewn techniques include single-layer interrupted or continuous with either absorbable or nonabsorbable sutures, or various double layer techniques. The advent of stapling devices in the last century has made a significant contribution to colorectal surgery. Stapling devices have been widely accepted by surgeons performing gastrointestinal surgery. Numerous studies have been conducted comparing the various anastomotic techniques. Debates have been raised comparing single *versus* double-layered closure, absorbable *versus* nonabsorbale sutures, sutures *versus* staples, and inverting *versus* everting techniques. None of the various methods of anastomosis has been proven to be superior to the others.

HISTORY

During the early 19th century, while writing on intestinal injuries, Travers stressed the uniform contact of cut ends of intestine utilizing everting sutures. Later that century, Lembert countered this idea, instead advocating inverting sutures with serosal to serosal contact. Halsted noted that the submucosal layer was the strength-bearing layer in intestinal anastomoses. By the time that Treves published "A System of Surgery" in 1895 "Lembert Sutures" were recommended in intestinal anastomoses. The first acclaimed mechanical device to create a non-sutured anastomosis was Murphy's button introduced in 1892. It consisted of two mushroom shaped pieces, which were secured within bowel ends by purse string sutures. The pieces were then joined together. The bowel would heal as an inverted anastomosis. The excess inverted tissue would slough and the intact "button" would pass per rectum. Murphy's button gained considerable acceptance for several decades.

Circular end-to-end stapling devices were developed in the Soviet Union during the 1950s. The KT, PKS and SPTU instruments were bulky and unwieldy but served as the prototypes for today's end-to-end staplers[6]. A Soviet instrument was brought to the United States by Ravitch in 1958. Subsequently, such devices have been manufactured in the United States but did not attain widespread use until the 1970s. Thus, today's surgeon has the option of suturing or stapling intestinal anastomoses.

RISK FACTORS

Numerous risk factors have been implicated as predisposing for anastomotic leaks. Schrock *et al*[7] performed a large retrospective analysis of factors relating to leakage of colonic anastomoses. Factors that were found to correlate with an increased leakage rate were older age, anemia, prior radiation therapy, intraperitoneal infection and anatomic level of anastomosis. Conversely, steroid use, nutritional status and experience of the operating surgeon did not significantly influence the anastomotic leak rate. Rullier *et al*[8] analyzed factors associated with leakage and reported male sex and level of anastomosis as independent risk factors. In the same study, low anastomoses in obese patients were reported as associated with higher risk of leak. A higher leak rate with low pelvic anastomosis has also been reported by other investigators[9].

In a more recent study, Makela *et al*[10] compared 44 patients with anastomotic leaks to 44 control patients matched for age, gender and indications for surgery. They found that malnutrition, weight loss, alcohol intake, lengthy operative times, peritoneal contamination, and blood transfusions were independent predictors for leaks. In addition, the presence of multiple risk factors increased the risk for anastomotic leaks.

Law *et al*[11] performed a prospective study to identify risk factors for anastomotic leak in 196 patients undergoing total mesorectal excision for rectal cancer ranging from 3 cm to 12 cm from the anal verge. The overall leakage rate was 10.2%. The leakage rate was significantly higher in men (13.4%) as compared with women (5.2%) ($P=0.049$). As expected, the presence of a proximally diverting stoma significantly decreased the leakage rate especially in patients with risk factors for anastomotic dehiscence and low pelvic anastomosis. Interestingly, ages, level of anastomosis, stage of disease, or techniques of anastomosis were not significant predictors of anastomotic leak.

Mechanical forces

Investigators have used strength measurements to assess colonic healing using either breaking strength or bursting strength[12]. The breaking strength represents the uniaxial force required to break a wound *in vitro* and is a test of the entire anastomotic line. The bursting strength is a multiaxial test that measures the weakest point of an intestinal anastomosis which is the most likely location of an anastomotic leak[13].

The mechanical strength of an anastomosis is related to whether an anastomotic leak occurs. The strength of an anastomosis is dependent on the deposition of collagen. The measurement of tissue collagen content is another tool used in experimental models[14]. Martens *et al*[15] demonstrated that increased production of collagen at the anastomotic site was present 12 h after surgery. Brasken *et al*[16] showed that large amounts of type I and III collagen were present on postoperative day 4 in the anastomosis. In support of Halsted's observations, it is generally appreciated that the ultimate strength of an anastomosis depends on the collagen content in the submucosa.

Nutrition

Bowel rest (with a low-residue diet) lowered the bursting strength of non-operated colon in rats[17]. Interestingly, however, it did not impair the strength of a healing anastomosis. Dietary protein depletion impairs colonic strength in healing rat colon. Data regarding the duration of protein depletion needed to impair colonic healing in rats is conflicting. While some studies suggest that as little as one week of protein depletion has a detrimental effect, others suggest that at least 7 wk of protein restriction are needed[18,19]. In a comparison of alimentation means, Kiyama *et al*[20] showed that colonic anastomoses in rats were stronger after enteral nutrition compared to parenteral nutrition.

Bowel preparation

The role of preoperative bowel preparation has become a matter of controversy. Poth EJ[21] in 1953 proposed use of neomycin and sulfathalidine for intestinal antisepsis with reduction in the postoperative complications. Nichols RL and Condon R[22] also suggested a historic reduction in mortality and morbidity with the use of bowel preparation in a collective review of literature. In a 1973 retrospective study, Irvin TT and Goligher JC[23] reported a significant decrease in anastomotic dehiscence with the use of mechanical bowel preparation than that without mechanical bowel preparation (7% *vs* 24%). Most of the reports favoring the use of mechanical bowel preparation are based on retrospective data. However, some randomized trials have reported significant differences in outcomes with use of oral antibacterial agents and mechanical preparation. Matheson *et al*[24] reported a significant reduction in the incidence of wound sepsis and anastomotic dehiscence using both a mechanical and antibiotic preparation.

In contrast, recent literature suggests no significant advantage utilizing aggressive mechanical preparations. To assess the need for mechanical preparation to decrease the rate of anastomotic leaks in elective colorectal surgery, a number of prospective randomized trials have been completed[25-28]. Recently, Guenaga *et al*[29] conducted a meta-analysis on the existing clinical trials which studied the effect of mechanical bowel preparations on the rate of anastomotic leaks. A total of 1204 patients were enrolled in the various studies. Patients were divided into 2 groups: Group 1 ($n=595$) which received a mechanical bowel preparation; and group 2 ($n=609$) without a mechanical bowel preparation. They showed that the rate of anastomotic leaks in group 1 was obviously higher (5.5%)

compared to group 2 (2.9%) ($P=0.02$). Clearly, controversy exists on whether mechanical bowel preparations influence the rates of anastomotic leaks in elective colorectal surgery. Recent meta-analysis and prospective trials have questioned the usefulness of mechanical bowel preparations and do not support its use.

Chemo-radiation

Preoperative chemo-radiation has been used in patients with rectal carcinoma and reductions in tumor size can be achieved with its use. Chemo-radiation may predispose to anastomotic problems in patients having colon surgery, particularly in patients with anastomosis in the pelvis. Many surgeons perform a temporary diverting stoma to minimize the consequences of anastomotic disruption in patients who have had pelvic radiation therapy[30]. Anastomotic leak and radiation therapy may contribute to the formation of pelvic fibrosis, rendering the neorectum stiff and noncompliant. After reconstruction, patients may suffer from tenesmus and fecal incontinence[31].

Since many colectomies are performed for cancer, the effects of common chemotherapeutic agents and external beam irradiation on colonic healing are of interest. Immediate post-operative administration of intravenous 5-flourouracil (5-FU) in rats undergoing colectomy resulted in more conflicting data. While 4-8 mg/(kg.d) for 10 d impaired breaking strength of rat colon[32], 20 mg/(kg.d) for 5 d had no significant effect compared to controls[33]. A third study on rats showed that 600 mg/m^2 of 5-FU in the early postoperative period had no effect on colon anastomotic bursting strength[34].

In a study on rats, preoperative vitamin A supplementation protected against impaired colonic healing caused by preoperative radiation therapy[35].

Del Rio *et al*[36] showed that chronic steroids (time released via subcutaneous route) impaired colonic anastomotic strength in rats. In contrast, a large retrospective review in humans suggested no steroid effect[7].

Surgical technique

The technique used to fashion a colorectal anastomosis is largely based on surgeon preference. In order to achieve an adequate colonic anastomosis with a low rate of post-operative anastomotic leak or stricture formation, certain basic surgical principles must be met. First, the technique utilized for the anastomosis must assure an adequate lumen. Second, an adequate blood supply must be maintained for both the proximal and distal colon after resection. Finally, the anastomosis must be performed so that there is no tension to pull it apart (i.e., the surgeon must assure adequate mobilization of the proximal and distal colon). Considerable investigation has been conducted during the last century to determine the best technique for colonic anastomoses. An intestinal anastomosis may be constructed by a variety of techniques, including single layered suture, double layered suture, interrupted or continuous sutures, absorbable or nonabsorbable sutures, stapling devices or with use of a biofragmentable ring. To date, no single technique, single layer suture, double layer suture or stapling has ever been definitely demonstrated to be superior in preventing anastomotic leaks[37,38].

Table 1 Prospective randomized trials comparing the effect of mechanical bowel preparation *versus* no preparation on anastomotic leaks in elective colorectal surgery (*n*, %)

Investigators	Number of patients	Bowel preparation group (leak rate)	No bowel preparation group (leak rate)	*P*
Miettinen *et al*[25]	267	4%	2%	0.28
Zmora *et al*[26]	380	3.7%	2.1%	0.50
Santos *et al*[27]	149	10%	5%	0.52
Burke *et al*[28]	186	7.8%	11%	0.90

Surgical technique has been extensively studied in animal models. When comparing inverting *versus* everting sutured techniques, the everting technique produced less inflammation and less stricture but the inverting technique was less likely to disrupt[39,40]. (Table 1) This was also supported by the work of Irvin *et al*[41] in both animal as well as human studies. In addition, they reported no difference in the two layered *versus* single layer inverting anastomosis technique when doing intestinal anastomosis[41,42]. With disruption being the most serious problem, the inverting technique is more commonly used.

Stapled *versus* various sutured anastomoses have been compared numerous times in animal models. In a detailed study in dogs, Chung *et al*[43] showed a single layered sutured anastomosis resulted in the least reduction in anastomotic blood flow. Stapled anastomoses reduced blood flow the most. Conversely, Kozol *et al*[40] showed that early anastomotic edema was greater in two layered sewn anastomoses than in stapled. It should be noted that in some clinical circumstances, the surgeon's choice of technique is limited. For example, it is generally accepted that for low pelvic colo-rectal anastomoses stapled techniques are easier to perform.

Numerous clinical studies have been performed to define the anastomotic leak rate using sutures (Table 2). The largest of these studies was conducted by Max *et al*[44] in 1000 patients. A retrospective study was performed in 1000 consecutive patients who underwent a single layer continuous polypropylene colorectal anastomosis. The clinical anastomotic leak rate was only 1%[44-47].

Similarly multiple studies have been performed utilizing the stapled technique for colorectal anastomoses (Table 3). The leak rates from these studies ranges from 1.5% to 11%[4,48-54]. The largest of these studies was conducted by Detry *et al*[48]. A prospective study was performed in 1 000 consecutive patients undergoing stapled colorectal anastomosis by a single surgical team. The clinical leak rate was 3.5%. Also, Hansen *et al*[53] performed a large prospective study in 615 patients who underwent stapled colorectal anastomoses by a total of 18 surgeons, showing only 1.5% clinical leak rate.

Specific studies have been performed comparing stapled and sutured colorectal anastomoses (Table 4)[55-58]. Docherty *et al*[55] conducted a randomized prospective multicenter trial in 732 patients undergoing either hand-sewn ($n=321$) or stapled ($n=331$) colorectal anastomoses. The location of the anastomosis included ileocolic, colocolic, colorectal, and colostomy closures. There was no difference in the stapled or sutured group with regards to rate of anastomotic leakage. Demetriades *et al*[58] conducted

Table 2 Clinical studies utilizing sutures for fashioning colorectal anastomosis

Investigators	Number of patients	Types of suture	Continuous *vs* interrupted	Leak rate (%)
Max *et al*[45]	1 000	Non-absorbable	Continuous	1
Mann *et al*[46]	320	Absorbable	Interrupted	3.4
Flyger *et al*[47]	105	Absorbable	Continuous	1
Deen *et al*[48]	26	Absorbable	Interrupted	3.9

Table 3 Clinical studies utilizing staples for fashioning colorectal anastomosis

Investigators	Study design	Number of patients	Leak rate (%)
Detry *et al*[49]	Prospective	1 000	3.5
Griffen *et al*[50]	Prospective	75	2.7
Cohen *et al*[51]	Prospective	26	3.8
Laitinen *et al*[52]	Prospective	39	5.3
Baran *et al*[53]	Retrospective	104	2.8
Karanjia *et al*[54]	Prospective	276	11
Hansen *et al*[55]	Prospective	615	1.5
Memon *et al*[56]	Prospective	218	3

Table 4 Comparison of stapled versus sutured colorectal anastomoses

Investigators	Number of patients	Staple technique leak rate (%)	Suture technique leak rate (%)	*P*
Docherty *et al*[57]	732	4.7	4.3	0.93
Fingerhut *et al*[58]	113	13	18.7	0.05
Everett *et al*[59]	100	0	2	NS
Demetriades *et al*[60]	207	6.3	7.8	0.69

NS = not significant.

a prospective multicenter trial comparing hand-sewn to stapled colonic anastomosis in the emergent penetrating trauma setting. A total of 207 patients were enrolled in the study from 19 different centers. All patients underwent colon resection with primary anastomosis. There were 128 hand-sewn anastomoses and 79 stapled anastomoses. The demographics of both groups were similar with respect to age, gender, mechanism of injury, associated injuries, and fecal contamination. They demonstrated that there was no statistically significant difference in the 2 groups with respect to anastomotic leaks.

Surgeons have attempted several intraoperative techniques in hopes of lowering anastomotic leak rates. One is "omentoplasty" which involves wrapping the anastomosis with omentum. This was prospectively studied by the French Associations for Surgical Research[59]. In their randomized study of 705 patients, omentoplasty did not decrease the anastomotic leak rate or the clinical severity of anastomotic leaks compared to the patients without omentoplasty. Some surgeons have routinely placed a pelvic drain after low anterior resections. In a prospective, randomized study of 319 patients, the same French investigators showed that routine pelvic drainage did not lower the rate or severity of anastomotic leaks[9,60].

Many surgeons utilize intraoperative air/water testing of colon anastomoses. With this technique, after completing the anastomosis, the patient is placed in reverse Trendelenburg position. The pelvis is filled with sterile saline solution and an assistant places a sigmoidoscope (flexible or rigid) into the rectum, below the anastomosis. The colon is then insufflated with air, and the surgeon views the pelvic saline bath for bubbling (a sign of an inadequate or leaky anastomosis). If bubbling is seen, the leak is identified and repaired with sutures. There are at least two studies of the efficacy of this technique. In a study of 145 patients, Beard *et al*[61] were able to lower the "radiologic" leak rate from 29% to 11% using air/water testing in order to plan the placement of additional sutures as needed. In a study of 82 patients, Pritchard *et al*[62] found the air/water test helpful in higher anastomoses but unreliable in very low anastomoses. This may be due to the difficulty in suture repairing very low anastomoses.

In many series, the leak rates were higher for anastomoses below the peritoneal reflection[8,63,64]. One large study revealed a 12.7% leak rate in colorectal anastomoses compared to 2.9% in colo-colonic anastomoses[65]. Anastomotic leak can be a serious complication of resection for low rectal resection. Several studies have been conducted to identify risk factors that contribute to anastomotic dehiscence in patients undergoing low anterior resection (LAR) and proctectomy with coloanal anastomosis. Leaks after coloanal anastomoses are no more frequent than with colorectal anastamoses with a range of 6% to 8%[66,67]. Certain risk factors are more frequently associated with rectal resection. Meade *et al*[68] reported that a distance of less than 5 cm from anal verge, male sex, alcoholism and smoking were the risk factors for anastomotic breakdown after low rectal resections. Similar results were reported by Rudinskaite *et al*[69].

Law *et at.* investigated operative results and oncological outcomes of anterior resection for rectal and rectosigmoid cancer. They reported a significantly higher leak rate (8.1%) in patients who underwent a total mesorectal excision than those who underwent partial mesorectal excision (1.3%). Additionally, they reported that higher anastomotic leakage rate was associated with the male gender, absence of stoma, and increased blood loss[70]. Recently, Matthiessen *et al*[71] reported similar results, but they did not report any advantage of performing a temporary stoma. It should be noted that the creation of a proximally diverting stoma to protect a low pelvic or technically inadequate anastomosis does not alter the risk for dehiscence but does ameliorate the septic effects of the leak[7,71,72].

Recently, emphasis on the quality of surgical care offered has increased tremendously. There is an increasing awareness of the outcomes of surgical care as a marker of quality. Dimick *et al*[73] reported lower mortality rates in patients undergoing surgery for colorectal cancer when these procedures were performed in high volume centers. Similarly, Hannan *et al*[74] suggested an inverse relationship between in-hospital mortality rates and case volume for patients undergoing certain procedures. They reported that individual physician volume has more significant influence on the mortality rates for certain procedures. The same

authors[75] recently reported a significant reduction in mortality of patients who underwent colectomy when these procedures were performed by high-volume surgeons at high-volume centers. Conversely, the data reported by Urbach *et al*[76] did not support superior outcomes when colon operations were done at high volume centers.

CLINICAL PRESENTATION AND DIGNOSIS

Anastomotic leakage typically occurs between the 3rd and the 6th post-operative days. The clinical manifestation of anastomotic dehiscence varies in magnitude from failure to thrive to profound sepsis. The presentations in a given patient depend, in part, on the location and magnitude of the leak, and whether any adjacent tissues such as omentum or small intestine contain the leak. Indeed, a less severe leak may be walled off by adjacent organs or omentum and may present with vague abdominal pain, failure to thrive, temperature elevation, tachycardia, prolonged ileus, diarrhea or intestinal obstruction. Recognition of this situation may be delayed as the nonspecific symptoms can be attributed to delayed recovery from a major operation rather than to an anastomotic failure. However, the physician must have a high index of suspicion to make an early diagnosis. Most patients with anastomotic dehiscence will have prolonged ileus, increased postoperative abdominal pain, fever, and leucocytosis. However, the spectrum can include sepsis, peritonitis and/or hemodynamic instability. Longo *et al*[77] described the initial symptoms in 56 patients with postoperative pelvic abscess that developed after colon surgery, showing that 93% had intestinal dysfunction and 4% were in shock.

The presence of the aforementioned risk factors should raise the index of suspicion for leaks. Suspicion of a leak should lead to diagnostic imaging. A gastrografin enema is a quick and inexpensive way to evaluate the integrity of a colonic anastomosis. A gastrografin enema is less useful for right colonic anastomoses because it becomes too dilute to accurately define the anastomosis. A CT scan with intravenous, oral and rectal contrast material may also be obtained in those patients with suspected anastomotic leak and should demonstrate any abscess or extravasation of contrast from the intestine. Barium enema should not be used in this circumstance because of the increase in morbidity and mortality associated with barium-induced peritonitis. Indium-labeled leukocyte scans are occasionally helpful to identify abdominal abscesses that are suspected but not seen using conventional imaging.

MANAGEMENT

The specific method of management of an anastomotic dehiscence depends on the manifestation of the leak and the clinical condition of the patient. As many as 36% to 49% of patients with a pelvic anastomosis will have a leak demonstrated when gastrograffin enemas are routinely used during the first postoperative week[78,79]. Most of these are "subclinical" leaks. In a patient with evidence of low-grade sepsis and documentation of a contained anastomotic leak with abscess, drainage of the abscess and broad-spectrum parenteral antibiotic therapy are required and may be sufficient therapy. Drainage of an abscess can be accomplished percutaneously or operatively. A CT scan of the abdomen and pelvis with intravenous, oral, and rectal contrast medium is advocated whenever an anastomotic leak and abscess is suspected. CT scan is highly sensitive and accurate (95%) in determining the presence of abdominal or pelvic abscess[80]. CT-guided percutaneous drainage is successful in as many as 85% of appropriately selected patients[81]. For a low colorectal anastomosis, abscess drainage can be accomplished through the anastomosis if the dehiscence is readily apparent at endoscopic examination. The defect can be gently enlarged to allow better drainage, and transrectal drains can be placed in the cavity for continuous or intermittent irrigation. Transvaginal and transperineal drainage can also be performed.

Clinically ill patients with sepsis, pain and tenderness will require reoperation. Creation of a proximal colostomy or ileostomy plus peritoneal lavage and placement of drains are indicated. Some studies have advocated proximal diversion without resection if the anastomosis has been used with good results[82,83].

Gross peritonitis requires laparotomy, resection of the anastomosis with end colostomy and mucous fistula or Hartmann pouch. Diversion alone without resection of the leaking anastomosis is not ideal because of persistent sepsis from the leaking anastomosis. In such cases, wide drainage of the anastomosis should be performed. Repair of the anastomosis, either alone or in combination with a proximal stoma, is not recommended because of the high risk of recurrent anastomotic failure and/or anastomotic stricture in the presence of intra-abdominal sepsis.

Unrecognized anastomotic leaks may present as enterocutaneous fistulas. A fistulogram and/or CT scan should be obtained to determine the site of the defect in the intestine and whether any undrained collection of pus is present. Any adjacent fluid collection should be drained to facilitate closure of the fistula. After control of the source of sepsis and in the absence of distal bowel obstruction or a foreign body, the majority of colocutaneous fistulas will close without operative intervention. Other important management guidelines include correction of anemia and fluid and electrolyte abnormalities, excellent wound care, and adequate nutrition. Bowel rest and total parenteral nutrition may be necessary to facilitate closure. A late manifestation of unrecognized anastomotic leaks is anastomotic stricture. Strictures may require endoscopic dilation. Refractory strictures will require surgical revision or resection and reanastomosis.

CONCLUSION

In summary, surgeons should be aware of risk factors for colonic anastomotic leaks. The ideal is avoidance of a colonic anastomotic leak by use of proper surgical technique. In fashioning a colorectal anastomosis, some basic surgical techniques must be followed to have an acceptable result. These include the presence of adequate blood flow to the anastomosis, minimal

contamination, absence of anastomotic tension, absence of active disease, and no distal obstruction. The utility of preoperative mechanical bowel preparation in decreasing the anastomotic leak rate has been questioned by findings from several recently performed randomized prospective studies. The use of sutures or staples to create a colorectal anastomosis has never been shown to significantly alter the anastomotic leak rate.

Even when excellent surgical technique is used, a small percentage of leaks are inevitable. Characteristics, such as male gender, obesity, level of anastomosis, peritoneal contamination, age, operative time and blood transfusions, have all been implicated as potential risk factors for anastomotic leakage in various studies[84]. The clinicians must have a high index of suspicion to diagnose an anastomotic leak early. If a leak occurs, it must be identified and treated expediently. Treatment is based on the patient's conditions and the magnitude of the leak.

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Pulmonary complications in patients with chronic obstructive pulmonary disease following transthoracic esophagectomy

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Abstract

AIM: To investigate the incidence of various types of postoperative pulmonary complications (POPCs) and to evaluate the significance of perioperative arterial blood gases in patients with esophageal cancer accompanied with chronic obstructive pulmonary disease (COPD) after esophagectomy.

MEHTODS: Three hundred and fifty-eight patients were divided into POPC group and COPD group. We performed a retrospective review of the 358 consecutive patients after esophagectomy for esophageal cancer with or without COPD to assess the possible influence of COPD on postoperative pulmonary complications. We classified COPD into four grades according to percent-predicted forced expiratory volume in 1 s (FEV1) and analyzed the incidence rate of complications among the four grades. Perioperative arterial blood gases were tested in patients with or without pulmonary complications in COPD group and compared with POPC group.

RESULTS: Patients with COPD (29/86, 33.7%) had more pulmonary complications than those without COPD (36/272, 13.2%) ($P < 0.001$). Pneumonia (15/29, 51.7%), atelectasis (13/29, 44.8%), prolonged O_2 supplement (10/29, 34.5%), and prolonged mechanical ventilation (8/29, 27.6%) were the major complications in COPD group. Moreover, patients with severe COPD (grade ⅡB, FEV1 < 50% of predicted) had more POPCs than those with moderate(grade ⅡA, 50%-80% of predicted) and mild (grade Ⅰ ≥ 80% of predicted) COPD ($P < 0.05$). PaO_2 was decreased and $PaCO_2$ was increased in patients with pulmonary complications in COPD group in the first postoperative week.

CONCLUSION: The criteria of COPD are the critical predictor for pulmonary complications in esophageal cancer patients undergoing esophagectomy. Severity of COPD affects the incidence rate of the pulmonary complication, and percent-predicted FEV1 is a good predictive variable for pulmonary complication in patients with COPD. Arterial blood gases are helpful in directing perioperative management.

Key words: Chronic obstructive pulmonary disease; Arterial blood gas; Esophageal cancer; Complication

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INTRODUCTION

Radical esophagectomy remains the most effective method in patients with esophageal cancer, and the five-year survival rate of 40% or higher can be achieved[1-3]. However, esophagectomy may be one of the greatest surgical operations. Postoperative pulmonary complications (POPCs) are common after esophagectomy[4,5].

Chronic obstructive pulmonary disease (COPD) is a common fatal disease in China. Postoperative pulmonary complication after thoracotomy is the major complication in patients with COPD[6-8].

COPD is considered as an important risk factor for pulmonary complications due to low cardiopulmonary reserve[9-12]. However, the incidence of each type of complications and its relationship with percent-predicted FEV1 after esophagectomy in patients with COPD are unclear.

Our study was to document the effect of COPD on complication rates and the incidence of various types of pulmonary complications in esophageal cancer patients with or without COPD following esophagectomy, to elucidate the relationship between percent-predicted FEV1 and pulmonary complications and to evaluate the change and significance of arterial blood gases after operation in patients with COPD.

MATERIALS AND METHODS

A total of 358 patients who underwent esophagectomy at Beijing Friendship Hospital and Peking University First Hospital between July of 2001 and March of 2005 were included in this study. Eighty-six of the 358 patients were diagnosed having COPD.

We divided COPD into 4 grades: grade Ⅰ: percent predicted FEV1%p ≥ 80 and FEV1/FVC< 70%(45 cases); grade Ⅱ A: 50% ≤ FEV1%p < 80%, FEV1/FVC < 70% (32 cases); grade Ⅱ B: 30% ≤ FEV1%p < 50%, FEV1/FVC < 70% (9 cases); gradeⅢ: FEV1%p < 30%, FEV1/FVC < 70% (0 case).

Table 1 summarizes the patient characteristics. The two groups were similar in terms of age, sex, presence of other medical conditions, type and duration of operation, cancer stage, anastomosis site, blood loss and serum albumin. All patients underwent radical resection of tumors in the middle or lower third thoracic esophagus which were confirmed to be squamous cell cancers of esophagus after surgery. In our study, patients with tumor of the cervical and upper third thoracic esophagus were excluded because of different oncological characteristics and treatment protocols.

Basic hematological and biochemical tests, pulmonary function tests, electrocardiograph, chest CT scan, abdominal ultrasonography, barium contrast study and endoscopy were carried for all patients.

Patients were advised to stop smoking and to quit of alcohol two weeks prior to operation. Patients with hypercapnia and pulmonary hypertension were excluded. All patients in our study did not receive preoperative chemotherapy and chemoradiotherapy.

Esophagectomy via a left thoracotomy approach or cervical left thoracotomy approach was performed. Reconstruction of intestinal continuity was restored with a stomach placed in the left thoracic cavity or via orthotopic route when the anastomosis was carried out in the neck. The circular stapler was used when anastomosis was performed in the thoracic cavity and a hand-sewn anastomosis was done when it was in the neck. All patients received intravenous nutrition and continuous gastrointestinal decompression after esophagectomy.

All patients were followed up after surgery and complications occurring during the patient hospitalization were recorded. For this study, pulmonary complications were defined to include: pneumonia (manifesting fever, productive cough, increased white blood cell count, and marked infiltration on chest roentgenogram), atelectasis (manifesting segmental or lobar's atelectasis on chest roentgenogram without bronchial stenosis), pulmonary abscess (displaying intrapulmonary air containing space on chest roentgenogram and purulent exudation in the pleural cavity with fever and increased white blood cell count requiring drainage and antibiotic therapy), prolonged O_2 supplement (protracted supplemental oxygen≥14 d), acute respiratory distress syndrome(ARDS; PaO_2:FiO_2 ratio less than 250 for more than 24 h with pulmonary infiltrates, without clinical suspicion of volume overload, deterioration of respiratory status needing mechanical ventilatory support), and prolonged mechanical ventilation ≥2 d[4,6,9,13]. Postoperative pulmonary complications studied included pulmonary parenchyma but not complications of pleural cavity such as hemothorax, pneumothorax, thoracic abscess, chylothorax, pleural effusion, mediastinal emphysema.

Table 1 Baseline characteristics of study population with or without COPD undergoing transthoracic esophagectomy

Characteristics	COPD (*n* = 86, %)	Non-COPD (*n* = 272, %)	*P* value
Age, yr	61.3±5.5	63.2±7.1	0.522
Sex (male:female)	63/23	191/81	0.638
Smoking history	79 (91.9)	214 (78.7)	< 0.01
Past medical history			
Hypertension	16 (18.6)	48 (17.6)	0.840
Cardiac disease	13 (15.1)	35 (12.9)	0.594
Diabetes	10 (11.6)	33 (12.1)	0.900
Cancer stage			
Ⅰ	17 (19.8)	56 (20.6)	0.869
Ⅱ	48 (55.8)	145 (53.3)	0.685
Ⅲ	21 (24.4)	71 (26.1)	0.115
Ⅳ	0	0	-
Site of anastomosis			
Neck	32 (37.2)	108 (39.7)	0.679
Chest	54 (62.8)	164 (60.3)	0.679
Duration of operation (min)	162.75±51.05	185.15±66.24	0.239
Blood loss (mL)	365.50±219.36	434.00±232.48	0.344
Low serum albumin (<35g/L)	25 (29.1)	89 (32.7)	0.526
Spirometry			
FEV1 (L)	1.6±0.3	2.3±0.5	< 0.01
FEV1,% predicted	51.5±10.5	80.4±13.1	< 0.01
FEV1/FVC,%	57.6±8.9	73.3±6.8	< 0.01
DLCO,% predicted	83.7±13.4	85.0±13.9	0.774

FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity; DLCO = diffusion capacity of the lung for carbon monoxide.

Arterial blood gases were tested daily from first preoperative day to the seventh postoperative day at 4-5pm. Patients with respiratory failure or mechanical ventilation 7 d prior to operation were excluded in order to avoid intervention. Statistical analysis was performed using *t-test*, *ANOVA* and *chi-square test*. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS statistical package (version 10.0, SPSS Inc., Chicago, IL).

RESULTS

Postoperative pulmonary complications such as pneumonia and atelectasis occurred early after operation. Pulmonary complications in the 358 patients with or without COPD are shown in Tables 2 and 3. Patients with COPD (29/86, 33.7%) had more pulmonary complications than those without COPD (36/272, 13.2%) ($P < 0.01$), Pneumonia (15/29, 51.7%), atelectasis (13/29, 44.8%), prolonged O_2 supplement (10/29, 34.5%), prolonged mechanical ventilation (8/29, 27.6%) were the major complications in COPD group.

The rate of mortality was 3.5% (3/86) in the COPD group and 66.7% (2/3) of deaths were due to respiratory failure. In patients without COPD, the rate of mortality was 1.5% (4/272) and two patients (50%) had respiratory failure as their cause of death.

Table 2 Pulmonary complications occurring in patients with or without COPD undergoing transthoracic esophagectomy

Pulmonary complications	COPD (n = 86)	Non-COPD (n = 272)	P value
Pneumonia	15	19	<0.01
Atelectasis	13	15	<0.01
Pulmonary abscess	4	5	0.227
Prolonged O_2 supplement	10	11	<0.01
Prolonged mechanical ventilation	8	10	<0.05
Acute respiratory distress syndrome	5	4	<0.05

Table 3 Degree of COPD and outcomes in patients with or without POPC in COPD group

Severity of COPD	POPCs (n = 29)	Non-POPCs (n = 57)	P value
FEV1,%predicted			
Grade Ⅰ (>80%)	10	35	<0.05
Grade Ⅱ A (50%-80%)	13	19	
Grade Ⅱ B (30%-50%)	6	3	
GradeⅢ (<30%)	0	0	

POPCs = postoperative pulmonary complications.

Table 4 Comparison of PaO_2 changes in the first postoperative week in patients with or without POPCs in COPD group (mean±SD)

Time	POPCs	Non-POPCs	P
1 preoperative day	82.17±7.37	82.75±4.45	0.758
1 postoperative day	68.38±5.24	76.00±8.28	<0.01
2 postoperative day	61.83±7.03	69.05±7.78	<0.01
3 postoperative day	61.75±6.14	67.20±7.35	<0.05
4 postoperative day	62.71±4.93	70.15±9.34	<0.01
5 postoperative day	61.13±5.62	72.75±10.30	<0.001
6 postoperative day	61.83±6.49	76.55±9.62	<0.001
7 postoperative day	61.63±6.31	80.45±7.50	<0.001

PaO_2 = partial pressure of oxygen.

Table 5 Comparison of $PaCO_2$ changes in the first postoperative week in patients with or without POPCs in COPD group (mean±SD)

Time	POPCs	Non-POPCs	P
1 preoperative day	42.85±4.10	42.37±6.03	0.766
1 postoperative day	44.42±4.61	41.24±4.93	<0.05
2 postoperative day	45.35±4.97	41.38±5.19	<0.05
3 postoperative day	46.02±4.88	42.45±5.62	<0.05
4 postoperative day	46.01±5.56	39.10±3.59	<0.001
5 postoperative day	44.63±5.31	40.62±5.44	<0.05
6 postoperative day	45.95±6.56	42.03±5.60	<0.05
7 postoperative day	45.18±5.80	40.49±6.13	<0.05

$PaCO_2$ = partial pressure of carbon dioxide.

Table 6 Comparison of SaO_2 changes in the first postoperative week in patients with or without POPCs in COPD group (mean±SD)

Time	POPCs	Non-POPCs	P
1 preoperative day	94.48±1.93	94.44±1.51	0.940
1 postoperative day	94.56±1.18	93.78±1.80	0.095
2 postoperative day	94.01±1.94	94.30±1.39	0.572
3 postoperative day	95.39±1.15	94.56±1.75	0.077
4 postoperative day	94.65±1.27	95.31±1.15	0.079
5 postoperative day	94.94±1.39	94.26±1.81	0.176
6 postoperative day	94.88±1.60	94.96±1.74	0.868
7 postoperative day	95.02±1.16	94.61±1.90	0.399

SaO_2 = arterial oxygen saturation.

Moreover, patients with severe COPD (Grade Ⅱ B, 30% ≤ FEV1%p < 50%) had more POPCs than patients with moderate (grade Ⅱ A, 50% ≤ FEV1%p < 80%) and mild (grade Ⅰ, FEV1%p ≥ 80%) COPD (P < 0.05).

The perioperative changes in arterial blood gases in patients with or without POPCs in COPD group are listed in Tables 4-6. In non-POPC group PaO_2 decreased in the first three days after operation and then gradually returned to its normal level. Values for $PaCO_2$, SaO_2 and pH were in normal range. However, in POPC group, PaO_2 dropped significantly, recovered more slowly, and failed to return to normal at the end of the first week compared with non-POPC group. $PaCO_2$ in POPC group was significantly higher than that in non-POPC group in the first 7d, reaching more than 6Kpa in first postoperative week. In addition, there was no significant difference in SaO_2 and pH between the two groups.

DISCUSSION

Transthoracic esophagectomy displays a remarkable effect on pulmonary function, including lung and chest wall compliance reduction, ventilation function reduction, increase of oxygen consumption[14]. Pulmonary complication is considered as one of the most serious and threatening complications after esophagectomy, and is associated with poor short- and long-term outcomes[9,15]. Postoperative pulmonary complications occur frequently after transthoracic esophagectomy for esophageal cancer, accounting for 7.3%-50%[9,13,16-18]. In addition to surgical techniques and perioperative management strategies, different definition criteria for pulmonary complications, patient selection and willingness of surgeons to undertake high-risk cases may influence the outcomes[4,19,20].

COPD is considered as a postoperative pneumonia risk index and is significantly associated with the occurrence of pulmonary complications[21,22]. According to our definition, a higher rate of pulmonary complication is associated with esophageal resection for esophageal cancer with COPD, particularly in patients with percent-predicted FEV1 less than 50%. Pneumonia, atelectasis' prolonged O_2 supplement and prolonged mechanical ventilation are the major complications after esophagectomy. The incidence rate of pulmonary complication in COPD group was higher than that in non-COPD group. The incidence rates of acute respiratory distress syndrome and pulmonary abscess were lower. Moreover, pulmonary complications are associated with postoperative mortality and regarded as the most common cause of operation death.

To assess the independent effect of COPD, we matched

patients with severe COPD to comparison groups of patients with moderate and mild COPD. The rate of pulmonary complication increased along with percent-predicted FEV1 reduction, suggesting that percent-predicted FEV1 is a good risk factor for anticipating postoperative pulmonary complications after esophagectomy.

Previous studies indicate that various factors predispose to pulmonary complications[4,10,23-27], including advanced age, history of smoking, cirrhosis and diabetes, abnormal chest radiograph or lung disease, blood loss and low serum albumin, preoperative chemoradiotherapy, general performance status, inadequate postoperative analgesia and stage of disease. Some cell factors have a relation with pulmonary complications after esophagectomy, such as secretary leukocyte protease inhibitor and angiotensin-converting enzyme[28,29]. Our analysis also demonstrated a correlation between percent-predicted FEV1 and pulmonary complication in patients with esophagectomy.

The changes of arterial blood gases coincided well with the timing of pulmonary complications in our patients which occurred in the first postoperative week. The pathophysiologic feature of this group was a further depression and patients had a poor prognosis. However, pH values and oxygen saturation had no remarkable change in the two groups.

Our study was not designed to determine if perioperative care could decrease the rate of pulmonary complications in patients with COPD after esophagectomy, but we believe that aggressive treatment is important in improving the outcome of pulmonary complication so that patients with COPD benefit from radical procedure. It was reported that effective treatment can reduce pulmonary complications[13]. Rehabilitation training, proper antibiotics and eliminating phlegm's drugs, atomization, stopping smoking, nutritional supplementation may have some benefits to patients with COPD before operation[30-32]. Regulation of intravenous transfusion volume can effectively prevent postoperative pulmonary edema and improve oxygenation[33].

In conclusion, chronic obstructive pulmonary disease is the critical factor for the occurrence of postoperative pulmonary complications in esophageal cancer patients undergoing esophagectomy. Pulmonary complications go up along with the severity of COPD. Percent-predicted FEV1 is a good predictor for postoperative pulmonary complications. Arterial blood gases are helpful in directing perioperative management.

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GASTRIC CANCER

Extremely well-differentiated adenocarcinoma of the stomach: Clinicopathological and immunohistochemical features

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Abstract

AIM: Minimal deviation carcinoma of the uterine cervix, otherwise known as extremely well-differentiated adenocarcinoma (EWDA), is characterized by its benign microscopic appearance in contrast to its aggressive behavior. In order to elucidate the clinicopathological features and biological behavior of the gastric counterpart of EWDA, we, using immunohistochemistry, analyzed nine lesions for the phenotypic expression, proliferative activity, and the expression of oncogene-associated products.

METHODS: Clinicopathological features, including preoperative biopsy diagnosis, were reviewed. Using immunohitstochemistry, Ki-67 labeling index and expression of p53 and c-erbB-2 protein in the gastric lesions were detected.

RESULT: Locations in the middle or upper third of the stomach and polypoid macroscopic features are characteristic of EWDA of the stomach. Although 4 of the 9 lesions showed only focal lymphatic or venous invasion, lymph node metastasis was not present and none of the patients died of the lesions (mean follow-up period, 56 mo). All 9 cases of EWDA could be classified into gastric phenotype (5 lesions) and intestinal phenotype (4 lesions). The former resembled gastric foveolar epithelium, mucous neck cells or pyloric glands, but their papillary structures were frequently elongated and the tumor cells and their nuclei were slightly larger and more hyperchromatic compared to normal epithelium. The latter resembled intestinal metaplasia with minimal nulcear atypia and irregular glands; two of these lesions demonstrated complete intestinal phenotype, while two demonstrated incomplete intestinal phenotype. Ki-67 labeling index was low and none of the cases revealed over-expression of p53 and c-erbB-2 protein.

CONCLUSION: Unlike minimal deviation carcinoma of the cervix, these findings suggest that EWDA of the stomach is a lesion of low-grade malignancy. This favorable biological behavior is supported by the data of a low Ki-67 labeling index and a lack of p53 or c-erbB-2 protein over-expression. Because of its resemblance to normal gastric mucosa or mucosa with intestinal metaplasia, EWDA is often misdiagnosed. To prevent the misdiagnosis of such lesions, the clinical and pathologic characteristics should be taken into consideration.

Key words: Stomach neoplasms; Extremely well-differentiated adenocarcinoma; Ki-67; p53; c-erbB-2

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INTRODUCTION

Silverberg and Hurt proposed the term "minimal deviation carcinoma" for extremely well-differentiated adenocarcinoma (EWDA) of the uterine cervix[1], which has a benign microscopic appearance yet shows an aggressive behavior[1-3]. This carcinoma is characterized by mucinous glands which resemble normal endocervical glands but invade the cervical stroma. Several similar cases of adenocarcinomas which show deceptively benign appearance have been reported in the stomach[4-12]. In those reports, the difficulty with histological diagnosis based on biopsy specimens is well discussed in detail; however, the biological behavior of the lesions still remains unclear.

Based on Lauren classification[13], gastric carcinomas

have been classified into two types, intestinal-type and diffuse-type. Following recent advances in mucin histochemistry and immunohistochemistry, it has been clarified that differentiated adenocarcinoma can be classified into two subtypes, these being gastric and intestinal phenotypes[14-20]. With regard to EWDA of the stomach, Endoh *et al*[11] reported eight cases of EWDA mimicking complete-type intestinal metaplasia, confirmed by phenotypic investigation using immunohistochemical methods. Most reported cases of EWDA of the stomach seem to be intestinal-type carcinomas, resembling complete or incomplete intestinal metaplasia[4-10]. In addition, we have encountered a few reports of cases of EWDA mimicking normal gastric mucosa, where the cases were considered to be lesions of the gastric phenotype. However, in these cases, there was very little objective investigation of the phenotypic expression.

In order to elucidate the characteristics of EWDA including its biological behavior, we describe herein the clinicopathological features of nine cases, including phenotypic expression, proliferative activity and expression of some oncogene-associated products.

MATERIALS AND METHODS

Patients

EWDA is defined as neoplastic lesions composed of highly differentiated neoplastic epithelium which mimicks the normal gastric mucosa or intestinal metaplastic mucosa with mild nuclear atypia, but has the ability to invade the gastric wall. We retrospectively reviewed 3106 cases from our old consecutive files that had been diagnosed as well-differentiated adenocarcinoma of the stomach, and found three (0.1%) cases of EWDA. Other 6 collected cases of EWDA were added, making a total of 9 cases for this study. One of the reported cases[7] was included in this study. Although we encountered some similar lesions restricted to the mucosa, these were excluded because of difficulty in diagnosing them as malignant.

The clinicopathological findings were principally based on the General Rules for Gastric Cancer Study as outlined by the Japanese Research Society for Gastric Cancer[21]. Eight specimens were obtained by surgery, and one was endoscopically resected. The resected specimens were fixed in 100 mL/L buffered formalin. The early lesions were cut many times throughout the entire tumors, whereas the advanced lesions were cut only once through their center. The sections were then embedded in paraffin. Then 4-μm thick sections were routinely stained with hematoxylin and eosin stain (H&E). In addition, pre-operative biopsy specimens were also reviewed.

Immunohistochemistry

The monoclonal antibodies against human gastric mucin (45M1, Novocastra, Newcastle-upon-Tyne, UK, diluted 1:50) as a marker for gastric foveolar cells[22,23], MUC6 (Novocastra, Newcastle-upon-Tyne, UK, diluted 1:200) as a marker of gastric mucous neck cells and pyloric glands[24,25], MUC2 (Novocastra, Newcastle-upon-Tyne, UK, diluted 1:200) as a marker for intestinal goblet cells[26-28], CD10 (Novocastra, Newcastle-upon-Tyne, UK, diluted 1:200) as a marker for the small intestinal brush border[29-31], Ki-67 (MIB-1, dilution 1:100; Immunotech, Marseille, France), p53 (PAb 1801, dilution 1:100; Oncogene Research Products, Cambridge, Massachusetts, USA) and c-erbB-2 (dilution 1:200; Nichirei, Tokyo) were used. Immunohistochemical staining was carried out using streptavidin-biotin-peroxidase complex method (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan) following antigen retrieval with microwave heating (citrate buffer, 30 min; phosphate-buffered saline, 10 min, respectively) utilizing an H2800 Microwave Processor (Energy Beam Sciences, Agawan, Massachusetts, USA) at 800 W. Sections were visualized with diaminobenzidine (DAB) and counterstained with methyl-green or hematoxylin. The negative controls consisted of substituting mouse normal serum for the primary antibodies.

Table 1 Phenotypic classification by immunohistochemical stains

		Human gastric mucin or MUC6	
		(-)	(+)
CD10 (+)		C - type	
CD10 (-)	MUC2 (+)	I - type	
	MUC2 (-)	U - type	G - type

C: complete intestinal, I: incomplete intestinal, G: gastric, U: unclassified.

Evaluation

The positivities of human gastric mucin (HGM), MUC6, MUC2 and CD10 were estimated as being significantly positive when more than 10% of the area was positive-stained. According to the combination of their expression, phenotypes were classified into four types: gastric type (G-type), incomplete intestinal type (Incomp. I-type), complete intestinal type (Comp. I-type), and unclassified type (Table 1)[20].

The Ki-67 (MIB-1) labeling index (LI) was defined as a percentage of MIB-1-positive nuclei, and was evaluated in the invasive areas. The MIB-1 LI was determined by counting at least 1000 nuclei in the selected fields at x400 magnification. p53 immunoreactivity was defined as positive when distinct nuclear staining was recognized in at least 10% of the cells, since most of the previously published studies employed this as the cut-off level. Cases with less than 10% positive cells were regarded as negative. c-erbB-2 was regarded as positive when there was membranous staining in more than 10% of the area of the tumor.

RESULTS

Histologic findings and phenotypic expression

All the EWDA had invaded the submucosa or even deeper; four were restricted to the submucosa, two had invaded the muscularis propria, and three had reached the subserosa beyond the muscularis propria. The EWDAs were classified into gastric phenotype (HGM+ or MUC6+/MUC2-/CD10-) containing 5 cases and

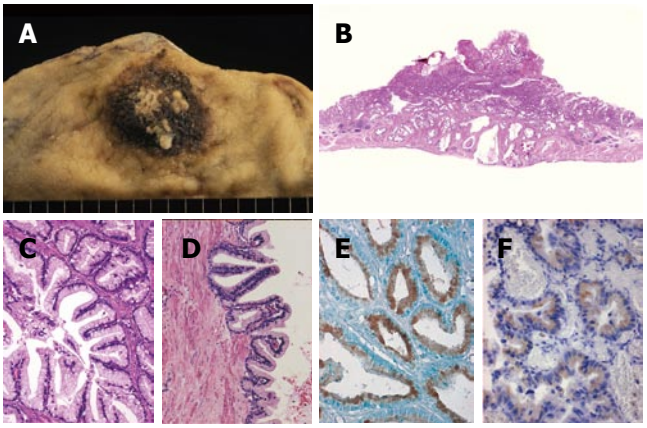

Figure 1 Extremely well-differentiated adenocarcinoma of the stomach, gastric-type (case 4). **A:** Macroscopic view showing a polypoid lesion with an irregular surface; **B:** cancer invasion of the whole thickness of the gastric wall (low-power view); **C:** carcinoma mimicking the normal gastric foveolar epithelium with basally located small nuclei (hyperchromatic nuclei) and abundant mucin; **D:** papillary projections occasionally seen in the carcinomatous glands; **E:** diffuse positive staining of human gastric mucin in carcinomatous glands; and **F:** focally positive staining of MUC6 in carcinomatous glands.

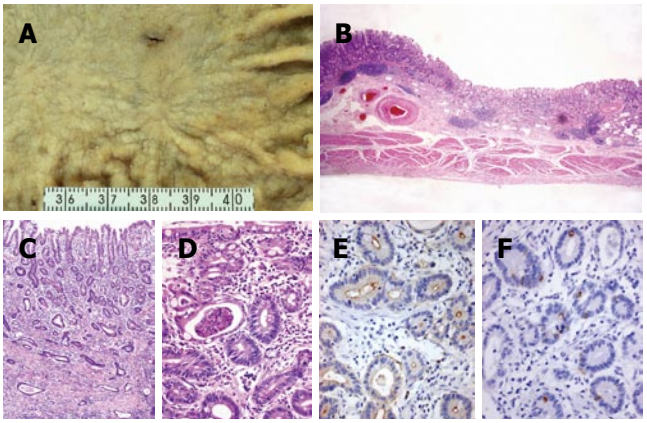

Figure 2 An early lesion of extremely well-differentiated adenocarcinoma of the stomach, complete intestinal-type (case 8). **A:** Macroscopic view showing a shallow depressed lesion with an irregular margin; **B:** carcinoma invasion to the submucosal layer (low-power view); **C** and **D:** carcinoma mimicking the intestinal metaplasia of complete-type with basally located small nuclei, eosinophilic cytoplasm and scattered goblet cells. Note the irregular arrangement of glands and intraluminal debris; **E:** CD10 positivity of carcinomatous glands along the luminal surfaces; and **F:** MUC2 positivity of scattered goblet cells.

intestinal phenotype containing 4 cases. The intestinal phenotype cases were further classified into complete intestinal phenotype (HGM- / MUC6- / MUC2 + / CD10+) and incomplete intestinal phenotype (HGM- / MUC6- / MUC2 + / CD10-), each phenotype contained 2 cases. With regard to MUC6 expression which indicates differentiation to the pyloric glands, MUC6 expression was only detected in 2 of 5 cases of the gastric phenotype.

The five lesions classified as gastric phenotype were composed of well-differentiated epithelium mimicking foveolar epithelium, mucous neck cells or pyloric glands with abundant clear cytoplasm and basally situated nuclei. With careful observation, the nuclei were seen to be slightly larger than those of normal gastric mucosa, and to be markedly hyperchromatic. The superficial area

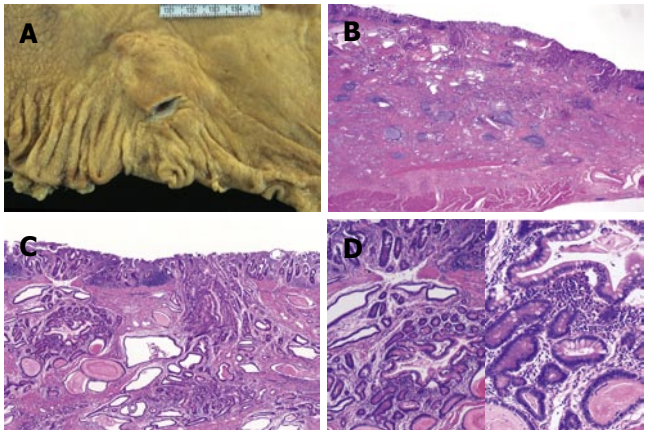

Figure 3 An advanced lesion of extremely well-differentiated adenocarcinoma of the stomach, complete intestinal-type (case 9). **A:** Macroscopic view showing a polypoid mass with an irregular surface, but unclear margin; **B:** cancer invasion of the whole thickness of the gastric wall (low-power view); **C:** carcinomatous gland infiltrating into the submucosa; **D:** carcinoma mimicking the intestinal metaplasia of complete-type with basally located small nuclei, eosinophilic cytoplasm and scattered goblet cells.

tended to resemble the foveolar epithelium while the deep area tended to resemble mucous neck cells or pyloric glands. Two lesions mainly showed remarkable papillary proliferation. The epithelium was lined with a single layer of columnar cells with abundant clear cytoplasm with basally situated nuclei. The glands in this phenotype showed intraluminal papillary projections with or without a fibrous core. In the invasive area, one of five revealed marked desmoplastic reaction, however, other four revealed only slight desmoplastic reaction. One of the cases of gastric-type EWDA is shown in Figure 1.

The four lesions classified as intestinal phenotype were composed of intestinal-type glands with various amounts of goblet cells and Paneth cells, focally showing an irregular shape. Brush border-like structures were occasionally seen, and were confirmed by CD10 staining in the two cases classified as complete intestinal phenotype. We found difficulty in diagnosing these lesions as neoplastic in the mucosa because their glands were somewhat regular in shape and their cytologic atypia was minimal. However, their glands were of varying sizes and showed irregular branching in the deep portion of the mucosa and the submucosa. The glands in the submucosa or proper muscle layer were surrounded by an acute, chronic inflammatory infiltrate with lymphoid follicles. Occasionally, cystically dilated gland was seen in the submucosa, and mucous which had partially leaked out into the stroma owing to destruction of the glands, was seen in these three lesions. In the invasive area, all the four cases revealed marked desmoplastic reaction. Two cases of intestinal-type EWDA, early and advanced, are shown in Figures 2 and 3.

Regarding the background mucosa of the tumors, the surrounding mucosa could not be examined in one gastric phenotypic lesion because the lesion had been endoscopically resected. In another gastric phenotypic lesion, no intestinal metaplasia was seen. As for the other seven cases, various degrees of intestinal metaplasia were

Table 2 Clinicopathological data

Case	Age	Sex	Loc	Size	Macro	Depth	ly	v	LN	Prognosis	Biopsy
Gastric phenotype											
1	81	m	M	5.5	0-I	sm	(+)	(-)	NA	5 mo, alive	benign
2	51	m	U	2.5	0-IIa	sm	(-)	(-)	(-)	23 mo, alive	NA
3	63	m	M	8	1	ss	(-)	(-)	(-)	66 mo, alive	benign
4	76	m	U	3.5	1	ss	(-)	(+)	(-)	30 mo, alive	Ca, susp
5	57	m	U	5	1	ss	(+)	(-)	(-)	48 mo, alive	NA
Intestinal phenotype (incomplete intestinal-type)											
6	65	f	M	1.5	0-IIa	sm	(+)	(-)	(-)	38 mo, alive	Ca, susp
7	45	m	M	2.4	1	mp	(-)	(-)	(-)	129 mo, alive	Ca, susp
(complete intestinal-type)											
8	54	m	M	2.2	0-IIc	sm	(-)	(-)	(-)	136 mo, alive	Ca, susp
9	65	m	M	4	1	mp	(+)	(+)	(-)	30 mo, alive	Ca, susp

Loc: location (U: upper third, M: middle third), Macro: macroscopic feature, Depth: depth of invasion (sm: submucosa, mp: muscularis propria, ss: subserosa), ly: lymphatic permeation, v: venous invasion, LN: lymph node metastasis, NA: not assessed, Ca, susp: carcinoma, suspected.

Table 3 Previously reported cases of gastric EWDA

Case	Author		Age	Sex	Macro	Location	Size	Depth	ly	v	n	Prognosis	Phenotype	Biopsy
1	Araki	(1984)	50	m	1	M	45	ss	1	0	0	?	Incomp-I?	benign
2	Satoh	(1987)	65	m	1	U	40	mp	0	0	0	?	Comp-I?	benign
3	Yaosaka	(1989)	53	m	1	M	80	mp	2	0	1	?	Comp-I?	benign
4	Matsunaga	(1995)	42	m	0-I	L	45	sm	2	0	0	?	?	reg. Atypia
5	Kobayashi	(1999)	55	m	1	M	20	ss	0	0	0	?	Comp-I	Ca, susp
6	Endoh	(1999)	60	f	0-IIa+IIc	M	10	sm	0	0	0	?	Comp-I	Ca
7	Endoh	(1999)	68	f	0-IIc+IIb	M	20	sm	0	0	0	?	Comp-I	Ca
8	Endoh	(1999)	70	m	0-IIb	M	27	sm	0	0	0	?	Comp-I	NA
9	Endoh	(1999)	62	f	0-IIc+IIa	M	15	sm	0	0	0	?	Comp-I	NA
10	Endoh	(1999)	59	m	0-IIa	M	15	sm	0	0	0	?	Comp-I	Ca
11	Endoh	(1999)	74	m	0-IIa+IIc	L	18	sm	0	0	0	?	Comp-I	NA
12	Endoh	(1999)	70	m	0-IIa+IIc	M	25	sm	0	0	0	?	Comp-I	benign
13	Endoh	(1999)	65	m	1	M	55	se	0	0	0	?	Comp-I	benign
14	Adachi	(2000)	54	f	0-I	M	40	sm	?	1	0	?	I?	benign
15	Sato	(2004)	50	m	2	M	48	ss	2	1	0	?	Mixed	benign

Incomp-I: incomplete intestinal, Comp-I: complete intestinal, I: intestinal, reg. atypia: regenerative atypia (benign), NA: not assessed, Ca: carcinoma, Ca, susp: carcinoma, suspected, Macro: macroscopic type Location (U: upper third, M: middle third, L: lower third) Depth: depth of invastion (sm: submucosa, mp: muscularis propria, ss: subserosa).

seen in the surrounding mucosa of both the gastric and intestinal phenotypes.

Patient characteristics

The clinicopathological findings of the nine patients with EWDA of the stomach are summarized in Table 2. The patients included eight men and one woman with ages ranged from 45 to 81 (average 62) years. There were no patients who were diagnosed as Peutz-Jeghers syndrome. None of the patients died or suffered recurrence during the follow-up periods which ranged from 5 to 136 (average, 56) mo.

Macroscopic findings

The tumors had a maximum diameter of 1.5 to 8 (average, 3.6) cm. Of the nine lesions, three tumors were located in the upper third of the stomach, the remaining six were located in the middle third. No lesions were present in the lower third. Among the four early lesions (restricted to the submucosa), two lesions were of superficial elevated (Type 0-IIa) type while the others were of superficial depressed (Type 0-IIc) type or protruding (type 0-I) type. All the advanced lesions (invading the muscularis mucosa and/or the subserosa) were of polypoid type (Type 1).

Pre-operative biopsy

Pre-operative biopsy specimens could be evaluated only in seven cases because of unavailability of specimens in two cases. Two of the seven cases were diagnosed as benign lesions, and the remaining five were initially suspected as being carcinomas, although there was difficulty in distinguishing whether they were neoplastic or regenerative lesions. Only one lesion could be finally diagnosed as a definite carcinoma through repeated biopsy (Case 9).

Ki-67, p53 and c-erbB-2 expressions

None of the cases of EWDA revealed over-expression of p53 or c-erbB-2. Regarding the proliferating activity, the mean value of Ki-67 LI of the EWDAs was 8.7% (range, 0.5%-23.9%).

DISCUSSION

Gastric carcinomas, based on Lauren classification, have been divided into two histologic types by standard hematoxylin and eosin (H&E) staining, such as

"intestinal" and "diffuse" types[13]. It has been considered that intestinal-type carcinoma is almost equivalent to differentiated type carcinoma and that diffuse-type carcinoma is almost equal to gastric or undifferentiated type carcinoma. However, gastric carcinomas are currently classified according to the expression of gastric or intestinal phenotypes, using immunohistochemical or mucin-histochemical methods[14,18]. Accordingly, we divided gastric carcinomas into three phenotypes (complete-intestinal type, incomplete-intestinal type and gastric type) according to the type of intestinal metaplasia, as suggested in previous studies[19,20], using immunohistochemical methods for CD10 (CALLA) which is considered to be expressed by the brush border of the small intestine[29,31], MUC2 which is considered to be expressed by intestinal goblet cells[26,28] and human gastric mucin (HGM) which is considered to be expressed by the gastric foveolar epithelial mucin [22,23]. Some authors have also reported that the phenotypic expression is related to the tumor growth pattern and aggressiveness[32,33], and that this classification is clearly in a good accordance with that of the background mucosa[19]. In this study, the phenotype of EWDA of the stomach was investigated using not only these three antibodies, but also MUC6 as a marker of gastric mucous neck cells and pyloric glands[24,25]. Our nine cases of EWDA could be classified into three phenotypes (complete-intestinal type, incomplete-intestinal type and gastric type).

Clinicopathologically, EWDA of the stomach had several characteristic features, in comparison with the previously reported cases of EWDA listed in Table 3[4-6,8-12]. The location and macroscopic features of the tumors are characteristic. Usually, more than 40% of the gastric carcinomas are located in the distal part of the stomach and polypoid type is rare (3.3%) among advanced gastric carcinomas, as reported by our previous study [34]. All EWDA in our study and most (13/15) ones of the previous reports were located in the middle and upper stomach. Macroscopically, the early lesions of EWDA were flatly elevated (0-IIa) or depressed (0-IIc), while all the advanced lesions were polypoid. The same tendency was also seen in the previous reports. This finding implies that the EWDA arises as a flat lesion but latter grows into a polypoid mass due to massive infiltration of carcinoma cells beneath the mucosa.

The gastric phenotype of gastric EWDA is more likely to be confused with normal gastric mucosa or hyperplastic polyps, whereas the intestinal phenotype of gastric EWDA is more likely to be confused with intestinal metaplastic epithelium. The high degree of differentiation and mild cellular atypia of these lesions result in frequent diagnostic difficulties especially with regard to biopsy specimens prior to surgery. In fact, pre-operative biopsies were negative in eight of 12 cases in previous reports and in two of our current seven cases. These highly differentiated lesions of the stomach have received relatively limited attention. Although EWDA of the stomach is very rare, it is important to take it into consideration when making a differential diagnosis of neoplastic or dysplastic lesions in the stomach.

The histological features of EWDA with regard to pre-operative biopsy specimens and surgical specimens were retrospectively reviewed. The most useful histological feature in diagnosing the intestinal phenotype of EWDA is the irregularity of the tubules in the deep portion of the mucosa and the submucosa. It is therefore important to obtain biopsy specimens from these areas. Moreover, endoscopic mucosal resection by means of which we can examine the entire thickness of the mucosal layer may be a suitable diagnostic procedure in diagnosing intestinal phenotypic lesions. In the cases of gastric phenotype of EWDA, the neoplastic epithelium resembled gastric surface mucous cells or pyloric glands. Their papillary structure was similar to that of normal foveolar epithelium and hyperplastic polyps, although some of them strikingly elongated. In addition, the individual cells and nuclei were obviously larger and their nuclei were more hyperchromatic than those in normal foveolar epithelium. Since the cellular atypism is minimal, it is important to compare their size and the amount of chromatin with that in the surrounding normal epithelium.

There have been no reports about the biological behavior of EWDA, although a low incidence of lymphovascular invasion and lymph node metastasis has been noted (Table 3). As for our cases, all the patients are currently alive. Three lesions revealed only focal venous or lymphatic invasion, but no lymph node metastasis was seen in our cases of EWDA. These findings suggest a favorable prognosis for EWDA of the stomach unlike the prognosis for minimal deviation adenocarcinoma of the uterine cervix, although it needs to be noted that our series was small with limited follow-up data. With regard to the correlation between phenotypes and clinicopathological features, there was no significant difference between the two except for tumor location. Three of the five EWDAs of gastric phenotype were located in upper third of the stomach, whereas all the EWDAs of intestinal phenotype were located in the middle third.

The proliferative compartment in normal gastric mucosa is known to be restricted to the middle layer of the mucosa. Several reports have indicated the relationship between a high Ki-67 LI and poor prognosis in cases of gastric carcinoma[35,36]. In our present study, the Ki-67 LI was lower (average, 8.7%) compared with the previously reported data(from 41.8% to 47.1%)[35-38]. A low Ki-67 LI (13%) in the submucosal invasive area has also been reported by Endoh *et al*[11], and this finding reflects the slow growth and reduced aggressiveness of EWDA of the stomach.

The reported prevalences of abnormal expression of p53 and c-erbB-2 protein have been shown to range from 47% to 60%[39-42] and from 5.7% to 33.0%[43-46], respectively. It has been reported that the over-expression of p53[37-39] and c-erbB-2 protein[43] is a marker of poor prognosis in gastric carcinoma. Fortunately, none of the lesions of EWDA showed over-expression of p53 or c-erbB-2 in our study. It seems reasonable to regard these lesions as having a low-grade malignancy, but an ability to invade downward into the gastric wall, a finding which is supported by the data of low Ki-67 LI and no over-expression of p53 or c-erbB-2. In addition, these immunoreactivities of p53 and c-erbB-2 seemed to be useless for the diagnosis of EWDA.

In the practical diagnosis of a stomach biopsy, it is important to bear in mind the existence of extremely well-differentiated adenocarcinoma (EWDA) of both intestinal and gastric types.

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Anticancer and cytotoxic properties of the latex of *Calotropis procera* in a transgenic mouse model of hepatocellular carcinoma

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Abstract

AIM: To evaluate the anticancer property of the dried latex (DL) of *Calotropis procera*, a tropical medicinal plant, in the X15-*myc* transgenic mouse model of hepatocellular carcinoma and to elucidate its mechanism of action in cell culture.

METHODS: The young transgenic mice were orally fed with the aqueous suspension of DL (400 mg/kg for 5 d/wk) for 15 wk and their liver was examined for histopathological changes at 20 wk. Serum levels of vascular endothelial growth factor (VEGF) were also measured in these animals. To characterize the active fraction, DL was extracted with petroleum ether followed by methanol. The methanolic extract was sub-fractionated on a silica gel G column using a combination of non-polar and polar solvents and eleven fractions were obtained. Each fraction was analysed for cytotoxic effect on hepatoma (Huh7) and non-hepatoma (COS-1) cell lines and non-transformed hepatocytes (AML12) using tetrazolium (MTT) assay. Finally, the mechanism of cell death was investigated by measuring the levels of Bcl2, caspase 3 and DNA fragmentation.

RESULTS: DL treatment of mice showed a complete protection against hepatocarcinogenesis. No adverse effect was observed in these animals. The serum VEGF level was significantly lowered in the treated mice as compared to control animals. Cell culture studies revealed that the methanolic extract of DL as well as its fraction 8 induced extensive cell death in both Huh-7 and COS-1 cells while AML12 cells were spared. This was accompanied by extensive fragmentation of DNA in Huh-7 and COS-1 cells. No change in the levels of canonical markers of apoptosis such as Bcl2 and caspase 3 was observed.

CONCLUSION: DL of *C. procera* has the potential for anti-cancer therapy due to its differentiable targets and non-interference with regular pathway of apoptosis.

Key words: *Calotropis procera*; Transgenic mice; Hepatocellular carcinoma; Cytotoxicity; Anticancer agent; Differential killing

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INTRODUCTION

The incidence of cancer is increasing worldwide and it is the single most common cause of deaths in both developed and developing countries[1,2]. Irrespective of the cause, the localized malignant tumors are best managed by surgical removal[3,4] while the treatment options for advanced and metastasized tumors include chemotherapy and radiotherapy[5-7]. However, in view of the side effects of drugs used in the chemotherapy of different cancers, traditional herbal medicine and complementary and alternative medicine (CAM) are becoming increasingly popular among cancer patients in the developed countries[8,9]. In the traditional Indian medicinal system, the Ak plant or *Calotropis procera* (Ait.) R. Br. (*Asclepiadaceae*) has been used for a variety of disease conditions that includes its use in the treatment of leprosy, ulcers, piles and tumors[10]. The root extract of *C. procera* has been found to produce a strong cytotoxic effect on COLO 320 tumor cells[11]. Recently, a hemi synthetic derivative of a cardenolide isolated from the root barks of *C. procera* shows a strong cytotoxic effect on several human cancer lines, a high *in vivo* tolerance to tumor growth and prolonged survival in the human xenograft models of nude mice[12]. The chloroform-soluble

fraction of its roots, ethanolic extract of its flowers and aqueous and organic extracts of its dried latex (DL) also exhibit a strong anti-inflammatory activity in animal models of acute and chronic inflammation[13-15]. Further, recent epidemiological studies have shown that daily intake of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen results in a significant exponential decline in the risk of different clinical cancers[16]. In view of this, we have evaluated the cytotoxic and anti-tumor activities of the latex of *C. procera* respectively on hepatoma and non-hepatoma cell lines, and in a transgenic mouse model of hepatocellular carcinoma (HCC).

MATERIALS AND METHODS

Collection of latex and its fractionation

C. procera growing in wild was identified by the Raw Materials, Herbarium and Museum Division, National Institute of Science Communication, New Delhi, where a voucher specimen is preserved (Voucher No. PID1739). The latex was collected from the aerial parts of the plant and dried under shade (DL). DL was extracted with petroleum ether (B.P. 40-60 ℃) and methanol in a sequential order. The methanol extract (ME) was subjected to silica gel G (mesh 60-120) step column chromatography using a combination of non-polar and polar solvents and eleven fractions were collected[17]. The fractions were evaporated to dryness and tested for cytotoxic properties.

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Caspase 3 assay kit were procured from Invitrogen (California, USA). Dimethylsulfoxide (DMSO) and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (Missouri, USA). Silica gel G, petroleum ether (PE) and other chemicals were bought from Merck, India.

Treatment of oncomouse

The X15-*myc* transgenic mice expressing HBx and c-*myc* genes in the liver and thereby resulting in hepatocellular carcinoma[18], were used for this study following approval of the Institutional Animal Ethics Committee. Animals of matched age (5 wk old)) of either sex were used in this study. The overnight fasted animals were fed with bread soaked in aqueous suspension of the DL at a dose of 400 mg/kg for 15 wk (5 d/wk, $n = 6$). The control animals were given bread alone ($n = 6$). At 20 wk, the animals of both groups were sacrificed and their liver was collected in buffered formalin (100 mL/L). Paraffin blocks of samples were made and the tissue sections were stained with hematoxylin and eosin to observe histopathological changes.

Estimation of vascular endothelial growth factor (VEGF)

The serum level of VEGF was measured in the mice using a mouse specific ELISA kit (Oncogene Research Products, Germany).

Cell culture and cytotoxicity assay

The human hepatoma Huh-7 cell line[19] was a kind gift from Dr. A. Siddiqui (University of Colorado, Denver). COS-1 (African green monkey kidney cell line, CRL-1 650) and AML-12 cells (mouse hepatocyte, CRL-2 254) were purchased from the American Type Culture Collection (Virginia, USA). All cultures were grown in DMEM without phenol red ($DMEM^{-PR}$) supplemented with fetal bovine serum (100 mL/L), L-glutamine, penicillin and streptomycin and maintained at 37℃ in a CO_2 incubator. The cytotoxic effect of ME and each DL fraction was evaluated in cell cultures where the cells were seeded at a density of 4×10^5 cell/60 mm dish and allowed to settle for 12 h. Then these cells were incubated with different fractions of DL at concentrations ranging from 0.1-10 mg/L for either 24h or 48h. Cell viability was analyzed by MTT colorimetric assay[20]. Briefly, after treatment with the DL fractions, the cells were washed with culture medium and incubated with MTT solution (1 mg/mL in $DMEM^{-PR}$) for 1h at 37 ℃. The medium was decanted, the formazan product was dissolved in 1mL DMSO and the absorbance was read at 560 nm.

DNA fragmentation assay

Extract of the cells treated with DL fraction 8 was prepared in TET buffer (10 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 5 mL/L Triton X100) and incubated with proteinase K (40 mg/L) at 37 ℃ for 1h. After extraction with an equal volume of phenol, followed by phenol and chloroform, DNA was precipitated using ethanol. The DNA pellet was resuspended in H_2O and incubated with RNase A (40 mg/L) at 37 ℃ for 1h before electrophoretic separation in a 15 g/L TBE agarose gel[21].

Bcl-2 and caspase assay

The intracellular level of Bcl-2 was measured by immunoprecipitation using anti-Bcl-2 antibody (Santa Cruz Biotechnology, California, USA). The relative caspase activity was measured using the caspase 3 assay kit (Invitrogen, USA). The cell extract was prepared as per supplier's protocol and the caspase activity was measured in 50 μL aliquots by incubating with the substrate for 2 h at 37 ℃ and the absorbance was read at 405 nm.

Statistical analysis

The values are expressed as mean ± SE ($n = 6$) and the statistical analysis was performed by Student's t test. $P < 0.05$ was considered significant.

RESULTS

DL exhibits in vivo chemopreventive effect

The chemopreventive effect of orally administered DL was studied in the X15-*myc* transgenic mice. Histological examination of the liver of untreated mice at 20 wk showed a marked nuclear atypia, hyperchromatia, necrosis and loss in sinusoidal architecture (Figure 1A). Treatment of mice with DL (400 mg/kg) for a period of 15 wk protected these mice from malignant changes occurring in the liver while sinusoidal architecture and cellular integrity was slightly disrupted as compared to normal and hydropic changes were observed (Figures 1B and 1C). We further

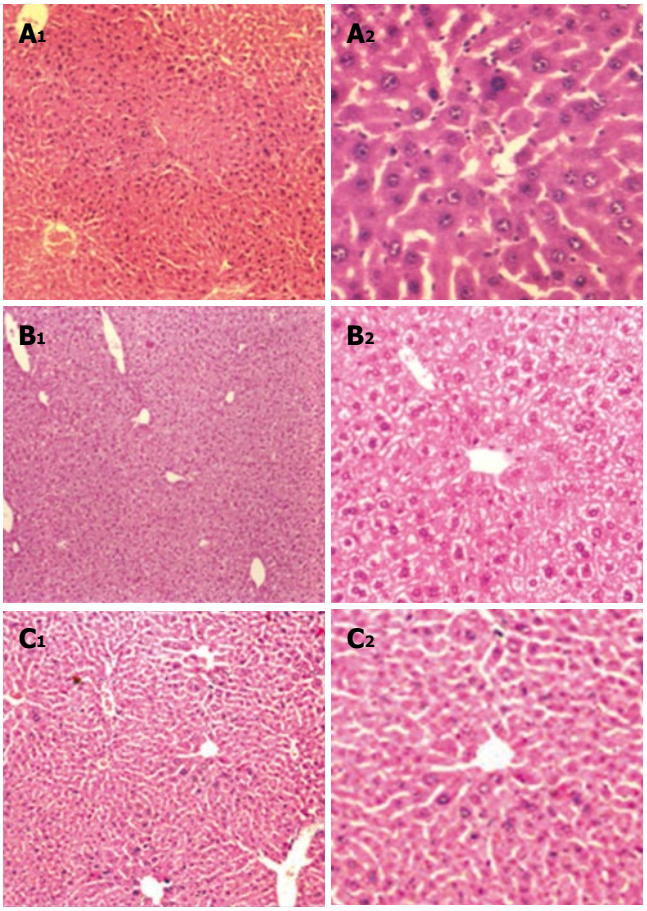

Figure 1 Histological analysis of mouse liver. **A**: X15-myc control; **B**: X15-myc mouse treated with DL suspension; **C**: Normal mouse liver. (**A**$_1$, **B**$_1$, **C**$_1$ = x100 and **A**$_2$, **B**$_2$, **C**$_2$ = x400).

measured the levels of VEGF, a marker of angiogenesis, in these mice. Treatment with DL produced a significant decrease in the serum VEGF levels of X15-*myc* mice from 12.18 ± 0.64 μg/L to 9.75 ± 0.27 μg/L (P = 0.002) while the level of VEGF in normal mice was 7.00 ± 0.55 μg/L.

DL is cytotoxic to cancer cells

Since the aqueous extract of DL showed a strong *in vivo* chemopreventive effect, the molecular basis of its action was investigated in cell culture. The ME of DL was evaluated for cytotoxicity using MTT assay on two different cell lines, viz., Huh-7 and COS-1 cells (Figure 2). ME induced cell death in both cell lines in a concentration dependent manner. Even at low concentration (0.1 mg/L), ME could induce cell death in both the cell lines. The effect was, however, more pronounced (-90% cell death) at higher doses of ME (1 and 10 mg/L).

A polar fraction of DL contributes to its cytotoxic effect

To identify the active component of DL, ME was subjected to silica gel G column chromatography and eleven fractions were collected[17]. Each fraction was evaluated for its cytotoxic activity on Huh-7 and COS-1 cells at 10 mg/L concentration and cell viability was determined by MTT assay (Figure 3). Out of 11 fractions, only fraction 8 exhibited a potent cytotoxic effect on both Huh-7 and COS-1 cells (~90% cell death) and the effect was comparable to ME (input). A marginal inhibitory effect was also seen with fraction 1 on Huh-7 cells (-24% cell death).

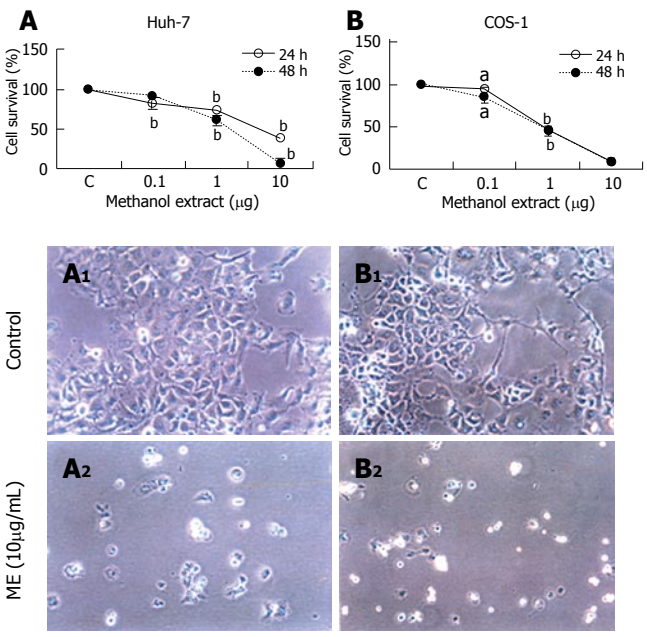

Figure 2 Cytotoxic effect of methanolic extract of DL on cancer cell lines. Huh-7 (**A**) and COS-1 cells (**B**) were incubated with different concentrations of ME and analyzed for cell viability at 24h or 48h (n = 6), mean ± SE. $^aP < 0.001$; $^bP < 0.01$; **A**$_1$ and **B**$_1$ are control Huh-7 and COS-1 cells; **A**$_2$ and **B**$_2$ are ME-treated Huh-7 and COS-1 cells (x200).

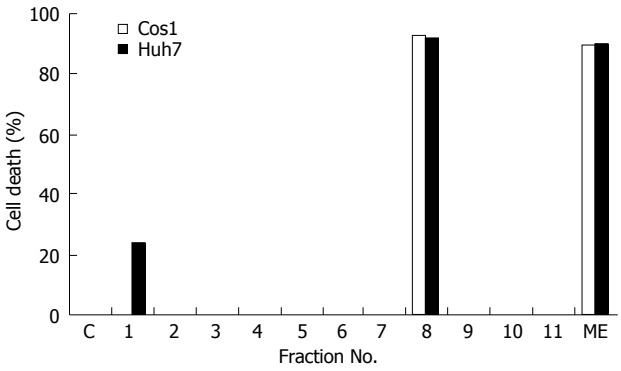

Figure 3 Cytotoxic effect of different fractions of the methanolic extract of DL on cancer cell lines. Huh-7 and COS-1 cells were incubated with either ME or its eleven fractions (all at 10 mg/L). Cell viability was measured at 48 h and results are expressed as % cell death.

We further investigated the relationship between the dose-response and cytotoxic effect of DL fraction 8 on Huh-7, COS-1 and non-transformed AML12 cells. At concentration ranging from 0.1 to 10 mg/L, it produced a dose-dependent decrease in the survival of Huh-7 and COS-1 cells leaving behind only 20%-30% living cells at 10 mg/L concentration. However, unlike the two cancer cell lines, AML12 cells showed a much better survival (-80%) in the presence of fraction 8 (Figure 4 A-C).

Fraction 8 of DL induces DNA fragmentation

To understand the mechanism of DL-induced cell death, the canonical markers of apoptosis like Bcl-2 and caspase 3 were measured in Huh-7 cells after treatment with fraction

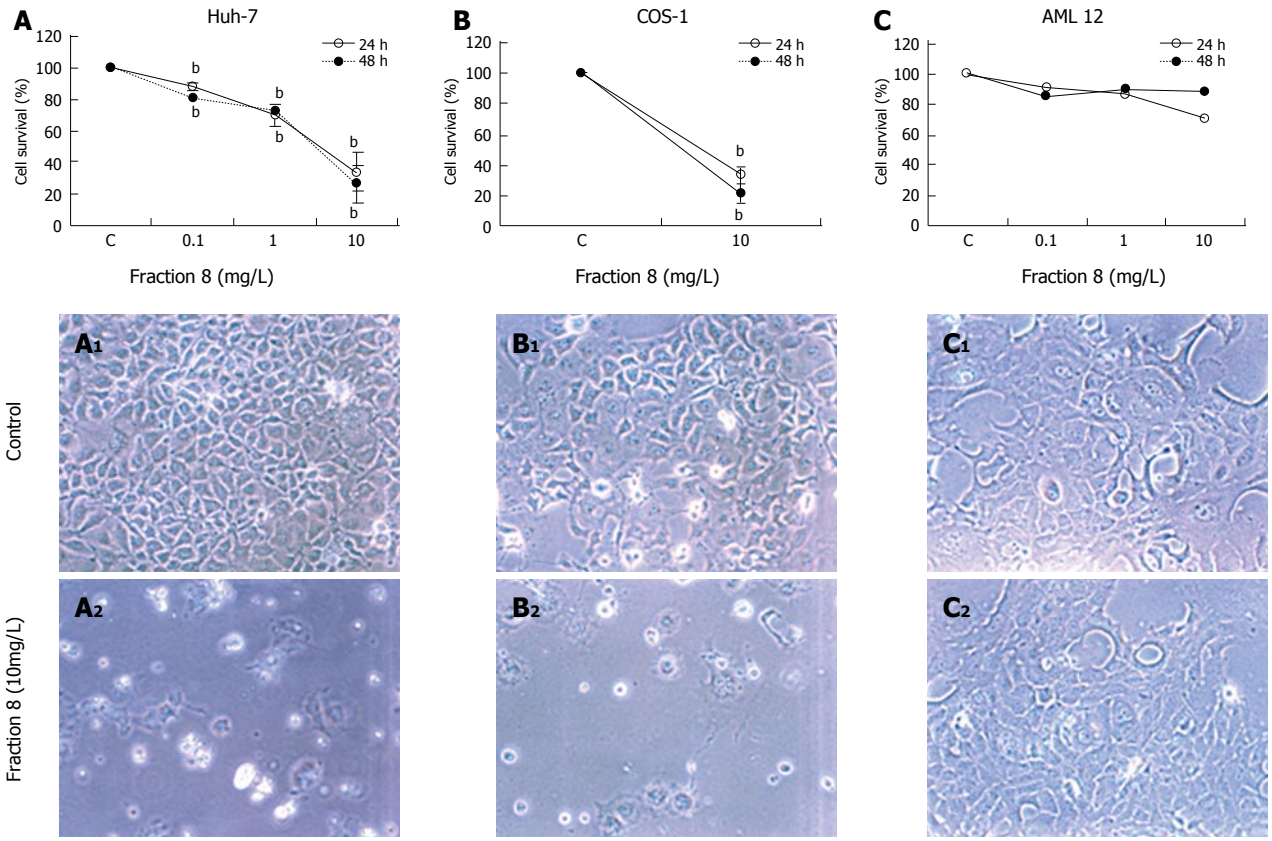

Figure 4 Cytotoxic effect of fraction 8 of the DL methanolic extract on different cell lines. Huh-7 (**A**), COS-1 (**B**) and AML12 cells (**C**) were incubated with different concentrations of fraction 8 and cell viability was measured at 24 and 48 h (mean ± SE, $^{b}P < 0.01$). **A**$_1$, **B**$_1$ and **C**$_1$ are control Huh-7, COS1 and AML12 cells; **A**$_2$, **B**$_2$ and **C**$_2$ are fraction 8 treated Huh-7, COS-1 and AML12 cells (x200).

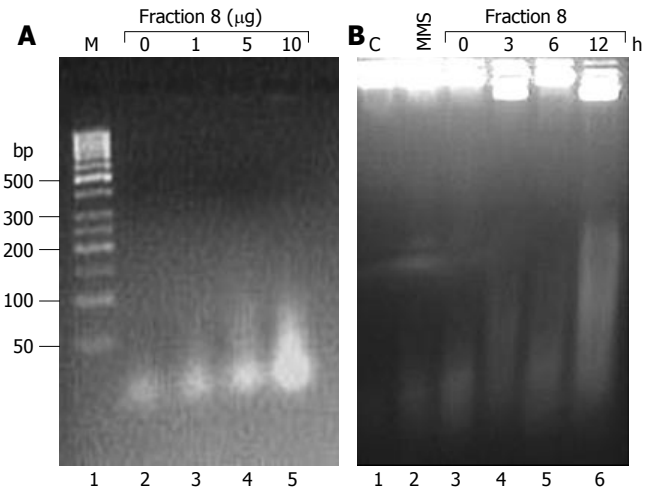

Figure 5 Effect of fraction 8 on DNA fragmentation in Huh-7 cells. Huh-7 cells were treated either with different concentrations (1, 5 and 10 mg/L) of fraction 8 for 3 h (**A**) or for 3, 6 and 12 h with 10 mg/L of fraction 8 (**B**). Total DNA was extracted after proteinase K and RNase A treatment and resolved by agarose gel electrophoresis. C: DNA from control cells; M: 100 base pair (bp) ladder; MMS, DNA from cells treated with methyl methanesulfonate (2 g/L) for 15 min.

8. Surprisingly, no change in the levels of both markers was observed (data not shown). Nonetheless, there was a marked increase in the fragmentation of cellular DNA that was both concentration as well as time dependent thereby suggesting a rise in the cellular nuclease activity (Figure 5).

DISCUSSION

The latex of *C. procera* has been shown to possess potent anti-inflammatory and antioxidant properties. In this study we have tested the latex for its chemopreventive and *in vitro* cytotoxic properties. The study was carried out in an mouse model of HCC developed by the integration of a chimeric transgene having hepatitis B virus X and murine c-*myc* genes exhibiting liver-specific neoplastic changes that are associated with inflammation and angiogenesis[18]. Five days a week oral administration of DL to the X15-myc mouse produced a marked chemopreventive effect as revealed by histological analysis. The mitotic changes produced by transgene expression were inhibited at the dose studied though some hydropic changes were observed. This was accompanied by inhibition of cellular infiltration. The DL might be producing chemopreventive effects through inhibition of different mediators of inflammation[15]. The pro-inflammatory cytokines and reactive oxygen and nitrogen species are known to activate signaling molecules involved in inflammation and carcinogenesis. These include nuclear transcription factor NF-κB, inducible nitric oxide synthetase (iNOS) and cyclooxygenase-2 (COX-2)[22]. The elevated levels of tumor COX-2 have been reported to correlate with invasiveness and elevated VEGF levels that brings about neovascularization and progression of HCC[23,24]. In fact, a recent study has revealed that COX-2 expression correlates with VEGF expression and microvessel density in HCC caused by hepatitis B virus[25]. It is interesting to note that DL not only inhibited

the inflammatory changes, but also produced a significant decrease in VEGF levels as observed with other anticancer drugs[26-28].

We further evaluated the cytotoxic effects of DL on hepatoma (Huh-7), non-hepatoma (COS-1) and non-cancerous (AML12) cell lines and observed that the cytotoxic activity was associated with one of the polar fractions of DL, i.e., fraction 8. Like total methanolic extract, fraction 8 also showed a strong cytotoxic effect on both transformed cell lines used here. However, a marginal effect on the killing of AML12 cells suggested a high degree of selectivity for transformed cells. Such differential killing of cancerous cells could relate to their altered metabolic status and/or membrane properties. In fact selective killing of cancerous cells by chemotherapeutic drugs like methotrexate and polyunsaturated fatty acids has been reported earlier as well[29,30]. Thus, it would be interesting to investigate the mechanism of such target selectivity by DL. The cytotoxic effect of DL was accompanied by intracellular fragmentation of target cell DNA. We observed a dramatic increase in the fragmentation of Huh-7 cells upon incubation with DL fraction 8. Though the action was rapid, it was not accompanied by expression of the common markers of intrinsic pathway of apoptosis such as Bcl-2 or caspase 3. Since Bcl-2 and caspases are markers of mitochondria-mediated apoptotic death, the genomic DNA can still undergo fragmentation by cellular DNase activity independent of mitochondrial pathway[31]. Hence a possible role of hypoxia and free radical-dependent activation of DNase activity or direct DNA damage and consequent degradation of cellular DNA[32] as evident here, cannot be ruled out. Thus, our study suggests that the latex of *C. procera* possesses an activity that has a chemopreventive action *in vivo* and cytotoxic action on cancer cell lines. Further study would be necessary to demonstrate its efficacy in HCC treatment.

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Usefulness of liver infiltrating CD86-positive mononuclear cells for diagnosis of autoimmune hepatitis

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Abstract

AIM: Although the pathogenic mechanism underlying autoimmune hepatitis (AIH) remains unclear, the immune system is thought to be critical for the progression of the disease. Cellular immune responses may be linked to the hepatocellular damage in AIH. Recently, much attention has been focused on the critical functions of costimulatory molecules expressed on mononuclear cells in the generation of effective T cell-mediated immune responses. Analysis of costimulatory molecule expressed on mononuclear cells from the patients with AIH may give us insight into the pathogenic mechanism of hepatocellular damage in AIH.

METHODS: Peripheral blood mononuclear cells (PBMC) were taken from the patients with AIH (34 cases) and healthy controls (25 cases). Liver infiltrating mononuclear cells (LIMCs) were taken from the patients with AIH (18 cases), the patient with chronic hepatitis C (CH-C) (13 cases) and the patients with fatty liver (2 cases). Using flow cytometry, the cells were analyzed for the expression of costimulatory molecules, such as CD80, CD86, and CD152 (CTLA-4). The results were compared with clinical data such as the level of gammaglobulin, histological grade, presence or absence of corticosteroids administration and the response to corticosteroids.

RESULTS: The levels of CD80+, CD86+ and CD152+ PBMC were significantly reduced in the patients with AIH as compared with healthy controls. By contrast, those cells were significantly higher in LIMC than in PBMC of the patients with AIH. Especially, the level of CD86+ LIMC showed a marked increase irrespective of the degree of disease activity in the patients with AIH, although CD86+ cells were rarely present in PBMC. The levels of CD86+ cells were present in significantly higher frequency in patients with AIH than in the patients with CH-C. Furthermore, the patients with AIH with high levels of CD86+ LIMC showed good responses to corticosteroids, whereas 2 cases of AIH with low levels of CD86+ LIMC did not respond well.

CONCLUSION: These results suggest that LIMC over-expressing costimulatory molecules such as CD80 and CD86 appears to play a role in the pathogenesis of AIH. Especially, CD86 molecule expressed on the LIMC may be useful for the diagnosis of AIH and for the prediction of the therapeutic effects of corticosteroids on AIH.

Key words: Autoimmune hepatitis; Costimulatory molecule; CD86 molecule; Peripheral blood mononuclear cells; Liver infiltrating mononuclear cells; Flow cytometry

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INTRODUCTION

In autoimmune liver diseases including autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC), autoimmune mechanisms are thought to affect the disease progression and manifestation. Although the mechanism of hepatocellular damage in AIH and PBC is still unclear, it is speculated that the immunological disorder is linked to host immunological targeting of hepatocytes in the liver[1-3]. It has been recently reported that polymorphism of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and the susceptibility to AIH and PBC were associated[4]. Among T cell-mediated immune responses, especially those mediated by CD4+ or CD8+ T cells, are likely to be associated with the hepatocellular damage in AIH[5,6]. We have previously reported that the expression of bcl-2, an anti-apoptotic molecule, in CD4+ Th1 cells was increased in peripheral blood and in the liver of the patients with

AIH[7]. Moreover, it was reported that T cells bearing certain T cell receptor clonotypes were expanded in the liver of the patients with AIH[8]. Although extensive efforts have been made to identify the target antigens expressed on the hepatocyes in AIH, the real target antigen has not been identified yet.

For the generation of effective T cell-mediated immune responses, much attention has been paid on the critical functions of a series of costimulatory molecules expressed on the cell surface of T or B cells. The T cell receives signals from antigen-presenting cells (APC) through the interaction of the T cell receptor (TCR) and major histocompatibility complex (MHC) in an antigen-dependent manner. However, these signals alone are insufficient to generate effective T cell-mediated immune responses and often lead to T cell anergy. The aberrant expression of costimulatory molecules was reported in some liver diseases other than AIH[9-14]. Although it is clear that the costimulatory molecules play a crucial role in T cell activation, few reports have addressed the role of these costimulatory molecules in AIH. In the present study, to elucidate the role of costimulatory molecules on mononuclear cells in AIH, some of the costimulatory molecules expressed on PBMC and LIMC were analyzed using flow cytometry and the results were evaluated in terms of the clinical status of the patients with AIH.

MATERIALS AND METHODS

Patients

Thirty-four patients with AIH serologically and histologically diagnosed at Kagawa University Hospital were enrolled in the present study. All patients had anti-nuclear antibody (ANA) in their sera and 19 of 34 (55.88%) patients showed hyper-gammaglobulinemia (> 2.0 g/dL). Based on the criteria proposed by International Autoimmune Hepatitis Group, all patients satisfied the score over probable AIH. PBMCs were analyzed in 34 patients with AIH. LIMCs were also analyzed in 18 of 34 patients at the liver biopsy for diagnosis. As a disease control, PBMC and LIMC of thirteen patients with chronic hepatitis C (CH-C) were analyzed. Twenty-five healthy individuals without any symptoms of liver injury were selected as normal controls for the analysis of PBMC. All of these studies were conducted with informed consent at the time of the enrollment for this study in all patients.

Flow cytometric analysis of PBMC

PBMCs from patients and healthy individuals were separated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Histopaque; Sigma Chemical Co., St Lois, MO). Immediately after separating PBMC, cells were washed twice in ice-cold phosphate-buffered saline (PBS), and adjusted to 4×10^5 cells per well of a 96-well U-bottom cell culture plate in flow microfluorometry medium [FMF medium: Hanks' balanced salt solution (without Ca, Mg and phenol red) containing 2 g/L bovine serum albumin, and 1 g/L sodium azide]. After centrifugation at 1200 r/min for 5 min, the supernatant was removed and cells were incubated for 30 min with FITC-conjugated or phycoerythrin (PE)-conjugated antibodies. The combinations of antibodies used were anti-CD80-FITC (Pharmingen, San Diego, USA)/anti-CD152-PE (Pharmingen), anti-CD86-FITC (Pharmingen). FITC-conjugated IgG (Dainippon Pharmaceutical Co., Ltd. Osaka, Japan) and PE-conjugated IgG (Dako A/G, Denmark) were used as the negative controls. After being washed twice, the pellets were resuspended with FMF medium, and the fluorescence detections were performed with a flow cytometer, COULTER EPICS XL. Prior to the analysis, FL1, FL2 and color compensation were adjusted so that no CD4CD8 double-positive cells were detected. The gate was set for accumulation of PBMC and ten thousand events were acquired for each analysis. Flow cytometric analysis was done immediately after isolating PBMC.

Flow cytometric analysis of LIMC

Liver specimens from the 18 patients with AIH, 10 patients with CH-C and 2 patients with fatty liver were obtained using a 16 G biopsy needle for diagnosis. Most of the patients with AIH underwent laparoscopy to obtain the liver specimen and observe the change of the liver surface for diagnosis. After incubating the liver biopsy specimen in RPMI-1640 containing 1 g/L collagenase for 2 h to destroy the connective tissue, LIMCs were separated by Ficoll-Hypaque density gradient centrifugation at 3000 r/min for 10 min. Isolated LIMCs were stained with FITC- or PE-conjugated antibodies and analyzed using flow cytometry. The cells positive for CD69, a marker for activated T cells, were also analyzed in LIMC. An anti-CD69-PE (Dako Japan A/G) was used. Three thousand events were acquired for each analysis.

Clinical markers

Levels of alanine aminotransferase (ALT) (IU/L) and gammaglobulin (g/dL) and titer of ANA were monitored. These laboratory data were compared with the level of LIMC positive for costimulatory molecules obtained in the present study. Inflammation and fibrosis in histological analysis was graded according to the classification documented by Knodell *et al*[15]. Histological staging and grading were classified based on the classification documented by Desmet *et al*[16].

Statistical analysis

Statistical analysis was performed using Macintosh software, Statview II (version 4.2) and a Mann-Whitney *U* test (non-parametric analysis). $P < 0.05$ was considered statistically significant.

RESULTS

Flow cytometric analysis of PBMC

For the analysis of costimulatory molecules, we focused on several costimulatory molecules critical for the induction of effective T and B cell-mediated immune responses. Initially, we examined the expression of those molecules on PBMC in patients with AIH. The level of positive cells for each surface molecule on PBMC was compared between the patients and healthy controls (Table 1). The results revealed that the patients with AIH had significantly

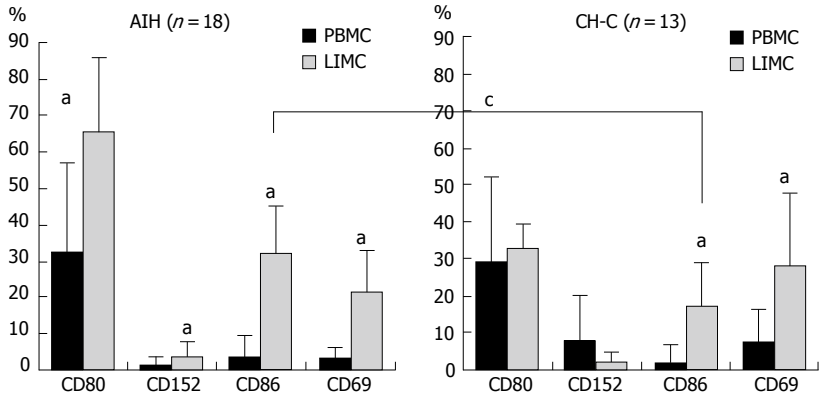

Figure 1 Isolation of PBMCs and LIMCs from peripheral blood and liver biopsy specimens in the patients with AIH and CH-C by using density gradient centrifugation. The cells were stained with anti-CD80, anti-CD86, anti-CD152 and anti-CD95 antibodies and analyzed using flow cytometry. The percentage of cells positive for those costimulatory molecules is shown. CD80+, CD86+ and CD152+ cells were significantly higher in LIMCs compared to PBMC in patients with AIH. The difference of CD86+ cells between LIMCs and PBMCs was more striking than that of CD80+ cells between LIMCs and PBMCs. (a) represents the significant difference ($P < 0.05$) from the data of PBMCs and (c) shows the significant difference ($P < 0.05$) of LIMCs between AIH and CH-C.

Table 1 Analysis of costimulatory molecules expressed on PBMCs (mean ± SD)

	Control n = 25	AIH Patients n = 34	P value
CD80	41.3 % ± 7.04 %	32.7 % ± 23.5 %	0.008
CD86	1.15 % ± 1.19 %	0.78 % ± 0.75 %	0.04
CD152 CTLA-4	13.8 % ± 10.9 %	2.32 % ± 2.80 %	0.01

The P values calculated by a Mann-Whitney's U-test are shown. Compared to the healthy controls, CD80+, CD86+ and CD152+ PBMC of the patients with AIH are shown to be significantly fewer.

fewer CD80+, CD86+ and CD152+ (CTLA-4+) cells in PBMC as compared with the healthy controls. Among the cells analyzed, CD86+ cells were present in low frequency in PBMC of both the patients and healthy controls.

Flow cytometric analysis of LIMCs

CD80 and CD86 are expressed on professional APC and activated lymphocytes, and have unique expression pattern[17]. In the patients with AIH, the predominant location of inflammation and damage is seen in the liver. Therefore, it might be reasonable to think that those decreased cells in the blood, such as CD80+, CD86+ or CD152+ cells, accumulate in the liver and are likely to be important to analyze the expression of costimulatory molecules on LIMCs as well as on PBMCs. Although it may be necessary to compare the expression of costimulatory molecules on LIMCs between the patients and healthy controls, it is ethically difficult to take mononuclear cells from the liver of healthy controls. Therefore, the frequency of LIMC positive for each costimulatory molecule was compared between PBMC and LIMC in patients with AIH and CH-C in the present study (Figure 1). In both AIH and CH-C, the ratios of CD69+ cells in LIMCs were significantly higher than that in PBMCs, suggesting infiltration of many activated T cells into the liver in both AIH and CH-C. In AIH, the ratios of CD80+ and CD86+ cells were significantly higher in LIMCs compared to PBMCs. The difference of CD86+ cells between LIMCs and PBMCs was more striking than that of CD80+ cells between LIMCs and PBMCs. Although the ratio of CD152+ cells was significantly higher in LIMCs than that in PBMCs, both the ratios were very low. In CH-C, the ratio of CD86+ cells was significantly higher in LIMCs than that in PBMC, but the ratios of CD80+ and CD152+ cells in LIMC were not significantly different from those in PBMC. Although the ratios of CD86+ cells were significantly higher both in AIH and in CH-C, the ratio of CD86+ cells in LIMCs was significantly higher in AIH than that in CH-C. Taken collectively, the most dramatic and apparent difference between PBMCs and LIMCs was the marked increase of CD86+ cells in LIMCs of AIH. By contrast, the levels of CD86+ cells in LIMCs of patients with fatty liver were low (Table 2).

Relationship between the level of CD86+ LIMCs and clinical markers

Since the most dramatic change of frequency between PBMCs and LIMCs was the increase of CD86+ cells in patients with AIH, relationship between the level of CD86+ LIMC and the clinical parameters was examined in patients with AIH (Table 2). In most of the patients with AIH tested, the levels of CD86+ LIMC were elevated by more than 20%. Three patients (No. 1-3 in Table 2) were already being treated with corticosteroids when the biopsy was performed. Even in these 3 patients, the aberrant expression of CD86 molecule on LIMCs was observed. The level of CD80+ or CD86+ LIMC did not show any significant correlation with that of ALT [correlation coefficient (r): CD80 *vs* ALT = -0.160; CD86 *vs* ALT = -0.166]. Furthermore, no significant correlation of the level of CD86+ LIMC with the level of serum gammaglobulin, ANA titer and HAI score was observed. These results suggested that LIMCs in patients with AIH are over-expressing CD80 or CD86 molecule irrespective of the degree of hepatocellular damage or disease activity. Among 18 patients analyzed for the expression of costimulatory molecule on LIMCs, clinical course after the administration of corticosteroids could be followed up in 10 patients. Administration of corticosteroids was effective in decreasing the level of transaminase in 8 of 10 patients. All of these 8 patients showed the high levels of CD86+ LIMC (> 20%). By contrast, 2 patients who did not respond satisfactorily to corticosteroids showed low levels of CD86+ LIMC (11.1% and 5.9%, respectively).

Representative AIH cases reactive and non-reactive to corticosteroids

Clinical course, laparoscopic appearance of the liver surface and liver histology of two AIH cases reactive and non-reactive to corticosteroids are shown in Figures 2 and 3. The

Table 2 Analysis of CD86+ cells in liver infiltrating mononuclear cells

	γ -globulin (g/dL)	ALT (U/L)	ANA	Histology	HAI	CD86 (%)	CS	Reactivity to CS
No. 1	2.1	34	×40	CH (A0, F2)	9	44.0	On	Reactive
No. 2	1.1	55	×8	CH (A0, F1)	3	43.5	On	Reactive
No. 3	1.6	44	×20	CH (A2, F3)	13	27.5	On	NT
No. 4	2.0	151	×160	CH (A3, F3)	16	42.2	Off	Reactive
No. 5	2.0	99	×40	CH (A0, F1)	3	43.7	Off	NT
No. 6	2.0	105	×40	CH (A2, F2)	3	33.8	Off	NT
No. 7	2.3	71	×640	CH (A1, F1)	5	41.2	Off	NT
No. 8	2.4	18	×320	CH (A1, F2)	2	34.0	Off	NT
No. 9	1.6	50	×320	LC (A1, F4)	18	19.1	Off	NT
No. 10	2.7	84	×1280	CH (A2, F2)	14	11.1	Off	Non-reactive
No. 11	1.3	131	×5120	CH (A2, F2)	14	28.9	Off	Reactive
No. 12	2.4	93	×640	CH (A2, F2)	12	40.0	Off	Reactive
No. 13	1.7	363	×2560	CH (A2, F2)	15	27.3	Off	Reactive
No. 14	1.4	123	×40	CH (A1, F1)	2	5.9	Off	Non-reactive
No. 15	1.4	50	×20	LC (A3, F4)	18	25.7	Off	NT
No. 16	2.1	34	×20	CH (A2, F2)	12	29.4	Off	NT
No. 17	2.3	65	×40	LC (A2, F4)	20	24.1	Off	Reactive
No. 18	1.9	17	×1280	CH (A2, F3)	12	23.0	Off	Reactive
No. 19	1.2	45	(-)	Fatty liver	NT	11.8	Off	NT
No. 20	1.3	47	(-)	Fatty liver	NT	10.7	Off	NT

Comparison of the level of CD86+ LIMC and clinical data in patients with AIH (18 cases) and fatty liver (2 cases). LIMCs taken from the liver biopsy specimen of patients with AIH (18 cases) and 2 patients with fatty liver were stained with antibody against CD86 and analyzed by using flow cytometry. Three thousand events were acquired for each analysis. Patients with AIH showed high levels of CD86+ LIMC irrespective of the levels of γ-globulin, ALT, histological activity, and the presence or absence of the administration of corticosteroids (CS). Response to CS in patients with AIH was shown to be associated with the level of CD86+ LIMC. NT: not tested.

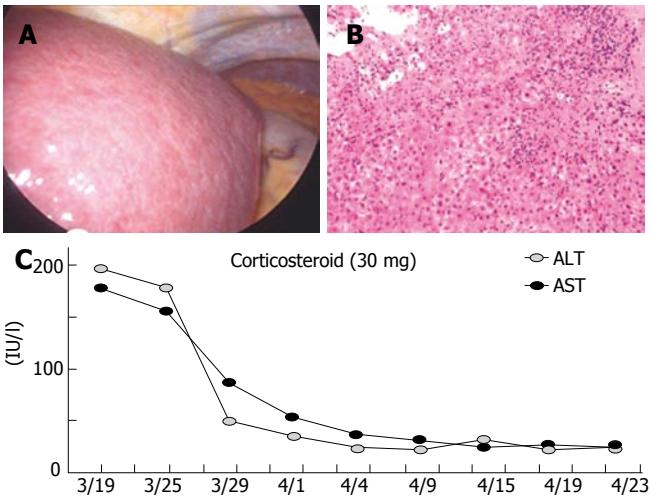

Figure 2 Laparoscopic findings, liver biopsy and clinical course after the administration of corticosteroids of a patient with AIH reactive to corticosteroids. **A:** Laparoscopic findings showing many reddish markings on the liver surface, indicating that the ongoing hepatitis was active; **B:** microscopic observation of the liver showing formation of rosette and bridging necrosis, infiltration of a large number of lymphocytes and plasma cells; and **C:** time-course of the levels of ALT and AST after the administration of corticosteroids. The levels of ALT and AST were rapidly decreased to normal ones after administration of corticosteroids.

former case was a 58-year-old female (No. 11 in Table 2) who showed a good response to the treatment of corticosteroids (Figure 2). At the time of diagnosis, she had ALT 131 IU/dL, AST 139 IU/dL, T-Bil 1.1 mg/dL, ALB 2.9 g/dL, IgG 2730 mg/dL and ANA ×5120. Laparoscopic findings of the liver showed many reddish markings on the surface of the liver, indicating that the ongoing hepatitis was active (Figure 2A). Histological findings were compatible to AIH showing the formation of rosette and bridging necrosis, infiltration of a large number of lymphocytes and many plasma cells (Figure 2B). The level of CD86+ LIMC was high, 28.9 %. The levels of ALT and AST were rapidly decreased to normal ones after the administration of corticosteroids (Figure 2C). By contrast, the latter case was a 59-year-old female (No. 10 in Table 2) who was not reactive to the treatment of corticosteroids (Figure 3). At the time of diagnosis, she had ALT 50 IU/dL, AST 29 IU/dL, T-Bil 1.9 mg/dL, ALB 2.9 g/dL, IgG 2880 mg/dL, and ANA ×1280. Laparoscopic findings of the liver showed many reddish markings and small lymph cysts on the surface of the liver (Figure 3A). Histological findings revealed the bridging necrosis, infiltration of a large number of lymphocytes and plasma cells (Figure 3B). The level of CD86+ LIMC was relatively low, 11%. Although the levels of ALT and AST were slightly improved after the administration of corticosteroids, those were elevated again despite the treatment.

As aforementioned, these 2 cases showed similar aspects of laboratory data, laparoscopic findings and histological findings. Nevertheless, one with a high level of CD86+ LIMC was responsive to corticosteroids, and the other with a low level of CD86+ LIMC was not. In these 2 cases, the differences were seen in the response to corticosteroids and in the level of CD86+ LIMC, but not in other clinical markers.

DISCUSSION

Although the mechanism of hepatocellular damage in AIH has not been well understood, many lines of evidence have demonstrated the presence of immunological disorders in AIH[5]. For example, wide ranges of circulating auto-antibodies are observed in the sera of patients with AIH[18-21]. Moreover, it has been shown that

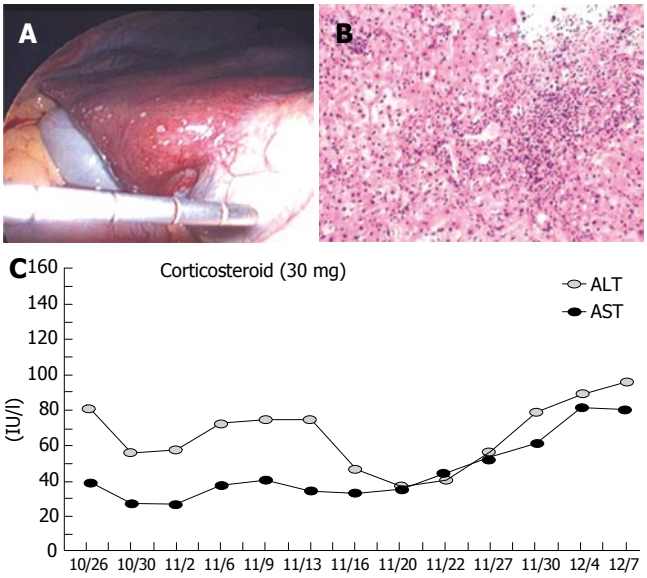

Figure 3 Laparoscopic findings, liver biopsy and clinical course after the administration of corticosteroids of a AIH patient non-reactive to corticosteroids. **A:** Laparoscopic view showing many reddish markings on the surface of the liver and small lymph cysts; **B:** microscopic findings of the liver showing bridging necrosis, infiltration of a large number of lymphocytes and plasma cells; and **C:** the time-course of the levels of ALT and AST after the administration of corticosteroids. The levels of ALT and AST were not improved to normal level after administration of corticosteroids.

the susceptibility to AIH was associated with HLA class II, DR3 and DR4 alleles[3,22,23]. Some reports have also suggested the association of cytotoxic T lymphocytes (CTL) with the hepatocellular injury in AIH using animal models[24-26].

Helper T cells (Th) receive a TCR signal from APC via HLA class II molecules. Helper T cells are divided into two subtypes, Th1 and Th2, according to their cytokine secretion profile[27]. Both Th1 and Th2 differentiate from Th0 cells. In the treatment of the patients with AIH, corticosteroids are effective in decreasing hepatocellular damage. This effect is thought to be due to the action of corticosteroids in inhibiting Th0 activation, leading to inhibition of CTL responses and antibody production[28,29]. CTL and Th receive signals from APC via the TCR-MHC complex[30]. However, this signal alone is not enough to activate T cells, and the expression of costimulatory molecules plays a crucial role in full T cell activation. Nevertheless, few reports have analyzed the expression of costimulatory molecules on mononuclear cells in AIH. In the present study, flow cytometry was used to analyze the expression of costimulatory molecules on mononuclear cells, because it is a sensitive method for detecting positive cells. PBMCs expressing CD80+, CD152+ (CTLA-4+) and CD86+ were shown to be significantly lower in the patients with AIH as compared with the healthy controls. However, in the patients with AIH, these cells existed more frequently in LIMCs than that in PBMCs. Interestingly, the most dramatic difference in frequency between PBMCs and LIMCs was detected in the ratios of cells positive for CD86 molecule, formerly designated as B7-2. Although CD86+ cells were barely detected in PBMCs obtained from the patients and healthy controls, they were considerably high in LIMCs obtained from the patients with AIH.

CD80 and CD86 molecules have unique expression patterns on professional APC and activated lymphocytes[31]. CD80 is constitutively expressed at low levels on lymphoid cells, whereas CD86 expression is rapidly increased upon activation[32]. Our results regarding the high level of CD86+ LIMC in the patients with AIH suggested that activated APC or lymphocytes might be enriched in the liver of the patients with AIH. Furthermore, the aberrant expression of CD86 was observed irrespective of the presence of hepatocellular damage and the treatment with corticosteroids, and the enhanced expression of CD86 was not observed in other liver diseases. These results suggest that the costimulatory molecules, such as CD80 and CD86, are continuously expressed on mononuclear cells in the liver even when the hepatocellular damage is not present. In addition, we often observe the relapse of liver dysfunction during the time course of tapering the dose of corticosteroids. Therefore, these lead us to speculate that excessive antigen presentation by APC to T cells, via the interaction between the CD80/86 and CD28 molecules, might contribute to the hepatocellular damage in AIH and administration of corticosteroids may block the signal transduction between APC and T cells. Indeed, it is important to identify which subtypes of PBMC or LIMC really express CD86 molecule. However, we have not identified it yet. Because the expression of CD86 on PBMCs was extremely low and the amount of LIMCs purified from a tiny liver specimen was quite a few, it was technically difficult to identity a specific subtype of the cells. The more detail analysis of this subtype and the function CD86+ cells in AIH awaits further elucidation in the next study.

Recently, extensive efforts have been made to elucidate the critical function of costimulatory molecules in various diseases. The aberrant expression of costimulatory molecules on mononuclear cells has been reported in a variety of autoimmune diseases, such as lupus, multiple sclerosis (MS), and experimental autoimmune encep halomyelitis[33-35]. The enhanced expression of CD80 and CD86 has also been reported in liver diseases, such as fulminant hepatic failure, primary biliary cirrhosis (PBC), primary sclerosing cholangitis, hepatitis C and hepatocellular carcinoma[9-14]. However, all of these reports focused on the CD80 or CD86 molecules expressed on either hepatocytes or bile ducts. There are few reports expressing the role of costimulatory molecules in AIH. The costimulatory molecules, such as CD80, CD86 and CTLA-4 (CD152), are essentially expressed on mononuclear cells. To our best of knowledge, this is the first descriptive report showing the aberrant expression of CD86 molecule on LIMCs of patients with AIH. Recently, anti-CD86 antibody or soluble CD152 (CTLA-4) was used as blocking agents for CD86 function in the treatment of autoimmune diseases[36-38]. Similarly, our data suggest that anti-CD86 antibody might be used to stabilize liver function for the treatment of AIH.

In PBC, presence of anti-mitochondria M2 antibody is a useful diagnostic marker[39,40]. In AIH, presence of ANA and the scoring system proposed by the International

Autoimmune Hepatitis Group are currently being used for the diagnosis of AIH[41]. However, some cases are still difficult to classify as definite or probable AIH. Actually, the cases with low levels of gammaglobulin (< 2.0 g/dL) were present in 8 of 18 cases whose LIMCs were examined in the present study. In contrast, all cases except No. 14 in Table 2 showed high levels of CD86+ LIMC. Furthermore, 2 patients who did not respond satisfactory to corticosteroids showed low levels of CD86+ LIMC. It is well known that non-response to the treatments of corticosteroids is uncommon in AIH. Therefore, these 2 cases might not be a real AIH although they were clarified as AIH by the scoring system. Thus, the detection of CD86+ LIMC may be useful for the diagnosis of AIH and this may lead to be helpful for the prediction of therapeutic effects of corticosteroids on AIH in the future.

In summary, our results showed lower proportions of CD80+, CD86+ and CD152+ (CTLA-4+) PBMC in the patients with AIH as compared with the healthy controls. These cells are present in greater frequency in LIMCs, suggesting that the aberrant expression of these costimulatory molecules on LIMCs might be associated with pathogenic mechanism of AIH. Especially, the level of CD86+ LIMC is likely to be helpful for the diagnosis of AIH and for the prediction of therapeutic effects of corticosteroids on AIH.

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VIRAL HEPATITIS

Antibody to E1 peptide of hepatitis C virus genotype 4 inhibits virus binding and entry to HepG2 cells *in vitro*

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Abstract

AIM: To analyze the neutralizing activity of antibodies against E1 region of hepatitis C virus (HCV). Specific polyclonal antibody was raised via immunization of New Zealand rabbits with a synthetic peptide that had been derived from the E1 region of HCV and was shown to be highly conserved among HCV published genotypes.

METHODS: Hyper-immune HCV E1 antibodies were incubated over night at 4℃ with serum samples positive for HCV RNA, with viral loads ranging from 615 to 3.2 million IU/ mL. Treated sera were incubated with HepG2 cells for 90 min. Blocking of viral binding and entry into cells by anti E1 antibody were tested by means of RT-PCR and flow cytometry.

RESULTS: Direct immunostaining using FITC conjugated E1 antibody followed by Flow cytometric analysis showed reduced mean fluorescence intensity in samples pre-incubated with E1 antibody compared with untreated samples. Furthermore, 13 out of 18 positive sera (72%) showed complete inhibition of infectivity as detected by RT-PCR.

CONCLUSION: In house produced E1 antibody, blocks binding and entry of HCV virion infection to target cells suggesting the involvement of this epitope in virus binding and entry. Isolation of these antibodies that block virus attachment to human cells are useful as therapeutic reagents.

Key words: Flow cytometry; Hepatitis C virus; E1 envelope; Therapeutic antibodies; Direct immuno-fluorescence; HepG2 cells

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INTRODUCTION

Hepatitis C virus (HCV) is the major etiology of non-A, non-B hepatitis that infects 170 million people worldwide. Approximately 70% to 80% of HCV patients develop chronic hepatitis, 20% to 30% of which progress to liver cirrhosis[1]. At present, there is no vaccine available to prevent HCV infection, and current therapies are not optimal. The initial steps of HCV infection (binding and entry) that are critical for tissue tropism, and hence pathogenesis, are poorly understood. Studies to elucidate this process have been hampered by the lack of robust cell culture systems or convenient small animal models that can support HCV infection. HCV is an enveloped, positive-stranded RNA virus that belongs to the *Flaviviridae* family. Based on the sequence heterogeneity of the genome, HCV is classified into six major genotypes and 100 subtypes[1]. The viral genome (9.6 kb) is translated into a single poly-protein of 3000 amino acids (aa). A combination of host and viral proteases are involved in poly-protein processing to give at least nine different proteins[2]. Like other enveloped viruses, E1 and E2 proteins most likely play a pivotal role in the assembly of infectious particle and in the initiation of viral infection by binding to its cellular receptor(s). It has been suggested that the humoral and cellular immune responses to the E1 envelope protein are largely impaired in patients with chronic active hepatitis C, and that such responses may be important for clearance of HCV[3]. Leroux-Roels *et al*,[4] have previously reported that cellular immune responses to the E1 envelope protein are almost absent in patients with chronic active hepatitis C, while long-term responders to IFN- therapy, on average, show higher levels of E1 antibodies[5]. Depraetere *et al*,[6] suggesting that E1 antibodies contribute, at least

partially, in viral elimination. Baumert *et al*[7] confirmed the presence of such higher antibody levels directed at the HCV envelope in sustained viral responders to IFN-based therapy. Maertens *et al*[8] have been able to show that therapeutic vaccination of chronically infected chimpanzees with the HCV E1 protein induces the appearance of T-helper immune responses and antibodies which are very rarely seen in patients[6,7] or chimpanzees[9] with chronic active hepatitis C. The use of a viral envelope protein has the advantage of potentially inducing not only T-cell responses, but also neutralizing antibodies and complement activation. The E1 protein was chosen as vaccine rather than the E2 protein not only because E2 has the disadvantage of displaying a very high strain-to-strain variation in the hypervariable region I (HVRI), but also because of the higher degree of inter-genotype cross-reactivity of E1 as compared to E2. The E2 hypervariable region is immunodominant and neutralizable[10]. However, strong anti-E2 vaccine responses directed against the HVR I do not cross-neutralize with the infecting strain[11,12]. Although the E1 antigen is also variable between genotypes, it shows a relatively high degree of conservation within the subtypes, such as subtype 1b[13], the most widespread genotype worldwide. In the present study, we aimed to examine the neutralizing -related activity of an in house made antibody against the most conserved region of HCV E1 protein, for blocking the entry of HCV virion to HepG2 cells.

MATERIALS AND METHODS

Infected Serum samples

We selected 28 serum samples which tested positive for HCV RNA at different viral loads (ranging from 615 to 3.2 million IU/ mL) for infection experiments. The presence of HCV RNA was determined by nested RT-PCR and genotyped using Innolipa system (Bayer, Germany). Viral loads were determined by branched DNA method (Bayer, Germany).

Design of E1 conserved synthetic peptides

Sequence analysis of HCV quasi-species in local patients (Data not shown), revealed several conserved regions within the core and the E1 proteins. We designed 4 core and one E1-specific peptides and analyzed their ability to detect circulating antibodies in infected patients. The results of these studies showed that only one core-peptide (C1) had reasonable sensitivity and specificity. However the rest of peptides including E1 peptide had poor reactivity with circulating antibodies[14]. In the present study, we raised HCV specific polyclonal antibodies against the 4 core and an E1 peptide as follows:

(C1) DVKFPGGGQIVGGVYLLPRR, (C2) PRLGVRATRKTSERSQPRG,
(C3) IPKARRPEGRTWAQPGY, (C4) IPKDRRSTGKSWGKPGY,
(E1) GHRMAWDMM

Production of polyclonal antibodies against core and Envelope regions of HCV

New Zealand rabbits were immunized independently (two rabbits per each peptide) with purified synthetic peptides coupled with KLH protein. Equal volume of diluted core and E1 synthetic peptides and Freund's complete adjuvant were emulsified and injected subcutaneously into the rabbits in three different sites. On d 15 and 28, the rabbits were immunized again with the same protein emulsified with Incomplete Freund`s adjuvant. On d 32 the rabbits were sacrificed and sera were separated and stored at -20 ℃. For direct immuno-fluorescence, immunized polyclonal antibodies were digested with pepsin A (porcine 1:60 000 grade (sigma P-7012) ST. Louis, Mo, USA) at acidic pH and the $F(ab)_2$ portion was labeled with fluorescence isothiocyanate-FITC according to Hudson and Hay[15].

Flow cytometric analysis of E1 binding to HepG2 cells

The interaction of E1 glycoprotein with cells was quantified using a fluorescence activated cell sorting (FACS) based assay. Surface labeling was performed by direct immuno-fluorescence. Twenty eight serum samples that tested positive for HCV RNA with broad range of viral loads (617-3.2×10^6 IU / mL) were incubated with anti E1 antibody (diluted 1:250 in cultured media) overnight at 4 ℃. The pretreated sera with E1 antibody were incubated with HepG2 cells for 90 min at 37 ℃ in CO_2 incubator. Cells were centrifugated and supernatants were removed. Cell pellets were washed 4 times with PBS and incubated with FITC labeled $F(ab)_2$ portion of HCV E1 antibody (at 1:1500 dilution) for 30 min at 4 ℃. Cells were washed 3 times with PBS containing 1% normal goat serum. Cells were suspended in 500 μL PBS and analyzed with flow cytometry (FACS Calibure, BD). The mean fluorescence intensity were determined using cell Quest software (Becton Dickinson)

Isolation and extraction of RNA from serum and HepG2 cells

RNA was isolated from serum samples and HepG2 cells as reported by Lohr *et al*[16]. Briefly, cells were precipitated and washed in the same buffer to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mmol/L sodium citrate, 0.5% sarcosyl and 0.1 mol/L β-mercaptoethanol. Cellular RNA was extracted using the single-step method described originally by Chomczynski and Sacchi[17].

PCR of genomic RNA strands of HCV

Reverse transcription-nested PCR was carried out according to Lohr *et al*.[16] with few modifications. Retro-transcription was performed in 25 μL reaction mixture containing 20 units of AMV reverse transcriptase (Clonetech, USA) with either 400 ng of total PBMCs RNA or 3 μL of purified RNA from serum samples (equivalent to 30 μL serum) as template, 40 units of RNAsin (Clontech, USA), a final concentration of 0.2 mmol/L from each dNTP (Promega, Madison, WI, USA) and 50 pmol of the reverse primer P1 (for plus strand) or 50 pmol of the forward primer P2 (for minus strand). The reaction was incubated at 42 ℃ for 60 min. and denatured at 98 ℃ for 10 min. Amplification of the highly conserved 5'-UTR sequences was done using two rounds of PCR with 2 pairs of nested primers. First round amplification was done in 50 μL reaction con-

Table 1 Neutralizing activity of E1 antibody on infectivity of HCV to HepG2 cells

n	Viral Load	Genotype	Pre-incubation	
			PBS	Anti E1 Ab
1	<615	4c/4d	positive	positive
2	1481570	4	positive	positive
3	28469	4c/4d	Cells not infected[1]	Cells not infected[1]
4	4030	4e	Cells not infected[1]	Cells not infected[1]
5	42154	4	positive	positive
6	114598	4	positive	negative
7	<615	4c/4d	positive	positive
8	-	1b	Cells not infected[1]	Cells not infected[1]
9	2835590	4a	positive	negative
10	110933	4c/4d	positive	negative
11	1052310	4c/4d	positive	negative
12	806482	4e	positive	negative
13	4721	4c/4d	positive	negative
14	817331	4	Cells not infected[1]	Cells not infected[1]
15	2077	4a	Positive	negative
16	177021	4	Positive	negative
17	147226	4c/4d	Cells not infected[1]	Cells not infected[1]
18	3284580	4c/4d	Positive	negative
19	1136230	4	Positive	negative
20	506773	4c/4d	Cells not infected[1]	Cells not infected[1]
21	380695	4	Cells not infected[1]	Cells not infected[1]
22	159725	4	Positive	negative
23	1216640	4e	Positive	negative
24	<615	4a	Cells not infected[1]	Cells not infected[1]
25	<615	4c/4d	Positive	positive
26	284962	4	Cells not infected[1]	Cells not infected[1]
27	336705	4a	Positive	negative
28	<615	4	Cells not infected[1]	Cells not infected[1]

The presence of HCV RNA was determined by nested RT-PCR and genotyped using Innolipa system (Bayer, Germany). Viral loads were determined by branched DNA method (Bayer, Germany).

PBS: phosphate buffer saline

[1]Cells not infected: HepG2 cells did not infected by positive HCV serum.

taining 50 pmol from each of P2 forward primer and P3 reverse primer, 0.2 mmol/L from each dNTP, 10 μL from RT reaction mixture as template and 2 units of Taq DNA polymerase (Promega, USA) in 1X buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min. at 94℃, 1 min at 55℃ and 1 min at 72℃ for 30 cycles. The second round amplification was done similar to the first round, except for use of the nested reverse primer P4 and forward primer P5 at 50 pmol each. A fragment of 172 bp was identified in positive samples. Primer sequences were as follows: P1: 5' ggtgcacggtctacgagacctc 3' P2 : 5' aactactgtcttcacgcagaa 3', P3: 5' tgctcatggtgcacggtcta 3', P4: 5' actcggctagcagtctcgcg 3', P5: 5' gtgcagcctccaggaccc 3'. To control for false detection of negative-strand HCV RNA and known variations in PCR efficiency[18,19], specific control assays and rigorous standardization of the reaction were employed as previously described[20]. These specific control assays were: (1) cDNA synthesis without RNA templates to exclude product contamination, (2) cDNA synthesis without RTase to exclude Taq polymerase RTase activity, (3) cDNA synthesis and PCR step done with only the reverse or forward primer to confirm no contamination from mixed primers. These controls were consistently negative. In addition, cDNA synthesis was carried out using only one primer followed by heat inactivation of RTase activity at 95℃ for 1 h, in an attempt to diminish false detection of negative-strand prior to the addition of the second primer.

Infection of HepG2 cells with HCV

Cells were grown for 48h to semi-confluence in complete DMEM medium, washed twice with FCS -free medium then inoculated with serum samples (500 μLl plus 500 μL FCS-free DMEM/3×10^6 cells) obtained from HCV infected patients (RT-PCR and antibody positives). The viral load in the used sera was quantitated by bDNA technology and the average copy number was 615-3.2×10^6 IU/mL. After 90 min, DMEM containing FCS was added to make the overall serum contents 10% in a final volume 8 mL including the volume of human serum used for infection and cells were maintained overnight at 37℃ in 50 mL/L CO_2. Next day, adherent cells were washed three times with culture medium to get rid of the remaining infectious serum and incubation was continued in complete medium containing 10% FCS with regular medium changes. An inhibition assay of viral absorption or attachment to presumed susceptible cells has been developed for assessing the neutralizing related capacity of antibodies[21]. To analyze the neutralizing-related activity of antibodies against E1 region of HCV as compared with antibodies against core peptides, serial dilutions of serum samples were incubated with equal volumes of different amounts of the studied specific polyclonal antibodies in PBS at 4℃ overnight. One hundred microliters of pretreated serum samples were incubated with 1 mL of the cell suspension of HepG2 cells containing 0.5 million cells. Appropriate controls included HCV RNA positive sera that have neither been treated with E1 nor with core antibodies as positive controls, HepG2 cells not infected with HCV RNA positive sera as negative controls. Retrotranscription-PCR was performed on intracellular HCV RNA of HepG2 cells under the above described circumstances.

RESULTS

Selection of highly conserved peptide sequence among various HCV genotypes

The most conserved 10 amino acid stretch within the N-terminal region of E1 protein derived from several reported HCV isolates is shown in figure 1. When this 10mer peptide (GHRMAWDMM) was synthesized and used for immunization of New Zealand rabbits, the reactivity of hyper-immune E1 antibody was confirmed by enzyme-linked immunosorbent assay (ELISA) and western blot for detection of E1 protein in infected sera and infected HepG2 cells (Data not shown).

Selection of HCV positive sera and infection of HepG2 cells

Twenty eight HCV RNA positive sera with different viral loads and various subtypes of genotype 4 were used for infection experiments (Table 1). Only 18 samples were able to infect HepG2 cells, thus producing 18 different cell lines of HCV infected HepG2 cells.

```
AB67036[H77]      GHITGHRMAWDMMMNWSPTAALVVAQLLRIPQAIMDMIAGAHWGVLAGIAYFSMVGNWAK 160
BAD73999[1b]      GHVSGHRMAWDMMMNWSPTTALVVSQLLRIPQAVVDIVVGAHWGVLAGLAYYSMVGNWAK 180
BAB08107[2b]      GHITGQRMAWDMMLNWSPTLTMILAYAARVPELVLEIVFGGHWGVVFGLAYFSMQGAWAK 180
CAA72338[4a]      GHITGHRMAWDMMMNWSPTTTLVLAQVMRIPTTLVDLLSGGHWGVLVGVAYFSMQANWAK 170
CAC16101[4d]      GHITGHRMAWDMMMNWSPTATLVLAQLMRIPGAMVDLLAGGHWGILVGIAYFSMQANWAK 179
CAC16106[4f]      GHITGHRMAWDMMMNWSPTTTLVLAQIMRVPAALVDMLAGGHWGVLAGMAFFSMQANWAK 179
CAA73640[5a]      GHITGHRMAWDMMMKWSPTTALLMAQLLRIPQVVIDIIAGGHWGVLLAAAYFASTANWAK 180
AAW56714[6a]      GHVTGHRMAWDMMMSWSPTTTLVLSSILRVPEICASVIFGGHWGILLAVAYFGMAGNWLK 180
                  **:::*:********:.**** :::::   *:*   .:: *.***:: . *::.  . * *
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Figure 1 Sequence alignment of amino acids no 311 to 370 (numbering starts from initiating methionin in the core protein of genotype 4a) of HCV E1 among representative genotypes. Eight sequences of different subtypes were aligned using ClustalW software, the output diagram is shown with the legend on the left having the accession number and the subtypes. Sequences shown in bold represent the highly conserved amino acid stretch (GHRMAWDMM) used for production of polyclonal antibody.

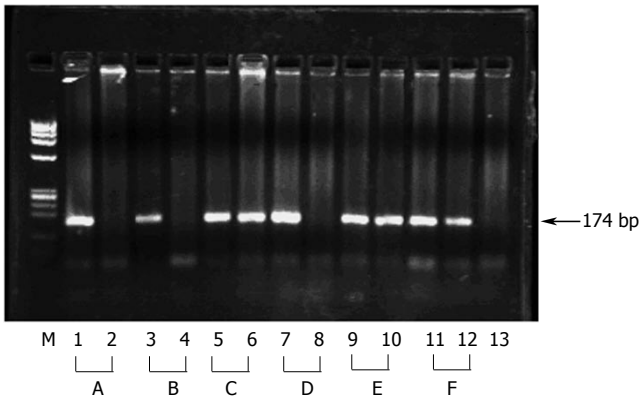

Figure 2 Inhibition of HCV entry into HepG2 cells by anti E1 Ab. Sera from 6 HCV infected patients (A to F) were used for infection of HepG2 cells before (Lanes 1,3,6,7,9 and 11) and after pre-incubation with anti E1 Ab (Lanes 2,4,6,8,10 and 12). RNA was detected in HepG2 lysates by nested HCV RT-PCR and the products were resolved on 2% agarose gel as described in materials and methods. The presence of a 174 bp band indicates presence of viral RNA while absence of the band indicates successful blocking of viral entry into cells. Lane M shows φx Hae III digest as a molecular weight marker, lane 13 shows RT-PCR of the PBS used for the last wash step of the cells.

Inhibition of HCV entry into HepG2 cells by specific E1 antibodies

Although an in vitro culture for HCV is not available, an inhibition assay of viral absorption or attachment to presumed susceptible cells has been developed by another laboratory for assessing the neutralizing related capacity of antibodies[21]. In the present study we used the HepG2 cell line to examine the biological function of E1 antibody. All 18 serum samples were positive for HCV RNA and have the ability to infect HepG2 cells as determined by PCR (Figure 2). After incubation of serum samples with E1 antibodies overnight at 4 ℃, only 5 out of 18 samples remained infectious to HepG2 cells while the remaining 13 samples did not infect HepG2 cells with an inhibition rate of 72% (Figure 2)

Inhibition of HCV binding to HepG2 cells by specific E1 antibodies

The mean fluorescence intensity of bound HCV particle was determined by flow cytometric analysis of HepG2 cells incubated with FITC labeled $F(ab)_2$ portion of HCV E1 antibody after subtraction of the nonspecific fluorescence value. Figure 3 showed a 6 fold (from 12% to 2%) reduction of mean fluorescence intensity in HepG2 cells treated with serum samples pre-incubated with specific anti E1 antibodies compared with cells incubated with untreated positive sera.

DISCUSSION

Although a detailed analysis of the viral genomic organization has led to the identification of various genetic elements[2] and the establishment of subgenomic replicons[22] in transfection experiments, the study of whole viral entry and infection is still hampered by the inability to propagate the virus efficiently in cultured cells and the limited animal tropism of the virus. The chimpanzee is the only nonhuman host serving as a model for HCV infection[23]. Binding of individually expressed recombinant glycoprotein E2 to human cell lines has been used as a surrogate model for binding of virus to host cells, allowing the study of antibody mediated neutralization of binding[24]. Using this surrogate assay, Pileri *et al*[25] have demonstrated that envelope glycoprotein E2 interacts with the large extracellular loop of cellular membrane protein CD81, a member of the tetraspanin family[26]. CD81 has been suggested as a candidate receptor for HCV[27]. Recently Brazzoli *et al*[28] suggested that productive folding of the major HCV spike protein E2 is assisted by E1. In the present study ,we developed, in house, a monospecific polyclonal antibody for an E1 peptide. The observed great homology within the N-terminal region of E1 suggests that this domain plays a major role in E1/E2 interaction and proper folding of envelop glycoproteins[29,30]. Therefore, a monospecific antibody against the amino terminal domain of E1 protein was expected to interfere with virus binding to membrane receptor. The immunogenicity of this anti E1 Ab was demonstrated by our laboratory in immunoassay techniques such as flow cytometry for detection of E1 glycoprotein in infected cells[31]. In vitro infection experiments, rather than the artificial replicon assays, was reported by others to mimic the intracellular events occurring *in vivo*[32,33]. Besides, study of the E1 Ab activity in blocking infection of cells by several infected sera is easier to accomplish via direct infection than the use of the laborious cloning to produce replicon (s) from each sample. However direct infection experiments does not facilitate 100% efficiency of HCV infection into HepG2 cells in all studied cases. In the current study, only 18 out

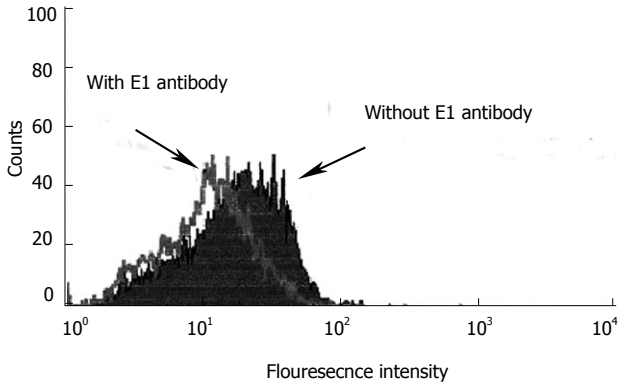

Figure 3 Neutralization of HCV infection into HepG2 cells by anti E1 antibody. The pretreated sera with anti E1 Ab or with PBS were incubated with HepG2 cells for 90 min at 37 °C in CO_2 incubator. Cell pellets were washed with PBS, incubated with FITC labeled $F(ab)_2$ portion of anti E1 Ab and analyzed with flow cytometry as described in Materials and Methods The mean fluorescence intensities decreased from 12.5% in cells incubated with PBS to 2% after treatment with anti E1 Ab using cell Quest software (Becton Dickinson).

of 28 positive samples (64%) had the ability to infect HepG2 cells as determined by intracellular detection of HCV RNA by RT-PCR. The reasons for the inability of the other ten serum samples to infect HepG2 cells is not clear. The quasispecies pool in each sample seems to play a significant role in determining viral entry in each case[34,35], Moreover, the competitive binding of viral particles and low density lipoproteins (LDL) toward the limited number of LDL receptors on cell membrane contributes in the sample to sample variation probably due to individual variations in LDL levels. Enjoji *et al*[36] reported that LDL competitively inhibit the infection of hepatocytes with HCV. On the other hand, our results showed that variations in viral counts appear not to be involved in determining the efficiency of HCV entry into HepG2 cells, a finding that agrees with earlier reports[37]**.** Anti E1 Ab could completely inhibit entry of viral particles into cells in 13 out of 18 (72%) samples. The reasons why the remaining 5 cases (28%) escaped the inhibitory effect of anti E1 Ab may be related to the relative protection of circulating viral particles by exosomes against neutralizing antibodies[38]. Alternatively, the concentration of E1 antibody may be not sufficient for complete inhibition of binding in all tested samples due to variations in the levels of circulating free E1 antigen.

The results of RT-PCR in HepG2 cells were confirmed by the results of flow cytometry. The direct immunostaining of E1 antibody conjugated with FITC and flow cytometric analysis showed reduced mean fluorescence intensity in the samples pre-incubated with E1 Ab compared with samples without E1 Ab. Shimizu *et al*[39] and Farci *et al*[11] demonstrated that a rabbit hyper-immune serum prepared against a peptide representing the 21 C-terminal amino acids of the HVR1 H77 of HCV 1a could neutralize the homologous virus *in vitro* and *in vivo*. These studies provided the first identification of a neutralization epitope on the surface of HCV. They also demonstrated that the neutralization was highly strain-specific and that minor variants of HCV bearing divergent sequences in the HVR1 were not neutralized and emerged in the cell culture and the chimpanzee as neutralization escape mutants. The development of a vaccine against HCV, based on stimulating neutralizing antibody to the HVR1, appeared to be a daunting task[40]. In the present study we provide alternative approach which may bear new hope for developing HCV vaccine based on conserved N-terminal region of E1 protein. Recently, Leroux-Roels *et al*[41] suggested that immunization of healthy individuals against HCV with the E1 protein as a prophylactic vaccine may not only raise useful (potentially neutralizing) anti-E1 antibodies but could also induce a strong T-cell response that might contribute to the prevention of chronic evolution in cases of acute hepatitis C.

In conclusion, in house produced anti E1 Ab that was raised in rabbits against the most conserved region among reported viral strains, blocks HCV infection to target cells suggesting the involvement of this epitope in virus binding and entry. Isolation of similar humanized antibodies that block virus binding and entry will be useful in providing potential therapeutic reagents and for vaccine development.

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BASIC RESEARCH

A comparison of gene expression in mouse liver and kidney in obstructive cholestasis utilizing high-density oligonucleotide microarray technology

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Abstract

AIM: To assess the effects of obstructive cholestasis on a wider range of gene expression using microarray technology.

METHODS: Male C57BL/6J mice underwent common bile duct ligation (BDL) and were matched with pair-fed sham-operated controls. After 7 d, the animals were sacrificed and total RNA was isolated from livers and kidneys. Equal amounts of RNA from each tissue were pooled for each group and hybridized to Affymetrix GeneChip®MG-U74Av2 containing a total of 12488 probe sets. Data analysis was performed using GeneSpring® 6.0 software. Northern analysis and immunofluorescence were used for validation.

RESULTS: In sham-operated and BDL mice, 44 and 50% of 12488 genes were expressed in livers, whereas 49 and 51% were expressed in kidneys, respectively. Seven days after BDL, 265 liver and 112 kidney genes with GeneOntology annotation were up-regulated and 113 liver and 36 kidney genes were down-regulated in comparison with sham-operated controls. Many genes were commonly regulated in both tissues and metabolism-related genes represented the largest functional group.

CONCLUSION: Following BDL, microarray analysis reveals a broad range of gene alterations in both liver and kidney.

Key words: Bile duct ligation; Cholestasis; Kidney; Liver; Microarray

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INTRODUCTION

Cholestasis, defined as impairment of bile secretion, is a feature of many hepatic disorders and systemic diseases. The recent cloning and functional characterization of different transport proteins for bile acids, organic anions and cations in hepatocytes and cholangiocytes have provided new insights into the molecular biology and physiology of bile formation and have increased understanding of the pathophysiology of cholestatic disorders[1]. Thus it is now established that a number of transport proteins in the basolateral and canalicular hepatocyte membrane undergo adaptive regulation in response to cholestatic liver injury to minimize the hepatic accumulation of toxic substances, such as hydrophobic bile acids[2-4]. Previous studies have indicated that in addition to the liver, adaptive regulation of these transporters in cholestasis also occurs in extrahepatic tissues, including the kidney[5] and the intestine[6]. Other alterations in cholestasis affect hepatic signal transduction[7,8], vesicular transport[7], apoptosis[9,10], metabolism[11], and the structure of the extracellular matrix[12,13].

Given the wide range of signaling, regulatory, and metabolic pathways, structural elements, and transport proteins which may be affected in cholestasis, much

further research will be necessary to more fully understand the extent of these adaptations. High-density DNA microarrays containing thousands of DNA fragments and oligonucleotides are a potentially promising approach to identify additional genes of interest that play a role in this pathophysiologic process. Based on their ability to monitor large numbers of genes at a time, high-density DNA microarrays are a sensitive, time-saving, and efficient tool in determining gene expression and finding regulatory pathways[14].

In the present study, we, therefore, have utilized high-density oligonucleotide microarray technology to screen for gene alterations in the liver and kidney following bile duct ligation (BDL) in mice, an established model of obstructive cholestasis. This study has allowed a comprehensive gene expression profile to be obtained in cholestatic mouse liver and kidney as well as it has highlighted a number of genes whose expression is particularly altered by this process.

MATERIALS AND METHODS

Animals and animal treatment

Male C57BL/6J mice (8-12-wk-old) purchased from Jackson Lab (Bar Harbor, ME) underwent BDL or sham-surgery as previously described[15]. The common bile duct was identified, ligated twice close to the liver hilum immediately below the cystic duct, and then divided between the ligatures. Control mice underwent sham-surgery in which the common bile duct was exposed but not ligated. Since sham-operated mice tend to consume more food than BDL mice and the expression of some genes may be affected by caloric intake, food intake of BDL mice was monitored daily and sham-operated mice were pair-fed so as to receive the same amount of food as BDL mice. Animals were sacrificed 7 d after surgery and livers and kidneys were harvested. The protocol was approved by the Yale Animal Care and Use Committee, and the animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, revised 1985).

Isolation of total RNA

Blood-free livers and kidneys were homogenized in GTC solution containing 4 mol/L guanidinium thiocyanate, 25 mmol/L Na-citrate, and 5 g/L N-lauroylsarcosine and subjected to CsCl gradient centrifugation. The recovered total RNA was further purified by phenol/chloroform extraction and ethanol precipitation. The RNA concentration was determined spectrophotometrically and the RNA quality was confirmed by formaldehyde-agarose gel electrophoresis. Equal amounts of liver and kidney, respectively, total RNA from each of four BDL and four sham-operated mice were pooled to minimize inter-animal variations and used for biotin-labeling.

DNA microarray hybridization and analysis

The biotin-labeled RNA from the different groups was hybridized with two replicates for each condition to individual high-density oligonucleotide microarray chips (GeneChip®MG-U74Av2) from Affymetrix (Santa Clara, CA) containing a total of 12488 probe sets. Microarray expression data were generated with Affymetrix Microarray Suite 5.0 software and further analysis was carried out with GeneSpring®6.0 (Silicon Genetics, Redwood City, CA). Raw intensity values from each chip were normalized to the 50th percentile of the measurements taken from that chip to reduce chip-wide variations in intensity. Each gene was normalized to the average measurement of that gene in the respective paired controls to enable comparison of relative changes in gene expression levels between different conditions. Cross-gene error model was active based on the replicates. Comparisons of gene expression data were made between BDL and sham-operated mice. Signal and detection flag from Microarray Suite 5.0 were used as quality controls. Only genes with a minimum signal intensity of 600, a detection flag present in both replicates in at least one of the comparison conditions, and a two-fold and above change in gene expression were used for further analysis. For identification of differentially expressed genes in the different groups, a one-sample *t*-test with a *P* value cutoff of 0.05 was performed to determine if the average log of the ratio of the replicates was significantly different from 1.0, which was the value of the control samples after normalization. Finally, genes were categorized into GeneOntology (GO) and annotated using NetAffx®, an analysis web interface from Affymetrix.

Northern analysis

To validate alterations in gene expression on the microarray, changes in the expression of selected genes were confirmed in aliquots of the same RNA samples used for the microarray by Northern analysis as previously described[16]. The following primers were used for the generation of specific probes: cytochrome P450 7b1 (GenBank accession number U36993): 5'-GAATCTC AGCTTAGAGAGTAAGAG-3' (sense), 5'-TTTGTA CCTAAAGGAGACGGCAG-3' (antisense); organic cation transporter 1 (Oct1) (GenBank accession number U38652): 5'-GCAGCCTGCCTCCTCATGATC-3' (sense), 5'-GGTAAATCGTGTTTTCTTTGGCC-3' (antisense); similar to putative integral membrane transport protein (GenBank accession number AI647632): 5'-TGATTACA AGAAATGTCAAGCAGG-3' (sense), 5'-CCTCTTCCTG ACTCCATCCATG-3' (antisense).

Immunofluorescence

Indirect immunofluorescence with a polyclonal antibody against Oct1[17,18] (dilution 1:100; kindly provided by Prof. Dr. H. Koepsell, Würzburg, Germany) was conducted on liver specimens from sham-operated and BDL mice as previously described[19].

RESULTS

Gene expression profile in mouse liver in obstructive cholestasis

Of the total of 12488 genes on the microarray chip, 44 and 50% were expressed in the livers of sham-operated and BDL mice, respectively. After 7 d of obstructive cholestasis 265 genes with GO annotation were up-regulated and 113 were down-regulated in livers of BDL

mice by a factor of two or more in comparison with sham-operated pair-fed controls. Metabolism-related genes represented the largest functional group among the altered genes after BDL in liver (Table 1). It should be noted that the grouping of the altered genes was primarily done to achieve a clearer arrangement for the reader. Since a considerable number of the encoded proteins have multiple, little characterized or even unknown functions, we want to point out that the classification provided is subject to the personal opinions and emphasis of the authors (Table 1). Upon request, a complete list of the altered genes including genes without GO annotation that are not mentioned here can be obtained from the authors. Alternatively, the complete list of altered genes can be accessed via http://livercenter.yale.edu/datalist.html.

Gene expression profile in mouse kidney in obstructive cholestasis

In the kidneys of sham-operated and BDL mice, 49 and 51% of the 12 488 genes on the microarray chip were expressed, respectively. Seven days after surgery, 112 genes with GO annotation were up-regulated and 36 were down-regulated in the kidneys of BDL mice at least two-fold when compared with the sham-operated pair-fed controls. Thus the number of altered genes in kidney seven days after BDL was considerably smaller than that in liver (148 *vs* 378). Of the 112 GO genes up-regulated in kidney after BDL, 53 were also up-regulated in cholestatic liver. In contrast, of the 36 genes down-regulated in kidney, 7 were also down-regulated in liver (Table 1). What was particularly striking is that many of the most highly up-regulated genes in liver were also the same genes that were most highly up-regulated in kidney, irrespective of their functional class (Table 1). This suggests that both the liver and the kidney may be responding to similar transcriptional signaling molecules in this cholestatic model. For example, the acute phase gene, *serum amyloid A3*, was up-regulated 10.0-fold in liver and 36.1-fold in kidney, the gene encoding *chemokine (C-X-C motif) ligand 1* was increased 9.6-fold in liver and 4.2-fold in kidney, and the gene encoding the transport molecule *lipocalin 2* was up-regulated 13.1-fold in liver and 66.5-fold in kidney. In addition, a number of cell adhesion and extracellular matrix genes were similarly up-regulated in both liver and kidney. However, only one membrane transporter gene was up-regulated in both tissues, the gene encoding the *β1 subunit of the voltage-gated sodium channel* (Table 1). Interestingly, several genes for nucleic acid binding proteins were also highly up-regulated in both liver and kidney including the genes encoding the transcription factors *FBJ osteosarcoma oncogene* (alias *c-Fos*), *CCAAT/enhancer binding protein (C/EBP), delta*, and *activating transcription factor 3*.

In contrast, only seven genes were commonly down-regulated in both liver and kidney. These included the *RIKEN cDNA 1700013L23 gene* and the genes encoding *similar to putative integral membrane transport protein, major urinary protein 2*, *transthyretin*, *cytochrome P450 7b1* (GenBank accession numbers AV141027 and U36993), and *thioether S-methyltransferase*.

Northern analysis of selected genes

Gene expression results from the microarray were confirmed by Northern analysis for selected genes that included *cytochrome P450 7b1*, *organic cation transporter 1 (Oct1; solute carrier family 22, member 1)* and *similar to putative integral membrane transport protein* from aliquots of the RNA samples utilized for the microarray (Figure 1).

Tissue immunofluorescence of Oct1 in liver

Indirect immunofluorescence was performed to illustrate the decreased expression of the organic cation transporter Oct1 in BDL mouse liver. Figures 2A and B demonstrate that the findings are consistent with the microarray and the Northern blot results and corroborate that obstructive cholestasis leads to a down-regulation of Oct1 in mouse liver similarly as demonstrated previously in rat liver following BDL[19,20].

DISCUSSION

Ligation of the common bile duct in rodents is a well-established model of obstructive cholestasis. While most previous studies have been limited to investigations of small numbers of genes and their encoded proteins, we have been able to simultaneously monitor the responses of large numbers of genes in this cholestatic model by using high-density oligonucleotide microarray technology. In contrast to a recent study which investigated gene expression in obstructive cholestasis only in the livers of BDL mice[21], we additionally monitored alterations of gene expression in the kidneys because the kidney is functionally closely linked to the liver and provides an alternative excretory route for cholephilic substances in cholestasis[5]. One of the interesting conclusions from this analysis is the finding that many of the most highly up-regulated genes were shared in both liver and kidney, possibly due to a common response to similar transcriptional signaling molecules in both tissues. The interpretation and discussion of our data is based on the assumption that changes in gene expression lead to changes in protein expression although it is known that changes at the mRNA level do not always result in changes in protein expression in certain time periods[22]. As others have done, we first evaluated the observed changes in gene expression in terms of what is already known about the effects of cholestasis. We then attempted to identify novel regulatory processes that have not yet been investigated[22].

For example, our microarray data largely confirm previous results obtained by conventional determination of transcription in obstructive cholestasis, such as the up-regulation of the canalicular cation transporter *multidrug resistance P-glycoprotein 1a* (*Mdr1a*, Abcb1a)[23] or the down-regulation of the basolateral *sodium-taurocholate cotransporting polypeptide* (*Ntcp*, Slc10a1)[24]. In addition, our gene expression profile obtained from cholestatic liver also closely matched the gene expression profile recently generated by Campbell *et al*[21], although there are a substantial number of additional gene alterations in our data set. This difference can be explained since Campbell

Table 1 Fold increase/decrease in liver and kidney, GenBank accession number, and classification of altered genes in mice 7 d after bile duct ligation in comparison with pair-fed sham-operated controls

Liver	Kidney	Accession number	Description
Cell death			
3.6		AF011428	CD5 antigen-like
3.2		AW046181	Serum/glucocorticoid regulated kinase
2.6		AV373612	Bcl2-associated athanogene 3
-2.6		X65128	Growth arrest specific 1
-2.7		AA770736	Induced in fatty liver dystrophy 2
	2.8	M61737	Fat-specific gene 27
	2.7	AV003873	Clusterin
	2.3	D14077	Clusterin
	-7.0	AJ000062	Deoxyribonuclease I
Stress response			
10.0	36.1	X03505	Serum amyloid A 3
5.0		M13521	Serum amyloid A 2
2.8		M12566	Orosomucoid 2
2.5		J04633	Heat shock protein 1, alpha
2.5		X60676	Serine (or cysteine) proteinase inhibitor, clade H, member 1
	7.4	M96827	Haptoglobin
	-2.2	Z36774	Serine (or cysteine) proteinase inhibitor, clade F, member 2
Immune and inflammatory response			
31.9	5.0	M94584	Chitinase 3-like 3
17.3		M19681	Chemokine (C-C motif) ligand 2
10.7		X53798	Chemokine (C-X-C motif) ligand 2
9.6	4.2	J04596	Chemokine (C-X-C motif) ligand 1
9.4		AW120786	Chemokine (C-X-C motif) ligand 14
9.2		U18424	Macrophage receptor with collagenous structure
8.4		AV370035	Chemokine (C-C motif) receptor 5
7.1		U56819	Chemokine (C-C) receptor 2
6.9	5.5	AF002719	Secretory leukocyte protease inhibitor
6.1		M18237	Immunoglobulin kappa chain variable 8 (V8)
5.9		U34277	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
5.4		M83218	S100 calcium binding protein A8 (calgranulin A)
3.6	3.7	X04673	Adipsin
3.6		AI844520	Interferon gamma inducible protein 30
3.6		AF081789	Complement component 1, q subcomponent, receptor 1
3.5		X12905	Properdin factor, complement
3.3		L32838	Interleukin 1 receptor antagonist
3.2		U96752	Histocompatibility 2, Q region locus 1
3.2	3.4	M22531	Complement component 1, q subcomponent, beta polypeptide
3.2		X15591	Cytotoxic T lymphocyte-associated protein 2 alpha
3.1		X63782	Lymphocyte antigen 6 complex, locus D
2.9		M58004	Chemokine (C-C motif) ligand 6
2.9		M21932	Histocompatibility 2, class II antigen A, beta 1
2.9		U16985	Lymphotoxin B
2.9		M14639	Interleukin 1 alpha
2.9	4.2	X58861	Complement component 1, q subcomponent, alpha polypeptide
2.8		U77461	Complement component 3a receptor 1
2.8		M31314	Fc receptor, IgG, high affinity I
2.8		X52643	Histocompatibility 2, class II antigen A, alpha
2.7		AB007599	Lymphocyte antigen 86
2.6		X15592	Cytotoxic T lymphocyte-associated protein 2 beta
2.6		AF013715	Periplakin
2.6	2.1	X66295	Complement component 1, q subcomponent, gamma polypeptide
2.5	3.1	L38444	T-cell specific GTPase
2.5		AA596710	Leukotriene B4 12-hydroxydehydrogenase
2.5		AB019505	Interleukin 18 binding protein
2.4		M34815	Chemokine (C-X-C motif) ligand 9
2.4	2.0	X00496	Ia-associated invariant chain
2.4	2.8	AJ007970	Guanylate nucleotide binding protein 2
2.3		D86382	Allograft inflammatory factor 1
2.3		L22181	Formyl peptide receptor 1
2.2		AF038149	Paired-Ig-like receptor B
2.1		AW060457	Immunoglobulin superfamily, member 7
2.1		U03003	Defensin related cryptdin 6
2.0		M29855	Colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)
2.0		AF003525	Defensin beta 1
-2.8		M29007	Complement component factor h

Liver	Kidney	Accession number	Description
-4.6		L22977	X-linked lymphocyte-regulated 3b
	13.6	U47810	Complement component factor i
	6.5	K02782	Complement component 3
	6.0	X06454	Complement component 4 (within H-2S)
	4.2	AI563854	Tumor-associated calcium signal transducer 2
	3.8	AA986114	T-cell immunoglobulin and mucin domain containing 2
	3.5	U49513	Chemokine (C-C motif) ligand 9
	3.0	Y08830	Tumor-associated calcium signal transducer 2
	2.4	AA270365	Cytokine receptor-like factor 1
	2.2	AI152789	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A
Signal transduction			
12.9		U88328	Suppressor of cytokine signaling 3
5.4		Z48043	Coagulation factor II (thrombin) receptor-like 1
5.0	2.7	M14044	Annexin A2
4.0	2.2	AJ001633	Annexin A3
3.6		AI641895	Shroom
3.6		U90715	Coxsackievirus and adenovirus receptor
3.6		AI317205	Mitogen activated protein kinase kinase kinase 1
3.4		J03023	Hemopoietic cell kinase
3.1		AW209098	IQ motif containing GTPase activating protein 1
3.0		AW049806	RIKEN cDNA 1700093E07 gene
3.0		X84797	Hematopoietic cell specific Lyn substrate 1
2.9	3.0	AB015978	Oncostatin M receptor
2.6		X93328	EGF-like module containing, mucin-like, hormone receptor-like sequence 1
2.3		D63423	Annexin A5
2.3	2.1	M69260	Annexin A1
2.2		M68902	Hemopoietic cell phosphatase
2.2		AF020313	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein
2.1	3.6	AV374868	Suppressor of cytokine signaling 3
2.1		AA608387	Interleukin 13 receptor, alpha 1
-2.0		AC002397	Gene rich cluster, C9 gene
-2.0		AW125649	Guanine nucleotide binding protein, alpha 12
-2.4		AI839138	Thioredoxin interacting protein
-2.6		AV321519	Sorting nexin 17
-2.7		AA691492	RIKEN cDNA D530020C15 gene
-5.6		D17444	Leukemia inhibitory factor receptor
-11.7		AV349152	Regulator of G-protein signaling 16
-15.3		U94828	Regulator of G-protein signaling 16
	2.3	AF084466	Ras-related associated with diabetes
	2.1	AF009246	RAS, dexamethasone-induced 1
	-2.1	AF054623	Frizzled homolog 1 (Drosophila)
	-2.2	D85605	Cholecystokinin A receptor
	-2.2	AI834895	Membrane progestin receptor alpha
	-2.3	AW046638	PDZ domain containing 1
Cell growth and maintenance			
8.7		M33960	Serine (or cysteine) proteinase inhibitor, clade E, member 1
6.9		X98471	Epithelial membrane protein 1
5.8	4.9	X66449	S100 calcium binding protein A6 (calcyclin)
5.4		AF055638	Growth arrest and DNA-damage-inducible 45 gamma
5.1		M17298	Nerve growth factor, beta
3.6		AI849928	Cyclin D1
3.5		X59846	Growth arrest specific 6
3.2		M64292	B-cell translocation gene 2, anti-proliferative
3.2		AW048937	Cyclin-dependent kinase inhibitor 1A (P21)
3.1		AF009366	Neural precursor cell expressed, developmentally down-regulated gene 9
2.7		M21019	Harvey rat sarcoma oncogene, subgroup R
2.7		X06368	Colony-stimulating factor 1 receptor
2.2		X81579	Insulin-like growth factor binding protein 1
2.1		AI851454	Cysteine rich protein 2
2.0		AA529583	Mortality factor 4 like 2
-2.1		X95280	G0/G1 switch gene 2
-2.2		M31680	Growth hormone receptor
-2.5		U15012	Growth hormone receptor
	3.4	AI852641	Nuclear protein 1
	2.8	M34094	Midkine
	2.8	AF058798	Stratifin
	2.1	X81580	Insulin-like growth factor binding protein 2
Protein biosynthesis			
2.3		Y11460	Integrin beta 4 binding protein

Liver	Kidney	Accession number	Description
2.1		NM_011690	Valyl-tRNA synthetase 2
-2.0		AV055186	Ribosomal protein, large, P1
Proteolysis and protein degradation			
7.6	2.4	X61232	Carboxypeptidase E
6.1		AW060527	Ubiquitin-conjugating enzyme E2 variant 2
4.0		AJ000990	Legumain
4.0	5.0	AJ223208	Cathepsin S
3.7		AL078630	Ubiquitin D
2.0		U35833	Ubiquitin-like 1 (sentrin) activating enzyme E1B
-2.2		AI844932	F-box only protein 8
-2.4		L21221	Proprotein convertase subtilisin/kexin type 4
-2.6		AV359471	Ubiquitin specific protease 15
	-2.2	J04946	Angiotensin converting enzyme
	-2.5	L15193	Meprin 1 beta
Protein amino acid phosphorylation and dephosphorylation			
3.2		D89728	Serine/threonine kinase 10
3.2		M97590	Protein tyrosine phosphatase, non-receptor type 1
2.6		D37801	Protein tyrosine phosphatase, non-receptor type 21
2.0		X61940	Dual specificity phosphatase 1
-2.1		L31783	Uridine monophosphate kinase
Cell adhesion and extracellular matrix			
24.7		L36244	Matrix metalloproteinase 7
20.6		U43525	Proteinase 3
10.7		M82831	Matrix metalloproteinase 12
10.4	2.3	D00613	Matrix gamma-carboxyglutamate (gla) protein
9.0	3.1	X16834	Lectin, galactose binding, soluble 3
8.9		L02918	Procollagen, type V, alpha 2
8.1		M31039	Integrin beta 2
7.2		M62470	Thrombospondin 1
6.2		X13986	Secreted phosphoprotein 1
5.9	2.4	U03419	Procollagen, type I, alpha 1
5.8		D14010	Regenerating islet-derived 1
4.9	2.1	X52046	Procollagen, type III, alpha 1
4.7	2.5	M90551	Intercellular adhesion molecule
4.2		X58251	Procollagen, type I, alpha 2
4.0		L57509	Discoidin domain receptor family, member 1
3.4	4.3	U12884	Vascular cell adhesion molecule 1
3.2	3.3	M84487	Vascular cell adhesion molecule 1
3.2		L29454	Fibrillin 1
3.0		Z22532	Syndecan 1
2.9		M23552	Serum amyloid P-component
2.8		X04017	Secreted acidic cysteine rich glycoprotein
2.7		M38337	Milk fat globule-EGF factor 8 protein
2.7		AA763466	Procollagen, type I, alpha 1
2.5		AA919594	Elastin
2.5		M70642	Connective tissue growth factor
2.5		D88577	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 13
2.3		M15832	Procollagen, type IV, alpha 1
2.2		X59990	Catenin alpha 1
2.2		U82624	Amyloid beta (A4) precursor protein
2.1		X53928	Biglycan
2.1		U89915	F11 receptor
2.0		X04647	Procollagen, type IV, alpha 2
2.0	2.2	V00755	Tissue inhibitor of metalloproteinase 1
2.0		X91144	Selectin, platelet (p-selectin) ligand
-2.1		AF101164	CEA-related cell adhesion molecule 2
-2.2		AI840501	Camello-like 1
	2.1	L19932	Transforming growth factor, beta induced
Cytoskeleton and structural elements			
5.0	7.3	M36120	Keratin complex 1, acidic, gene 19
4.8		V00830	Keratin complex 1, acidic, gene 10
4.5		U38967	Thymosin, beta 4, X chromosome
3.6	2.3	AI852553	Thymosin, beta 10
3.6		U42471	Wiskott-Aldrich syndrome homolog (human)
3.4		U29539	Lysosomal-associated protein transmembrane 5
3.4		M22479	Tropomyosin 1, alpha
3.2	2.2	M28739	Tubulin, beta 2
3.2		AW215736	RIKEN cDNA 2310057H16 gene

Liver	Kidney	Accession number	Description
3.2	4.5	M22832	Keratin complex 1, acidic, gene 18
3.1		AI505453	Myosin heavy chain IX
3.0		X15662	Keratin complex 2, basic, gene 8
2.8		X60671	Villin 2
2.7		D49733	Lamin A
2.7		AW125446	Golgi phosphoprotein 2
2.6		AW050256	Tubulin, beta 3
2.6		AI839417	Moesin
2.4		AW125698	Myosin heavy chain IX
2.4		AW212775	Actin-related protein 2/3 complex, subunit 1B
2.4		AV356071	Lysosomal-associated protein transmembrane 5
2.2		M28727	Tubulin, alpha 2
2.2		AI835858	Tropomyosin 4
2.2		M12347	Actin, alpha 1, skeletal muscle
2.1		D88793	Cysteine and glycine-rich protein 1
2.1		AF020185	Dynein, cytoplasmic, light chain 1
2.1		AI837625	Cysteine and glycine-rich protein 1
2.1	3.2	X54511	Capping protein (actin filament), gelsolin-like
2.0	2.1	X04663	Tubulin, beta 5
2.0		AI841606	Actin-binding LIM protein 1
2.0		M21495	Actin, gamma, cytoplasmic
2.0		AI849152	Clathrin, light polypeptide (Lcb)
2.0		M60474	Myristoylated alanine rich protein kinase C substrate
-2.2		AW123904	Gamma-aminobutyric acid (GABA(A)) receptor-associated protein-like 1
	3.3	AB000713	Caudin 4
	2.6	AA755126	Keratin complex 2, basic, gene 7
	2.6	AF087825	Claudin 7
	2.3	AI195392	Actinin, alpha 1
Transport			
13.1	66.5	X81627	Lipocalin 2
12.1	2.0	L48687	Sodium channel, voltage-gated, type I, beta polypeptide
7.2		U04827	Fatty acid binding protein 7, brain
3.7		M24417	ATP-binding cassette, sub-family B (MDR/TAP), member 1A
3.4		AI842825	Glycolipid transfer protein
3.2		NM_033444	Chloride intracellular channel 1
3.2		X99347	Lipopolysaccharide binding protein
2.9		L13732	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
2.8		U72680	FXYD domain-containing ion transport regulator 5
2.8		X60367	Retinol binding protein 1, cellular
2.5		U27315	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4
2.4		AI842065	Expressed sequence AW538430
2.3		AI849583	RIKEN cDNA 6330416G13 gene
2.3		AI852578	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2
2.1		D87661	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide
2.1		U28960	Phospholipid transfer protein
-2.0	-2.2	AA670737	RIKEN cDNA 1700013L23 gene
-2.1		M16360	Major urinary protein 5
-2.1		AF072757	Solute carrier family 27 (fatty acid transporter), member 2
-2.1		M16358	Major urinary protein 4
-2.1		L28836	ATP-binding cassette, sub-family D (ALD), member 3
-2.3		U38652	Solute carrier family 22 (organic cation transporter), member 1
-2.3		M16357	Major urinary protein 3
-2.3		U95131	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1
-2.3	-2.3	AV355798	Major urinary protein 2
-2.3		AV104178	Serine (or cysteine) proteinase inhibitor, clade A, member 6
-2.4		U95132	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1
-2.4		M16359	Major urinary protein 1
-2.4		AB028737	ATP-binding cassette, sub-family C (CFTR/MRP), member 6
-2.6	-2.0	D00073	Transthyretin
-2.9		AJ011080	Afamin
-3.8	-3.8	AI647632	Similar to putative integral membrane transport protein
-4.4		AI255271	Major urinary protein 2
-6.4		Y14660	Fatty acid binding protein 1, liver
-6.6		X70533	Serine (or cysteine) proteinase inhibitor, clade A, member 6
	4.0	M55413	Group-specific component
	2.7	AF047838	Chloride channel calcium activated 1
	2.7	AI849587	Protein distantly related to the gamma subunit family
	2.6	D00466	Apolipoprotein E
	2.4	AI661431	Aquaporin 2
	2.3	AI197481	Amiloride binding protein 1 (amine oxidase, copper-containing)
	-2.1	AI606956	Solute carrier family 2 (facilitated glucose transporter), member 5

Liver	Kidney	Accession number	Description
	-2.1	AW122706	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
	-2.2	AI120514	Solute carrier family 26 (sulfate transporter), member 1
	-2.3	AI837530	Solute carrier family 9 (sodium/hydrogen exchanger), member 8
Cell surface markers and membrane proteins			
12.9		X13333	CD14 antigen
6.7		X97227	CD53 antigen
6.4		M65027	Glycoprotein 49 A
5.9		D16432	CD63 antigen
5.3	2.3	AW209486	Prostate stem cell antigen
5.0	3.4	AF024637	TYRO protein tyrosine kinase binding protein
3.6		M58661	CD24a antigen
3.3		U37438	Deleted in malignant brain tumors 1
3.3		M55561	CD52 antigen
3.3		AI854863	RIKEN cDNA 1200015A22 gene
3.0		AF039663	Prominin 1
2.7		AI849180	Integral membrane protein 2C
2.6		AI787183	RIKEN cDNA 0610011I04 gene
2.5	2.5	X68273	CD68 antigen
2.2		AB031386	RIKEN cDNA 1810009M01 gene
2.0		L11332	CD38 antigen
-2.5		AI843959	RIKEN cDNA 5730403B10 gene
	3.3	AW261569	RIKEN cDNA D630035O19 gene
	2.0	AI847784	CD34 antigen
	-2.5	L23108	CD36 antigen
Transcription factors and nucleic acid binding proteins			
8.1	7.2	V00727	FBJ osteosarcoma oncogene
6.2	3.6	AW124113	Brain abundant, membrane attached signal protein 1
4.9	2.2	AW049031	Core promoter element binding protein
4.0		M90397	B-cell leukemia/lymphoma 3
3.8		M31885	Inhibitor of DNA binding 1
3.8	3.0	AA614971	Molecule possessing ankyrin-repeats induced by lipopolysaccharide
3.2	3.6	X61800	CCAAT/enhancer binding protein (C/EBP), delta
3.2		AF017258	Ribonuclease, RNase A family, 2
2.7		AB016424	RNA binding motif protein 3
2.6	2.4	U19118	Activating transcription factor 3
2.5		AF016294	E74-like factor 3
2.4		L03215	SFFV proviral integration 1
2.3		AI642098	RIKEN cDNA 4921515A04 gene
2.3		U20735	Jun-B oncogene
2.2		M60523	Inhibitor of DNA binding 3
2.2		D26089	Minichromosome maintenance deficient 4 homolog (S. cerevisiae)
2.1	2.2	U20344	Kruppel-like factor 4 (gut)
-2.0		U36799	Retinoblastoma-like 2
-2.0		AF038995	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6
-2.1		L20450	Zinc finger protein 97
-2.1		X77602	Upstream transcription factor 2
-2.2		AF064088	TGFB inducible early growth response 1
-2.2		U95945	One cut domain, family member 1
-2.4		U62674	Histone 2, H2aa1
-2.4		AA002843	Nuclear factor I/X
-2.7		AI834950	Nuclear receptor subfamily 1, group D, member 1
-2.8		AW047343	D site albumin promoter binding protein
-3.4		X57638	Peroxisome proliferator activated receptor alpha
	4.7	AI840339	Ribonuclease, RNase A family 4
	2.7	M28845	Early growth response 1
	2.3	X16995	Nuclear receptor subfamily 4, group A, member 1
Metabolism			
8.5		M13018	Cysteine-rich protein 1 (intestinal)
6.1		AV327760	Stearoyl-Coenzyme A desaturase 2
6.0	37.5	X51547	P lysozyme structural
5.9		AW046124	Cytochrome b-245, alpha polypeptide
5.1	4.6	M21050	Lysozyme
4.9		X97047	Pyruvate kinase, muscle
4.2		AV368209	Pyruvate kinase, muscle
4.1		U43384	Cytochrome b-245, beta polypeptide
4.1		AA726364	Lipoprotein lipase
4.0		AI846517	Cytochrome b-561
4.0		AI854821	RIKEN cDNA 0610041P13 gene
3.8		U13705	Glutathione peroxidase 3
3.8		U12961	NAD(P)H dehydrogenase, quinone 1

Liver	Kidney	Accession number	Description
3.6	2.1	M26270	Stearoyl-Coenzyme A desaturase 2
3.6		M34141	Prostaglandin-endoperoxide synthase 1
3.6		X07888	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
3.5		M31775	Cytochrome b-245, alpha polypeptide
3.5		AI847162	RIKEN cDNA 1300017C10 gene
3.4		U87147	Flavin containing monooxygenase 3
3.4		AA690863	ATPase, class VI, type 11A
3.4		J04696	Glutathione S-transferase, mu 2
3.3		X56824	Heme oxygenase (decycling) 1
3.0		AJ238894	Acyl-Coenzyme A thioesterase 3, mitochondrial
3.0		D42048	Squalene epoxidase
2.9		AW060927	Lanosterol synthase
2.8		J03953	Glutathione S-transferase, mu 3
2.4		U49350	Cytidine 5'-triphosphate synthase
2.4		AI594518	Chitinase, acidic
2.2		J02980	Alkaline phosphatase 2, liver
2.2		U27455	Serine palmitoyltransferase, long chain base subunit 2
2.2		AI327450	Phospholipase A2, group IB, pancreas
2.2		AF077527	Syndecan binding protein
2.2		AA710635	Colipase, pancreatic
2.1		M62766	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
2.1		AW049778	Mevalonate (diphospho) decarboxylase
2.1		AF057368	7-dehydrocholesterol reductase
2.0		U49385	Cytidine 5'-triphosphate synthase 2
-2.0		AW123316	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)
-2.0		AA824102	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7
-2.0		AF098009	Fatty acid amide hydrolase
-2.1		AV216468	Expressed in non-metastatic cells 1, protein
-2.1		L42996	Dihydrolipoamide branched chain transacylase E2
-2.1		AI846934	Lipin 1
-2.1		AV071102	Cytochrome c oxidase, subunit VIc
-2.1		AI839995	Sarcosine dehydrogenase
-2.1		X61397	Carbonic anhydrase 8
-2.1		AF022894	Sulfotransferase family 1B, member 1
-2.1		U24493	Tryptophan 2,3-dioxygenase
-2.2		AA675075	Proline dehydrogenase (oxidase) 2
-2.2		AV276715	Aldehyde dehydrogenase family 3, subfamily A2
-2.3		L11333	Esterase 31
-2.3		L11163	Acyl-Coenzyme A dehydrogenase, short chain
-2.3		AI840013	Peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase
-2.4		M27347	Elastase 1, pancreatic
-2.4		U32684	Paraoxonase 1
-2.4		M77015	Hydroxysteroid dehydrogenase-3, delta<5>-3-beta
-2.4		AF030343	Enoyl coenzyme A hydratase 1, peroxisomal
-2.4		AF047542	Cytochrome P450, family 2, subfamily c, polypeptide 37
-2.4		AF047727	Cytochrome P450, family 2, subfamily c, polypeptide 40
-2.4		Z14050	Dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenyme A isomerase)
-2.5		D17674	Cytochrome P450, family 2, subfamily c, polypeptide 29
-2.5		AI844846	2,4-dienoyl CoA reductase 1, mitochondrial
-2.6		X83202	Hydroxysteroid 11-beta dehydrogenase 1
-2.6		U14390	Aldehyde dehydrogenase family 3, subfamily A2
-2.6		AW012588	3-ketoacyl-CoA thiolase B
-2.7		AI530403	Acetyl-Coenzyme A acyltransferase 1
-2.7		X51971	Carbonic anhydrase 5a, mitochondrial
-2.7		AF031170	Hydroxysteroid dehydrogenase-6, delta<5>-3-beta
-2.8		AI266885	RIKEN cDNA 1700124F02 gene
-2.9		AF030513	Retinol dehydrogenase 6
-3.0		U15977	Fatty acid Coenzyme A ligase, long chain 2
-3.0		X04283	Cytochrome P450, family 1, subfamily a, polypeptide 2
-3.4		X63349	Dopachrome tautomerase
-3.6		M15268	Aminolevulinic acid synthase 2, erythroid
-4.0		D63764	Pyruvate kinase liver and red blood cell
-4.1		AF026074	Sulfotransferase related gene X1
-4.1		Y14004	Cytosolic acyl-CoA thioesterase 1
-4.3	-3.8	AV141027	Cytochrome P450, family 7, subfamily b, polypeptide 1
-4.3		AJ132098	Vanin 1
-4.6		AW226939	Carboxylesterase 3
-5.1		U49861	Deiodinase, iodothyronine, type I
-6.1	-3.4	U36993	Cytochrome P450, family 7, subfamily b, polypeptide 1
-6.4		U12791	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
-6.6	-2.8	M88694	Thioether S-methyltransferase
-6.9		AF090317	Cytochrome P450, family 8, subfamily b, polypeptide 1

Liver	Kidney	Accession number	Description
-14.0		AB018421	Cytochrome P450, family 4, subfamily a, polypeptide 10
-17.9		Y11638	Cytochrome P450, family 4, subfamily a, polypeptide 14
-28.0	2.5	AJ006474	Carbonic anhydrase 3
-37.8		M21855	Cytochrome P450, family 2, subfamily b, polypeptide 9
-93.8		L41519	Hydroxysteroid dehydrogenase-5, delta<5>-3-beta
	6.4	AB006034	Cytochrome P450, family 27, subfamily b, polypeptide 1
	4.8	U49430	Ceruloplasmin
	3.0	AF032466	Arginase type II
	2.9	J05277	Hexokinase 1
	2.6	Z19521	Low density lipoprotein receptor
	2.6	U04204	Aldo-keto reductase family 1, member B8
	2.6	AI848668	Sterol-C4-methyl oxidase-like
	2.6	U31966	Carbonyl reductase 1
	2.5	U49915	Adipocyte complement related protein
	2.4	AW124337	Microsomal glutathione S-transferase 1
	2.3	U18975	UDP-N-acetyl-alpha-D-galactosamine:(N-acetylneuraminyl)-galactosylglucosylceramide-beta-1, 4-N-acetylgalactosaminyltransferase
	2.2	L06047	Glutathione S-transferase, alpha 4
	2.1	AA718169	Resistin
	2.1	D88994	AMP deaminase 3
	2.0	AA710564	N-acetylneuraminate pyruvate lyase
	-2.0	U19265	Glucosaminyl (N-acetyl) transferase 1, core 2
	-2.0	AB005450	Carbonic anhydrase 14
	-2.1	M75886	Hydroxysteroid dehydrogenase-2, delta<5>-3-beta
	-2.1	AB020239	Adenylate kinase 4
	-2.2	U48896	UDP-glucuronosyltransferase 8
	-2.2	U89352	Lysophospholipase 1
	-2.2	M12330	Ornithine decarboxylase, structural
	-2.3	U90535	Flavin containing monooxygenase 5
	-2.3	AF009605	Phosphoenolpyruvate carboxykinase 1, cytosolic
	-2.3	AB015426	Fucosyltransferase 9
	-2.4	U89906	Alpha-methylacyl-CoA racemase
	-2.5	AA840463	Lysophospholipase 1
	-3.5	X06358	UDP-glucuronosyltransferase 2 family, member 5
Other			
18.6	8.2	U69488	G7e protein
10.9	2.4	X67644	Immediate early response 3
7.6		U78770	Trefoil factor 2 (spasmolytic protein 1)
2.9		AI117936	Mus musculus 11 days embryo head cDNA, RIKEN full-length enriched library, clone: 6230409N14 product:unknown EST, full insert sequence
2.7		AI852545	Transgelin 2
2.6	2.0	AW121336	RIKEN cDNA 1600023A02 gene
2.6		X58196	H19 fetal liver mRNA
2.5	2.2	U25844	Serine (or cysteine) proteinase inhibitor, clade B, member 6a
2.4		AA980164	SPARC related modular calcium binding 2
2.4		D38410	Trefoil factor 3, intestinal
2.2		U44426	Tumor protein D52
2.1		U22262	Apolipoprotein B editing complex 1
2.1	4.6	AW230891	Leucine-rich alpha-2-glycoprotein
-2.1		U32170	Regucalcin
-2.3		AI854813	Mus musculus 3 days neonate thymus cDNA, RIKEN full-length enriched library, clone: A630086H07 product:RAS GTPASE-ACTIVATING-LIKE PROTEIN IQGAP2 homolog [Homo sapiens], full insert sequence
-2.3		AW049373	RIKEN cDNA 2310016A09 gene
-2.8		AI326963	Angiopoietin-like 4
-3.0		AA797604	Angiopoietin-like 4
-3.4	10.7	AB011030	Protein related to DAN and cerberus
	9.8	AA986050	Fibrinogen, gamma polypeptide
	6.8	M64086	Serine (or cysteine) proteinase inhibitor, clade A, member 3N
	5.0	AA880891	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10
	2.6	AI876446	Fibrinogen, alpha polypeptide
	2.1	X61597	Serine (or cysteine) proteinase inhibitor, clade A, member 3C
	2.0	X59520	Cholecystokinin
	2.0	D13003	Reticulocalbin
	-2.1	AI314227	RIKEN cDNA 0610006H10 gene
	-2.2	AW122036	Mus musculus transcribed sequence with strong similarity to protein ref:NP_005351.2 (H.sapiens) v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian); v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog; Avian musculoaponeurotic fibrosarcoma (MAF) protooncogene [Homo sapiens]
	-3.4	M93264	Pregnancy zone protein

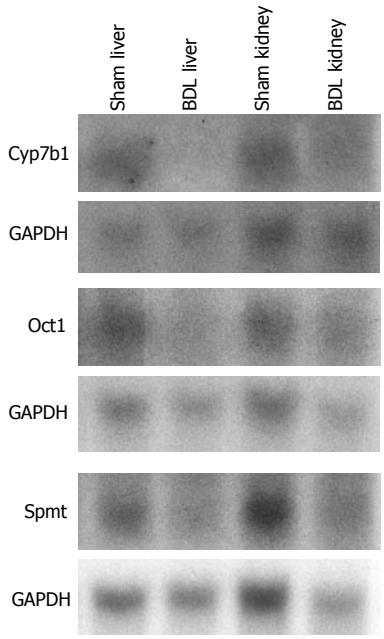

Figure 1 Confirmation of microarray data by Northern analysis of selected genes. Northern blots were performed with aliquots of pooled RNA from livers and kidneys from each 4 pair-fed sham-operated and 4 bile duct ligated mice, 7 d after surgery. Cyp7b1: Cytochrome P450 7b1; Oct1: Organic cation transporter 1; Spmt: Similar to putative integral membrane transport protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Sham: Sham-surgery; BDL: Bile duct ligation.

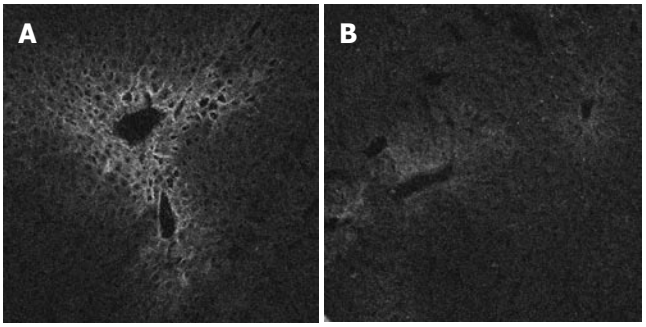

Figure 2 Indirect immunofluorescence of organic cation transporter 1 in murine liver sections. **A:** A low magnification view (x 20) shows antibody labeling at the basolateral membranes of hepatocytes of the pericentral zone of the liver lobule in the liver section of a sham-operated mouse, 7 d after surgery; **B:** In contrast, there is only a weak signal for organic cation transporter 1 after bile duct ligation.

et al[21] excluded genes with expression levels of less than 1 000, whereas we included genes with a mininum signal intensity of 600 and above. This approach led to the identification of a number of novel gene alterations of functional significance for the cholestatic phenotype. For instance, the decrease in expression of the gene encoding *Oct1* in BDL liver in the present microarray, an alteration not reported by Campbell *et al*[21] but previously reported by Ogawa *et al*[20] in the rat, led us to study this important basolateral cationic drug transporter in more detail. We were subsequently able to demonstrate that Oct1 is indeed down-regulated in rat liver, but not in kidney, in obstructive cholestasis at the mRNA as well as the protein levels and that this decrease results in reduced hepatic uptake of the Oct1 substrate tetraethylammonium[19]. Northern analysis and immunofluorescence microscopy of hepatic Oct1 performed in the present study indicated a similar pattern in mouse and confirmed the results of our microarray.

A number of other observations emerge from this analysis that deserve further study. For example, among the cell growth-related genes, the number of genes up-regulated in liver after BDL surpassed by far the number of down-regulated genes, a pattern which might reflect the extensive fibroproliferative process and tissue remodeling that takes place in this model of obstructive cholestasis. Similarly, a large number of genes related to cell adhesion, the extracellular matrix, and the cytoskeleton were found to be altered that have not been identified yet. We presume that many of these genes may play an important but as yet to be identified role in the fibrogenic response of the liver to bile duct obstruction. Alterations in the composition of the extracellular matrix are typical features of hepatic fibrosis[13], including substantial increases of collagens and non-collagenous components[25,26]. Accordingly, we observed a uniform up-regulation of genes encoding the *procollagen types* $I\alpha_1$, $I\alpha_2$, $III\alpha_1$, $IV\alpha_1$, $IV\alpha_2$, and $V\alpha_2$ in this mouse model of obstructive cholestasis. In addition, two members of the matrix metalloproteinase family, the *matrix metalloproteinases 7* and *12*, were up-regulated more than ten-fold following BDL when compared with the sham-operated controls. Matrix metalloproteinases represent a group of calcium-dependent enzymes involved in physiological and pathological degradation of extracellular matrix and tissue-remodeling[27]. *Matrix metalloproteinase 7 (matrilysin)*, an enzyme which is associated with poor prognosis in hepatocellular[28] and cholangiocellular carcinomas[29], has been closely related to the fibro-proliverative process in chronic hepatitis C[30] but not in cholestatic liver diseases. In contrast, *matrix metalloproteinase 12*, to our knowledge, has not been associated with liver fibrosis before and deserves future attention. Interestingly, the genes encoding *tissue inhibitor of metalloproteinase 1*, *vascular cell adhesion molecule 1* and *intercellular adhesion molecule* were up-regulated both in liver and kidney of BDL mice. Genes encoding the *procollagen types* $I\alpha_1$ *and* $III\alpha_1$ were also increased in the kidney of BDL mice although at lower levels than in the liver. The up-regulation of fibrosis-associated factors in kidney following BDL might be due to a paracrine action of fibrogenic mediators such as *connective tissue growth factor* whose hepatic expression is increased in cholestasis as previously described[31,32] and confirmed in our microarray. However, the functional relevance of the increased expression of these fibrotic genes in the kidney remains to be determined. Alternatively, the simultaneous up-regulation of important regulators of transcription following BDL such as *FBJ osteosarcoma oncogene*, *core promoter element binding protein*, and *activating transcription factor 3* in both liver and kidney supports the idea of coordinated gene regulation in different tissues as response to a specific stimulus. Another non-collagenous component of the extracellular matrix which was up-regulated in BDL liver is the gene for the matricellular protein *secreted acidic cysteine rich glycoprotein*. Matricellular proteins are a group of matrix-associated factors that mediate cell-matrix interactions but do not serve primarily as structural elements[13]. In particular, the expression of *secreted acidic cysteine rich glycoprotein* has been associated with cell proliferation, migration, and extracellular matrix remodeling in tissues, and *secreted acidic cysteine rich glycoprotein* has been found to be increased in different models of hepatic fibrosis[33].

The expression of a number of genes encoding

membrane proteins and transporters that were not previously known to be affected by cholestasis was also of interest. For example, the gene encoding the *β1 subunit of the voltage-gated sodium channel* which is important for the maturation and function of this channel[34] was up-regulated in liver as well as in kidney of BDL mice. In contrast, the expression of the gene encoding the ATP-binding cassette transporter *multidrug resistance-associated protein 6* (*Mrp6,* Abcc6) was reduced in cholestatic mouse liver as previously described for the rat[20]. Since mutations of human MRP6 are associated with pseudoxanthoma elasticum, a disorder characterized by calcification of the elastic fibres and abnormalities of the collagen fibrils[35], it is tempting to speculate that reduced hepatic Mrp6 expression in cholestasis might have functional implications for the development of liver fibrosis. Other genes up-regulated in cholestatic liver were the genes encoding the macrophage receptor markers *CD14 antigen* and *CD68 antigen*. Hepatic expression of both markers is increased in patients with biliary atresia[36], and expression of *CD68 antigen* may be an indicator of prognosis[37]. The functional significance of the concomitant *CD68 antigen* elevation in BDL kidney is unclear at the moment but illustrates again the close linkage between liver and kidney in this model of cholestasis and supports again a concept of coordinated gene regulation in different tissues.

In accordance with previous studies[38], obstructive cholestasis decreased the expression of a number of genes encoding *cytochrome P450* isoenzymes in liver. Since BDL results in an increase in liver concentrations of bile acids[15], the down-regulation of the *cytochrome P450 7b1 (oxysterol 7α-hydroxylase)* and *cytochrome P450 8B1 (sterol 12α-hydroxylase)* genes, that encode key enzymes in the conversion of cholesterol to bile acids[39], may represent adaptive responses to minimize the liver levels of cytotoxic bile salts. The increase of the gene encoding *cytochrome P450 27b1 (25-hydroxyvitamin D_3 1α-hydroxylase)* in BDL kidney is another interesting observation. *Cytochrome P450 27b1* catalyzes the conversion of 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3, the last step in vitamin D activation, which takes place in kidney[40]. Thus the increase in renal *cytochrome P450 27b1* expression may reflect an adaptive response to compensate for 25-hydroxyvitamin D deficiency in cholestasis. This may be a pathophysiologically important mechanism since patients with primary biliary cirrhosis often present with deficiencies of 25-hydroxyvitamin D but normal or even elevated levels of 1, 25-dihydroxyvitamin D[41].

In summary, the present study provides a comprehensive gene expression profile from mouse liver and kidney in obstructive cholestasis. Changes in gene expression were validated by Northern analysis, immunofluorescence, or comparison with the literature. The findings in this study provide new insights for generating novel hypotheses concerning the adaptive responses of gene expression in this mouse model of cholestasis.

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CLINICAL RESEARCH

Impact of lymph node micrometastasis in hilar bile duct carcinoma patients

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Abstract

AIM: To immunohistochemically examine micrometastasis and VEGF-C expression in hilar bile duct carcinoma (HBDC) and to evaluate the clinical significance of the results.

METHODS: A total of 361 regional lymph nodes from 25 patients with node-negative HBDC were immunostained with an antibody against cytokeratins 8 and 18 (CAM 5.2), and immunohistochemical staining of VEGF-C was performed in 34 primary resected tumors.

RESULTS: Lymph node micrometastasis was detected in 6 (24%) of the 25 patients and 10 (2.8%) of the 361 lymph nodes. Patients with micrometastasis showed significantly poorer survival rates than those without ($P = 0.025$). VEGF-C expression was positive in 17 (50%) of 34 HBDC, and significantly correlated with lymph node metastasis ($P = 0.042$) and microscopic venous invasion ($P = 0.035$).

CONCLUSIONS: It is suggested that immunohistochemically detected lymph node micrometastasis has an impact on the outcome of HBDC. VEGF-C expression is highly correlated with lymph node metastasis in HBDC and might therefore be a useful predictor.

Key words: Hilar bile duct carcinoma; Lymph node metastasis; Micrometastasis; Vascular endothelial growth factor-C

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INTRODUCTION

Hilar bile duct carcinomas (HBDC) are one of the most difficult to cure malignant gastroenterological tumors[1-4] and curative resection is essential for long-term survival. Because hilar bile duct tumors are in close proximity to vital structures in the hepatic hilum, such as the hepatic artery and portal vein, and since they tend to spread to the proximal biliary tract and perineural and perilymphatic spaces, hepatectomy with thorough systematic extended lymph node dissection is frequently required for curative resection. However, even with margin-negative resection, the prognosis after curative resection remains poor. One possible reason for the poor outcome is existence of occult lymph node metastasis that cannot be detected by conventional hematoxylin and eosin (HE) staining at the time of surgical resection. Immunohistochemical and molecular techniques have, however, made it possible to identify lymph node micrometastasis missed by traditional methods. Recently, immunohistochemical and/or genetic detection of lymph node micrometastases of various tumors, including carcinomas of the breast[5,6], lung[7,8], esophagus[9,10], stomach[11-14], colorectum[15,16] and gallbladder[17-19], has been reported. However, we were able to find only one report documenting this in HBDC[20].

Vascular endothelial growth factor C (VEGF-C) is a member of the highly glycosylated vascular endothelial growth factor (VEGF) family that regulates vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis and vascular permeability, and has been implicated in many physiological and pathological processes[21,22]. Overexpression of VEGF-C cDNA in the skin of transgenic mice has been shown to selectively induce lymphatic endothelial cell proliferation and hyperplasia of the lymphatic vasculature[23]. It was also recently reported that a VEGF-C-transfected tumor cell line implanted into the stomach of nude mice gave rise to numerous lymph node metastases[24]. The most prominent VEGF-C expression has been detected in the human heart, placenta, muscle, ovary, and small intestine[25], and a positive correlation between expression and various clinicopathological factors, especially lymph node metastasis, has been reported in a number of tumors,

including carcinomas of the thyroid[26], breast[27], lung[28], esophagus[29], stomach[30,31], colorectum[32], prostate[33] and pancreas[34]. However, no investigations have been conducted with regard to VEGF-C expression in HBDC and possible clinicopathological associations. In this study, we examined lymph node micrometastasis and VEGF-C expression in patients with HBDC and evaluated the clinical significance of the results.

MATERIALS AND METHODS

Patients and specimens

From January 1981 to August 2000, 61 patients with HBDC underwent surgical resection plus systematic lymph node dissection in the First Department of Surgery, Mie University School of Medicine. Of these patients, 34 underwent macroscopic and microscopic margin-negative resection. Patients consisted of 21 males and 13 females with a mean age of 64.4±11.0 years (range: 37-89 years). The median follow-up period was 31.8 mo (minimum: 1.0 mon). No lymph node metastases were detected in 25 (73.5%) of the 34 patients by traditional pathologic examinations with HE staining.

Hepatectomy was performed in 29 (85.3%) of the 34 patients: extended right hepatectomy in 9 patients, left hepatectomy in 8, resection of segments 4a and 5 in 5, hilar resection in 4, extended left hepatectomy in 2, and caudate lobectomy only in 1. All 29 patients underwent combined resection of the caudate lobe. Two patients underwent combined resection of the portal vein, and 3 underwent pancreatoduodenectomy (PD) or pylorus-preserving pancreatoduodenectomy. Another 5 patients were treated with bile duct resection alone, including 2 patients who underwent combined PD.

A total of 361 lymph nodes dissected from 25 node-negative patients were examined immunohistochemically by staining with an antibody against cytokeratins 8 and 18, and all 34 primary tumors were immunohistochemically stained for VEGF-C. Tumor specimens and lymph nodes were collected from pathology files after obtaining informed consent from all patients in accordance with institutional guidelines.

Lymph node groups and resected margin status

Identification of the sites of lymph node metastasis were performed in accordance with the TNM Classification of Malignant Tumors proposed by the International Union Against Cancer (UICC)[35], which defines regional lymph nodes as the cystic duct, pericholedochal, hilar and peripancreatic (head only), periduodenal, periportal, celiac and superior mesenteric nodes, N0 as no regional lymph node metastasis and N1 as regional lymph node metastasis.

Evaluation of resected margin status was performed in accordance with the General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract (The 5th Edition) proposed by the Japanese Society of Biliary Surgery (JSBS)[36], which defines pEM0 as no tumor invasion within 5 mm of the resected margin, pEM1 as tumor invasion within 5 mm of the resected margin and pEM2 as distinct tumor invasion of the resected margin. pEM0 and pEM1 resections were defined as margin-negative in this study.

Immunohistochemical staining

Tissue samples were fixed in 10% formaldehyde with phosphate-buffered saline (PBS) and embedded in paraffin. Lymph node tissue was cut into six 5-μm thick sections, and primary tumor tissue was cut into a single 5-μm thick section. Briefly, the sections were deparaffinized with xylene and rehydrated through graded concentrations of ethanol. For antigen retrieval, sections were placed in 0.1*mol*/L citrate buffer (pH 6.0) and heated three times for 3 min each in a microwave oven (500 W). Lymph node sections were then incubated with a mouse monoclonal antibody (CAM 5.2; Becton Dickinson, San Jose, CA) specific for cytokeratins 8 and 18, and tumor sections were incubated with affinity-purified goat polyclonal antibodies (IBL, Fujioka, Japan) to VEGF-C at 1:30 dilution. Immunohistochemical detection of CAM 5.2 and VEGF-C was performed by a standard avidin-biotin method on an automated Ventana ES immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions[37].

We examined 6 sections per lymph node and diagnosed micrometastasis when tumor cells were detected immunohistochemically, after being missed by routine histologic examinations with HE staining. VEGF-C immunoreactivity was mainly present in the cytoplasm of cancer cells and/or in the connective tissue around cancer cells. For evaluation of VEGF-C immunostaining, we examined at least 200 cancer cells per case. Cases in which at least 10% of the cancer cells were immunoreactive were defined as VEGF-C positive. All immunohistochemical evaluations were performed by an experienced histopathologist unaware of the clinicopathological features of the patients.

Statistical analysis

All statistical calculations were carried out using StatView-J 5.0 statistical software (SAS Institute, USA). Results are expressed as the means±SD. Statistical analysis for comparisons of VEGF-C expression and clinicopathological factors (age, gender, lymphatic vessel invasion, microscopic venous invasion, perineural invasion and lymph node metastasis) were performed using the chi-square test and Fisher's exact probability test. Analysis for comparisons of VEGF-C expression and other factors (pT classification and histopathological grading) was performed using the Mann-Whitney *U*-test. The Kaplan-Meier method was used to estimate postoperative survival rates, and the generalized log-rank test was used to compare differences in survival rates. All P values were two-sided and $P<0.05$ was considered statistically significant.

RESULTS

Patient outcomes

Of the 34 patients with margin-free resected HBDC, 4 died of other causes; three of multiple organ failure including 1 postoperative death (within 1 mo), and 1 of unknown causes. In addition, 15 (50.0%) of the remaining 30 patients died of disease. Recurrence sites were the

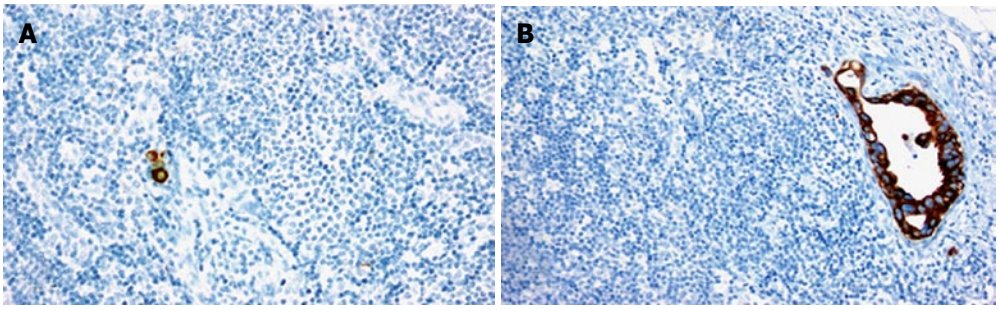

Figure 1 Immunohistochemical staining of lymph node micrometastasis with the monoclonal antibody CAM 5.2. **A:** Micrometastasis consisting of a single cell (original magnification, × 200). **B:** Micrometastasis consisting of a small cluster of tumor cells (original magnification, × 100).

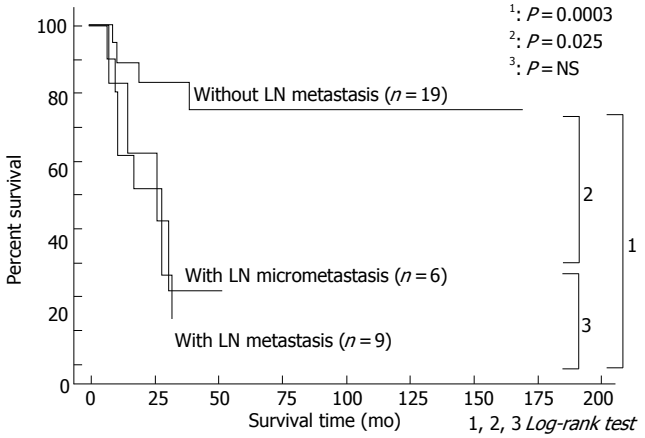

Figure 2 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis, including micrometastasis.

liver in 2 patients including 1 patient with combined lung recurrence, the peritoneum in 1 patient, and local regions in 11 patients. Of these 11 patients, 3 showed combined recurrence in other sites; 1 showed combined liver metastasis, 1 showed combined lung metastasis, and 1 showed combined peritoneum recurrence.

Detection of lymph node micrometastasis

Micrometastasis was detected in 6 (24.0%) of the 25 node-negative patients and 10 (2.8%) of the 361 lymph nodes by immunohistochemical examination with CAM5.2. Lymph node micrometastasis was present in the form of a single-cell metastasis (Figure 1A) or a small cluster of tumor cells (Figure 1B). Of the 6 patients with lymph node micrometastasis, 5 had regional lymph node micrometastasis and 1 had regional lymph node with para-aortic lymph node micrometastases.

Impact of lymph node micrometastasis on survival

Cumulative survival rates were compared according to nodal status: the without lymph node metastasis group versus lymph node micrometastasis group versus HE diagnosed (overt) lymph node metastasis group (Figure 2). The 3- and 5-year survival rates of the 19 patients without lymph node metastasis were 81.6 and 72.5%, respectively, as opposed to 20.8 and 20.8%, respectively, in the 6 patients with micrometastasis and 29.6% and 0.0%, respectively, in the 9 patients with overt lymph node metastasis. Patients with lymph node micrometastasis showed significantly worse survival rates than those without ($P = 0.025$), and moreover, patients with overt lymph node metastasis showed worse survival rates than those without ($P = 0.0003$). There were no statistical differences between patients with lymph node micrometastasis and those with overt lymph node metastasis ($P = 0.469$). Five patients died of disease without overt lymph node or micrometastasis. Of these, 4 died of local recurrence, including 1 patient with combined liver metastasis. The remaining patient died of liver and lung metastasis. Follow-up revealed that 3 patients with lymph node micrometastasis survived with no evidence of disease for 11.7 and 36.7 and 60.3 mon after surgical resection, respectively.

To further evaluate the impact of lymph node micrometastasis on survival, survival rates were compared according to two groups: patients without lymph node metastasis versus those with overt lymph node and micrometastasis (Figure 3), and patients without lymph node metastasis and those with lymph node micrometastasis versus those with overt lymph node metastasis (Figure 4). The 3- and 5-year survival rates of the 19 patients without lymph node metastasis were 81.6 and 72.5%, respectively, as opposed to 25.5 and 8.5%, respectively, in the 15 patients with overt lymph node and micro metastasis ($P = 0.0004$). On the other hand, the 3- and 5- year survival rates of the 25 patients without lymph node metastasis and those with lymph node micrometastasis were 66.9 and 60.2%, respectively, as opposed to 14.8 and 0.0%, respectively, in the 9 patients with overt lymph node metastasis ($P = 0.0015$).

VEGF-C expression and clinicopathological factors

VEGF-C expression was observed in 17 (50.0%) of the 34 primary tumors (Figure 5). The correlations between VEGF-C expression and clinicopathological factors are shown in Table 1. Microscopic venous invasion ($P = 0.035$) and lymph node metastasis ($P = 0.042$) were significantly correlated with VEGF-C expression.

Prognostic factors for hilar bile duct carcinoma

To identify useful prognostic factors, we performed univariate analysis of the following possible independent prognostic factors: age (above 60 years versus 60 years or less), gender, operative procedure (hepatectomy versus bile duct resection), histopathological grading (well differentiated versus moderately or poorly differentiated), lymphatic vessel invasion, microscopic venous invasion, perineural invasion, microscopic resection margin (em0 versus em1), VEGF-C expression, lymph node metastasis (including micrometastasis) and lymph node metastasis (excluding micrometastasis) (Table 2). Ultimately, 4 independent variables (microscopic resection margin ($P = 0.040$), VEGF-C

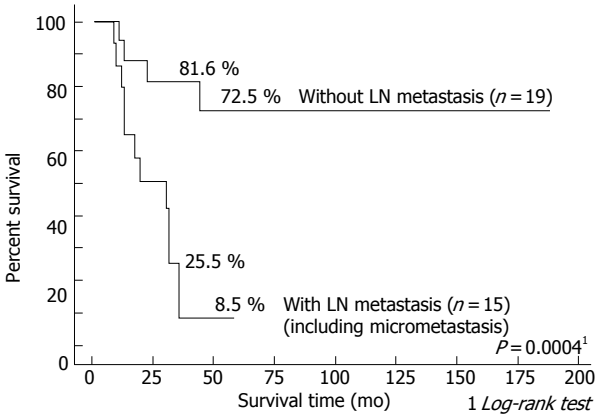

Figure 3 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis: patients without lymph node metastasis versus those with overt lymph node and micro metastasis.

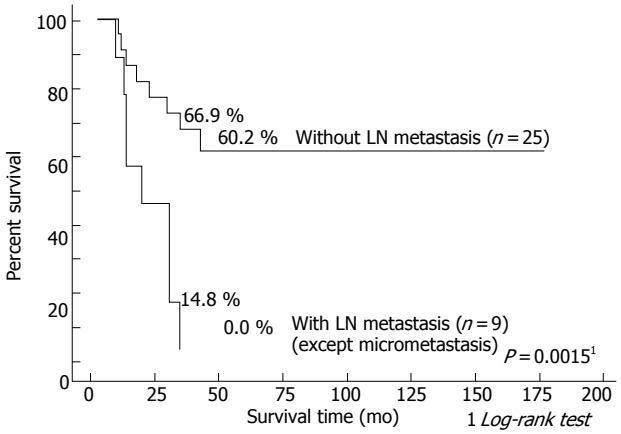

Figure 4 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis: patients without lymph node metastasis and those with lymph node micrometastasis versus those with overt lymph node metastasis.

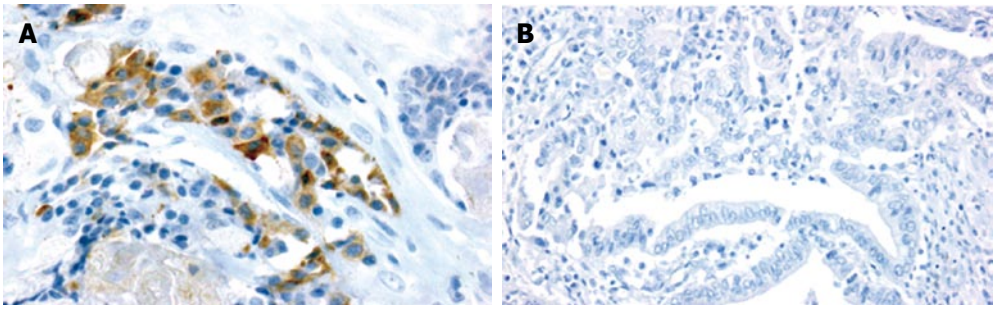

Figure 5 Immunohistochemical staining of primary tumors with VEGF-C polyclonal antibody. **A:** VEGF-C positive (original magnification, ×400). **B:** VEGF-C-negative (original magnification, ×200).

Table 1 Clinicopathological factors and VEGF-C expression

		VEGF-C expression		
		Positive (n = 16)	Negative (n = 18)	P Value
Age		64.7±11.6	64.1±10.8	0.870
Gender (M / F)		10 / 6	11 / 17	0.999
pT classification[1]	pT1	1	4	0.56
	pT2	8	11	
	pT3	7	3	
Histopathological Grading[1]	G1	10	15	0.225
	G2	6	2	
	G3	0	1	
Lymphatic vessel invasion	(presence)	14 (87.5 %)	13 (72.2 %)	0.405
Venous invasion	(presence)	10 (62.5 %)	4 (22.2 %)	0.035
Perineural invasion	(presence)	14 (87.5 %)	10 (55.6 %)	0.063
Lymph nodes metastasis	Metastasis (-)	6	13	0.042
	Metastasis (+) (including micrometastasis)	10	5	

[1]According to the TNM staging system. pT classification: pT1: Tumor confined the bile duct; pT2: Tumor invades beyond the wall of the bile duct; pT3: Tumor invades the liver, gallbladder, pancreas, and/or unilateral tributaries of the portal vein (right or left) or hepatic artery (right or left); pT4: Tumor invades any of follwing: main portalvein or its tributaries bilatellary, common hepatic artery, or other adjacent structures, e.g., colon, stomach, duodenum, abdominal wall. Histopathological Grading: G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated.

expression (P = 0.036), lymph node metastasis (including micrometastasis) (P = 0.0004) and lymph node metastasis (excluding micrometastasis) (P = 0.0017)) were identified as statistically significant predictors of survival.

DISCUSSION

Lymph node metastasis is a well known important predictor of prognosis with a wide variety of malignant tumors, and some studies have reported a significant relationship

Table 2 Univariate analysis of survival

Variable		5-yr survival (%)	*P* Value
Age	<60 *vs* ⩾60	40.0 *vs* 44.9	0.912
Gender	male *vs* female	42.3 *vs* 45.5	0.872
Operative procedure	hepatectomy *vs* bile duct resection	42.2 *vs* 60.0	0.430
Histopathologic Grading[1]	G2, G3 *vs* G1	37.5 *vs* 46.0	0.393
Lymphatic vessel invasion	present *vs* absent	37.4 *vs* 75.0	0.076
Venous invasion	present *vs* absent	36.4 *vs* 49.7	0.185
Perineural invasion	present *vs* absent	36.3 *vs* 68.6	0.064
Microscopic resection margin[2]	em1 *vs* em0	31.7 *vs* 83.3	0.040
VEGF-C expression	positive *vs* negative	23.3 *vs* 70.5	0.036
Lymph node metastasis (Including micrometastasis)	positive *vs* negative	8.5 *vs* 72.5	0.0004
Lymph node metastasis (except micrometastasis)	positive *vs* negative	0.0 *vs* 60.2	0.0017

[1] According to the TNM staging system. Histopathological Grading: G1 Well differentiated, G2 Moderately differentiated, G3 Poorly differentiated.

[2] According to the Japanese Society of Biliary Surgery. General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract. em0: no tumor invades within 5 mm from resected margin; em1: tumor invades within 5mm from resected margin.

between lymph node metastasis and prognosis of HBDC patients[38-40]. However, patients with early stage carcinoma and no apparent lymph node metastasis sometimes die of metastasis after surgery despite complete resection of the primary lesion. One of the possible reason for the poor outcome in these patients is occult lymph node metastasis not identified by conventional HE staining at the time of surgical resection.

Numerous studies on the incidence and significance of lymph node micrometastasis in cancer patients have been conducted in recent years. A number of investigators have proposed the prognostic significance of lymph node micrometastasis for various tumors including lesions of the lung, esophagus, stomach and colon, while others have suggested that lymph node micrometastasis is not significant for patient outcome. Thus, there is no consensus on the clinical significance of lymph node micrometastasis. However, we were able to find only one report documenting this in HBDC. Tojima *et al*[20] investigated 954 nodes from 45 patients with pN0 hilar cholangiocarcinoma after curative resection, and found micrometastasis in 13 (1.4%) nodes from 11 (24.4%) patients. Their data yielded similar survival curves for patients with and without lymph node micrometastasis (5-year survival rates: 43.6% *vs* 42.1%, respectively).

In this study, we demonstrated significant differences between outcomes of HBDC patients with and without lymph node micrometastases. Interestingly, a stronger correlation was recognized when patients with lymph node micrometastasis were treated as lymph node metastasis positive, compared to when they were treated as lymph node metastasis negative ($P=0.0004$ versus $P=0.0017$) (Figures 3 and 4). This might suggest the need to consider lymph node micrometastasis as overt lymph node metastasis.

One possible reason for the above-mentioned differing results is the number of sections examined. The number of sections immunohistochemically stained is considered an important factor in the diagnosis of lymph node micrometastasis. Many investigators examine lymph node micrometastasis using various sections of different thickness for immunohistochemical staining; however, the total thickness examined tends to range from 3 to 30 μm[6,8,10,13-20]. Sasaki *et al* examined the correlation between the number of CAM 5.2 sections and cumulative positive rate of lymph node metastasis[41]. They found that positive metastasis detection reached a plateau when over 9 sections (total thickness 27 μm) were examined. In this study, to identify lymph node micrometastasis, we examined six 5-μm sections (total thickness 30 μm) per lymph node by immunohistochemical staining. When we examined only one to four sections per lymph node, we found fewer lymph node micrometastases (data not shown).

Another possible reason for the differing results is the criteria of lymph node micrometastasis. In many studies, including ours, micrometastasis is defined as tumor cells detected only by immunohistochemical staining. However, some authors set size criteria for micrometastasis, such as deposits less than 2[42] or 0.5 mm in diameter[20,43]. Recent progress in molecular biological techniques has led to the development of genetic methods for detecting micrometastasis, including RT-PCR. RT-PCR is capable of detecting more micrometastasis foci than immunohistochemical staining[44]. Five patients without overt lymph node or micro metastasis died of disease recurrence in this study. If we use RT-PCR to detect lymph node micrometastasis, we will be able to evaluate lymph node micrometastasis in more detail, and the significance of lymph node micrometastasis will potentially increase. Therefore, further examinations using RT-PCR appear necessary.

VEGF-C is a specific ligand of VEGFR-3 and VEGFR-2, and has been shown to stimulate lymphangiogenesis and angiogenesis both *in vitro* and *in vivo*. Nakashima *et al*[45] investigated VEGF-C expression in 52 patients with gallbladder carcinoma and found that expression was significantly stronger ($P<0.001$) in patients with lymph node metastasis than those without, and that the VEGF-C-positive group showed poorer outcomes than the negative group ($P<0.001$).

Our study revealed a significant correlation between VEGF-C expression and both the presence of lymph node metastasis (HE detected and micrometastasis) and outcome of HBDC. These results suggest that VEGF-C expression might play an important role in causing lymph node metastasis in HBDC, consistent with the findings of previous studies regarding other malignant tumors.

In conclusion, our findings suggest that immunohistochemical detection of lymph node micrometastasis provides very useful information of survival rates after surgery for HBDC. However, considering that 1 patient with lymph node micrometastasis survived for more than 5-years with no evidence of tumor recurrence, long-term survival is thus possible for some patients with lymph node micrometastasis; therefore, extended lymph node dissection is necessary in HBDC patients. Although further study is needed, VEGF-C seems to be a useful predictor of overt and micro lymph node metastasis.

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CLINICAL RESEARCH

Evaluation of thermal water in patients with functional dyspepsia and irritable bowel syndrome accompanying constipation

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Abstract

AIM: To evaluate the efficacy of water supplementation treatment in patients with functional dyspepsia or irritable bowe syndrome (IBS) accompanying predominant constipation.

METHODS: A total of 3872 patients with functional dyspepsia and 3609 patients with irritable bowel syndrome were enrolled in the study by 18 Italina thermal centres. Patients underwent a first cycle of thermal therapy for 21 d. A year later patients were re-evaluated at the same centre and received another cycle of thermal therapy. A questionnare to collect personal data on social and occupational status, family and pathological case history, life style, clinical records, utilisation of welfare and health structure and devices was administered to each patient at basal time and one year after each thermal treatment. Sixty patients with functional dyspepsia and 20 with IBS and 80 healthy controls received an evaluation of gastric output and oro-cecal transit time by breath test analysis. Breath test was performed at basal time and after water supplementaton therapies. Gastrointestinal symptoms were evaluated at the same time points. Breath samples were analyzed with a mass spectometer and a gascromatograph. Results were expressed as $T_{1/2}$ and T-lag for octanoic acid breath test and as oro-cecal transit time for lactulose breath test.

RESULTS: A significant reduction of prevalence of symptoms was observed at the end of the first and second cycles of thermal therapy in dyspeptic and IBS patients. The analysis of variance showed a real and persistant improvement of symptoms in all patients. After water supplementation for 3 wk a reduction of gastric output was observed in 49 (87.5%) of 56 dyspeptic patients. Both $T_{1/2}$ and T-lag were significantly reduced after the therapy compared to basal values [91 ± 12 ($T_{1/2}$) and 53 ± 11 (T-lag), Tables 1 and 2] with results of octanoic acid breath test similar to healthy subjects. After water supplementation for 3 wk oro-cecal transit time was shorter than that at the beginning of the study.

CONCLUSION: Mineral water supplementation treatment for functional dyspepsia or conspipation accompanying IBS can improve gastric acid output and intestinal transit time.

Key words: Mineral water; Constipation; Dispepsia; Thermal therapy

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INTRODUCTION

Functional dyspepsia and irritable bowel syndrome accompanying predominant constipation (IBSc) are two of the most prevalent diseases in the industrialized world. These disturbances are among the first diseases for a gastroenterologic examination with high social and economical costs. In particular, dyspepsia is the first cause of specialized blood and invasive examination in Europe and USA[1]. Dyspepsia may be organic when associated with specific gastrointestinal or liver diseases. To diagnose functional dyspepsia gastroenterologists need specific criteria known as "Roma criteria"[2]. Prevalence of dyspepsia in general population is variable in different studies between 14% and 41%[3-5]. About 65% of dyspeptic patients result from functional dyspepsia[6]. About 35%

of patients with functional dyspepsia present a delayed gastric output which is related to clinical symptoms[7,8] or other disturbances of gastrointestinal motility[9,10]. On the contrary, the link between gastric *H pylori* infection and functional dyspepsia has not been clarified yet[6] because the data on the effect of *H pylori* eradication on dyspeptic symptoms are discordant[11,12].

Treatment of dyspepsia is based on drugs that inhibit the gastric acid secretion (such as proton pump inhibitors or H2 blocker agents) or drugs that stimulate gastrointestinal motility (prokinetics agents). However, the efficacy of such therapies is often unsatisfactory in particular for the short duration of the improvement of symptoms. It should be underlined that a prolonged treatment with these drugs is related to a high incidence of side effects. Although there is no scientific evidence, several dietetic regimens have been used in combination with drugs by dyspeptic patients.

A high enrichment of fibers in diet is the first therapeutic steps for constipation[13]. In fact, a high fiber intake is related to an increased fecal mass in healthy subjects (with high interindividual variability)[14,15]. The efficacy of this treatment seems to be time-related, probably for an intestinal adaptation to the high intake of fibers[16]. IBSc is a chronic functional disorder associated with psychological, environmental, emotional, social factors (drugs, stress, lifestyle)[17]. A reduction of water intake may play an important role in the pathogenesis of constipation and water supplementation is often suggested by a general practitioner in clinical practice to IBSc patients[18,19]. Recently, it has been described that an increased water intake up to 1.5 liters of mineral water is able to increase the effect of a diet with fiber enrichment in patients with constipation[20]. In Europe, especially in Italy, Germany and France, a large number of thermal centers stress on the real role of a treatment with thermal mineral water in functional gastrointestinal diseases. For this reason we need international studies or controlled trial to evaluate the effect of mineral water on functional dyspepsia or IBSc. These studies may allow a medical prescription by physicians and evaluate the socio-economical impact on public health systems of these treatments compared to pharmacological therapies. The efficacy of thermal treatment with mineral water on gastrointestinal diseases[21] remains to be further clarified.

This study was to evaluate the economic and health indicators for the assessment of the efficacy of thermal therapies in reducing the health costs. Moreover, the effect of water supplementation therapy on functional dyspepsia and IBSc was also evaluated.

MATERIALS AND METHODS

Patients

A total of 3872 patients with functional dysepsia and 3609 patients with IBSc were enrolled in the study from 1999 to 2000 by the medical staff of 18 thermal centres distributed throughout Italy (Bagni di Lucca, Chianciano, Comano, Franciacorta, Pejo e Rabbi, Recoaro, Sangemini, Sant'Andrea Bagni, San Carlo, S. Elena, SanPellegrino, Sarnano, DI Stabia, Vallio, Vulpacchio, Montecatini, Angolo, Boario). Patients with a history of gastrointestinal, liver, pancreatic, gall bladder, neurological, muscular, rheumatological, autoimmunitary and immunological diseses were excluded from the study. Moreover, patients with severe high blood pressure (diastolic>110, systolic>180), cancer, recent surgical resection, and pregnant women were also excluded from the study. All enrolled patients underwent abdominal ultrasonography and fecal occult blood test. Only patients negative in both tests were enrolled in the study and underwent a first cycle of thermal therapy for a standard period (2000 mL of mineral water for 21 d). Compliance was evaluated by the percentage of empty bottles returned by patients at the end of the cycle. Before the first cycle of thermal therapy was started, an informed consent was obtained from all enrolled subjects. The anamnestic and clinical data were collected by submitting a questionnaire. The questionnaire included approximately 1400 closed-answer questions (Table 1) for identifying the exposure variables and risk indicator, with special reference to the social-demographic and clinical variables including different sections (personal data, social and occupational status, family and pathological case history, life style, clinical records and social data, also including indicators of the standard living, utilisation of welfare and health structure and devices). According to the first study plan, the patients were re-examined at the same thermal centre one year after the first therapeutic cycle and received a second cycle of thermal treatment. A second follow-up was made after another year. During the follow-up the effect of the treatment was evaluated by assigning a score on side effects, personal opinion of the treatment and overall tolerability of therapy (Table 1). Moreover a retrospective assessment of the clinical follow-up, drug intake and utilisation of welfare and health service between the first and second cycles was carried out. Statistical analysis was performed using Bowker's symmetry test and ANOVA to compare uncontinuos variables.

Table 1 Main items included in the questionnaire

Anagraphic data
Blood pressur, heart rate
Occupation
Physical activity[1]
Disease duration
Main symptoms[1]
Admission to hospital (d)[1]
Missed work days[1]
Clinical relapse[1]
Drugs comsumption[12]
Igienic-dietary habit[1]
Personal opinions on therapy[2]
Reasons of Good opinions on therapy[2]
Side effects[2]

[1]During past 12 mo; [2]Evaluation after treatment.

Methods

Sixty patients (30 females, 30 males, mean age 44±6 years) with functional dyspepsia and 60 healthy controls (30 males, 30 females, the mean age of 41±5 years) were enrolled. All patients underwent upper digestive endoscopy and abdominal ultrasonography to exclude peptic ulcer

disease, gastroesophageal reflux disease, liver, pancreatic and gall bladder diseases. Diagnosis of functional dyspepsia was made based on the Roma II criteria. All patients underwent ^{13}C octanoic acid breath test (OBT) and filled in a questionnaire for evaluation of gastrointestinal symptoms (post-prandial fullness, epigastric pain, bloating, heartburn, nausea, vomiting).

After OBT, all patients started a diet supplemented with mineral water (2000 mL of mineral water/d for 3 wk). During the mineral water treatment, patients were controlled with a standardized diet (similar caloric and fiber intake)[20]. Compliance to thermal therapy was evaluated by the number of empty bottles returned at the end of the study (20% or more of full bottles returned were considered as the indicator of in adequate compliance). After 3 wk of mineral water supplementation treatment, the OBT was repeated and a new questionnaire for evaluation of gastrointestinal symptoms was administered. Patients after an overnight fasting had 91 mg of 13-C octanoic acid dissolved in an egg with a standardized meal (50 g ham, 150 mL fruit juice, 100 g white bread and 100 mL water) in 10 min. Breath samples were collected in a test tube before and every 15 min for 4 h after ingestion of the labeled substrate.

Analysis of ^{13}C in the breath was performed using a mass spectrometer (Breath Mat; FinniganMat; Bremen, Germany). $0T_{1/2}$ and T-lag values were used to express the results after a regression analysis of exhaled air curves. Results were expressed as $T_{1/2}$ and Tlag. T-test for coupled or uncoupled data was used to compare the difference between groups. Difference in symptoms was evaluated by χ^2 test or Fisher's exact test. $P<0.05$ was considered statistically significant.

Twenty patients (10 females, 10 males, mean age 41 ± 5 years) with IBSc and 20 sex and age matched healthy controls (10 females, 10 males, mean age 40 ± 7 years) were enrolled. Diagnosis of IBSc was made based on the Roma II criteria. All patients underwent H_2-lactulose breath test (LBT) to evaluate the oro-cecal transit time. Then, all patients started a controlled diet with standard fiber and caloric intake supplemented with 2000 mL of mineral water. After 3 wk the diet was stopped and LBT was performed. All enrolled patients filled in a questionnaire for evaluation of gastrointestinal symptoms (bloating, hard stools, number of evacuations in a week, incomplete evacuation) before and after mineral water supplementation. Compliance to thermal therapy was evaluated by the number of empty bottles returned at the end of the study (20% or more of full bottles returned were considered as the indicator of in adequate compliance).

LBT was performed after 20 g of lactulose dissolved in 100 mL of water was administered. Breath samples were collected at basal time and every 15 min for 4 h in a specific test tube after the assumption of lactulose. The presence of hydrogen in the breath samples was evaluated by gascchromatography (Quintron Milwaukee, Wisconsin USA). The oro-cecal transit time was evaluated by curves of hydrogen exhaled during the test.

The Student t test for coupled or uncoupled data was used to compare the difference between groups. Difference in symptom prevalence was evaluated by χ^2 test or Fisher's exact test as appropriate. $P<0.05$ was considered statistically significant.

Table 2 Patients enrolled and re-evaluated after the first and second cycles of thermal therapy with mineral water

Water Type	Pathology	1st yr	2nd yr	3rd yr
	Dyspepsia			
Bicarbonate		1667	966	110
Salse		1282	979	30
Solfate		923	923	24
Total		3872	2868	164
	IBSc			
Bicarbonate		1471	861	59
Salse		1181	701	19
Solfate		957	949	30
Total		3609	2511	108

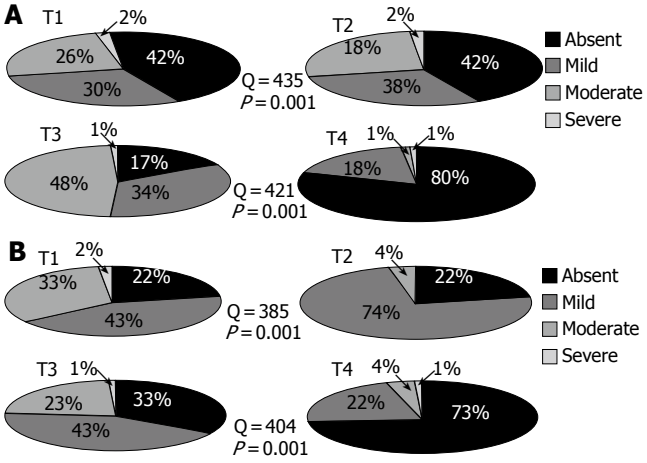

Figure 1 Prevalence of epigastric pain and heartbum (**A**) and postprandial gastric fullness (**B**) at the 4 time points of follow-up in dyspeptic patients.

RESULTS

A totae of 3872 patients with functional dyspepsia and 3069 patients with IBSc were enrolled in the first part of the study. After a year 74% and 69.5% came for follow up visit and to perform a second cycle of thermal therapy (Table 2). No difference among different mineral content of water supplemented was observed.

Figures 1A and 1B show the prevalence of dyspeptic symptoms (epigastric pain and post-prandial fullness) during the two cycles of thermal therapy. A significant reduction in the prevalence of dyspeptic symptoms was observed both at the end of the first and second cycles. In particular, 80% of the patients were symptom free after the first cycle of thermal therapy. Moreover, the score during the pretreatment period was similar between the two cycles. However, ANOVA showed reduction of symptoms when the whole follow-up period was considered. Similar results were observed for post-prandial fullness. The analysis of variance showed a real and persistant improvement of symptoms in all patients.

When the main symptoms of IBSc (bloating, hard stools, incomplete evacuation) were considered, significant improvement was observed with ANOVA (Figures

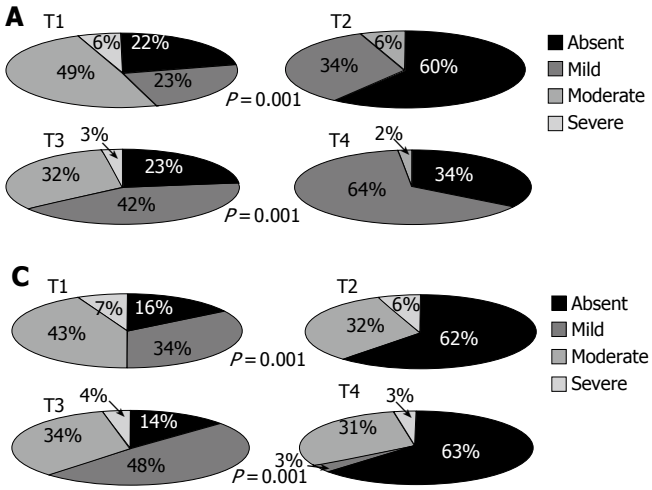

Figure 2 Prevalence of bloating (**A**), incomplete evacuation (**B**), and epigastric hard stool (**C**) at the 4 time points of follow-up in patients with IBS-c.

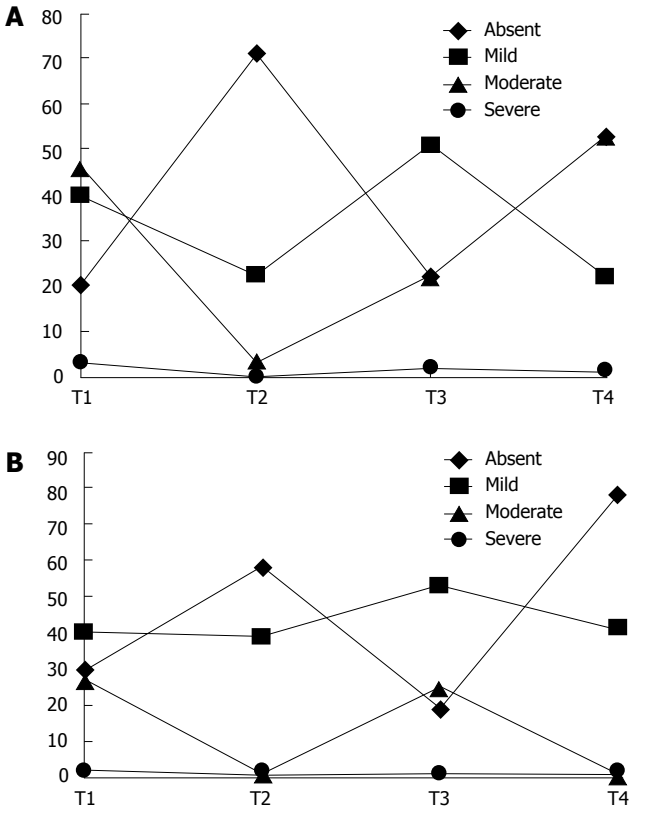

Figure 3 Mean score of symptoms in dyspeptic (**A**) and IBSc (**B**) patients at the 4 time points of follow-up.

2A-2C).

To better underline the temporal evolution of syptoms we reported the mean of scores for symptoms of dyspepsia and IBS at the 4 time points (Figures 3A and 3B).

The data on the number of hospitalization, days of absence from work and the clinical recrudescence before and after two cycles of thermal therapy were decreased by about 30% (Table 3). Moreover, data on overall tolerablity of treatment and reasons of acceptance of this treatment are shown in Table 4. About 80% of patients appreciated the treatment and about 95% gave a positive result of the treatment

Sixty patients and 60 healthy controls were enrolled in the study. Fifty-eight patients had a slow gastric output

Table 3 Dyspepsia-IBSc: relapse after two cycles of thermal therapy

	1 cycle (%)[2]	2 cycles (%)[2]
Admission to hospital[1]	5.6	2.0
Work lack[2]	3.4	2.2
Clinical relapse	29.6	21.9

[1] In previous 12 mo.
[2] On yearly working days.

Table 4 Subjective evaluation of dyspepsia and IBSc

Personal opinions on therapy (%)		Reasons of good opinion (%)	
Excellen	42	Care	95.6
Good	41.8	Relax	68
Lean	1.2	Stay	55.8
Null	6.3	Climate	45.3
No comment	8.4		

measured by OBT (mean $T_{1/2}$: 131 ± 18; Table 2). Patients with a normal gastric output were excluded. One patient and 2 controls refusing to give their consent were excluded. One patient was excluded for low compliance with the treatment (more than 20% of full bottles returned).

Dyspeptic patients presented an alterated gastric output and a significant difference at enollment compared with healthy controls when both $T_{1/2}$ (131 ± 18 *vs* 81 ± 7; $P < 0.01$) and T-lag (92 ± 11 *vs* 51±10; $P < 0.01$) were considered.

After mineral water supplementation for 3 weeks, a reduction of gastric output was observed in 49 (87.5%) of 56 dyspeptic patients. Both $T_{1/2}$ and T-lag were significantly reduced after the therapy compared to the basal values (91 ± 12 for $T_{1/2}$ and 53 ± 11 for T-lag, Table 5) with OBT similar to that in healthy controls. In controls and 7 patients, the gastric output did not change after mineral water supplementation treatment.

The prevalence of dyspeptic symtoms was significantly lower after the therapy than at enrolment. The prevalence of bloating and gastric fullness was significantly lower after mineral water supplementation treatment (Table 6). The

Table 5 Parameters of gastric emptyng rate (^{13}C octanoic acid breath test) in dyspeptic patients before and after mineral water supplementation therapy (mean ± SD)

	Pre-treatment	Post-treatment	*P*
$T_{1/2}$	131±18	91±12	<0.001
T-lag	92±11	53±11	<0.001

Table 6 Prevalence of gastrointestinal symptoms before and after mineral water supplementation therapy in dyspeptic patients

Symptomatology	T0 % (*n/n*)	T2 % (*n/n*)	*P*
Gastric fullness	86 (48/56)	25 (14/56)	<0.0001
Epigastric pyrosis	52 (29/56)	30 (17/56)	<0.05
Bloating	52 (29/56)	23 (13/56)	<0.005
Epigastric pain	24 (12/56)	11 (6/56)	NS
Nausea	5 (3/56)	0.2 (1/56)	NS
Vomiting	0.2 (1/56)	0 (0/56)	NS
Overall	100 (56/56)	68 (38/56)	<0.0001

Table 7 Oro-cecal transit time in IBC patients before and after mineral water based diet (mean ± SD)

	Pre treatament	Post treatament	*P*
Patients	1205±12	97±8	<0.001
Controls	85.5±14	81±8	NS
P	<0.001	<0.001	

Table 8 Gastrointestinal symptoms before and after mineral water supplementation therapy in patients with IBS

Symptoms	T0 % (*n/n*)	T21 % (*n/n*)	*P*
Bloating	90 (9/10)	20 (2/10)	<0.005
Abdominal Pain	40 (4/10)	20 (2/10)	NS
	T0 Media	T21 Media	
Evacuation/wk (*n*)	1.7	3.3	<0.001

T0: Basal time
T21: After mineral water supplementation therapy
NS: Not significant

overall prevalence of gastrointestinal symptoms was reduced after mineral water supplementation treatment too. At the basal point, a mean global score of symptoms was 15±4. After 30 d of mineral water supplementation treatment, the score was 7±3. A lower prevalence of abdominal pain, nausea, vomiting was also observed. No severe side effects were reported by patients. Only one control experienced mild diarrhea but treatment was not stopped. Five patients and 7 controls reported an increased number of evacuations during treatment. No effects of mineral water supplementation trealment on blood pressure, glycaemic control and heart rate were observed.

Oro-cecal transit time was longer in patients with IBSc than in controls (Table 7). All patients and 3 healthy controls had an abnormal oro-cecal transit time. After minere water supplementation treatment for 3 wk, the oro-cecal transit time was shorter than at the beginning of the study. A slight reduction in transit time was observed in healthy controls especially in those presenting a pathological transit time at the start of the treatment. The number of evacuations in a week was increased and bloating was reduced during mineral water supplementation treatment in patients with IBSc (Table 8). No side effects were reported by patients and controls.

DISCUSSION

Several diseases have been treated with thermal therapies for a long time in different countries. However, whether thermal therapy should be considered as pertinent to alternative medicines is a matter of debate and largely depends on the different cultural settings in which this practice is performed. In the Scandinavian, British and North American countries the therapeutic value and benefits of thermal (spa) treatment are seen with scepticism and looked at as an alternative and unorthodox practice. On the contrary, spa has been considered a credible medical treatment and supported by official undergraduate and postgraduate university teaching in most countries of the continent, Southern and Eastern Europe (France, Germany and Italy). It should also be mentioned that, differently from various forms of alternative medicine, spa therapy is firmly maintained in medical hands and undergoes orthodox medical control. As a consequence, thermal medicine appears as a supportive rather than alternative practice. Thus in the mentioned countries, spa therapy cannot be labelled as "alternative" medicine, but should be defined as "complementary" or "auxiliary" medicine. Nevertheless, also in these settings, an alignment to the Anglo-Saxon scepticism towards spa therapies has recently developed within the medical and academic community, although not all do so among patients and within the "civic" society.

Such scepticism is based on the scant number of studies published in medical journals of good reputation, apt to investigate with correct methodology and design the real benefits of spa therapies in various clinical conditions in term of efficacy and cost-effectiveness. Even in those countries in which "hydrology" is a recognized medical speciality with an academic background (as in Italy) most researcher work done in the past has been characterized by an approach mainly pathophysiologic and pharmacological, aimed at investigating the mechanism and biological effects of the mineral water rather than at assessing the related clinical and health economic effects with appropriate methods.

As in a number of European countries, variable kinds of financial support (public and private) have been provided for different forms of thermal treatment applied to various diseases. This issue is not a simple question of medical and academic relevance but has great implications from the socioeconomic point of view. In Italy, almost 340 thermal industries are crucial economics and social factor for many geographic areas. The yearly financial turnover related to the thermal activities amounts to 300 million dollars and 2000 million dollars as for health aspects and

linked activities, respectively, and is a key element of the national economy.

The evaluation of literature on the efficacy of spa therapies in the international bibliography can clearly show how wide the gap is in this context, if compared to traditional clinical domains and other nonconventional therapies. The limited impact of scientific research on the efficacy of spa therapies accounts the reservations of the scientific world to the actual efficacy of spa therapies, thus paving the way in Italy to discuss the public funding of these activities. On these bases in 1995, the Association of Thermal Industries and Curative Mineral Waters (Federterme", which officially represents all 340 Italian "medical" spa centres) have developed the epidemiological, health and cost-effectiveness aspects related to spa activities to assess the efficacy of thermal therapies in reducing the health costs.

The observational study has given very important results. The high number of patients who decided to come back for a second period of thermal treatment suggests a very good impact of the therapy on symptoms evaluted by ANOVA test. The data cannot be ascribed only to a placebo effect. We however, cannot conclude that thermal therapies are able to influence the natural history of studied diseases (dyspepsia and IBSc). It is possible that the beneficial effet of a single thermal treatment can influence the perception of symptoms as less severe even in a long follow-up period. This hypotesis was supported by reduction of day and number of hospitalization and day of absence by work during the follow-up period. It should be important to evaluate the economical impact of such results but it was not possible in this study. However, all cited parameters showed a reduction of about one third during the follow-up perion when compared to the former years. No reduction in drug use was observed (data not shown). It is possible to conclude that mineral water supplementation treatment for functional dyspepsia and IBSc can improve symptoms and reduce the medical cost as well as deserves further attentions. However, a best detailed analysis on cost/effectiveness should be performed.

Our data showed that mineral water supplementation therapy could reduce the gastric output of solid food and improve symptoms in patients with functional dyspepsia. The improvement of both studied parameters ($T_{1/2}$ and Tlag) suggests that mineral water can normalize both gastric output time and redistribution of alimentary bolus in the stomach. The pathophysiologic mechanism causing these effects are unknown. Our study demonstrated that mineral water supplementation therapy could improve symptoms and gastric acid output in functional dyspeptic patients evaluated by a questionnaire and OBT. The effects exerted by mineral water include stimulation of chemoreceptors and baroceptors in the gastric walls. The well known effect of water on intestinal motility (due to osmotic properties) with increased intestinal transit time induces an early duodenal transit of food with an earlier relaxation of pylorus and a faster transit of bolus from stomach to duodenum. Moreover, the presence of liquid in the stomach accelerates food disintegration and solubilization. The chemical content of particular water plays a role in the stimulation of specific gastric receptors that increases the gastric motility by secreting local hormones (gastrin, secretin, vasoactive intestinal peptide). The evidence that the effect of water is limited in patients suggests that hormones may be dysregulated in such patients. The presence of large amounts of calcium and magnesium in mineral water may directly stimulate the gastric smooth muscle to increase its motility and relax pylorus, thus producing its effect on gastric output and symptoms. The restoration of a correct gastric output reduces the time of exposition of bolus to intestinal bacteria, thus reducing the intensity of bloating due to intestinal bacteria overgrowth.

Constipation predominant irritable bowel sindrome is one of the frequent gastroenterological diseases in general population. Several pharmacological treatments have been proposed and used. Although therapies for constipation are efficacious, most of them are self-prescribed by patients with a high cost. Laxatives for example are widely used without medical control and may produce side effects. Moreover, the efficacy of laxative treatment is temporary and induces patients to increase dosage of drugs. A diet containing high fibers has been demonstrated to be a valid alternative to drugs for chronic constipation[20].

In our study, mineral water supplementation theray (2000 mL) for 3 wk accelerated oro-cecal transit time and improved symptoms in patients with IBSc. Compliance to therapy was excellent. The mechanisms of action of mineral water are not completely clear. Others iones present in mineral water may directly or indirectly (via neuroendocrine secretion of vasointestinal active peptides) stimulate smooth muscle to increase its motility. These actions reduce the transit time and increase the number of evacuations in a day with improvement of symptoms in IBSc patients. The decreased transit time improves bloating and reduces the time of contact between intestinal content and saprophytic flora with reduction in gas production.

In conclusion, mineral water supplementation theray can improve gastiric acid output, oro-cecal transit time and symptoms in patients with functional dyspepsia or IBSc. Mineral water supplementation therapy seems to be a simple, well-tolerated, cheap therapy for functional dyspepsia or IBSc and should be taken into account by physicians in the treatment of dyspepsia ad IBSc.

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RAPID COMMUNICATION

Interleukin-6 and its soluble receptor in patients with liver cirrhosis and hepatocellular carcinoma

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Abstract

AIM: To evaluate the immunohistochemical localization of interleukin-6 (IL-6) and IL-6 receptor (IL-6R) on tumor tissue specimens from patients with hepatocellular carcinoma (HCC) and the serum levels of IL-6 and sIL-6R in a group of patients with HCC as well as liver cirrhosis (LC) in a group of patients with LC alone and in a control group.

METHODS: Three groups of subjects were studied: group I ($n = 83$) suffering from HCC and LC, group II ($n = 72$) suffering from LC alone and group III ($n = 42$) as healthy controls. All patients had hepatitis C virus infection. Serum IL-6 and IL-6R levels were determined using a commercially available ELISA kit. Immunohistochemistry was performed using the streptavidin-biotin complex and rabbit polyclonal antibodies against IL-6 and IL-6R.

RESULTS: Immunohistochemistry analysis showed a medium to strong cytoplasmic and membrane reactivity for IL-6 and IL-6R respectively, in at least 40% of cases of HCC, whereas liver cirrhosis patients and controls were negative for IL-6 or showed a very mild and focal dot-like cytoplasmic reaction for IL-6R. Serum IL-6 levels in HCC group were significantly higher than those in LC and control groups ($P < 0.0001$). There was no significant difference in sIL-6R concentrations among 3 groups. When the patients with HCC were divided into groups according to Okuda's classification, a significant serum increase of IL-6 and sIL-6R level was observed from stage I to stage III ($P < 0.02$, $P < 0.0005$). When HCC and LC patients were divided into 3 classes of cirrhosis severity according to Child-Pugh, values in HCC patients were significantly higher than those in LC patients for each corresponding class ($P < 0.01$).

CONCLUSION: IL-6 serum levels in HCC patients are higher than those in LC patients and controls, suggesting an increased production of this cytokine by neoplastic cells. sIL-6R values are similar in all groups, increasing only in stage III HCC patients. These data suggest that they have a closer relationship with the neoplastic mass rather than with the residual functioning hepatic mass.

Key words: Interleukin-6; Cytokine; Chronic liver disease; Immunohistochemistry

Soresi M, Giannitrapani L, D'Antona F, Florena AM, La Spada E, Terranova A, Cervello M, D'Alessandro N, Montalto G. Interleukin-6 and its soluble receptor in patients with liver cirrhosis and hepatocellular carcinoma. *World J Gastroenterol* 2006; 12(16): 2563-2568

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INTRODUCTION

Interleukin-6 (IL-6) is a proinflammatory cytokine which plays an important role in the host defence mechanism. Serum IL-6 levels are low in physiological conditions, but increase considerably in pathological conditions such as trauma, inflammation and neoplasia. In tumors, IL-6 may be involved in promoting the differentiation and growth of target cells. In fact, several neoplastic cell lines (such as esophageal cancer, renal cell carcinoma, multiple myeloma, prostate and ovarian cancer) have been shown to produce high *in vitro* levels of IL-6[1-5], and high concentrations of this cytokine are associated *in vivo* with a poor outcome of the disease in many types of tumours[6-12]. It has also been hypothesized that activation of the IL-6 gene is responsible for the derangement of some events which can lead to neoplastic degeneration[13].

IL-6 activity is mediated through the binding to its membrane receptor (IL-6R), which in turn promotes the

interaction with another receptor component, gp130, able to transduce IL-6 signalling at the intracellular level[14]. High concentrations of soluble IL-6R, like IL-6, are present in serum and other biological fluids in different pathological conditions, because it is released from cells expressing it on their surface[15].

Many works have reported high serum levels of IL-6 in various liver diseases, such as acute hepatitis[16], alcoholic cirrhosis[17], HBV-associated chronic hepatitis, primary biliary cirrhosis (PBC)[18], chronic hepatitis and HCV-correlated liver cirrhosis[19,20] and in hepatocellular carcinoma (HCC)[21-24].

Studies on animal models have shown that transgenic mice expressing high levels of IL-6 and sIL-6R develop hepatic nodular hyperplasia and signs of sustained hepatocyte proliferation, suggesting that IL-6 and sIL-6R could provide the primary stimulus to cell proliferation and are involved in development of HCC[25].

This study aimed to evaluate the immunohistochemical expression and localization of IL-6 and sIL-6R on tissue specimens from patients with HCC-associated liver cirrhosis and liver cirrhosis alone, and the serum levels of IL-6 and sIL-6R in patients with HCC- associated liver cirrhosis (LC) and to compare them in patients with LC alone and healthy controls.

MATERIALS AND METHODS

Patients

The study was performed in 207 subjects divided into three groups. Group I included 93 patients with HCC (61 males, 32 females, mean age 62.2 years, range 43-76 years). Diagnosis was made in 41 cases based on biopsy or cytological findings, diagnosis of the remaining cases was made on the basis of multiple, concordant imaging techniques (ultrasound, helicoidal computed tomography (CT), lipiodol-CT, selective angiography) and biochemical examination (AFP > 400 ng/mL). Some of the patients known as cirrhotics were enrolled in a prospective study for HCC screening, and others were referred to our center diagnosed as HCC. HCC was associated with the presence of serum HCV antibodies in all cases. The patients were then divided into the 3 stages of Okuda's classification[26] which as well as neoplasia size were also taken into account of serum values of bilirubin and albumin and the presence of ascites. The last three parameters were used to evaluate the hepatic functioning mass and the severity of the underlying cirrhosis. In brief, stage I was an initial stage in which the neoplasm (or the sum of the nodules) measured less than 50% of the whole liver section on CT scan. There was no ascites, albumin levels were over 3 g/dL and bilirubin levels were below 3 mg/dL. Stage II was moderately advanced, with two or more indices of advanced disease. Stage III was very advanced, with three or all indices of advanced disease. Group II included 72 patients (48 males, 24 females, mean age 56.5 years, range 36-75 years) suffering from liver cirrhosis, consecutively selected from out- or in-patients examined at our hospital. Diagnosis was made in 46 cases based on biopsy findings and diagnosis of the remaining cases was made on the basis of unequivocal clinical, biochemical and instrumental data. A post-study follow-up for at least 6 mo excluded the existence of neoplasia. The disease was associated with hepatitis C virus infection in all cases. The control group was composed of 42 healthy asymptomatic subjects (31 males, 11 females, mean age of 54.9 years, range 45-61 years recruited from donors at the blood bank of our hospital. Liver disease was excluded on the basis of anamnestic, biochemical and instrumental data. There were no cases of neoplastic disease, evaluated by a follow-up for at least six months. Daily alcohol consumption of above 30 g/d was found in none of the three groups.

Methods

Blood samples were taken after overnight fasting. After centrifugation part of the sera was used to assay the main parameters of liver function by routine methods. The remainder was frozen at - 40 ℃ for IL-6 and sIL-6R assay. Serological testing for anti-HCV was performed using the third generation of enzyme-linked immunosorbent assay (ELISA) (Orthodiagnostic System, Raritan, New Jersey, USA) in accordance with the manufacturer's instructions. Anti-HCV reacting samples were confirmed using the third generation of anti-HCV recombinant immunoblot assay (RIBA III, Chiron Corporation, Emeryville, CA, USA). Markers of HBV were tested using the Abbott RIA kit.

Liver biopsy samples were obtained for diagnostic purposes percutaneously according to the Menghini technique using 1.0-1.2 mm diameter needles (Surecut, Hospital Service, Rome, Italy). In some cases HCC was diagnosed with a thin needle (20 gauge, Surecut) guided by ultrascan using a Toshiba SSA 240 A apparatus with a 3.5 MHz probe.

Serum IL-6 and sIL-6R levels were determined using a commercially available ELISA kit (Quantikine, human IL-6 and sIL-6R, R & D Systems, Minneapolis, USA) in accordance with the manufacturer's instructions.

Immunohistochemistry analysis was performed on ten different HCC samples, five cirrhotic and two normal liver samples. Histologically normal liver tissues were obtained from patients during surgery for cholelithiasis. Written informed consent was obtained. The specimens were fixed in formalin and embedded in paraffin. Five- μm thick sections were cut and dewaxed, hydrated and incubated in 3% hydrogen peroxide for 20 min. Sections were then heated in microwave oven and non-specific binding was blocked by incubation with 3% rabbit normal serum for 20 min. Immunohistochemistry was performed using the streptavidin-biotin complex (StreptABC) with the following antibodies: rabbit polyclonal antibody against IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against IL6R (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 overnight in a 4 ℃ moist chamber and mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, Dako, Santa Barbara, CA) at a dilution of 1:100 for 30 min at room temperature. Sections were then incubated for 30 min at room temperature with biotinylated anti-rabbit and anti-mouse immunoglobulin diluted in phosphate-buffered

Table 1 Median and range of serum values of IL-6 (pg/mL) and sIL-6R (ng/mL) in controls, liver cirrhotoc (LC) and hepatocellular carcinoma (HCC) patients and divided according to stage of disease

	Controls	LC	HCC	HCC Stage I	HCC Stage II	HCC Stage III	Z =	*P*
	(*n* = 42) median (range) (a)	(*n* = 72) median (range) (b)	(*n* = 93) median (range) (c)	(*n* = 28) median (range) (d)	(*n* = 46) median (range) (e)	(*n* = 19) median (range) (f)		
IL-6	3.6 (3-17)	6.4 (3-155)	14 (3-301)	6.5 (3-270)	12 (3-301)	32 (3-110)	b-c: z = 3.1	0.002
							a-c : z = 5.4	0.0001
							a-b : z = 2.3	0.03
							d-f : z = 2.8	0.005
							e-f : z = 2.7	0.01
							b-d : z = 1.9	0.05
sIL-6R	34.8 (7.2-80)	39.2 (4.8-80)	42.4 (17.6-80)	34.2 (17.6-65.6)	39.6 (18-80)	56 (30-80)	a-c : z = 2.1	0.04
							b-f : z = 2.7	0.01
							d-f : z = 3.2	0.002
							e-f : z = 2.9	0.004

Table 2 Median and range of serum values of IL-6 (pg/mL) and sIL-6R (ng/ml) in HCC and LC patients divided into groups according to Child-Pugh classes

	Class A		Class B		Class C	
	LC	HCC	LC	HCC	LC	HCC
	(*n* = 20) median (range)	(*n* = 44) median (range)	(*n* = 33) median (range)	(*n* = 30) median (range)	(*n* = 19) median (range)	(*n* = 19) median (range)
IL-6	3[a] (3-52)	6.5[a] (3-270)	8.5 (3-155)	14 (3-301)	8[c] (3-132)	28[c] (3-301)
sIL-6R	49.8[b] (21.2-80)	36.8[b] (17.6-67.2)	37.2 (4.8-72)	42.8 (18-72.8)	6.8[d] (24.4-57.2)	56.2[d] (24-80)

[a]$P < 0.04$ *vs* sIL-6R ; [b]$P < 0.04$, [d]$P < 0.03$ *vs* IL-6.

saline (PBS), with streptavidin-biotin complex for 50 min at room temperature and colour was developed with diaminobenzidine (DAB) for 40 min at room temperature, counterstained with Mayer haematoxylin for 1 min. The expression of IL-6 and IL-6R was considered positive when >30% of cells showed cytoplasmic or membrane staining. The percentage of PCNA- stained nuclei was calculated by counting the number of stained nuclei out of 1000 cells per high-power field for 10 different tumor sections.

Statistical analysis

The data were expressed as mean ± SD. Groups were compared using the Mann-Whitney *U* test. χ^2 test was used for the frequency analyses. Simple linear regression test and Spearman's rank correlation test were used where appropriate. The cut-off values IL-6 and sIL-6R were calculated as the value of the maximized likelihood ratio (LR) obtained using the following formula: LR = probability of true positives + probability of true negatives/probability of false positives + probability of false negatives. $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the median, range and ratio of serum IL-6 and sIL-6R values in the 3 study groups, and also in the HCC group divided according to Okuda's classification. Analysis performed with the Mann-Withney *U* test showed that the HCC group had significantly higher IL-6 values than the LC ($z = 3.1$, $P < 0.002$) and control group ($z = 5.4$, $P < 0.0001$). However, the LC patients had significantly higher IL-6 serum levels than controls ($z = 2.3$, $P < 0.03$). A significant difference was found in sIL-6R serum levels between the HCC group and controls ($z = 2.1$, $P < 0.04$), but not between the HCC and LC groups.

Analysis of the values after division of the HCC group according to Okuda's classification showed that serum values of IL-6 were significantly higher in stage III patients than in stage II and stage I patients ($z = 2.7$, $P < 0.01$ and $z = 2.8$, $P < 0.005$, respectively). Moreover, IL-6 values in stage I HCC patients were also significantly higher than those in the LC patients ($z = 1.9$, $P < 0.05$). There was no significant difference in sIL-6R values between stage I and stage II patients and the LC patients. The only significant difference was found between the stage III HCC patients and the LC patients, stage I and stage II HCC patients ($P < 0.004$).

Spearman's rank correlation test showed a significant increase in IL-6 and sIL-6R levels from stage I to stage III ($r = 0.28$, $P < 0.02$; $r = 0.39$, $P < 0.0005$, respectively).

Table 2 shows the median and range of IL-6 and sIL-6R in the HCC and LC groups divided according to Child-Pugh classes. The median IL-6 value was higher in HCC group than in the LC group, but the difference was significant only in classes A and C ($P < 0.0005$ and < 0.04, respectively) when compared to the corresponding LC groups. The median sIL-6R value decreased from class A to C in the cirrhotic group, but increased in the HCC group. Consequently, in the LC group the median sIL-6R value was significantly higher in class A ($P < 0.04$), while in the HCC group it was higher in class C ($P < 0.03$).

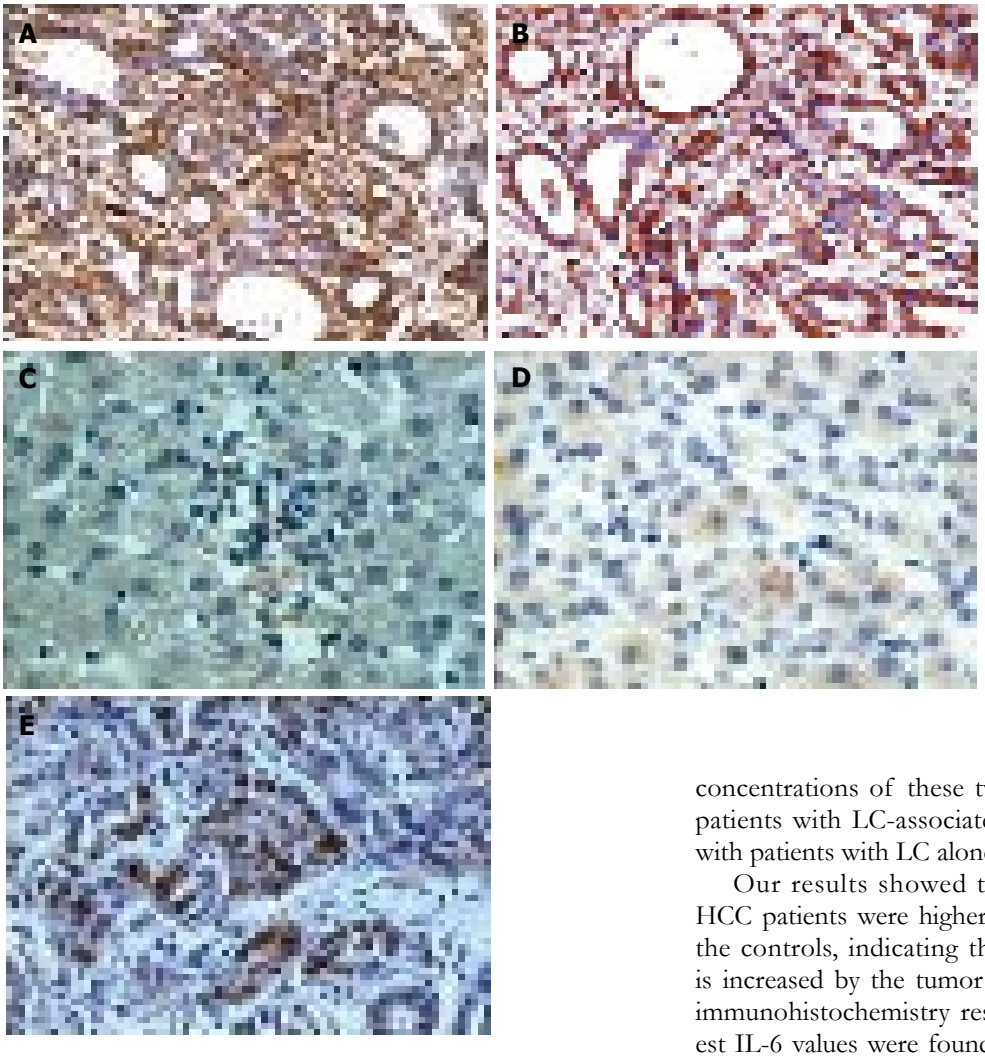

Figure 1 Results of immunohistochemical analysis of cytoplasmic and membrane reaction of IL-6 and IL-6R in HCC (**A, B**), normal liver (**C, D**), and cirrhotic (**E**) patients.

Figure 1 shows the results of the imunohistochemistry analysis. In the HCC group (ten cases), moderate to strong cytoplasmic and membrane reactivity for IL-6 and IL-6R respectively, was observed in at least 40% of cases (Figures 1A, 1B), whereas control cases of normal liver (two cases) were negative (Figure 1C) or showed a very mild and focal dot-like cytoplasmic reaction for IL-6R (Figure 1D). In liver cirrhosis group (ten cases) immunohistochemistry was negative on the whole or similar to control cases. PCNA immunoreactivity was observed in some nuclei of neoplastic cases with a mean value of 50% in the evaluated areas (Figure 1E).

DISCUSSION

Circulating IL-6 levels are elevated in patients with chronic viral[18-20] and alcoholic hepatitis[17]. Increased IL-6 is correlated with the stage of disease in liver cirrhosis [27]. Higher levels of IL-6 correlated with tumor size and cancer aggressiveness in patients with HCC[22].

At present, there are no data about the behaviour of circulating sIL-6R in patients with HCC and very few for other neoplasms. However, in multiple myeloma higher sIL-6R values correlate with a poor outcome of the disease[28]. Therefore, the aim of this study was to evaluate the expression and localization of IL-6 and sIL-6R in pathological liver tissue specimens as well as the serum concentrations of these two cytokines and their ratio in patients with LC-associated HCC and to compare them with patients with LC alone and healthy controls.

Our results showed that the median IL-6 levels in HCC patients were higher than those in LC patients and the controls, indicating that production of the cytokine is increased by the tumor cells. This is supported by the immunohistochemistry result and the fact that the highest IL-6 values were found in Okuda's stage III, in which the neoplastic mass is the most extensive. However, LC patients also had higher median IL-6 values than controls, but only for Child's classes B and C, while values in class A were close to control levels. In classes B and C, increased IL-6 serum levels compared to control values might reflect a response of the residual hepatic cells to cytolysis and to the attempt to recover the liver mass[25] associated with a clear impairment. In contrast, in HCC patients, whatever class of the disease, the median values were higher than those in LC patients, indicating that IL-6 serum levels increase when patients pass from cirrhosis to HCC. This was not true in the sIL-6R values, because the median value in HCC patients was significantly higher than that in controls ($P < 0.04$), and similar to that of LC patients. When the patients with HCC and LC were divided according to Child-Pugh's classification, the median serum values of sIL-6R tended to decrease from class A to class C in LC patients, while the opposite occurred in HCC patients, indicating that in cirrhotic patients sIL-6R production and release decrease as the disease progresses owing to the reduction of the liver parenchymal mass. On the other hand, in HCC patients, increased sIL-6R serum concentration might be due to the increasing tumor mass.

When the patients with HCC were divided according to Okuda's classification, IL-6 values significantly increased as the disease worsened. At all the stages the median IL-6 values were significantly elevated compared to the cirrhotic patients, indicating that neoplastic degeneration even in its

initial stages, causes variations in IL-6 levels, which could enable us to discriminate cirrhotic from HCC patients. Interestingly, sIL-6R levels were elevated only in patients with a more severe disease (stage III).

The high serum levels of IL-6 and sIL-6R were corroborated by the immunohistochemistry analyses, which showed the marked expression of both cytokines and their receptor in the tumor. This expression correlates to cell proliferation, as demonstrated by the concomitant presence of a high percentage of PCNA positive nuclei. The existence of such a relationship has also been reported for colorectal cancer[29]. On the whole, our results suggest that HCC cells, especially in advanced stages of the disease, may produce and secrete IL-6 and sIL-6R to stimulate their growth by an autocrine/paracrine mechanism as suggested by previous reports concerning cells derived from hepatomas or other types of cancer[30-34].

IL-6 production by tumor cells might also contribute to systemic complications such as induction of cachexia in the host[11] and local immunosuppression rather than immunopotentiation[35]. Thus, the present study might highlight the potential therapeutic benefits of approaches such as use of anti-sense nucleotides or IL-6R super antagonists, which can overcome the adverse effects of IL-6 in HCC, as in hepatoma cells, multiple myeloma, prostate cancer and other tumors[31, 34-36].

In conclusion, the IL-6 levels in patients with LC-associated HCC are higher than in patients with LC alone and controls, indicating that production of this cytokine is increased by tumor cells. This has been confirmed by the higher IL-6 values in stage III than in stages I and II of the disease. These results might be of help in differentiating cirrhosis from LC-associated HCC suggest that measures aimed at blocking adverse effects of IL-6 may be of potential clinical utility in HCC.

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RAPID COMMUNICATION

Rabeprazole test for the diagnosis of gastro-oesophageal reflux disease: Results of a study in a primary care setting

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Abstract

AIM: To determine the diagnostic value of the rabeprazole test in patients seen by general practitioners.

METHODS: Eighty-three patients with symptoms suggestive of GERD were enrolled by general practitioners in this multi-centre, randomized and double-blind study. All patients received either rabeprazole (20 mg bid) or a placebo for one week. The diagnosis of GERD was established on the presence of mucosal breaks at endoscopy and/or an abnormal esophageal 24-h pH test. The test was considered to be positive if patients reported at least a "clear improvement" of symptoms on a 7-point Likert scale.

RESULTS: The sensitivities of the test for rabeprazole and the placebo were 83% and 40%, respectively. The corresponding specificity, positive and negative predictive values were 45% and 67%, 71% and 71%, and 62% and 35%, respectively. A receiver operating characteristics (ROC) analysis confirmed that the best discriminatory cut-off corresponded to description of "clear improvement".

CONCLUSION: The poor specificity of the proton-pump inhibitor (PPI) test does not support such an approach to establish a diagnosis of GERD in a primary care setting.

Key words: Gastro-oesophageal reflux disease; Diagnostic tool; Rabeprazole; Proton pump inhibitors; Primary care

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most common disorders observed by primary care physicians. In this setting, an accurate, non-invasive and safe diagnostic test would be of great use. Proton-pump inhibitors (PPIs) are the most potent suppressors of gastric acid secretion and represent the mainstay of GERD treatment, with a therapeutic effect throughout the spectrum of the disorder. Therefore, in clinical practice, many physicians consider that rapid symptom relief after a short course of PPI therapy is a valuable marker for a diagnosis of GERD. This represents the basis for the development of so-called PPI tests, the value of which has previously been assessed by a number of different investigators, using various molecules which were tested in assorted referral populations, mainly in a secondary or tertiary care setting. The various PPIs and dosages, the duration of the PPI course and the way the results are interpreted are likely to be responsible for the conflicting results previously reported in the literature[1]. Rabeprazole is a more recently developed PPI with specific pharmacological properties such as a high pKa which may lead to both rapid accumulation in the acidic compartment of the parietal cell and more effective control of acidity during the first day of administration[2,3]. Similarly, when the target population for such PPI tests is considered, the lack of drug interference and the safety profile are both of most importance. In these respects, rabeprazole also displays some pharmacological advantages due to a partly non-hepatic metabolism and a linear response, which result in more predictable effects in terms of acid suppression[4,5].

We therefore aimed to determine the diagnostic value of the rabeprazole test in a population of patients followed up by general practitioners (GPs) for symptoms suspected to be reflux-related.

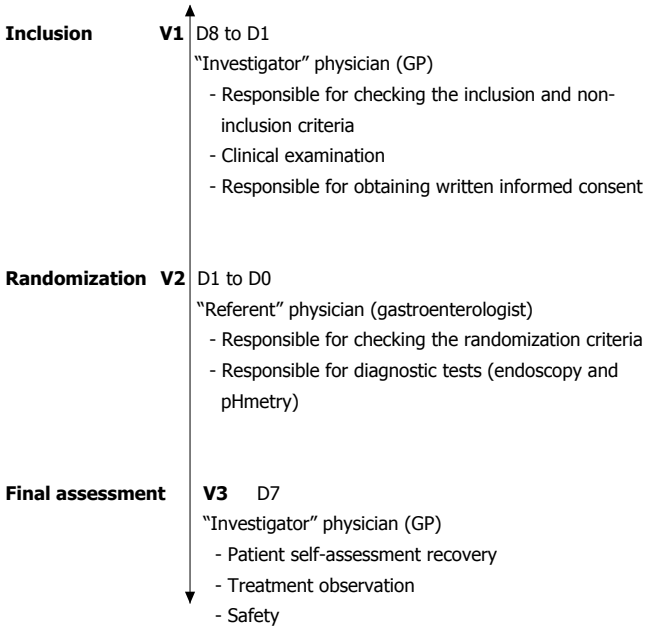

Figure 1 Diagrammatic illustration of the different phases of the study. Two visits were carried out by the investigator physician (V1: inclusion; and V3: final assessment). The second visit (V2) was carried out by the referent physician to check the inclusion criteria, to perform endoscopy (unless the patients had already undergone one endoscopy during the previous 6 months) and 24-h esophageal pH monitoring.

MATERIALS AND METHODS

Global design of the study

The study was conducted using a multi-centre, randomized and double-blind design. In order to maintain the double-blind nature of the study, each patient was examined by an "investigator" physician and a "referent" one. The investigator was a GP responsible for patient recruitment, inclusion and monitoring at the end of study assessment. The referent was a gastroenterologist with experience of endoscopy and esophageal pH monitoring, responsible for diagnostic testing, checking the randomization criteria, and prescribing treatment. The design of the study consisted three phases (Figure 1).

Phase 1 The investigator enrolled patients into the protocol according to the following inclusion criteria: (a) presence of at least 3 mo of typical (heartburn or regurgitation) or atypical (ascending burning epigastric pain, recurrent nausea, post-prandial digestive discomfort, dysphagia) gastrointestinal or extra-gastrointestinal symptoms suspected to be reflux-related; (b) occurrence of a particular symptom on at least 2 occasions during the 3 d prior to inclusion, the intensity of which being rated as "moderately uncomfortable or worse" using a 7-point Likert verbal analogue scale; (c) lack of previous investigations demonstrating esophagitis, such as esophageal pH monitoring and upper GI endoscopy; (d) absence of previous effective anti-reflux therapy, including PPIs, full-dose H_2-receptor antagonists or cisapride during the previous month. Conversely, the following patients were excluded from the trial: (a) women who were either pregnant, breast feeding or not using an effective method of contraception; (b) patients with a malignant condition or an uncompensated chronic disease, particularly uncompensated cardiac, liver or renal disease; (c) patients who had previously undergone a vagotomy or surgery that might alter gastric acid secretion; and (d) patients who were considered unable to comply with the conditions of the protocol, particularly with respect to follow-up and self-assessment questionnaires.

Written consent was obtained from all patients before inclusion. The study was approved by the Local Ethical Committee ('CCPPRB des Pays de la Loire n°2').

Patients were given a self-assessment form at the inclusion visit. On each day of the study, patients had to report their dominant symptom (i.e., the symptom which had led them to consult the investigator and that induced most discomfort for the patient). A consultation with the referral doctor was scheduled between 4 and 10 d after the inclusion visit. During this period patients were requested not to take any treatments that could be used to treat GERD, apart from the symptomatic treatment they had been given (Co-magaldrox, Maalox®).

Phase 2 The referent examined patients 4 to 10 d after their inclusion. The inclusion criteria were checked; notably the presence of at least two episodes of the dominant symptom in the 3 d prior to the consultation, rated as being at least "moderately uncomfortable" on the self-assessment form. Endoscopy was performed in all patients according to the usual practice of each centre. In patients with a normal endoscopy from the previous 3 mo, the results were considered not to contribute to a diagnosis of GERD and the investigation was not repeated. Twenty-four hour pH monitoring was performed immediately after the endoscopy. At the end of this second phase, patients who fulfilled the inclusion criteria and without any exclusion factors were randomly allocated to receive either a placebo or rabeprazole (20 mg bid) before breakfast and dinner for 1 wk. The investigators and patients were blinded to the administered treatments. Patients were not informed of the investigation results and the data was sent to a central database until the trial had been completed.

Phase 3 Patients were then received by the investigator at an end of study consultation after 7 ± 1 d of treatment. The investigator was unaware of the results of the endoscopy and pH monitoring. At this visit patients completed the response assessment form, rating any change in their symptoms. A rating according to 3 descriptions ("better", "roughly the same", or "worse") was firstly performed. Secondly, in cases where a symptom had improved, patients assessed the change using a Likert 7-point adjectival scale ("very slight improvement", "slight improvement", "clear improvement", "very great improvement", "near complete resolution", and "resolution"). The investigator recorded the number of tablets remaining, any adverse effects and any alteration in the patient's treatment over the period. According to the results of the rabeprazole test, the investigator classified the patient as a 'refluxer' or a 'non-refluxer'.

Esophageal pH monitoring

Esophageal pH monitoring was conducted using an ambulatory pH recording device (Synectics Mark II or III, Medtronics, Paris, France). The antimony electrode was positioned 5 cm above the cardia, located using the pH

step-up method[6] and an ambulatory recording was made. Patients were not given any particular lifestyle or dietary recommendations and were encouraged to behave as normally as possible. Patients were asked to record meals and sleeping periods, and the time of onset of symptoms. Patients returned to the referral centre 24 h later to stop the recording and remove the electrode. The data from the pH monitor were downloaded onto a computer, and the results were analyzed using a specific programme (EsopHogram Synectics software, Medtronics, Paris, France). For symptoms which had occurred during the recording period, analysis included symptom index determination (percentage of the total number of reported symptoms that were reflux-related) and probability of association as previously described[7,8]. Symptoms were considered to be reflux-related if they had occurred during the acid reflux event itself (pH < 4) or within 2 min after it had ended[9].

Evaluation and analysis of the results

Patients were classified as 'refluxers' or 'non-refluxers' according to the results of both upper GI endoscopy and pH monitoring. A diagnosis of reflux was established if one of the following criteria was present: (a) esophageal acid exposure (time below pH 4 during the 24-h period) greater than 4.2%; (b) statistically significant association between symptoms and reflux episodes ($P < 0.05$ or symptom index > 50%); and (c) presence of mucosal breaks at endoscopy.

The test (rabeprazole or a placebo) was considered to be positive or negative on the basis of the symptom response evaluated by the patient him/herself at the end of the one-week trial period. For this purpose, a 7-point adjectival Likert scale was used and the responses were dichotomised according to the cut-off descriptor of at least a "clear improvement". To further document the validity of that particular cut-off, a receiver operating characteristics (ROC) analysis of sensitivity was performed using different thresholds for the definition of a positive symptom response (CLINROC software - Metz Software, Chicago, IL, USA).

Sensitivity was defined as the proportion of 'refluxers' who had a positive test. Specificity was defined as the proportion of 'non-refluxers' for whom the test result was negative. The positive predictive value was defined as the proportion of 'refluxers' among patients with a positive test, whereas the negative predictive value was defined as the proportion of 'non-refluxers' among patients with a negative test.

Finally, all adverse events reported during the study period were also recorded for safety assessment.

Statistical analysis

As the study was mainly exploratory in nature, no prior forecast of subject number was made. The study period (January 2001 - May 2002) was also determined in order to reflect clinical practice in a primary care setting. We used mainly descriptive statistics and the results presented on the basis an intention-to-diagnose' (ITD) analysis. Student's *t*-test was used for quantitative variables and the Chi square test for qualitative variables. $P < 0.05$ was considered statistically significant.

Table 1 Characteristics of the intention-to-diagnose population in the placebo and rabeprazole groups (*n*, mean ± SD)

	Placebo (*n* = 39)	Rabeprazole (*n* = 33)	*P* value
Men/women (%)	46/54	33/67	0.269
Mean age (yr)(mean±SD)	47.1±11.8	49.1±11.9	0.4
BMI (kg/m^2) (mean±SD)	25.6±4.4	26.1±5.2	0.8
Smoker (%)	17.9	30.3	0.219
Previous endoscopy (%)	33.3	30.3	0.783
Time from first symptoms[1] (mo)	24.3±51.5	20.2±27.0	0.956
Post-prandial symptoms (%)	30.8	33.3	0.232
Nocturnal symptoms (%)	12.8	15.1	0.232
Hiatus hernia (%)	47.4	27.3	0.082
Esophagitis (%)	30.8	33.3	0.816
Barrett's oesophagus (%)	2.6	6.1	0.474
Diagnosis of GERD[2] (%)	67.6	62.1	0.642

[1]Suggesting the possibility of GERD; [2]Established by the presence of esophagitis and/or pathological exposure to acid during the 24-h period and/or significant association between symptoms and reflux.

RESULTS

Demographics and characteristics of population at inclusion

Ninety-one patients were selected. Of these patients, 83 were included, and 72 were randomized at the phase 2 visit. Among these 72 patients who completed the study and constituted the ITD population, 39 were in the placebo arm and 33 in the rabeprazole arm. Fourteen patients had at least one major deviation either at inclusion or during the course of the study (principally non-compliance with the intervals between visits and/or less than 6-d of treatment). The per-protocol population (PP), therefore, included 58 patients (33 in the placebo arm and 25 in the rabeprazole arm). In the two cohorts (ITD and PP), the most common predominant symptoms were epigastric pain, heartburn and regurgitation. At inclusion, the distribution of the predominant symptoms within the 2 groups was not significantly different. Approximately one third of patients in both groups had esophagitis, which was not of a severe grade in 90% of these subjects. None of the patients included had either stenosis or an ulcer at endoscopy. Compliance with treatment was good as 91% of tablets were taken; there was no significant difference between the two groups (Table 1). Since the results were very similar in the 2 cohorts, only the results from the ITD population are presented here. The characteristics of this population are shown in Table 1.

Six patients were unclassifiable due to pH monitoring technical failure. As a result, a definitive diagnosis of GERD or absence of GERD was made for 66 patients, 37 in the placebo group and 29 in the rabeprazole group. Forty-three were considered to be 'refluxers' (25 in the placebo arm and 18 in the rabeprazole arm) and 23 'non-refluxers' (12 in the placebo arm and 11 in the rabeprazole arm).

Diagnostic value of the rabeprazole test versus placebo test

In 'refluxers', the rabeprazole test was positive in 15 of 18 patients (sensitivity: 83.3%) whilst the placebo test was positive in 10 of 25 (sensitivity: 40.0%) ($P = 0.011$). In the 'non-refluxers', the rabeprazole test was negative in 5 of 11 patients (specificity: 45.5%) whilst placebo test was negative in 8 of 12 (specificity: 66.7%) ($P > 0.05$).

The positive and negative predictive values for the rabeprazole test were 71.4% and 62.5%, respectively. The corresponding predictive values for the placebo test were 71.4% and 34.8%, respectively ($P > 0.05$). The percentages of patients who were correctly classified by the rabeprazole test and the placebo test were 69.0% and 48.6%, respectively ($P > 0.05$).

The results of the ROC analysis are illustrated in Figure 2. The categories that were closest to the slope of the tangent to the 45° curve were those of "slight improvement" and "clear improvement". Nevertheless, the best compromise between sensitivity and specificity corresponded to the description "clear improvement" that was adopted for the former analysis.

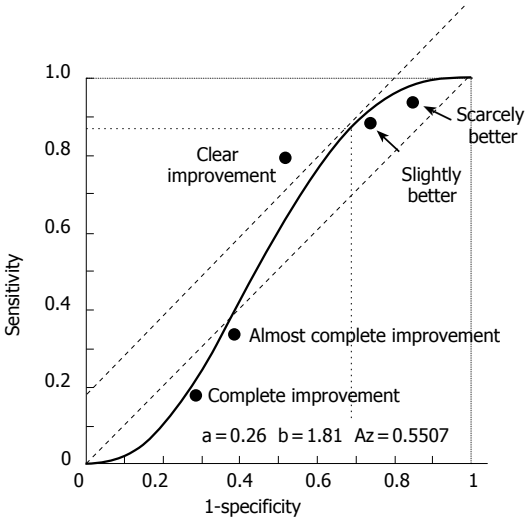

Figure 2 Receiver operating characteristics (ROC) curve. This was determined according to various satisfaction criteria (from "very slight improvement" to "resolution" of the symptoms) for patients with symptoms compatible with gastro-esophageal reflux. The symptoms were assessed after one week of treatment with a double dose of rabeprazole (20 mg bid) or a placebo.

Adverse events

Six patients (4 in the placebo arm and 2 in the rabeprazole arm) reported 7 adverse events (placebo: vomiting, diarrhoea, insomnia, dyslipidemia, urinary infection; rabeprazole: lymphadenitis, drug eruption) (non significant). No adverse event was considered to be serious.

DISCUSSION

The results of this study conducted in a primary care setting and with a placebo-controlled design showed that the rabeprazole test had high sensitivity (83.3%) but low specificity (45.5%). As compared with the placebo test, the rabeprazole test showed a superior sensitivity and negative predictive value, but non-significant differences with regard to specificity and positive predictive value.

This study is one of the first attempts to evaluate the diagnostic yield of a PPI test in primary care conditions with assessment of the results by GPs. The drug was administered for 7 d only for both practical and scientific reasons. In fact, a recent study using esomeprazole showed that although the sensitivity of the test increased each day following the start of the test, a plateau was reached after 5 d of administration, with no further improvement beyond that time point[10]. The reference tests (i.e. 24-h pH monitoring and endoscopy) were performed by independent physicians (referent) and the GP (investigator) remained blind to the results until response to PPI had been determined. In addition to acid exposure measurement, symptom analysis was also an important parameter of pH monitoring interpretation. This seems particularly relevant in a population of patients with normal endoscopy in approximately two thirds of cases. Indeed, nearly half of patients with endoscopy-negative GERD are known to have acid exposure within the normal range[11]. The use of symptom analysis permits an increase in the sensitivity of pH monitoring and therefore decreases the risk of missing genuine 'refluxers'. In addition, only patients with moderate to severe symptoms present on at least 2 occasions in the 3 d prior to inclusion were enrolled. The outcome of these rather rigorous inclusion criteria and design was a difficulty in fulfilling our initial goal of recruiting a large cohort of patients. Finally, from a number of candidate PPIs, we chose rabeprazole, since this drug has a number of potential advantages: (a) a rapid onset of action as shown by gastric pH monitoring studies[3,12,13]; (b) a good safety profile; (c) a lack of drug interactions due to its specificity in terms of hepatic and non-hepatic metabolic pathways[4,5]; and (d) its effectiveness in the treatment of some symptoms associated with GERD[14].

The results of our study are consistent with other studies of PPI tests which have without exception demonstrated the high sensitivity and poor specificity of this diagnostic approach[1]. This rather disappointing finding has recently been confirmed by the meta-analysis reported by Numans *et al*[15], which reached a similar conclusion concerning short-term trials of PPIs in GERD. The poor performance of PPI test is further reinforced by the comparison with the placebo-test which adequately 'classified' nearly half of the patients (random results). However, these results are not entirely surprising as a good placebo response has also been reported in short-term trials in GERD[10,16-18]. In addition, the above negative conclusions should be further balanced as far as negative predictive values are considered. Indeed, in the test conditions, a negative response to rabeprazole could exclude a diagnosis of GERD in 2 of 3 symptomatic patients (as compared to only one in three following the placebo). As the negative predictive value is influenced by the prevalence of the disease, our results may in fact be an underestimation and higher negative predictive values could be expected in a more representative sample of patients consulting for upper GI symptoms, supposing a 30% prevalence of GERD (i.e. approximately half of that observed in both the placebo and rabeprazole

arms of our study). Finally, our study design did not allow an appropriate evaluation of the cost-effectiveness of the PPI test as a whole and of the rabeprazole test in particular. The poor specificity of the test should, however, lead to some caution in terms of guidelines concerning long-term management strategy in GERD; there is a potential risk that 'non-refluxers' will continue to receive PPI treatment far beyond the initial test week if the symptomatic response is good. Whether such an empirical approach to acid-sensitive disorders is justified or potentially dangerous is presently unknown. Nevertheless, our data supports the conclusions of the French-Belgian consensus conference, which did not recommend the use of PPI tests for the diagnosis of GERD in clinical practice before the availability of further scientific information[19].

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RAPID COMMUNICATION

Progastrin-releasing peptide and gastrin-releasing peptide receptor mRNA expression in non-tumor tissues of the human gastrointestinal tract

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Abstract

AIM: To investigate the expression of gastrin-releasing peptide (GRP) and GRP-receptor mRNA in non-tumor tissues of the human esophagus, gastrointestinal tract, pancreas and gallbladder using molecular biology techniques.

METHODS: Poly A^+ mRNA was isolated from total RNA extracts using an automated nucleic acid extractor and, subsequently, converted into single-stranded cDNA (ss-cDNA). PCR amplifications were carried out using gene-specific GRP and GRP-receptor primers. The specificity of the PCR amplicons was further confirmed by Southern blot analyses using gene-specific GRP and GRP-receptor hybridization probes.

RESULTS: Expression of GRP and GRP-receptor mRNA was detected at various levels in nearly all segments of the non-tumor specimens analysed, except the gallbladder. In most of the biopsy specimens, co-expression of both GRP and GRP-receptor mRNA appeared to take place. However, expression of GRP mRNA was more prominent than was GRP-receptor mRNA.

CONCLUSION: GRP and GRP-receptor mRNAs are expressed throughout the gastrointestinal tract and provides information for the future mapping and determination of its physiological importance in normal and tumor cells.

Key words: Gastrin releasing peptide (GRP); Gastrin-releasing peptide receptor (GRPR); mRNA expression; Morphogenesis; Gastrointestinal tract

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INTRODUCTION

Gastrin-releasing peptide (GRP) is a member of the bombesin family of neuropeptides. Bombesin was originally isolated from the skin of the amphibian *Bombina bombina*, whereas GRP is the homologous peptide in mammals, including humans[1]. GRP and GRP-receptor are widely expressed in the central and enteric nervous systems (ENS). GRP is known to stimulate secretion of gastrin, gastric[2] and pancreatic juice[3] and hormones[4,5] to regulate the immune system[6], and to modulate smooth muscle contractility[7,8]. The direct expression of GRP and its receptor, and thus the exact mechanism behind its actions in gastrointestinal tissues, are only sparsely examined. Immunocyochemistry revealed the expression of GRP in submucosal cells of the ileum[9]. In colon, *in vitro* autoradiography showed the GRP-receptor expression in the myenteric, but not submucosal, plexus as well as on smooth muscle cells[10]. Examination of mucosal biopsies revealed GRP-receptor mRNA in cells lining the gastric antrum, but not in any other epithelial cells of the gastrointestinal tract[11].

GRP and GRP-receptor are frequently expressed in the gastrointestinal cancer cells, such as gastric adenocarcinoma[12,13], duodenal cancer[14], and colorectal cancer[15,16,17]. Cuttitta *et al*[18] have demonstrated that human cell lines derived from small-cell lung carcinomas of the lung (SCLC) proliferate in response to autocrine release of GRP. In gastrointestinal tumors, GRP-receptor activation only modestly increased tumor cell proliferation, but regulated tumor cell appearances or differentiation and, therefore, should be considered to act as a morphogen[1,16].

Based on these findings, it appears important to establish whether or not GRP and GRP-receptor mRNAs

Table 1 Biopsy specimens, sex, age, and β-actin, GRP and GRP-receptor PCR amplicons detected after exposure to X–ray films for one or five days

Experimental number	Tissue origin	Sex	Age	β-actin	GRP	GRPR	
				1 d	1 d	1 d	5 d
1	Esophagus	M	64	+	+	+	+
2	Ventricle	M	64	+	-	+	+
3	Ventricle	M	84	+	+	weak	weak
4	Duodenum	M	75	+	-	-	weak
5	Duodenum	M	63	+	+	-	weak
6	Ileum	F	78	+	+	+	+
7	Caecum	M	72	+	+	+	+
8	Colon ascendens	F	68	+	-	-	weak
9	Colon ascendens	M	83	+	-	-	weak
10	Colon ascendens	F	74	+	+	-	weak
11	Colon ascendens	M	72	+	+	-	weak
12	Colon transversum	M	79	+	+	+	+
13	Colon transversum	F	79	+	+	+	+
14	Colon transversum	F	79	+	+	+	+
15	Colon transversum	F	74	+	+	-	-
16	Colon descendent	M	79	+	+	+	+
17	Colon sigmoideum	M	69	+	+	-	-
18	Colon sigmoideum	M	86	+	+	-	weak
19	Colon sigmoideum	M	48	+	+	+	+
20	Colon sigmoideum	M	83	+	+	+	+
21	Rectum	M	81	+	+	-	weak
22	Rectum	F	54	+	-	-	-
23	Rectum	M	83	+	+	+	+
24	Gallbladder	M	58	+	-	-	-
25	Pancreas	Control		+	+	+	+
26	Stomach	Control		+	+	+	+

are expressed in non-tumor gastrointestinal tissues. In this study, we analyzed GRP and GRP-receptor mRNA expressions in the human esophagus, gastrointestinal tract, pancreas, and gallbladder by means of a reverse-transcription polymerase chain reaction (RT-PCR) technique and Southern blot analysis of the PCR amplicons.

MATERIALS AND METHODS

mRNA isolation and PCR amplification

The collection, origin and status of full thickness biopsies from the human gastrointestinal tract and surrounding tissues from surgically removed biopsies from patients undergoing surgery for gastric diseases has been described elsewhere in detail[19]. In all, 24 biopsy specimens and two control cDNAs (Table 1) were processed and analyzed for the expression of GRP and GRP-receptor mRNA. In subsequent PCR amplification experiments, mRNA and ss-cDNA preparations used were from a previous study and prepared as described recently[19].

β-actin amplification was performed in two rounds of PCR (30 and 25 cycles each time, respectively) with the same primers under cycle conditions as described above. Due to the positioning of the primers, cDNA and genomic DNA will yield β-actin fragments of different sizes (288 bp for cDNA and 400 bp for gDNA PCR amplicons, respectively). This allows monitoring for DNA contamination in ss-cDNA preparations and to assess for the integrity of the ss-cDNA used[19]. Control cDNA derived from pancreas and stomach mRNA was purchased from Clontech (BD-Biosciences, Clontech, Stockholm, Sweden).

PCR- amplification of GRP and GRP-receptor ss-cDNA

PCR was performed using a HotStarTaq Master mix kit (Qiagen, Hilden, Germany) in a final reaction volume of 25 μL. Each reaction contained 2 μL of the cDNA synthesis reaction as template. Quick-clone human pancreas and stomach cDNA (Clontech, BD Biosciences Stockholm, Sweden) were used as positive PCR amplification controls, whereas HotStar PCR amplification mix without ss-cDNA addition was used as a negative control. PCR amplification conditions, annealing temperature and primers used are shown in Table 2 and were taken from the study by Uchida *et al*[20]. For increased sensitivity, nested PCR amplifications were carried out for the detection of GRP and GRP-receptor cDNA. First round PCR amplicons were purified using a GFX PCR and Gel Band DNA Purification Kit (Amersham Biosciences, Uppsala, Sweden) and, subsequently, 1 μL was used in a nested PCR amplification. As positive PCR amplification controls, commercially available human stomach and pancreas cDNAs (Clontech, BD Biosciences Stockholm, Sweden) were included in the study. Negative PCR amplification controls (PCR mix without DNA template addition) were included to monitor possible contaminations.

Southern blot analysis of PCR amplicons

PCR amplicons were electrophoretically separated on a

Table 2 PCR-primers, expected fragment sizes and PCR amplification conditions

Primer	Sequence, 5′ to 3′ orientation	Size in bp	PCR conditions	
			[1]Cycles	Tannealing
hGRP-SE/1	AGTCTCTGCTCTTCCCAGCCTCT			
hGRP-AS/1	GCAGAACTCAGTCTCTTAGGGGT	558	30	55 ℃
hGRP-SE/2	CGTGCTGACCAAGATGTACC			
hGRP-AS/2	TCATTGCTGGTTCAGCTGGG	349	30	62 ℃
hGRPR-SE/1	AGCCCGGCATAGATCTTATCTTC			
hGRPR-AS/1	AGGGGGCAAAATCAAGGGTCAAT	1477	30	55 ℃
hGRPR-SE/2	CTCCCCGTGAACGATGACTGG			
hGRPR-AS/2	ATCTTCATCAGGGCATGGGAG	388	30	62 ℃
β-actin-SE	GCATGGAGTCCTGTCGCATCCACG			
β-actin-AS	CGTCATACTCCTGCTTGCTGATCCA	[2]288/400	30/25	55 ℃

[1]Numbers of cycles in first and second round PCR amplifications; [2]cDNA and gDNA PCR amplicon sizes, respectively.

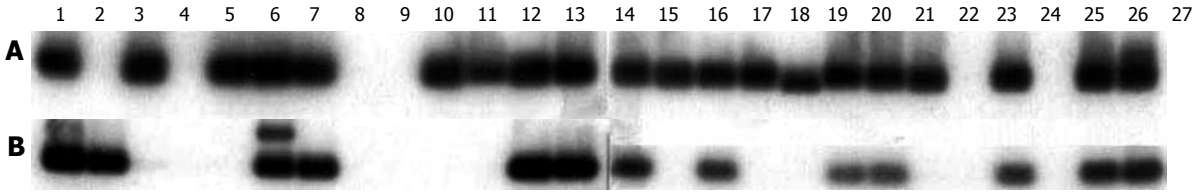

Figure 1 Southern blot hybridization analysis of nested PCR amplicons derived from ss-cDNA 1 to 26 as specified in Table 1. Lane 27 represents a negative PCR control (no ss-cDNA template added to the PCR master mix). Exposure to X-ray films was for 1 d using an intensifier screen at -70 ℃. **A:** hGRP-PCR amplicons; **B:** hGRP receptor PCR amplicons.

15 g/L agarose gel and Southern blot analysis was performed using 10 pmoles of [^{32}P]-5'-end-labelled nested primers hGRP-SE/2 and hGRP-AS/2 or hGRPR-1/SE and hGRPR-2/AS primers as hybridisation probes (Table 2) under the conditions described elsewhere[19].

RESULTS

Integrity of ss-cDNA

All analyzed ss-cDNA yielded fragments of the expected length (288 bp) after two rounds of β-actin PCR amplification and Southern blot analysis, indicating that the ss-cDNA used was essentially free of DNA contamination[19].

Differential tissue expression of GRP and GRP-receptor mRNA

Initially, first round and nested GRP and GRP–receptor PCR amplification conditions were optimized by means of annealing temperature and cycle conditions using two established, premade PCR amplification mixes (puReTaq Ready-To-Go PCR beads, Amersham Biosciences and HotStarTaq Master mix kit, Qiagen). Essentially, the HotStarTaq Master mix kit was used under the conditions described in Table 2.

Southern blot analysis (one-day exposure) of nested GRP-PCR amplicons derived from ss-cDNA revealed PCR bands of the expected size in 18 of 24 (75%) biopsy specimens and in the human stomach and pancreas control cDNA's. It appeared that GRP-PCR amplicons of two distinct sizes were present. These GRP-PCR amplicons were similar in sizes to earlier described GRP-PCR amplicons, derived from alternatively spliced GRP-mRNA[20]. However, no attempts were made to further investigate this point. Furthermore, 11 of 24 (46%) biopsy specimens and the human stomach and pancreas control cDNA yielded GRP-receptor PCR amplicons of the expected size (Figure 1, Table 1). After five-day exposure, an additional 9 of 24 (37%) biopsies yielded weak GRP-receptor PCR amplicons as judged by Southern blot analysis (Table 1), indicating a low level expression of GRP-receptor mRNA. Similarly, two tissues revealed the presence of an extra and larger GRP-receptor PCR amplicon. Its nature has not been further investigated.

No GRP and GRP-receptor PCR amplicons could be detected in the gallbladder tissue, indicating a lack of expression of these mRNAs. However, only one gallbladder biopsy was analyzed and, therefore, the result may not be conclusive since variable GRP and GRP-receptor mRNA expressions were observed in ventricle, duodenum, colon ascendens and rectum biopsies (Figure 1, Table 1). More specifically, GRP mRNA appeared to be expressed in 2 of 4 colon ascendens biopsies. In contrast, a weak band corresponding to GRP-receptor expression was detected in 4 of 4 colon ascendens biopsies after 5-d exposure, indicating a low level of GRP-receptor mRNA expression in these tissues (data not shown). Remarkably, PCR amplicons corresponding to GRP mRNA expression were detected in 4 of 4 transverse colon and 4 of 4 sigmoid colon biopsies (Figure 1). Similarly, GRP-receptor mRNA seemed to be co-expressed in 3 of 4

transverse colon and 3 of 4 sigmoid colon (Table 1).

DISCUSSION

Biopsy specimens were collected from various sites of the human gastrointestinal tract and surrounding tissues. Efforts were made to collect the biopsies from fresh, histologically normal tissues (Table 1). Our results showed that GRP and GRP-receptor mRNAs were widely expressed in the human gastrointestinal tract and surrounding tissues. It is tempting to speculate that the variation in GRP and GRP-receptor mRNA levels observed (Figure 1) may reflect a real-time mRNA expression situation. However, we can not exclude the possibility that artifacts based on sample selection (site of collecting and biopsy sizes) may contribute to the observed mRNA level variations.

Co-expression of GRP and GRP-receptor mRNA in the same tissue as observed could lend support to speculations about the existence of an autocrine and/or paracrine loop. For paracrine signaling, the communicating cells need to be in close proximity in order to establish such loops. Expressions of GRP and its receptor mRNA could be originating from different cell types in opposing parts of the tissue collected. To verify the existence of such an autocrine and/or paracrine loop, cellular co-expression of GRP and GRP-receptor mRNA and its subsequent translation into biologically active proteins must be confirmed. This could be achieved either by *in situ* hybridization or histochemistry techniques, using GRP and GRP-receptor specific hybridization probes or antibodies, respectively.

In lack of the results of such studies, the physiological importance of GRP and GRP-receptor mRNA expression in gastrointestinal tissues can only be speculated upon. However, based on earlier studies, it seems likely that GRP could act on the human colon via receptors on smooth muscle cells and gastric epithelial cells as well as on cells of the ENS[11,12]. GRP has been shown to be the primary transmitter of motor neurones to gastrin cells[2]. Pharmacological doses of GRP showed a concentration-dependent increase in the rhythmic activity of the ileocecum region[21] and evoked contractions of isolated muscle cells from jejunum[7]. Accordingly, inhibition of endogenous GRP delayed gastric emptying and gallbladder contraction[22]. In contrast, small bowel transit was prolonged by the same antagonist[22]. Thus, the effect on the small intestine may be important for mixing movements and not so much for the peristalsis. In our study, the presence of GRP and its receptor throughout the gastrointestinal tract, in addition to earlier studies that showed no expression of the peptides in the epithelial layer except gastric antrum[12] but on colonic smooth muscle cells and ENS[11], raises the hypothesis that the peptide may affect the motility along the entire GI tract. This is further underlined by the effects on smooth muscle cells[7,21]. Disturbed tissue levels of GRP have been described in patients with idiopathic intestinal pseudo-obstruction[23]. The physiology and pathophysiology behind intestinal motility and dysmotility are in many aspects unknown. It is difficult to study the physiological effect of one single peptide alone, as the ENS contains many different peptides with an important balance between them. However, GRP seems to be one of the interesting peptides in the regulation of gastrointestinal motility.

To best of our knowledge, this is the first study that describes the presence of GRP and its receptor in the human pancreas. It is in accordance with the observed effect of GRP on the secretion of pancreatic juice and pancreatic hormones[3-5]. Earlier animal studies have described that GRP is released from vagal, pancreatic nerves after stimulation[4,24]. GRP then acts by binding to a specific member of the 7 transmembrane spanning, G protein-coupled receptor superfamily where activation by GRP-receptors is coupled to phospholipase C and phospholipase D[24,25].

We were not able to detect GRP or GRP-receptor mRNA expression in the gallbladder. This may be explained by the fact that only one patient was examined. The earlier described effect of a GRP antagonist to inhibit gallbladder contraction suggests that GRP receptors are expressed in the gallbladder[22]. However, antagonists may antagonise more than one receptor, and the PCR technique in the present study was specifically examining the GRP-receptor, not similar receptors in the same family.

The effect of GRP in gastrointestinal carcinogenesis is unclear. Most of resected colon cancers aberrantly express GRP receptor mRNA[26], whereas immunohistochemically, less than three-quarters of human tumors express this protein[16]. Furthermore, not all of these receptors are functional when expressed, as only a minor amount of resected human colon cancers have been found to bind (^{125}I-Tyr^{4}) bombesin when studied pharmacologically[27]. The discrepancy between GRP-receptor mRNA and protein expression may be due to the frequency with which the coding sequence for this receptor is mutated[17]. Some authors suggest that GRP acts as a mitogen and increases tumor cell proliferation[18], while others have found that GRP/GRP-receptor co-expression in cancer promotes the development of a well-differentiated phenotype and is therefore more a morphogen than a mitogen[1]. Multiple studies suggest that the presence of these two peptides confers a survival advantage[14,16].

In conclusion, GRP and GRP-receptor mRNA appear to be expressed throughout the human gastrointestinal tract. This provides information for the future mapping of GRP and GRP-receptor expression at the cellular level, and thereby further determination of its physiological importance in normal and tumor cells.

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RAPID COMMUNICATION

Effects of ranitidine for exercise induced gastric mucosal changes and bleeding

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Abstract

AIM: To evaluate the effect of ranitidine on gastric mucosal changes and on GI bleeding in long distance runners.

METHODS: Twenty-four long distance runners (M: 16, F: 8, age: 18.2 ± 1.5 years) participated in this study. A symptom questionnaire, stool hemoccult test, and upper gastrointestinal (GI) endoscopy were performed on the subjects prior to the study. The subjects took oral ranitidine (150 mg, b.i.d.) for two weeks. The upper GI endoscopy and stool Hemoccult tests were repeated after the treatment.

RESULTS: Twenty-two of the 24 runners had at least one upper GI mucosal lesion before the medication. The Endoscopic improvements were seen in eleven of the 14 cases of erosive gastritis and four of the 5 cases of esophagitis. Six subjects were Heme occult positive prior to the study, but only one was positive after the medication.

CONCLUSION: Gastric mucosal lesions and GI bleeding in long distance runners seem to be associated to acid-related factors mediated by the high level of regular running. Ranitidine seems to be and effective prophylaxis to prevent gastric mucosal lesions and GI bleeding.

Key words: Endoscopy; Exercise; Gastrointestinal bleeding; Ranitidine

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INTRODUCTION

Long-distance running has become a popular exercise during the past decade, and the number of people participating in endurance racing has increased. It has been reported that troublesome gastrointestinal (GI) symptoms associated with endurance running events has been occurring more frequently[1,2]. Although the magnitudes of these symptoms are unknown, incidence rate varies from 10 to 81%, depending on factors such as the type, duration, and intensity of the exercise studied[3].

GI bleeding is not an uncommon finding after long distance races. Some of runners[3,4] who experienced macroscopic fecal blood and microscopic bleeding has been found in 8 to 87% of the runners[5,6]. Running-related GI mucosal changes such as gastric ulcer[7], hemorrhagic gastritis[8], erosive gastritis[9], ischemic colitis[10] were observed by endoscopy. These changes may contribute to runner's anemia and, if sustained, may possibly result in an iron deficiency that may potentially affect the recovery and performance of competitive runners. The mechanism by which hemorrhagic gastritis and ischemic colitis develop in runners is unknown, but it is usually attributed, in part, to ischemia. One report showed that running reduced visceral blood flow by up to 80% of pre-exercise level[11]. Another possible mechanism for the hemorrhagic gastritis is acid secretion[12]. Moderate-intensity running is not generally thought to alter gastric acidity[13] whereas heavier exertion paradoxically reduces acid secretion[14]. But some reports have suggested that acid reducing agents, i.e. cimetidine or ranitidine, might be useful in preventing running-associated

gastrointestinal bleeding and symptoms[6,15]. However, there has been only one case study regarding running related GI mucosal changes before and after treatment[15]. We, therefore, performed an observational trial to evaluate the effect of ranitidine on gastric mucosal changes and GI bleeding in long distance runners by endoscopy.

MATERIALS AND METHODS

Study subjects

Twenty-four professional long distance runners (16 male, 8 female, age range: 16-19 years) participated in this study. The Human Investigations Committee of Wonkwang University Hospital approved the protocol and each subject signed an informed consent prior to the study.

The subjects were well trained and had several years of experience in competitive running. They underwent a strict training program of 200 km of running per week and participated in three long-distance running competitions per year. All subjects were instructed to avoid nonsteroidal anti-inflammatory drugs, red meat, alcohol, iron and vitamin supplementation for at least 2 wk prior to the study

Symptom questionnaire

The questionnaire was designed with yes/no questions and a graded value scale (0: none = no symptom, 1: mild = intermittent symptoms but not interfere with the running, 2: moderate = want treatment for symptoms but rarely interfere with the running, 3: severe = want treatment for symptoms and interfere with the running) for the following symptoms: diarrhea, abdominal cramps, epigastric discomfort, epigastric pain, nausea, vomiting, and regurgitation related to running.

GI bleeding test

The presence of GI bleeding was tested before and after the study with hemeoccult test (LA Hemochaser, MIZUHO, Japan) by looking for the presence of agglutination after mixing the feces with a latex reagent within a 3 min.

Study protocols

Gastroscopy was performed on all subjects prior to the study. No pre-medication was used except for lidocaine (2 mL of viscous Xylocaine) as a local anesthetic. Two endoscopists performed the upper GI endoscopy with a flexible gastroscope (Pentax EG-2730, 9.0 mm diameter, Japan). Macroscopic observations of gastric mucosal damage were noted but not graded. *Helicobacter pylori* (*H pylori*) infection was evaluated by a rapid urease test of the one biopsy specimen obtained during endoscopy. The subjects with abnormal endoscopic findings took 150 mg of oral ranitidine (30 min postprandial, b.i.d.). The subjects commenced regular training for 2 wk while taking the medication. A second endoscopy was given to the subjects who had abnormal upper GI mucosal findings before the treatment.

Table 1 Gastrointestinal symptoms in runners (n = 24)

	None	Mild	Moderate	Severe	Percentage of symptomatic subjects (%)
Diarrhea	9	13	1	1	63
Abdominal pain	10	12	2		58
Epigstric discomfort	10	12	2		58
Epigastric pain	12	10	2		50
Nausea	14	8	2		42
Regurgitation	16	8			33
Vomiting	19	4	1		21

RESULTS

Symptom questionnaire

The results of the symptom questionnaire are summarized in Table 1. Only two subjects had no GI symptoms related to running. The most frequently experienced GI symptom was diarrhea (n = 15), followed by abdominal pain (n = 14), epigastric discomfort (n = 14), and epigastric pain (n = 12).

Gastroscopy findings

The results of the endoscopic examination before and after the treatment are listed in Table 2. Twenty-two of the 24 runners had at least one upper GI mucosal lesion, including erosive gastritis (n = 17), esophagitis (n = 8), alkaline reflux gastritis (n=8), and gastric ulcer (n = 1). Of the seventeen subjects with mucosal erosions, four subjects had stigmata of bleeding. Mucosal erosions were localized to the antrum (n = 11), gastric body (n = 8), fundus (n = 6), and gastric angle (n = 2). Sixteen out of 22 runners who had abnormal upper GI mucosal findings consented to have a second endoscopy after medication. Gastroscopic improvements were seen in eleven of the 14 cases of erosive gastritis, four of the 5 cases of esophagitis, but only one of the 5 cases of alkaline reflux gastritis.

GI bleeding

Six of the 24 participants were heme occult positive before the medication. Only one was positive after the medication (Table 2).

Helicobacter pylori status

Nine of the 24 participants were rapid urease test positive (Table 2). But there was no relation between *H pylori* status and endoscopic findings.

DISCUSSION

This study reported a high incidence of running related GI symptoms and the results confirmed the previous studies[2]. Moses reported that symptoms of the lower GI tract were more prevalent than those from the upper GI tract in most endurance events[16] and others found that frequency of GI symptoms were much higher during marathon running and/or triathlons than during other endurance sports,

Table 2 Endoscopic findings and hemoccult test before and after treatment with ranitidine

Subject/Sex	Before treatment (n = 24)			After treatment (n = 16)	
	Endoscopic findings	Hp status	OB	Endoscopic findings	OB
1/M	Alkaline reflux gastritis Erosive gastritis (upper body)	–	+	Procedure refused	–
2/M	Esophagitis, LA class A Alkaline reflux gastritis Erosive gastritis (fundus)	–	–	Erosive gastritis (antrum and body)	–
3/M	Esophagitis LA class A Erosion with blood clot (antrum)	–	+	Normal finding	–
4/M	Esophagitis, LA class A Alkaline reflux gastritis	+	+	Alkaline reflux gastritis	+
5/M	Erosive gastritis (fundus, body, antrum)	+	+	Normal finding	–
6/M	Esophagitis, LA class A Alkaline reflux gastritis	–	–	Procedure refused	
7/M	Erosive gastritis (fundus, body)	+	–	Procedure refused	
8/M	Gastric ulcer, Erosion with blood clot (midbody)	–	+	Normal finding	–
9/M	Erosive gastritis (fundus)	+	–	Normal finding	–
10/M	Erosive gastritis (fundus)	+	–	Erosive gastritis (body)	–
11/M	Esophagitis, LA class A Alkaline reflux gastritis	+	–	Procedure refused	
12/M	Erosive gastritis (body, antrum) Duodenitis	–	–	Procedure refused	
13/M	Normal finding	–	–	Procedure not performed	
14/M	Esophagitis, LA class A	+	–	Procedure refused	
15/M	Erosive gastritis (antrum)	–	–	Normal finding	–
16/M	Erosive gastritis (body, antrum)	–	–	Erosive gastritis (antrum)	–
17/M	Alkaline reflux gastritis, Erosive gastritis with blood clot (angle)	–	–	Alkaline reflux gastritis	–
18/F	Duodenitis	–	–	Normal finding	–
19/F	Esophagitis, LA class A Alkaline reflux gastritis Erosive gastritis (antrum)	–	–	Alkaline reflux gastritis	–
20/F	Erosion with blood clot (midbody)	–	+	Normal finding	–
21/F	Esophagitis, LA class A Erosive gastritis (fundus)	–	–	Esophagitis, LA class A	–
22/F	Alkaline reflux gastritis Erosive gastritis (antrum)	–	+	Alkaline reflux gastritis	–
23/F	Normal finding	+	–	Procedure not performed	
24/F	Erosive gastritis (antrum)	+	–	Normal finding	–

M, male; F, female; Hp, *Helicobacter pylori*; OB, occult blood; +, positive; -, negative; LA, Los Angeles.

i.e. cycling, rowing, and swimming[3,17].

The duration of the exercise can be an important factor. Peters *et al*[18] showed that during a protocol of alternately cycling and running, the number of subjects with GI symptoms increased exponentially with time. They also reported that running induced more GI symptoms when compared to cycling[18]. Another factor thought to influence the exercise induced GI symptoms is the intensity of the exercise. It has been reported that as intensity increases, gastric emptying is delayed[13,19] and splanchnic blood flow decreased[11]. Other factors such as mechanical trauma of the gut[20], intestinal permeability[21], and absorption[22] may also be affected by the intensity of the exercise.

The existence of gastrointestinal bleeding associated with endurance running has previously been demonstrated[2,23]. Sullivan[2] reported bloody stools after endurance events, and another study demonstrated the high frequency of occult GI bleeding, in which 23% of runners converted from blood test negative to blood test positive using the Hemeoccult card[23]. Stewart *et al*[24] reported that 83% of runners had positive stool occult blood after competitive running and the presence of iron-deficiency anemia possibly caused by multiple etiologies including GI bleeding.

While GI blood loss can be clearly attributed to running, its etiology or pathophysiology is not readily apparent. McMahon *et al*[25] suggested an ischemic etiology for the blood loss. They studied 32 runners after a marathon, and showed that six runners, who developed hemoccult-positive stools after a marathon race, were significantly younger and had faster race times when compared to those

with hemoccult-negative stool.

Another possible mechanism for the development of GI bleedings may be mucosal damages caused by gastric acid secretion[12]. Very little data exists regarding acid secretion during exercise and immediately following exercise due to the technical difficulties in such measurements during the exercise. Several reports have suggested that cimetidine or ranitidine might be useful in preventing running associated GI bleeding[6,8,15]. Our study found that six out of 24 participants had positive heme occult positive before medication and each of these 6 subjects had abnormal endoscopic findings before medication. After 2 wk of medication, five out of these 6 subjects became heme occult negative and had improved endoscopic findings (3 normal, 2 partial improvement, 1 dropout). These findings strongly suggest that GI bleeding induced by long distance running is closely related to gastric acid secretion, and reducing acid output with ranitidine seems to be an effective treatment.

With the wide spread popularity of this kind of sport, these complications might be more frequently observed than one would expect, and physicians should be aware of them when evaluating runners with occult GI bleeding with anemia.

The actual site of GI bleeding associated with running is uncertain, but some reports have identified bleeding lesions in the stomach and colon by endoscopy[7-10]. The most frequently reported endoscopic abnormalities in runners are erosive gastritis with bleeding stigmata in the stomach[8,9] and erosion with hemorrhage in the colon[10]. GI mucosal erosions have mainly been found in the corpus region of the stomach[9,15] where stress or shock associated gastritis is normally seen[26]. In contrast, one study has shown abnormal histological features in gastric antral mucosa with decreased mucosal secretion[27]. To our knowledge, only one case report using endoscopy has demonstrated that exercise induced hemorrhagic lesions were improved after taking a therapeutic course of cimetidine[15].

Our study shown that twenty-two of the 24 runners had at least one upper GI mucosal abnormality, of which seventeen subjects had mucosal erosions, and four had stigmata of bleeding. These mucosal erosions were found mainly in the antrum, but also in the gastric body, fundus, and angle. Sixteen of 24 runners had follow-up endoscopy after 2 wk medication of ranitidine. The endoscopic improvements were seen in eleven subjects with erosive gastritis.

Gastroesophageal reflux occurs more frequently with exercise than at rest[28]. Some GI symptoms occurring with exercise can be of esophageal origin, including chest pain and heartburn. These phenomena might be caused by reduced salivary secretion, which results in poor acid clearance[30]. In addition, esophageal ischemia, due to a decrease in visceral blood flow during exercise, may play an important role in development of esophagitis[11]. Esophageal acid exposure may be reduced by a histamine H2-receptor antagonist during running[29]. However, there are no reports describing exercise-induced esophagitis assessed by endoscopy. Long-term implications of gastro-esophageal reflux in the habitual runner or exerciser are also unknown. Our study showed that eight of the 24 runners had esophagitis before treatment and four out of 5 who received a second endoscopy had improvements after medication. This suggests that gastroesophageal reflux associated with running is also closely related to acid secretion.

In conclusion, this is the first study that was evaluated by endoscopy on running associated GI symptoms before and after acid reducing medication. Our results have shown that mucosal abnormalities, such as erosive gastritis and esophagitis in long distance runners are related to gastric acid. Therefore, acid suppressive agent is an effective treatment for running related GI symptoms.

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RAPID COMMUNICATION

Risk factor analysis for metaplastic gastritis in Koreans

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Abstract

AIM: To conduct a retrospective study to determine the risk factors for development of metaplastic gastritis in Korean population.

METHODS: The database of 113449 subjects who underwent a gastroscopy for the purpose of a regular check-up at center for health promotion, Samsung medical center during 5 years was collected and retrospectively analyzed. Among them, 5847 subjects who had endoscopically diagnosed as a metaplastic gastritis or 10076 normal as well as answered to questionnaire were included for present study. The subjects were divided into 2 groups; Group I, normal and Group II, metaplastic gastritis. Age, gender, *Helicobacter pylori* (*H pylori*) seropositivity, body mass index (BMI), family history of cancer, smoking, alcohol consumption, total daily calories, folate and salt intake and dietary habit (out-eating, overeating, irregular eating) were retrieved from questionnaire or electronic medical record and compared between group I and group II.

RESULTS: The prevalence of group II was 11% (13578/113449) increasing its prevalence with age ($P = 0.000$). But, there was no significant association between 2 groups in BMI, family history of cancer, alcohol consumption, total daily calories, folate and salt intake and dietary habit (out-eating, overeating, irregular eating). Old age ($P = 0.000$), male gender ($P = 0.000$), *H pylori* seropositivity ($P = 0.010$) and current smoker ($P = 0.000$) were significantly more common in group II at multiple logistic regression model.

CONCLUSION: Our data suggested that old age, male gender, *H pylori* seropositivity and smoking were risk factors for metaplastic gastritis, precancerous lesion of gastric cancer.

Key words: Intestinal metaplasia; Risk factors

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INTRODUCTION

Gastric cancer was recognized as the first leading cause of cancer death in Korea[1] and attention turned to epidemiologic associations between risk factors and gastric cancer. In the early 1970s, Correa formulated a multi-step model of gastric cancer, which postulated a temporal sequence of pathologic changes that led from chronic gastritis to atrophic gastritis, intestinal metaplasia and dysplasia and the eventual development of gastric cancer[2]. Chronic gastric inflammation seems to be the critical common cause of gastric cancer[3].

The purpose of this paper was to determine the risk factors for development of metaplastic gastritis, precursor of gastric cancer in Korean population

MATERIALS AND METHODS

Data collection

The database of 113449 subjects who underwent a gastroscopy for the purpose of a regular check-up at center for health promotion, Samsung medical center from January 2001 through June 2004 was collected and retrospectively analyzed. Among them, 13578 subjects were endoscopically diagnosed as a metaplastic gastritis and 10521 subjects was endoscopically diagnosed as a normal. But, only 5847 subjects who had endoscopically diagnosed as a metaplastic gastritis were answered to questionnaire whereas 10076 subjects who had endoscopically diagnosed as a normal were answered to questionnaire. Both 5847 metaplastic gastritis and 10076 normal were included for present study. Subjects with peptic ulcer or erosion of stomach were excluded in this study population. The subjects were divided into 2 groups; Group I, normal and Group II, metaplastic gastritis. Demographic data [age, gender, body mass index (BMI), family history of cancer] and life style data [smoking, alcohol consumption, total daily calorie intake, folate intake, salt intake, dietary habit (out-eating, overeating, irregular eating)] were retrieved from question-

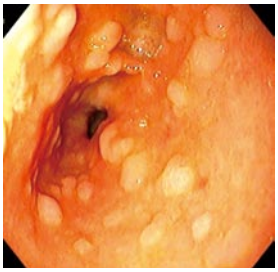

Figure 1 Endoscopic finding showed that atrophic mucosa with surface nodularity was generally diagnosed as a metaplastic gastritis.

naire or electronic medical records and compared between group I and group II. Those who have family history of any cancer were those who had a reply to yes in the question ("Do you have a blood relation who has a experience of diagnosis as cancer by a doctor?"). Data including smoking, alcohol consumption (frequency and amount of alcohol), dietary habit (out-eating, overeating, irregular eating) were collected from self administered questionnaire. Patients were asked whether they were active, past or never smoker. The subjects were divided into two groups (current smoking group and non-smoking group) by current smoking status. Nonsmoking group is composed of past or non-smoking. The subjects were divided into two groups by frequency and amounts of liquors (namely, *so-ju)* consumption in questionnaire. Over or equal 3-4 frequencies a week and $\geqslant$ 80 g once alcohol consumption is defined as the group of heavy alcohol consumption. Below 3-4 frequencies a week or $<$ 80 g once alcohol consumption is classified as the other group. Total amount of calorie, folate intake and fat intake were obtained in diet surveys. *Helicobacter pylori (H pylori)* seropositivity was retrieved from electronic medical record. *H pylori* infection was determined by measuring serum *H pylori* IgG antibodies. Specific anti- *H pylori* antibodies were measured with an enzyme-linked immunosorbent assay (ELISA) kit using an antigen (RADIM SpA, Pomezia, Italy). The sensitivity and specificity of this assay was reported to be 79% and 83%, respectively[4].

Criteria for metaplastic gastritis

Atrophic mucosa with surface nodularity on the endoscopic finding was diagnosed as metaplastic gastritis (Figure 1). 3354 individuals who showed endoscopically metaplastic gastritis had also a presence of metaplasia histologically on the updated Sydney system.

Statistical analysis

Logistic regression analysis was conducted by fixing the each group as a dependent variable and risk factors as independent variables. Continuous variables such as age, BMI, total daily calorie intake, folate intake and salt intake were analyzed by *t*-test. Gender, smoking, alcohol consumption, family history of cancer, dietary habit (out-eating, overeating, irregular eating) and *H pylori* seropositivity were analyzed by χ^2 test. The relative risk to develop metapalstic gastritis was calculated with odds ratio with 95% confidence interval. Risk factors were examined by multiple logistic regression analysis. Statistical significance was assumed at $P<0.05$. Statistical analyses were performed with SAS version 8.1 (SAS Institute Inc, Cary, NC, USA).

Table 1 Comparison of characteristics of normal and metaplastic gastritis (simple logistic regression model)

	Normal (%)	Metaplastic gastritis (%)	*P* value
Age	42.46±10.15	53.20±9.00	0.000
Sex			0.000
Men	3804 (36)	3983 (68)	
Women	6272 (64)	1864 (32)	
BMI[1]	24.52±2.36	23.97±2.86	0.208
Family history of cancer	160 (1)	125 (2)	0.065
Smoking			0.000
None or past	2933(84)	3308 (74)	
Current	550 (16)	1130 (26)	
Alcohol			0.059
Heavy alcoholics[2]	553 (15)	747 (16)	
Out-eating	2477 (23)	1535 (26)	0.110
Over eating	1743 (16)	912 (15)	0.598
Irregular eating	1824 (17)	1152 (19)	0.228
Total calories	2102.94±470.17	2226.46±486.22	0.515
Folate	246.34±91.24	289.51±108.55	0.211
Salts	22.48±7.27	23.02±4.97	0.319
H pylori seropositivity[3]	4165 (39)	3566 (60)	0.007

[1]BMI, body mass index.
[2]Over or equal 3-4 frequencies a week and $\geqslant$ 80 g once alcohol consumption is defined as the group of heavy alcoholics.
[3]*Helicobacter pylori* is abbreviated as *H pylori*.

RESULTS

Characteristics of study subjects (Table 1)

The prevalence of group II was 11% (13 578/113 449) increasing its prevalence with age ($P=0.000$). But, there was no significant association between 2 groups in BMI, alcohol consumption and family history of cancer. Male gender was a risk factor for metaplastic gastritis and current smokers were more likely to have metaplastic gastritis than none or past smokers. Neither dietary composition (folate, salts, calories) nor dietary habits (out-eating, overeating, irregular eating) was associated with metaplastic gastritis. *H pylori* seropositivity was more common in the group II.

Multiple logistic regression analysis (Table 2)

Finally, we conducted stepwise multiple logistic regression analysis in which above significant variables were used as independent variables. Number of subjects entered into the stepwise multiple logistic regression model were 4438 in group II and 3483 in group I. Entered variables were age, gender, BMI, family history of cancer, smoking, alcohol consumption, total daily calorie intake, folate intake, salt intake, dietary habit (out-eating, overeating, irregular eating), and *H pylori* seropositivity.

Old age ($P=0.000$), male gender ($P=0.000$), *H pylori* seropositivity ($P=0.010$) and current smoker ($P=0.000$) were significantly more common in the group II at multiple logistic regression model.

DISCUSSION

Gastric cancer was recognized as the first leading cause of

cancer death in Korea[1] and many epidemiologic studies about risk factors for gastric cancer were reported[5,6]. So far, we had no a large-scale epidemiologic studies about metaplastic gastritis, precursors of gastric cancer in the Korean population.

In the early 1970s, Correa formulated a multi-step model of gastric cancer, which postulated a sequence from chronic atrophic gastritis, intestinal metaplasia, dysplasia and gastric cancer[2]. Chronic gastric inflammation leads to repetitive injury and repair resulting in hyperplasia[3]. Whereas acute injury and inflammation associated with healing are usually self-limited, chronic injury or inflammation leads to a sustained expansion of tissue proliferation[7-10]. Sustained tissue proliferation is generally accepted as a risk factor for cancer[3]. As is well known, metaplastic gastritis is precursors of gastric cancer.

Our understanding of gastritis and cancer underwent a marked shift with rediscovery of *H pylori*[7-13]. *H pylori* is now thought to account for most of gastritis[13] whereas *H pylori* infection is not an only important factor for development for gastric cancer[5]. But, it is not clear whether *H pylori* infection is also important for development for metaplastic gastritis. Our study demonstrated that *H pylori* seropositivity is an independent risk factor for the metaplastic gastritis.

In the intestinal type of gastric cancer, environmental factors other than *H pylori* infection seem to play a part in the carcinogenesis[12]. Environmental factors may facilitate the development of atrophic gastritis and intestinal metaplasia[12,14]. Based on epidemiologic studies of dietary histories, the first step in the Correa pathway was believed to be initiated by a diet rich in salt and nitrates/nitrites as well as deficiencies in fresh fruits and vegetables[5]. Dietary factors and continued effects of chronic inflammation were felt to be responsible for the progression from gastritis to atrophy, metaplasia, dysplasia and carcinoma[12]. Ingestion of sodium chloride is thought to promote gastric carcinogenesis[14]. Exposure to N-nitroso compounds probably facilitates advancement of chronic atrophic gastritis and intestinal metaplasia in adulthood[5]. Our study showed that neither dietary habits, salts nor folate intakes is a risk factor for the metaplastic gastritis. The major limitation of our study is that diet survey used in our study is carried out by not-validated questionnaire.

Both superficial and chronic atrophic gastritis are common in alcoholics[6]. Alcohol consumption can also cause acute gastritis[15]. Our study demonstrated that alcohol is not a risk factor for the metaplastic gastritis. Male dominance of the metaplastic gastritis can be explained, in which male gender tends to have more dangerous environmental factors such as smoking. But, our study demonstrated that male gender and smoking are independent risk factors for the metaplastic gastritis, respectively.

First, the limitation of present study is selection-bias. The substantial numbers of subjects did not have information about all items of the questionnaire. Numbers of subjects entered into the multiple logistic regression model were 4438 (4438/13578, 32%) in group II and 3483 (3483/10521, 33%) in group I. The other limitation of our study is inter-examiner or intra-examiner bias of endoscopic diagnosis about metaplastic gastritis and normal. To ascertain the precision in endoscopic diagnosis of gastritis, we undertook a pilot study on 10 individuals. Endoscopic finding was obtained 2 times on 10 individuals by 2 examiners. We examined the inter-examiner bias between 2 examiners and intra-examiner bias between 2 examinations by same examiner. Kappa value of intra-examiner was 1.0 ($P=0.002$), 0.737 ($P=0.016$), respectively. Kappa value of inter-examiner was 0.875 ($P<0.001$).

Our data suggested that old age, male gender, *H pylori* seropositivity and smoking were risk factors for metaplastic gastritis, precancerous lesion of gastric cancer.

Table 2 Multiple logistic regression analysis about risk factors for development of metaplastic gastritis

Variable[1]	Odds ratio (95% CI[2])	*P* value
Age	8.945 (7.204-11.105)	0.000
Male gender	1.144 (1.133-1.155)	0.000
Current smoking	2.137 (1.693-2.697)	0.000
H pylori[3] seropositivity	1.223 (1.049-1.426)	0.010

[1]All variables in the model are as follows: age, sex, body mass index, family history of cancer, smoking, alcohol consumption, total daily calorie intake, folate intake, salt intake, dietary habit (out-eating, overeating, irregular eating), and *H pylori* seropositivity.
[2]CI, confidence interval.
[3]*Helicobacter pylori* is abbreviated as *H pylori*.

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RAPID COMMUNICATION

Gastric cancer patients at high-risk of having synchronous cancer

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Abstract

AIM: To identify patients with a high-risk of having a synchronous cancer among gastric cancer patients.

METHODS: We retrospectively analyzed the prospective gastric cancer database at the National Cancer Center, Korea from December 2000 to December 2004. The clinicopathological characteristics of patients with synchronous cancers and those of patients without synchronous cancers were compared. Multivariate analysis was performed to identify the risk factors for the presence of a synchronous cancer in gastric cancer patients.

RESULTS: 111 of 3291 gastric cancer patients (3.4%) registered in the database had a synchronous cancer. Among these 111 patients, 109 had a single synchronous cancer and 2 patients had two synchronous cancers. The most common form of synchronous cancer was colorectal cancer (42 patients, 37.2%) followed by lung cancer (21 patients, 18.6%). Multivariate analyses revealed that elderly patients with differentiated early gastric cancer have a higher probability of a synchronous cancer.

CONCLUSION: Synchronous cancers in gastric cancer patients are not infrequent. The physicians should try to find synchronous cancers in gastric cancer patients, especially in the elderly with a differentiated early gastric cancer.

Key words: Synchronous cancer; Gastric cancer; Colorectal cancer

Lee JH, Bae JS, Ryu KW, Lee JS, Park SR, Kim CG, Kook MC, Choi IJ, Kim YW, Park JG, Bae JM. Gastric cancer patients at high-risk of having synchronous cancer. *World J Gastroenterol* 2006; 12(16): 2588-2592

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INTRODUCTION

Gastric cancer is the most common form of cancer in Korea[1]. The overall age-standardized incidence rates of gastric cancer in 2002 were 69.6 per 100 000 among males and 26.8 per 100 000 among females. Despite the improved prognosis of gastric cancer resulting from early diagnosis, radical operations, and the development of adjuvant therapy, gastric cancer remains the second most common cause of cancer death worldwide[2,3]. In 1995, early gastric cancer (EGC) accounted over 30% of patients who underwent gastric cancer surgery in Korea and this percentage continues to increase[4]. As the prognosis of patients with EGC is excellent and age at diagnosis for gastric cancer is increasing, there is a greater risk that patients will also have a second primary cancer[5,6].

Second primary cancer influences the prognosis of gastric cancer patients, and because primary or secondary prevention is the best way to cure cancer, some investigators have focused on the characteristics of second primary cancers in gastric cancer patients[7-10]. However, few studies have been performed in this regard, and most of these studies are limited to metachronous cancers or the treatment-related second primary malignancies of gastric cancer patients[11,12]. The detection of synchronous cancers gives us the opportunity to treat both cancers simultaneously using less invasive techniques and thus to beneficially influence the prognosis and quality of life of these patients.

The aim of this study was to find a means of identifying gastric cancer patients at risk of having a synchronous cancer.

MATERIALS AND METHODS

Subjects

We retrospectively analyzed the prospective gastric cancer database at the National Cancer Center (NCC), Korea from December 2000 to December 2004. A total of 3291 gastric

Table 1 Clinicopathological features of patients with or without synchronous cancer

Characteristics	Synchronous cancer With *n* (%)	Synchronous cancer Without *n* (%)	*P* value
Age (yr, mean±SD)	64.6±9.5	59.4±12.5	<0.001
Sex			0.002
Male	90 (81.1)	2144 (67.4)	
Female	21 (18.9)	1036 (32.6)	
Multiplicity			0.009
Single	106 (95.5)	3146 (98.9)	
Multiple	5 (4.5)	34 (1.1)	
Preoperative Stage			<0.001
Early gastric cancer	62 (55.4)	1135 (35.7)	
Advanced gastric cancer	50 (44.6)	2045 (64.3)	
Differentiation			<0.001
Differentiated	94 (84.7)	1383 (43.4)	
Undifferentiated	17 (15.3)	1797 (56.6)	

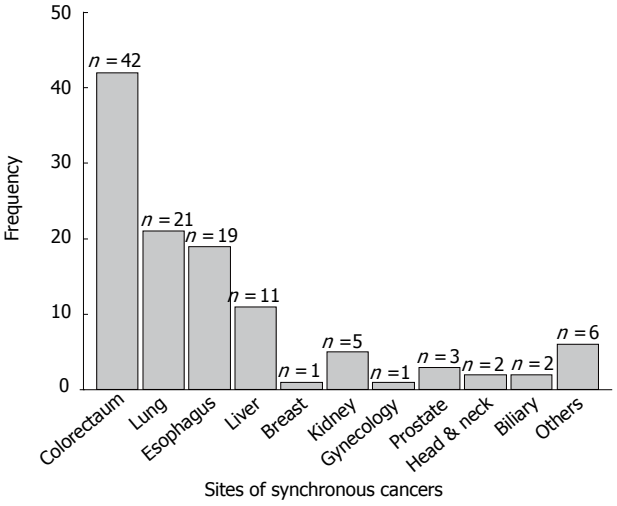

Figure 1 Site distribution of synchronous cancers.

cancer patients were registered (the registered patients were consecutive patients who had ever visited out patient clinic in the Center for Gastric Cancer with a diagnosis of gastric cancer or who were diagnosed as having gastric cancer at our center) during the study period. Gastric and synchronous cancers were all pathologically confirmed.

Methods

Synchronous cancers were defined as cancers detected in organs other than the stomach, which were diagnosed at the time of or within 6 mo of the first cancer diagnosis[13]. For each synchronous cancer we ruled out of the possibility that synchronous cancer represented metastasis of gastric cancer by histologic examination. Clinicopathological characteristics including age, sex, histological classification, a preoperative diagnosis of early or advanced gastric cancer, and multiplicity of gastric cancer were compared for patients with and without synchronous cancers. As for histological classifications, tubular carcinoma and papillary adenocarcinoma were classified as differentiated types, whereas poorly differentiated adenocarcinoma, mucinous adenocarcinoma, and signet ring cell carcinoma were classified as undifferentiated types[14]. The characteristics of synchronous cancers including operability and when they were diagnosed were separately analyzed.

Statistical analysis

All statistical analyses were performed using the statistical software 'Statistical Package for Social Sciences' (SPSS) version 10.0 for Windows (SPSS, Inc, Chicago, IL). Inter-group (for patients with and without synchronous cancer) comparisons of clinicopathological variables were made using the Student t-test for continuous variables and using the two-tailed Chi-square test for discrete variables.

Risk factors influencing synchronous cancers were determined by logistic regression analysis. Hazard ratio as determined by multivariate analysis, was defined as the ratio of the probability that an event (with synchronous cancer or not) would occur to the probability that it would not occur. The predicting powers of covariates were expressed by calculating hazard ratios with a 95% confidence interval. The accepted level of significance was $P < 0.05$.

RESULTS

Clinicopathological characteristics of the patients

Among the 3291 gastric cancer patients, 111 patients (3.4 %) had a synchronous cancer other than in the stomach; 2 patients (2/111 patients, 1.7%) had three cancers and the others had a single synchronous cancer. The mean age of patients with a synchronous cancer was higher than that of patients without (64.6 ± 9.5 *vs* 59.4 ± 12.5, t = -0.453, $P < 0.001$) and male patients were more common among those with synchronous cancers (χ^2=9.81, P=0.002). Differentiated type and early gastric cancer were more common among patients with synchronous cancers (χ^2=73.97, $P < 0.001$, χ^2=19.65, $P < 0.001$ respectively, Table 1).

Characteristics of synchronous cancers

The most common synchronous cancer was colorectal cancer (42 cases, 37.2%), followed by the lung (21 cases, 18.6%), esophagus (19 cases, 16.8%), and liver cancer (11cases, 9.7%) (Figure 1, Table 2). Gastric cancer was the most common form of cancer among colorectal cancer patients with a synchronous cancer. Of the 2 triple cancer patients, one patient had esophageal cancer and prostate cancer and the other patient had lung cancer and colorectal cancer. Forty-three patients (38.1%) were diagnosed with gastric cancer and synchronous cancer simultaneously and 45 patients (39.8%) were diagnosed with synchronous cancers before gastric cancer, and 25 cases (21.1%) were diagnosed after receiving a diagnosis of gastric cancer.

Operation of gastric cancer

Of the 111 patients with synchronous gastric cancers, 73 (64.6%) underwent gastric cancer surgery. Despite having a potentially resectable gastric cancer, 38 patients (34.2%) were treated non-surgically because of advanced

Table 2 Distribution of synchronous cancer according to gastric cancer location and diagnosis

Location	Time interval between the diagnoses of synchronous and gastric cancers			
	6 mo ~ (n=45)	Simultaneous (n=43)	~ 6 mo (n=25)	Total (n=113[1], %)
Colorectal	28	6	8	42 (37.2)
Lung	3	11	7	21 (18.6)
Esophagus	8	11	0	19 (16.8)
Liver	2	8	1	11 (9.7)
Breast	0	0	1	1 (0.9)
Kidney	0	2	3	5 (4.4)
Gynecologic	0	1	0	1 (0.9)
Prostate	1	0	2	3 (2.7)
Head and neck	1	1	0	2 (1.8)
GB, bile duct	0	2	0	2 (1.8)
Others	2	1	3	6 (5.3)

[1]Two patients have double synchronous cancers.

synchronous cancer (19 patients, 50.0%), an advanced gastric cancer (6 patients, 15.8%), or patient refusal (11 patients, 28.9%), and other causes (2 patients).

Logistic regression analysis of risk factors

Logistic regression analysis identified age at gastric cancer diagnosis, differentiation, and early or advanced gastric cancer as independent risk factors of synchronous gastric cancer. The incidence of synchronous cancer in elderly patients (≥60 years) with differentiated early gastric cancer was 9.3% (43/418 patients), while that in other patients was 2.4% (68/2 830 patients). Moreover, the incidence of colon cancer in elderly patients (≥60 years) with differentiated early gastric cancer was 3.5% (15/418 patients, Table 3).

Table 3 Logistic regression analyses of risk factors

Covariate	β	SE	RR (95% CI)	*P* value
Age, yr (< 62 *vs* ≥ 62)	0.471	0.223	1.601 (1.035-2.477)	0.035
Sex (Male *vs* Female)	-0.430	0.250	0.650 (0.398-1.061)	0.085
Histology (Diff. *vs* Undiff.)	- 1.691	0.276	0.184 (0.107-0.316)	< 0.001
Multiplicity (Single *vs* Multiple)	0.288	0.412	1.334 (0.595-2.994)	0.484
Stage (EGC *vs* AGC)	-0.431	0.202	0.650 (0.437-0.966)	0.033

SE: Standard error; RR: Relative risk; CI: Confidence interval.

DISCUSSION

The main findings of this study are; 1) That synchronous cancer has an incidence of 3.4% (111/3 291 patients) in gastric cancer patients; 2) That the most common type of synchronous cancer in gastric cancer patients is colorectal cancer (42 patients, 37.2%), followed by lung cancer (21 patients, 18.6%); and 3) That age at diagnosis, a differentiated gastric cancer, and early gastric cancer are risk factors of synchronous cancer in gastric cancer patients. The incidence of synchronous cancer has been reported to vary from 0.7% to 3.5%[7-12]. Reasons for these wide ranges of incidence are attributed to different study populations and methods. The lowest incidence of synchronous cancers in gastric cancer patients reported was 0.7%, and this study was a population-based study, whereas, the other studies were institution-based, like the present study. The relatively high incidence of synchronous cancer in our study (3.4%), despite the inclusion of patients with EGC and AGC, may be due to time. Most studies were performed before 2000, whereas the patients included in this study were diagnosed as having gastric and synchronous cancer from 2001. Thus, radiologic diagnostic tools, such as, computed tomography (CT) and positron emission tomography (PET) were considerably developed and diagnostic accuracy improved over the intervening period[15,16]. Another possible explanation might be that this study was conducted as a retrospective analysis of a prospective database, and that our institution is a cancer center and as such diagnostic efforts are more focused on the detection of cancers.

In the present study, colorectal cancer was the most common cancer among gastric cancer patients with a synchronous cancer. On the contrary, gastric cancer was the most common form of cancer among colorectal cancer patients with a synchronous cancer. This association between colorectal cancer and gastric cancer may be incidental; however, there is some basis to support the existence of such a relation. It is well known that gastric cancer is the second most common extra-colonic malignancy associated with hereditary non-polyposis colorectal cancer (HNPCC) syndrome[17]. Moreover, a defect in the mismatch repair system has been suggested to play a role in the development of multiple cancers, but mechanistic basis for the development of synchronous cancers is unclear[18]. Preoperative endoscopy is a routine procedure in the Center for Colorectal Cancer in NCC for the patients with a colorectal cancer.

Most patients with synchronous colorectal cancers in this study were diagnosed within one month prior to receiving a diagnosis of gastric cancer, or were diagnosed while gastric cancer diagnosis. Patients with colorectal cancer frequently have symptoms of obstruction or bleeding, whereas the symptoms of gastric cancer patients are rare and vague[19,20]. In addition, esophagogastrodu odenoscopy (EGD) in colorectal cancer patients is one of our institutional policies. Thus, it is frequently the cases that the stages of gastric cancer and associated colorectal cancer are early and advanced, respectively. The incidences of most cancers are higher in men than in women and tend to increase with age, and patients with synchronous cancer in this study were more commonly elderly males. These findings are consistent with those of previous studies and might be associated with recent trends in gastric cancer epidemiology in Korea and characteristics of gastric cancer[1,2,4]. The high incidence of synchronous cancers found among early gastric cancer patients in the present study might be associated with the increasing incidence of early gastric cancer in Korea, and the fact that patient diagnosed as having colorectal cancer undergo routine gastroscopy. However, the reason for the higher incidence of differentiated

type in gastric cancer patients with synchronous cancer is unclear, and requires further investigation.

The incidence of synchronous cancer in those with multiple gastric cancers tended to be higher than for those with a single gastric cancer. Thus, genetic instability such as microsatellite instability might be involved in the development of synchronous cancer and multiple gastric cancers. However, the multiplicity of gastric cancer proved not to be a risk factor of synchronous cancer by multivariate analysis.

Regardless of the fact that they had potentially curable gastric cancer, 38 (35.4%) patients did not undergo an operation, and the presence of an advanced synchronous cancer was a principal reason. The major factor influencing treatment plans was cancer stage. Therefore, urgent efforts should be made to identify those at high-risk of having a synchronous cancer.

Multivariate analysis showed that age at gastric cancer diagnosis of, tumor differentiation, and preoperative stage are risk factors for the presence of synchronous cancer in gastric cancer patients. Elderly patients ($\geqslant$ 60 years) with differentiated type of early gastric cancer had a synchronous cancer incidence of 9.3% and a synchronous colon cancer incidence of 3.5%. This result suggests that elderly patients with differentiated type of early gastric cancer should be assessed for the presence of a synchronous cancer. Most synchronous cancers, such as, colorectal, lung, esophageal, and liver cancers can be discovered during routine preoperative staging work up using EGD and radiologic examinations, such as, chest X-ray or abdomino-pelvic CT. However, the early detection of colorectal cancer, which proved to be the most common synchronous cancer type, requires colonoscopy.

Despite the fact that this study was conducted based on an analysis of a prospective database, not all patient information was recorded, because almost one third of the patients were not treated at our institution. Therefore, the preoperative stage was not always the same as the pathological stage. However, considering that the accuracy of preoperative staging for EGC or AGC is $>80\%$, we believe that our results would have been comparatively unchanged had we adopted pathological stage[21].

Colonoscopy is not routinely performed at our center in gastric cancer patients scheduled to undergo an operation or endoscopic mucosal resection. Because the indications for endoscopic mucosal resection are a differentiated cancer of <2cm in diameter, those patients that undergo EMR present a group at high-risk of having a synchronous cancer[22]. Moreover, it is likely that the incidence of synchronous colon cancer may have been higher if we had performed routine colonoscopy in these patients. We now plan to perform a routine colonoscopic examination on those determined by this study to be at high-risk.

In conclusion, synchronous cancers in gastric cancer patients are not infrequent. Considering the trend that the peak age of gastric cancer patients and the incidence of early gastric cancer are increasing, the present study cautions that physicians should try to find synchronous cancers in gastric cancer patients at risk, such as, in elderly patients with differentiated early gastric cancer.

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RAPID COMMUNICATION

An inherent acceleratory effect of insulin on small intestinal transit and its pharmacological characterization in normal mice

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Abstract

AIM: To study an inherent effect of insulin on small intestinal transit and to explore involvement of various systems/mechanisms in normal mice.

METHODS: Insulin at the doses of 2 μU/kg, 2 mU/kg, 2 U/kg or vehicle was subcutaneously administered to four groups of overnight fasted normal male mice. Blood glucose (BG) levels were measured 2 min before insulin administration and 2 min before sacrificing the animals for the measurement of small intestinal transit (SIT). Charcoal meal was administered (0.3 mL) intragastrically 20 min after insulin administration and animals were sacrificed after 20 min and SIT was determined. For exploration of the various mechanisms involved in insulin-induced effect on SIT, the dose of insulin which can produce a significant acceleration of SIT without altering BG levels was determined. The following drugs, atropine (1 mg/kg), clonidine (0.1 mg/kg), ondansetron (1 mg/kg), naloxone (5 mg/kg), verapamil (8 mg/kg) and glibenclamide (10 mg/kg), were administered intravenously 10 min prior to the administration of insulin (2 μU/kg).

RESULTS: The lower doses of insulin (2 μU/kg and 2 mU/kg) produced a significant acceleration of SIT from 52.0% to 70.7% and 73.5% without lowering blood glucose levels ($P < 0.01$), while the highest dose of insulin (2 U/kg) produced a fall in blood glucose levels which was also associated with significant acceleration of SIT ($P < 0.01$). After pretreatment of insulin (2 μU/kg) group with atropine, insulin could reverse 50% of the inhibition produced by atropine. In clonidine-pretreated group, insulin administration could reverse only 37% of the inhibition produced by clonidine and inhibition of SIT was significant compared with vehicle + insulin-treated group, i.e. from 74.7% to 27.7% ($P < 0.01$). In ondansetron-pretreated group, insulin administration could produce only mild acceleration of SIT (23.5%). In naloxone-pretreated group, insulin administration could significantly reverse the inhibition of SIT produced by naloxone when compared with naloxone *per se* group, i.e. from 32.3% to 53.9% ($P < 0.01$). In verapamil-pretreated group, insulin administration could only partially reverse the inhibition (65%). In glibenclamide-pretreated group, insulin administration produced further acceleration of SIT (12.2%).

CONCLUSION: Insulin inherently possesses an acceleratory effect on SIT in normal mice. Adrenergic and cholinergic systems can play a significant role. Calcium channels and opioidergic system can play a supportive role; in addition, enhancement of endogenous insulin release can augment the effect of exogenously administered insulin on SIT.

Key words: Adrenergic system; Blood glucose levels; Ca^{2+} channels; Cholinergic system; Insulin; Intestinal transit; Opioid system; Serotonergic system

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INTRODUCTION

Insulin is the drug of choice in the management of elevated blood glucose level in type 1 and sometimes in type 2 diabetes mellitus[1]. In addition to its effect on glucose metabolism, insulin is reported to act as a neuromodulator in the central nervous system[2] and as a

mild analgesic[3]. Takeshita and Yamaguchi[4] characterized an inherent antinociceptive response for insulin when administered subcutaneously in mice. Their study has also shown that the antinociceptive response is independent of hypoglycemic action of insulin.

Gastrointestinal (GI) disorders are common in diabetic patients[5]. About 75% of these patients suffer from GI disorders due to GI neuropathy leading to considerable morbidity[6,7]. These GI disorders include nausea, gastric stasis, constipation, fecal incontinence and diarrhea[8,9]. Clinical studies are available that report insulin therapy increases the gastric emptying through hypoglycemic effect[10,11], but no detailed report is available in normal experimental animals on its effect on small intestinal transit and mechanism involved. In this study, we, therefore, investigated an inherent effect of insulin on small intestinal transit in normal mice and explored the involvement of possible mechanisms. The outcome of this experiment may provide valuable insights into the effect of insulin on normal intestinal transit.

MATERIALS AND METHODS

Animals

Adequate number of randomly bred normal/healthy adult Swiss albino male mice, weighing between 20-25 g, were obtained from Central Animal House, JIPMER, Pondicherry. One week before the study, the animals were housed at Departmental Animal House in polypropylene cages under standard laboratory conditions. Animals were fasted overnight prior to the experiment in cages with mesh bottom and had free access to water. Experiments were performed during the day time (09:00 to 18:00). The experimental protocol was approved by JIPMER Institutional Animal Ethics committee.

Drugs and chemicals

Atropine injection IP (S K Parenterals Pvt. Ltd., Tetali), clonidine HCl (C.H. Boehringer Sohn Ingelhein, Germany), glibenclamide (Hoechst India Ltd., Bombay), gum acacia IP (Hikasu Chemicals, Mumbai), insulin injection IP (purified bovine insulin, 40 U per milliliter; Knoll Pharmaceuticals Ltd., Aslai, India), naloxone HCl (Endo Labs, USA), ondansetron (Cipla Ltd., Mumbai), verapamil (Torrent Laboratories Pvt. Ltd., Ahmedabad), wood charcoal (SD Fine Chemicals, Boisar) were used in this study. All the drugs were dissolved in sodium chloride injection IP except glibenclamide which was dispersed in 50 g/L Tween 80 in water for injection before administration.

Administration of insulin

Mice were randomly divided into four groups, each group consisted of 6 mice. Each group was subcutaneously (sc) administered insulin 2 μU/kg, 2 mU/kg, 2 U/kg or vehicle. Blood glucose level was recorded before insulin administration. Charcoal meal was administered 20 min after insulin administration and SIT was determined after 40 min.

Measurement of small intestinal transit

The small intestinal transit (SIT) was determined by identifying leading front of intragastrically (ig) administered marker in small intestine of an animal[12,13]. Charcoal meal marker was freshly prepared by dispersing 10 g of wood charcoal in 50 g/L gum Acacia mucilage. After 20 min of insulin administration, each mouse received 0.3 mL of this suspension intragastrically using metallic oral cannula. After 20 min, animals were sacrificed by intravenous administration of sodium pentobarbital (100 mg/kg), abdomen opened, the leading front of marker was identified in the small intestine and tied immediately to avoid movement of marker. The entire length of small intestine was isolated by cutting at pyloric and ileocaecal ends. The distance travelled by the charcoal meal and the total length of the intestine were measured in cm. The SIT was expressed as percentage (%) of the distance travelled by the charcoal meal to length of the intestine. This was carried out in the animals 40 min after insulin administration.

Measurement of blood glucose

Blood glucose (BG)[14] was measured by placing a drop of blood obtained by tail venipuncture, over an appropriate glucostix, read by Advantage Glucometer (Boerhinger Mannheim Corporation, Indianapolis, USA) and expressed as %, change in the glucose level considering the initial value of that animal as 100. This estimation was done 2 min before insulin/vehicle administration and 2 min before sacrificing the animal for measuring small intestinal transit.

Mechanisms of insulin-induced acceleration of small intestinal transit

Insulin at 2 μU/kg dose significantly accelerated (35.7%) small intestinal transit without affecting the blood glucose level; hence this dose was selected to evaluate the mechanism of insulin-induced intestinal hypermotility (Table 1). For exploring the various systems/mechanisms involved in insulin-induced effect on SIT, the antagonists or agonists (agents) of the following systems were attempted (Figure 1).

Cholinergic system

Involvement of cholinergic system was evaluated by using a well studied non-specific cholinergic antagonist atropine. The dose selection was based on a study reported by Chaudhuri *et al*[15]. Atropine (1 mg/kg) was injected intravenously (iv) 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT. Another group was treated similarly but with vehicle + insulin (Figure 1).

Adrenergic system

Involvement of adrenergic system was evaluated by using clonidine (0.1 mg/kg iv). The dose selection was based on studies reported by Donoso *et al*[16] and DiTullio

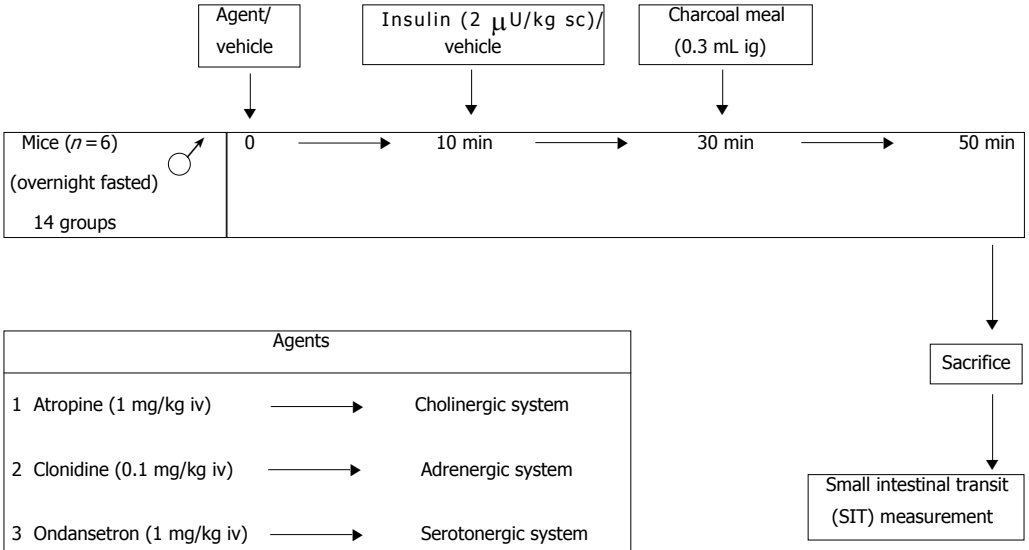

Figure 1 Experimental design carried out to explore various systems/mechanisms involved in insulin-induced acceleration of small intestinal transit in normal mice.

Table 1 Effect of insulin administration on small intestinal transit and blood glucose levels in normal mice

Treatment Insulin (U/kg sc)	% SIT[1]	Blood glucose (%)[2]
Vehicle	52.06 ± 1.48	103.73 ± 4.19
2 μ	70.77 ± 0.62[a]	99.47 ± 1.59
2 m	73.53 ± 0.63[a]	94.54 ± 4.62
2	80.10 ± 1.93[a]	42.09 ± 1.78[a]

Each value represents the mean ± SE (n = 6) sc: subcutaneous; SIT: small intestinal transit; [a]P < 0.01 *vs* vehicle treated group; [1]Values are % SIT considering the total length of the intestine as 100, SIT was determined 40 min after insulin administration; [2]Blood glucose was estimated 2 min before insulin/vehicle administration and 2 min before sacrificing the animals for the measurement of small intestinal transit (SIT), final blood glucose expressed as % considering the initial blood glucose of each animal as 100.

et al[17]. Clonidine (0.1 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Serotonergic system

Involvement of serotonergic system was evaluated by using ondansetron (1 mg/kg iv). The dose selection was based on a study reported by Nagakura *et al*[18]. Ondansetron (1 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Opioidergic system

Involvement of opioidergic system was evaluated by using naloxone (5 mg/kg iv). The dose selection was based on studies reported by Stein *et al*[19] and Peana *et al*[20]. Naloxone (5 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc). After 40 min of insulin injection, the animals were sacrificed to measure SIT (Figure 1).

Calcium channels

Involvement of calcium channels was evaluated by using verapamil (8 mg/kg iv). The dose selection was based on studies reported by Ramaswamy *et al*[21] and Amos *et al*[22]. Verapamil (8 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc). After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Insulin secretogogue

Influence of elevated endogenous insulin levels on SIT was evaluated by using glibenclamide (10 mg/kg iv). The dose selection was based on studies reported by Ramaswamy *et al*[23] and Ojewole *et al*[24]. Glibenclamide (10 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Statistical analysis

Results are expressed as mean ± SE and analyzed statistically using ANOVA, followed by Dunnett's multiple comparisons test. A P < 0.05 was considered statistically significant.

RESULTS

Effect of insulin on small intestinal transit

Insulin administration at lower doses (2 μU/kg and 2 mU/kg) produced a significant acceleration of SIT without altering the blood glucose levels (P < 0.01 Table 1), while the higher dose of insulin (2 U/kg) produced a profound fall in blood glucose levels (P < 0.01) which was associated with an acceleration of SIT.

Cholinergic system

Atropine (1 mg/kg) *per se* produced an attenuation of SIT by 47.0% when compared with vehicle treated group. In

atropine-pretreated group, insulin administration (2 μU/kg) could not completely reverse the inhibition produced by atropine. Insulin could overcome only 50% of the inhibition produced by atropine (Table 2 and Figure 2).

Adrenergic system

Clonidine (0.1 mg/kg) *per se* produced an inhibition of SIT by 72.0%. Conversely, in clonidine-pretreated group, insulin administration (2 μU /kg) could partially reverse (37%) the inhibition produced by clonidine but the inhibition of SIT was still significant (P <0.01) when compared with vehicle + insulin-pretreated group (Table 2 and Figure 2).

Serotonergic system

Ondansetron (1 mg/kg) *per se* could not alter the SIT when compared with vehicle-treated group. Conversely, in ondansetron-pretreated group, insulin administration (2 μU/kg) could produce only mild acceleration of SIT (23.5%) (Table 2 and Figure 2).

Opioidergic system

Naloxone (5 mg/kg) *per se* produced a significant inhibition of SIT by 41.2% when compared with vehicle-treated group. In naloxone-pretreated group, insulin administration (2 μU/kg) could significantly reverse the inhibition produced by naloxone (P < 0.01) when compared with naloxone *per se* group (66.4%). However, the reversal of inhibition was still significantly lower than insulin *per se* group (P <0.01) (Table 2 and Figure 2).

Calcium channels

Verapamil (8 mg/kg) *per se* produced a significant deceleration of SIT by 26.0% when compared with vehicle-treated group. In verapamil-pretreated group, insulin administration (2 μU/kg) could only partially reverse the deceleration produced by verapamil (65%) (Table 2 and Figure 2).

Insulin secretogogue

Glibenclamide (10 mg/kg) *per se* produced a significant acceleration of SIT by 43.8% when compared with vehicle-treated group. In glibenclamide-pretreated group, insulin administration (2 μU/kg) produced a further acceleration of SIT by 12.2% (Table 2 and Figure 2).

DISCUSSION

Recent experiments in streptozotocin (STZ)-induced diabetes in rats have demonstrated deleterious effects on the neuromuscular junction as well as on muscle itself. Actions at both sites may contribute to neuropathy and functional alterations in muscle contractile properties[25,26]. Abnormalities in gastric emptying and small intestinal motor function were also reported in STZ-treated rats[27,28]. This may be ascribed to the ability of STZ, which not only destroys β-cells of pancreas leading to diabetic state, but also affect the nervous system function which maintains the tone and motility of GI smooth muscles. Hence, STZ-induced experimental diabetic model became untenable to explore the effect of any agent on SIT, instead normal or healthy animals are appropriate for exploring inherent effect of any substance on SIT. Therefore, the data obtained can reflect the true changes and are devoid of the influence of degenerative changes induced by STZ in laboratory animals.

Our study with insulin administration demonstrated an interesting finding that after 40 min of its administration in normal mice, lower doses of insulin (2 μU/kg and 2 mU/kg) significantly accelerated the small intestinal transit (P< 0.01) without lowering blood glucose levels. On the other hand, the highest dose of insulin (2 U/kg) produced a significant fall in blood glucose levels which was also associated with an acceleration of SIT (P<0.01). The available literature indicated that insulin-induced hypoglycemia accelerates gastric emptying of solids and liquids in long-standing type 1 diabetes[29] or stimulates gastric vagal activity causing an increase in gastric emptying in healthy volunteers[30]. These effects on gastric emptying were dependent on blood glucose levels[29,30]. Moreover, these findings were observed with normal or higher doses of insulin. In our experiment, the blood glucose levels were not affected by the lower doses of insulin (2 μU/kg or 2 mU/kg) but produced a significant acceleration of SIT. The sub-hypoglycemic doses of insulin were used to avoid the hypoglycemic effect on intestinal motility. We suggest that the blood glucose levels may not play a significant role in accelerating SIT at least in the lower doses of insulin, indicating an inherent acceleratory effect of insulin on SIT. Our findings are partly in agreement with Takeshita and Yamaguchi[4] who reported antinociceptive effect of insulin was independent of blood glucose level in normal mice. It seems that insulin therapy may bring about an additional benefit in diabetic patients by normalizing the derangement of the gastrointestinal motility.

The gastrointestinal tract is in a continuous state of contraction, relaxation and secretion. These functions are controlled by neurohumoral systems, which in turn are regulated by various receptor systems, such as cholinergic, adrenergic, serotonergic, opioidergic and cell surface channels[31]. Many drugs affect GI transit by acting as agonists or antagonists at specific cellular receptors[32,33]. Acceleration of GI motility can be achieved by direct stimulation of gastrointestinal muscle, by activation of excitatory neural pathways or by inhibition of inhibitory pathways. Deceleration can be produced by direct relaxant effect on smooth muscle, by inhibiting the excitatory neural pathways or by activation of inhibitory pathways. Insulin's inherent acceleration of SIT can be evaluated by exploring the following systems.

Cholinergic system

Atropine is frequently used as a tool for identifying mechanisms involving cholinergic pathways[34]. It is a non-specific competitive antagonist of acetylcholine for muscarinic receptors and abolishes the effects of acetylcholine completely on the GI tract. Both in normal subjects and in patients with GI diseases, full therapeutic doses of atropine (0.5-1 mg) produce definite and prolonged inhibitory effect on the motor activity of the

Table 2 Influence of various systems / mechanisms on insulin-induced acceleration of small intestinal transit in normal mice

Pretreatment Agent/vehicle (mg/kg iv)[1]	Treatment Insulin (2 μU/kg sc)[2]	% SIT mean ± SE	% Acceleration	% Inhibition
Vehicle	Vehicle	55.10 ± 1.46	--	--
Vehicle	Insulin	74.76 ± 1.40[a]	35.68[3]	--
Atropine (1)	Vehicle	29.19 ± 3.28[a]	--	47.02[3]
Atropine (1)	Insulin	37.32 ± 3.58[b]	--	50.08[4]
Clonidine (0.1)	Vehicle	15.50 ± 1.46[a]	--	71.86[3]
Clonidine (0.1)	Insulin	27.71 ± 4.78[b]	--	62.93[4]
Ondansetron (1)	Vehicle	51.69 ± 1.13	--	6.18[3]
Ondansetron (1)	Insulin	63.84 ± 5.88	--	14.60[4]
Naloxone (5)	Vehicle	32.39 ± 2.56[a]	--	41.21[3]
Naloxone (5)	Insulin	53.90 ± 2.95[b]	--	27.90[4]
Verapamil (8)	Vehicle	40.72 ± 1.72[a]	--	26.09[3]
Verapamil (8)	Insulin	48.61 ± 3.27[b]	--	34.97[4]
Glibenclamide(10)	Vehicle	79.23 ± 1.52[a]	43.79[3]	--
Glibenclamide(10)	Insulin	88.88 ± 3.52	18.88[4]	--
Glibenclamide(10)	Insulin	88.88 ± 3.52	61.30[3]	--

Each value represents the mean ± SE (n = 6) or %.
[a]P < 0.01 *vs* vehicle + vehicle group.
[b]P < 0.01 *vs* vehicle + insulin group.
[1]Agent/vehicle administered 50 min before SIT measurement.
[2]Insulin/vehicle administered 40 min before SIT measurement.
[3]compared with vehicle + vehicle group.
[4]compared with vehicle + insulin group.

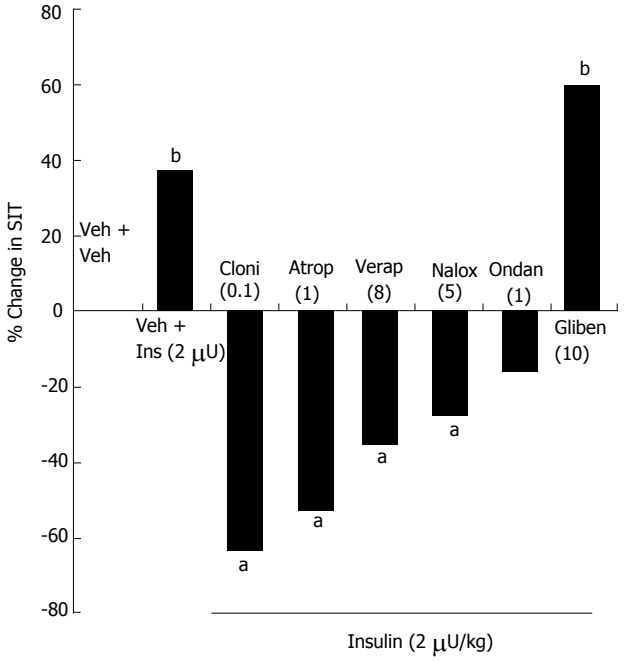

Figure 2 Involvement of various mechanisms in insulin-induced acceleration of SIT in normal mice. Each value represents % acceleration of SIT. [b]P < 0.01 *vs* vehicle + vehicle-treated group [insulin (2 μU/kg); glibenclamide (10 mg/kg)] or % inhibition of SIT. [b]P < 0.01 *vs* vehicle + insulin-treated group (2 μU/kg) (data was derived from Table 2). Cloni (0.1): clonidine (0.1 mg/kg); Atrop (1): atropine (1 mg/kg); Verap (8): verapamil (8 mg/kg); Nalox (5): naloxone (5 mg/kg); Ondan (1): ondansetron (1 mg/kg).

stomach, duodenum, jejunum and ileum[35]. Our study also confirmed inhibitory effect of atropine on SIT in normal mice. When atropine-injected group (1 mg/kg) was treated with insulin (2 μU/kg), insulin failed significantly to reverse the inhibition of SIT induced by atropine when compared with insulin *per se* treated group (Table 2). This finding indicates that insulin acts directly through muscarinic receptors to accelerate the SIT. Since atropine at the given dose could produce 50% inhibition of the insulin effect on SIT, this indicates a possibility that insulin could partly produce acceleratory effect by some other pathways in addition to cholinergic pathways as atropine could not completely prevent the acceleratory effect of insulin (Figure 2).

Adrenergic system

Clonidine has presynaptic α_2 receptor agonistic activity. Stimulation of α_2 receptors which are present on excitatory cholinergic intramural neurons in the intestine[36,37] attenuates the release of acetylcholine presynaptically, thereby producing depression of intestinal motility. In our study, we observed a significant attenuation of SIT in clonidine (0.1 mg/kg) *per se* treated group when compared with vehicle-treated group (P < 0.01), thus confirming the inhibitory effect of clonidine on SIT in mice.

When clonidine-injected group (0.1 mg/kg) was treated with insulin (2 μU/kg), the SIT was inhibited by 63% as compared with vehicle + insulin-treated group (Figure 2), thereby indicating that even though insulin could partially reverse the SIT (37%), the inhibitory effect of clonidine was still significant when compared with insulin *per se* treated group (P < 0.01) (Table 2). This finding indicates α-adrenergic pathways may be dominant in producing acceleratory effect of insulin on SIT. Insulin might have interfered with presynaptic α_2 receptors and facilitated the release of acetylcholine from excitatory neurons in the intestine. Since clonidine could not completely inhibit the SIT, we suggest that in addition to α-adrenergic pathways, insulin action may be associated with any other pathways but playing a minor role.

Serotonergic system

5-Hydroxy tryptamine (5-HT) receptors are broadly classified into five subtypes: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7[38]. Of these principal receptors, 5-HT_3 receptor has been suggested to play a role in the regulation of gastrointestinal motor function in many animal species and humans[39]. In the mouse ileum, 5-HT_3 receptors modulate neuronal activity within the enteric nerves leading to the contraction of the smooth muscle. The selective antagonists of 5-HT_3 receptors block the depolarizing actions of 5-HT on vagal afferents in gastrointestinal tract and have been proved to be a major breakthrough in the control of chemotherapy and radiotherapy-induced emesis[40,41]. In our study, a selective 5-HT_3 receptor antagonist, ondansetron (1 mg/kg), *per se* could not alter the normal SIT. Indeed, we selected the dose of ondansetron (1 mg/kg) which had significantly increased whole gut transit time in mice[18]. On the other hand, in ondansetron-pretreated group, insulin administration (2 μU/kg) slightly accelerated SIT (23.5%) (Table 2). This finding indicates that at this dose, ondansetron might rather partially inhibit the insulin-induced acceleration on SIT (14.6%) (Figure 2). Ondansetron (1 mg/kg) might have a prominent effect on the large intestine of mice. Hence, we propose that dose-

dependent studies are required to show the involvement of serotonergic systems with ondansetron in small intestinal motility and to evaluate insulin's acceleratory effect on SIT in this animal model.

Opioidergic system

Opioid neurons constitute the largest population of peptide-containing neurons in the myenteric plexus of the gut[42]. Opioid receptors are present on enteric nerves, epithelial cells and muscle cells[43,44]. It is known that opioid agonists slow intestinal transit particularly at the proximal portion and also reduce luminal secretions[45]. The administration of naloxone will antagonize or reverse the actions of opioids and of similar agents[44]. Therefore, the use of naloxone is expected to produce normal SIT, followed by administration of any opioids. In contrast, we observed that naloxone (5 mg/kg) *per se* produced significant attenuation of SIT ($P < 0.01$) in this animal species. This finding indicates that naloxone acts similar to opioids (Table 2).

In support of this finding, we came across similar contrasting reports but on gastric emptying. Champion *et al*[46] reported that naloxone (2 mg) delayed gastric emptying of radio-opaque material in healthy volunteers. They suggested that naloxone inhibited endogenous opiate system which normally stimulates gastric emptying and they had used the dose of naloxone two to three times greater than those usually given to reverse narcotic-induced respiratory depression and in large doses, naloxone itself may inhibit gastric emptying. Similarly, Asai and Power[47] also reported naloxone (0.01-10 mg/kg) *per se* significantly inhibited gastric emptying in rats. These studies revealed effect of naloxone on gastric emptying, but our study reveals a similar finding in the small intestine. Our study with naloxone *per se* may also suggest that naloxone may inhibit a subset of endogenous opiate system which normally stimulates movement of the contents of the intestine in this animal species. We used higher dose of naloxone to block all the receptors, thereby masking the effects of endogenous opioids, and to explore whether any subset of opioid receptors were involved in insulin-induced acceleration of SIT.

When naloxone-injected group (5 mg/kg) was treated with insulin, the SIT was significantly reversed as compared with naloxone *per se* treated group ($P < 0.01$) (Table 2). However, the naloxone-induced inhibition of SIT (28%) in insulin-treated group was still significant when compared with insulin *per se* treated group (Figure 2). This finding suggests that insulin may act through a subset of opioid receptors in the intestine to accelerate the SIT and that might be inhibited by naloxone treatment.

Calcium channels

Calcium is involved in the initiation of contraction of smooth muscle[48]. The visceral smooth muscle has a poorly developed sarcoplasmic reticulum and the increase in intracellular calcium concentration is primarily due to Ca^{2+} influx from the extracellular fluid via voltage-gated Ca^{2+} channels[49]. The L-type calcium channel is present in many cells and it is the main source of Ca^{2+} for contraction of smooth muscle[50]. This channel is blocked by dihydropyridines such as nifedipine, and other drugs such as verapamil and diltiazem[50]. Verapamil, a phenylalkylamine derivative, blocks the calcium channels on the surface of smooth muscle cells and relaxes the smooth muscle, thereby attenuating the intestinal motility[45,51]. The objective of this experiment was to evaluate the involvement of Ca^{2+} in insulin-induced acceleration of SIT. Verapamil at the given dose (8 mg/kg) *per se* significantly inhibited SIT, indicating the involvement of Ca^{2+} channels in normal physiology of small intestinal motility (Table 2). In verapamil-pretreated group, insulin administration could reverse the inhibition produced by verapamil by 65%. This finding may indicate that as insulin could not completely reverse inhibition of calcium channels, some other systems are also involved in acceleratory effect of insulin action on SIT.

Insulin secretogogue

Glibenclamide, an oral hypoglycemic drug, acts by stimulating insulin release from β-cells of pancreas[1]. The objective of this experiment was to find whether endogenously released insulin can potentiate the action of exogenously administered insulin effect on SIT. Glibenclamide (10 mg/kg) *per se* produced a significant acceleration of SIT by 43.8% (Table 2). This finding may indicate that endogenous release of insulin by glibenclamide increases the SIT.

When insulin-injected group was pretreated with glibenclamide, the SIT was further accelerated by 12% (Figure 2). This observation indicated that insulin from endogenous sources might have contributed to the additional acceleration of SIT by exogenously administered insulin.

Figure 2 indicates the significant involvement of following systems in decreasing order of acceleratory effect of insulin on SIT: adrenergic system > cholinergic system > calcium channels > opioidergic system ($P < 0.01$). In addition, release of endogenous insulin augments the effect of exogenously administered insulin on SIT.

In conclusion, the sub-hypoglycemic doses (2 μU/kg or 2 mU/kg) of insulin accelerate the small intestinal transit in normal mice without markedly changing blood glucose levels. It can be assumed that therapy of type 1 diabetes with insulin can simultaneously relieve at least one of the diabetic GI complications, such as constipation or sometimes may aggravate the diabetic neuropathy-associated diarrhoea. Furthermore, we explored influence of various systems, channels and endogenous insulin in acceleratory effect of exogenously administered insulin. Based on these observations, we report that adrenergic and cholinergic pathways play a significant role in hypermotility of small intestine induced by insulin administration in mice. Calcium channels and opioidergic pathways play supportive role; in addition, enhancement of endogenous insulin release can augment the effect of exogenously administered insulin on SIT.

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RAPID COMMUNICATION

Comparison of clonogenic assay with premature chromosome condensation assay in prediction of human cell radiosensitivity

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Abstract

AIM: To determine whether the number of non-rejoining G2-chromatid breaks can predict the radiosensitivity of human cell lines.

METHODS: Cell lines of human ovary carcinoma cells (HO8910), human hepatoma cells (HepG2) and liver cells (L02) were irradiated with a range of doses and assessed both of cell survival and non-rejoining G2-chromatid breaks at 24 h after irradiation. Cell survival was documented by a colony assay. Non-rejoining G2-chromatid breaks were measured by counting the number of non-rejoining G2 chromatid breaks at 24 h after irradiation, detected by the prematurely chromosome condensed (PCC) technique.

RESULTS: A linear-quadratic survival curve was observed in three cell lines, and HepG2 was the most sensitive to γ-radiation. A dose-dependent linear increase was observed in radiation-induced non-rejoining G2-PCC breaks measured at 24 h after irradiation in all cell lines, and HepG2 was the most susceptible to induction of non-rejoining G2-PCC breaks. A close correlation was found between the clonogenic radiosensitivity and the radiation-induced non-rejoining G2-PCC breaks ($r = 0.923$). Furthermore, survival-aberration correlations for two or more than two doses lever were also significant.

CONCLUSION: The number of non-rejoining G2 PCC breaks holds considerable promise for predicting the radiosensitivity of normal and tumor cells when two or more than two doses lever is tested.

INTRODUCTION

An important goal of current research in radiation oncology is the development of an assay or a combination of assays to predict the radiation response of individual human tumors and normal tissues. Accurate prediction of response to conventional therapy could lead to treatment modification when necessary. The important radiobiological factor that has a significant effect on a tumor's response to radiotherapy is intrinsic radiosensitivity, which, determined with a colony assay, is significantly associated with treatment outcome after radiotherapy[1,2]. However, it is unlikely to use colony formation as a routine predictive assay, as it takes several weeks to complete. This is usually too long for patients to wait for appropriate treatment. Other assays should therefore be developed which can be used routinely in a clinical setting. Such assays should give a direct or indirect measure of cell killing, and chromosomal radiosensitivity is one of the potential endpoints for assessing cellular intrinsic radiosensitivity.

Researchers have studied the metaphase chromosome aberrations using conventional techniques, and found a close relationship between radiation-induced chromosome aberrations and cell kill[3,4]. However, it is difficult to obtain enough metaphase for cytogenetic analysis because of the cell cycle delay or interphase cell death after irradiation. These problems might be solved by studying the interphase cell aberration directly. Thus, PCC technique using phosphatase inhibitors is optimal. Because the method involves non complex procedures and generates results in the non-cycling state, it is an advantage for measuring chromatin damage by qualitatively different types of radiation because one can observe chromatin

damage in the situation in which the effects on an LET-dependent cell-cycle delay and interphase cell death are removed, and PCC technique is very useful for measuring the radiation-induced chromatid breaks in all of the cell cycles, especially in G2 phase[5-8].

It has been shown a linear dose response in the same dose range used for cell-survival experiments in different *in vitro* cell systems using PCC assay to measure chromosomal damage[9-12]; and the repair kinetics of radiation-induced chromatid breaks has been studied[9,13,14]. Furthermore it was demonstrated that there is a good correlation between the induction of non-rejoining PCC breaks in interphase cells and cellular radiosensitivity[15-17]. However, it is well established that the radio-sensitivity is dependent of cell cycle progression, and among each stage of interphase, G2 phase is the most radiosensitive, G1 phase the second radiosensitive, and S phase the least radiosensitive; while G0 phase is radio-resistant for lack of oxygen and enough repairing time[18-20]. Previous studies led to the conclusion that G2 phase is a very important stage. However, there were only a few reports on the chromosome aberrations in G2 phase and little is known of the relationship between G2 chromosomal aberration and cellular clonogenic radiosensitivity.

The purpose of this study was to explore the chromosome aberrations in G2 phase. The non-rejoining G2-PCC breaks after 24 h of post-irradiation incubation by γ-rays of two human tumor cell lines were measured using PCC technique. In order to evaluate the potential of the PCC assay as a predictor of cellular radiosensitivity, the correlation between radiation-induced non-rejoining G2-PCC breaks and the cellular clonogenic radiosensitivity was studied. Furthermore, the results were analyzed together with the author's previous results of human normal liver cells L02[21].

MATERIALS AND METHODS

Cell culture and irradiation

Human ovary carcinoma cells (HO8910) and human hepatoma cells (HepG2) were grown in RPMI-1640 medium supplemented with 100 mL/L foetal calf serum, 100 kU/L penicillin and 100 g/L streptomycin at 37 ℃ in a 50 mL/L CO_2 atmosphere with 950 mL/L humidification.

Irradiation was generated from ^{60}Co source (Radiology Department, Affiliated No.1 Hospital, Lanzhou University, Lanzhou). HO8910 cells and HepG2 cells were irradiated at doses of 0, 0.5, 1.0, 2.0, 4.0, 6.0 or 8 Gy, with a dose rate of 0.2 Gy/min.

Survival assay and PCC induction

After irradiation, cells were plated at a density of about 100 surviving cells per 6 cm culture dish and incubated for about 14 d, and then fixed and stained with a solution of Giemsa.

Calyculin A, a specific inhibitor of protein phosphatase types 1 and 2A, which can induce PCC in various types of cells with high efficiency[5,22], was purchased from Wako Chemicals (Osaka, Japan), dissolved in 100% ethanol as a 1 mmol/L stock solution and stored at -20 ℃. After irradiation, cells were incubated at 37 ℃ in a 50 mL/L CO_2 atmosphere. Twenty-four hours later calyculin A was added (to give a final concentration of 50 nmol/L) to the cell cultures and cells were further incubated for 30 min. Chromosome spreads were then harvested by cell swelling in 75 mmol/L KCl for 20 min at 37 ℃ and fixed with Carnoy's fixation. A final wash and fixation in the same fixative was completed before dropping cells onto a glass slide and hot humidity drying.

Table 1 Parameters of cell survival and non-rejoining G2-chromatid breaks of the three cell lines after exposure to γ-rays irradiation

Cell line	$\alpha(Gy^{-1})^1$	$\beta(Gy^{-2})^1$	$SF2^2$ (mean ± SE)	$Slope^3$ (mean ± SE)
HepG2	0.2	0.075	0.455±0.06	2.17±0.225 (r=0.974)
HO8910	0.08	0.065	0.652±0.07	1.83±0.013 (r=0.987)
L02	0.04	0.05	0.863±0.09	1.134±0.06 (r=0.992)

[1]α and β values were determined from the linear quadratic equation.
[2]Survival fraction at 2 Gy was measured from the raw data.
[3]Slope from the fitted linear regression represents non-rejoining G2-chromatid breaks per cell per unit dose.

Observation and data evaluation

Colonies of more than 50 cells were counted as survivors. The number of colonies per dish was counted and the surviving fractions were calculated as the ratio of plating efficiencies for irradiated and non-irradiated cells. Plating efficiency was defined as the colony number divided by the number of cells plated. The survival data were fitted to the linear-quadratic model: $LnS = -\alpha D - \beta D^2$, where S is the survival fraction and D is the radiation dose.

Chromosome was stained with 50 g/L Giemsa for 20 min. Fifty well-spread G2 phase cells were scored under oil immersion with a light microscope for each dose point according to the standard criteria[23]. Briefly, chromatid discontinuing, misalignment of the distal to the lesion, or a non-stained region longer than the chromatid width were classified as a break. Iso-chromatid breaks were scored two breaks. The total chromatid breaks were calculated by summing the production of chromatid-type breaks and iso-chromatid breaks. The mean aberration values for the three separate experiments were obtained. These mean values and the standard error of the means were plotted and fitted by linear regression, and the slopes of the dose-response curves were calculated.

RESULTS

Cell survival

Cell survival parameter from the linear quadratic model fitted to the survival data is shown in Table 1. SF2 was measured from the raw data also shown in Table 1. For radiosensitivity, three cell lines displayed the different potency, in the order of, HepG2 > HO8910 > L02.

Non-rejoining G2-chromatid breaks

All cell lines showed a dose-related increase in the induction of non-rejoining G2-chromatid breaks (Figure 1). The dose-response curves were fitted by linear regression and the slopes of the dose-response curves were evaluated.

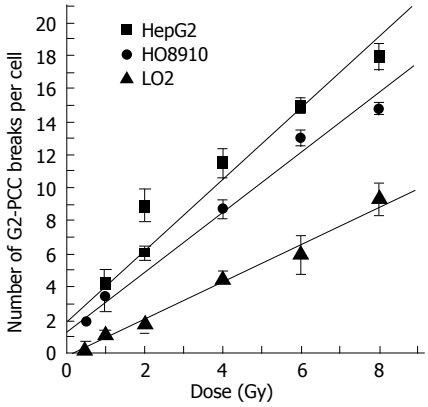

Figure 1 Dose-response curves for the induction of non-rejoining G2-PCC breaks 24 h after irradiation.

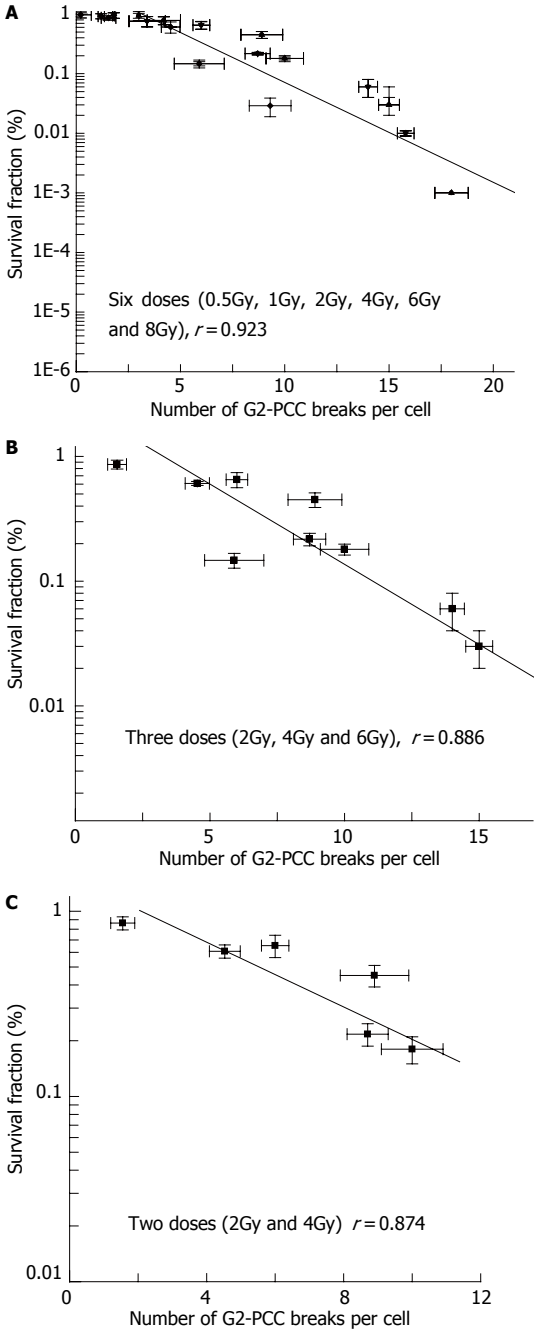

Figure 2 The relationship between survival fraction and non-rejoining G2-PCC breaks 24 h after irradiation.

Table 2 Correlation between non-rejoining G2 chromatid breaks and cell survival

	Dose (Gy)	*r*	*P*
Multiple	2+4	0.874	0.023
	2+4+6	0.886	<0.01
	0.5+1+2+4+6+8	0.923	<0.001

The slope of HepG2 was the steepest, and that of HO8910 the second and L02 the least. Namely, the order of the slopes was HepG2 > HO8910 > L02. The values for the slopes of the curves are given in Table 1.

Correlation between cell survival fraction and non-rejoining G2 chromatid breaks

To examine the correlation between clonogenic radiosensitivity and the non-rejoining G2-chromatid breaks, the non-rejoining G2 chromatid breaks were plotted against the surviving fractions (Figure 2A). Correlation coefficient was 0.923 for three cell lines. The relationships for non-rejoining G2 chromatid breaks at different multiple doses (Figure 2) were investigated. Correlation coefficients are shown in Table 2. It is clear that increasing combining doses improves the correlation coefficients. This is true for any combination of two doses or more than two doses (data not shown).

DISCUSSION

Consistent with previous studies[21], fitted cell survival curve of three cell lines were linear quadratic in the present study. Among the three cell lines, survival fraction of L02 cells was the highest after absorbing same dose of radiation. These suggested that human normal liver cells were of much lower sensitivity to γ-rays than both human ovary carcinoma cells and human hepatoma cells. These may be because reductive glycogens synthesized in normal liver cells which interact with free radicals reduce the radiation injury[21]. In addition, the theoretic basis of radiotherapy is that the normal cells are of low radiosensitivity, and our results further confirmed this.

It has been shown that the rejoining of radiation-induced DNA double strand breaks (dsbs) was saturated within 2-4 h in mammalian cell lines[24,25] and that the number of residual dsbs did not change between 4 and 20 h using the neutral filter elution[26]. So it is considered that the most important parameter related to clonogenic radiosensitivity is the residual dsbs for 4 h or more after irradiation[27]. The PCC technique measures the chromatid breaks caused by the dsbs[28,29], and the processes of chromosomal breaks rejoining contain a fast component and a slow component[5]. The G2-PCC breaks were rejoined with a half-time of 5 min for the fast component and about 3 h for the slow component after irradiation[5], implying that the rejoining of chromosome breaks occurred mainly during the early hours after irradiation. Furthermore, Borgmann[29] reported that the number of PCC fragments induced by radiation plateaued at 4-6 h after irradiation, and did not change for about 10 h. In

addition, Suzuki[13] reported that the repairing process of radiation-induced chromosome aberration occurred during the early 10 h after irradiation. Based on previous studies, most kinds of cells end their spontaneous repair process within 24 h if they are injured, and after 24 h, injured and normal cells without any artificial factors will be stable and continue synthesizing, mitosis processes. Therefore, it is reasonable that the non-rejoined excess fragments examined after incubation for 24 h after irradiation may be the most important parameter related to clonogenic radiosensitivity. Based on these, the non-rejoining G2-PCC breaks were examined after incubation for 24 h post-irradiation in this study, and a good linear relationship was found between dose and the non-rejoining G2 chromatid breaks after the cells were exposed to ^{60}Co γ-rays (Figure 1). In addition, we found that cells which were more sensitive to the cell killing were similarly more susceptible to induction of non-rejoining G2 chromatin breaks (Table 1 and Figure 1). Suzuki[15-17] and Borgmann[29] found similar results. Additionally, a good linear relationship was also found between dose and the initial chromatid breaks[7,21,30]. It is easy to study the radiation-induced chromatid damage with the PCC technique[11,31,32], and the data obtained thereby may accurately reflect the radiation damage for the chromatin level without the competition as a result of cell cycle and/or interphase cell death. Therefore the PCC technique is very useful for detecting chromatin damages by qualitatively different types of radiation. Furthermore, it is faster in obtaining the result by the PCC technique than by most of other techniques.

In the present study we have attempted to validate the use of radiation-induced non-rejoining G2 chromatid breaks as a measure of cellular radiosensitivity. One of the prerequisites for the potential use of such a rapid assay would be that the relationship with clonogenic survival, the Gold Standard and most relevant assay for tumor control, should be high. In this research, a good correlation (r=0.923) was found between cell survival and the number of non-rejoining G2-chromatid breaks in the three cell lines (Figure 2A). It implies that radiosensitivity can be determined by the induction of non-rejoining G2-PCC breaks.

To our knowledge, few researches have looked at the relationship between non-rejoining G2 chromatid breaks and cell killing, and only a few studies have investigated the relationship between the induction of non-rejoining chromatid breaks in interphase detected by PCC technique and cell killing. Ofuchi[17] showed a strong correlation between non-rejoining chromatid breaks and cell survival in human hepatoma cells. Suzuki[11] showed a good correlation between induction of non-rejoining PCC breaks and cell death in normal human cells, in human tumor cell lines[12], and in 6 primary cultured cells[15]. All these suggest one possibility that radiosensitivity can be determined by the induction of non-rejoining PCC breaks.

Dependent on the amount of primary tumor material (biopsy), it might not be feasible to obtain a complete dose-response curve consisting, for example, of four or more different doses. It is therefore more likely that only one or two doses could be studied with any statistical significance. Because of the limited number of cell lines in this study, it was not reliable to analyze the correlation between cell survival and the induction of non-rejoining G2-chromatid breaks for individual doses. For multiple doses, highly significant and good correlation were observed if two or more doses were used (Figure 2 and Table 2). Coco Martin[3] found similar correlation coefficients as reported here for 13 human tumor cell lines at two or more radiation doses using metaphase cytogenetic techniques. It indicates that two doses can be applied on fresh tumor tissue to obtain a relative high correlation. As for the individual doses, it is necessary to expand the number of cell lines to confirm the correlation between cell survival and the induction of non-rejoining G2-chromatid breaks.

In conclusion, it is suggested that the PCC technique is useful for determining chromosome aberration in G2 cells, and the amount of non-rejoining G2-PCC breaks induced by radiation can evaluate the cellular clonogenic radiosensitivity. To assess the general applicability of this approach, further studies are needed to expand the number of human cell lines.

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RAPID COMMUNICATION

Clinical characteristics of remote Zeus robot-assisted laparoscopic cholecystectomy: A report of 40 cases

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Abstract

AIM: To summarize the performing essentials and analyze the characteristics of remote Zeus robot-assisted laparoscopic cholecystectomy.

METHODS: Robot-assisted laparoscopic cholecystectomy was performed in 40 patients between May 2004 and July 2005. The operating procedures and a variety of clinical parameters were recorded and analyzed.

RESULTS: Forty laparoscopic cholecystectomy procedures were successfully completed with Zeus robotic system. And there were no post-operative complications. Total operating time, system setup time and performing time were 100.3 ± 18.5 min, 27.7 ± 8.8 min and 65.6 ± 18.3 min, respectively. The blood loss and post-operative hospital stay were 30.6 ± 10.2 mL and 2.8 ± 0.8 d, respectively. Camera clearing times and time used for operative field adjustment were 1.1 ± 1.0 min and 2.0 ± 0.8 min, respectively. The operative error was 7.5%.

CONCLUSION: Robot-assisted laparoscopic cholecystectomy following the principles of laparoscopic operation has specific performing essentials. It preserves the benefits of minimally invasive surgery and offers enhanced ability of controlling operation field, precise and stable operative manipulations.

Key words: Zeus; Robotic surgical system; Laparoscopic cholecystectomy

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INTRODUCTION

During the past two decades, the benefits of laparoscopic surgery for the patients are less trauma and pain, a shorter post-operative hospitalization, a better cosmetic result and a faster return to normal activity[1]. However, although there are clear benefits to the patients, surgeons face distinct disadvantages. Working through fixed entry points limits maneuverability of the instruments inside the body cavity to five degrees of freedom (DOF). Looking at a two-dimensional screen, surgeons are handicapped by the loss of the visual perception of depth, and the need for a human assistant to hold and move the camera makes surgeons loss the independent ability of controlling operation field [2]. Reduced dexterity and impaired visual control are considered the major burdens of laparoscopic surgery and the attempts in developing robotic telemanipulation systems aim at overcoming these problems. Nowadays both Zeus and da Vinci robotic telemanipulation systems are available to patients. After the success of the first laparoscopic cholecystectomy with Zeus robotic surgical system of our hospital on 26 April 2004, we performed the same operation in the other 40 patients between May 2004 and July 2005, and acquired some experience.

MATERIALS AND METHODS

Patients

Between May 2004 and July 2005, forty patients (24 females and 16 males), with average age 36 years (range 14-50 years) underwent the robot-assisted laparoscopic cholecystectomy in our hospital. Six cases were cholecystopolyp and the others were cholecystolithiasis combined with chronic cholecystitis. Informed consents were obtained from all patients before operation.

Operation

Zeus robotic surgical system (Computer Motion Com-

pany, IEC 60601, Class III, Type CF) consists of Aesop (automatic endoscopic system optimal position), Hermes (acoustic center), Zeus surgical system (three separate robotic arms, operator console, optic console), and Socrates (remote co-operating system).

All the patients in supine position were under the total intravenous combined inhalation anesthesia. The left and right robotic arms were placed at each side of the patient's head. Aesop arm was placed beside the left hip. All robotic arms were attached to the sidebars of the operating table. The movable lower limits of the three arms were set at an optimal distance that a palm was able to insert between the robotic arm and the patient's abdominal wall, and then the instrument adapter was installed onto its robotic arms. Pneumoperitoneum was established after the puncturation with Verres needle at the umbilicus and the cavity pressure was maintained at 12 mmHg. The first 10-mm trocar was introduced at the umbilicus and the 10-mm 30º laparoscope was inserted to explore the abdominal cavity. Under laparoscopic vision, another 10-mm trocar and a 5-mm trocar were respectively introduced at 5-cm below the xiphoid process and 5-cm below the right costal margin in anterior axillary line. A dissection clamp and a grasper were inserted through the trocars. The laparoscope, a grasper and a dissection clamp were engaged with the instrument adapters of Aesop arm, left robotic arm and right robotic arm. Then the three robotic arms were adjusted in a double 90º position, i.e., the angle between the forearm and the upper arm of a robotic arm was 90º, and the angle between the laparoscopic instruments in the adapters and the forearm was also 90º. The condition of double 90º offered the largest range of motion. The surgeon wearing a microphone sat before the operator consol which was placed about 5 m far from the operating table, and steered two egg-shaped manipulators to control the left or right robotic arm. In the mean time, Aesop was acoustically controlled to move up and down, left and right, forward and backward, so as to offer the optimal operative field. An assistant standing by the operating table prepared to adjust the tiny regulator of Aesop or another two robotic arms. An instrument nurse also stood by the table to replace the instruments at any moment. The surgeon steered the grasper of the left robotic arm to retract the neck of gallbladder to expose the Calot's triangle and manipulated the dissection clamp of the right robotic arm to dissect the cystic duct and cystic artery. The cystic artery and duct were sheared after double ligation with clips separately, and then gallbladder was cut down from the liver with the diathermy hook. The gallbladder was pulled out by the assistant from the port below xiphoid process[3].

All the operation procedures were recorded on videotape for later analysis. Time parameters included total operative time, system setup time and performing time. The total operative time was defined as the time from disinfection of the operative field to skin closure. The system setup time was defined as the time from disinfection to the start of grasping the neck of gallbladder. The performing time was defined as the time from the start of grasping the neck of gallbladder to the moment the gallbladder was completely freed from the liver. Minimally invasive parameters included the blood loss, post-operative complications and post-operative hospital stay. Parameters of operative field included camera clearing time and time used for operative field adjustment. Camera clearing time was defined as the time that the laparoscope was taken out to clear the contaminant on the camera during operation. Time used for operative field adjustment mean the time which was taken for the surgeon to stop perform to adjust the operative field or clear the camera. The rate of operative error mean the percent that the cases in which operative errors, such as hepatic laceration or gallbladder perforation happened, occupied among the 40 cases. Conversion cases mean the number of procedures converted to other procedures, such as open procedure or conventional laparoscopic procedure.

RESULTS

Forty laparoscopic cholecystectomy procedures were successfully completed with Zeus robotic system and there were no conversion cases. Total operating time, system setup time and performing time were 100.3 ± 18.5 min, 27.7 ± 8.8 min and 65.6 ± 18.3 min, respectively. The blood loss and postoperative hospital stay were 30.6 ± 10.2 mL and 2.8 ± 0.8 d, respectively. Camera clearing time and time used for operative field adjustment were 1.1 ± 1.0 and 2.0 ± 0.8 min, respectively. The operative error was 7.5%. All the patients began to intake diet at 6-9 h after operation. Their wound healed well without oozing blood and infection, and there were no post-operative complications in any of the patients.

DISCUSSION

At the beginning of 1999, two US companies, Computer Motion and Intuitive Surgical, received European CE accreditation for the clinical application of Zeus and da Vinci robotic surgical systems that were independently invented. In 2000, the two companies received FDA accreditation too, which indicated that robotic surgical systems began to be formally available to patients in the world[4]. In 2001 September 7, a medical team led by French doctor Marescaux accomplished the famous Zeus robot-assisted cholecystectomy, cross over Atlantic Ocean, named as "Lindbergh operation", which created a precedent of remote operation[5]. Up to now, the safety and feasibility of robotic surgical systems applied in general surgery, thoracic/vascular surgery and gynaecology/urology have been demonstrated. Until 2000, more than 6000 robot-assisted procedures were performed, thirty-seven percent among them were in general surgery, mainly cholecystectomy[6]. In the late two years, the technique of robot-assisted operation became more and more mature and wide, and the number of procedures increased quickly. After the success in the first Zeus robot-assisted laparoscopic cholecystectomy in Chinese mainland by our hospital, the other 40 cases were accomplished between May 2004 and July 2005. Based on these, the robot-assisted operative characteristics and performing essentials were analyzed.

Robotic surgical system is a new achievement which is resulted from the medical application of highly developed automatic technique, computer image technique and con-

trol technique. The advanced system possesses the enormous superiority over conventional laparoscopic surgery and forms its characteristics. During Zeus robot-assisted procedures, there was no need for a human assistant to hold the camera, who often provides error and unstable operative field or contaminates camera by touching tissue. The surgeon directly control the camera engaged with Aesop arm by voice, which restores his capability to master operative field as open operation. Moreover, the arm cannot shake and has the ability to memory the previous position. Therefore, the adjustment of operative field is quick and convenient and the operative field is direct-viewing and stable[7]. In our study, camera clearing time and time used for operative field adjustment were merely 1.1 ± 1.0 and 2.0 ± 0.8 min, respectively. There was no vibration of laparoscopic instruments in all the 40 procedures, which benefits from the mechanism and working principle of Zeus robot. First, with the surgeon sitting at a remote and ergonomically designed workstation, Zeus robotic system eliminates the need to twist and turn in awkward positions, which is in favour of the long and precise operation[8]. Second, it is the 12- to 15-fold magnification of image by the camera of Zeus system that conduces to accurate operation[9]. Third, it provides adjustable motion scaling and tremor reduction. Motion scaling reduces the surgeon' s motion at the console to finer movements within the patient. When the system is set to 5:1, a 5-cm sweep by surgeon's hand is a 1-cm sweep within the abdomen. This promotes the accuracy of operation. Tremor reduction can completely eliminate any tremor from the surgeon's hand, which increases the stability of operation, thereby decreasing operative errors[10]. The operative error was lower than that of early conventional laparoscopic surgery. The endowrist at the tip of instrument provides the surgeon with six degrees of freedom inside the patient's body. The additional degrees of freedom increase the dexterity and create the sense of actually having the surgeon's hand within the abdominal cavity during laparoscopic surgery. This vastly simplifies tasks such as suturing, tying and complex dissection, all of which are extremely challenging for most surgeons with standard laparoscopic equipment[11]. Nio *et al*.[12] selected 20 medical students without any surgical experience to perform at random a set of laparoscopic tasks either manually or robot-assisted (Zeus). This task consisted of dropping beads into receptacles, running a 25-cm rope, capping a hypodermic needle, suturing, and performing a laparoscopic cholecystectomy on a cadaver liver of a pig. The dropping beads exercise and the laparoscopic cholecystectomy required more time when performed with robotic assistance, as compared with manual performance. Grasping the beads, the rope, and either the needle or the cap were tasks that required fewer actions to complete when performed with robotic assistance. As compared with the robot-assisted rope-passing exercise, more failures were made in the manually performed procedure, mainly caused by unintentional dropping of the rope. Therefore, robot-assisted laparoscopic surgery by participants without any surgical experience might require more time, but actions can be performed more precisely as compared with manual laparoscopic surgery. Zeus robot-assisted laparoscopic cholecystectomy preserves the benefits of minimally invasive surgery. Robotic surgical system overcomes the technical limit of conventional laparoscopic surgery and expends the field of minimally invasive surgery into cardiac surgery. Kappert *et al*.[13] performed 29 off-pump totally endoscopic coronary bypass (TECAB) on a beating heart with the Da Vinci system and an endoscopic stabilizer (Intuitive Surgical Company). Patients were operated upon via four 1-cm chest incisions using the da Vinci robot for the internal thoracic artery (ITA) harvesting and for performance of anastomoses on the beating heart. In this series, they had a 100% survival rate. Conversion rate to a median sternotomy was 3.4%; time of harvesting was 26 min; time of anastomosis was 29 min; and operating time was 130 min. Post-operative time in ICU was 17 h; and post-operative hospital stay was 7 d. In robotic surgery, extra time is needed to setup and position the robotic arms and instruments before starting of the actual dissection[14]. However, the setup time decreased from 40-50 min to 20-30 min with increasing experience. If the robotic systems are placed at independent operating room and arms are fixed on the operating table the total operative time will reduce.

Zeus robot-assisted cholecystectomy is performed by the surgeon who controls the robotic arms via manipulators to accomplish the operation. It is not a job fulfilled 'automatically' by the robot according to a certain input program. The surgeon should follow the operative principle of conventional laparoscopic cholecystectomy, be familiar with the working rational of robotic systems, and continue to summarize the performing essentials as the accumulation of experience: (1) Installation of the robotic arms. Installation should depend on the body height and body type of the individual patient. Generally, the left and right robotic arms were placed at each side of the patient's head. Aesop arm was placed beside the left hip. The movable lower limits of the three arms were set to insert a palm between the robotic arm and the patient' s abdominal wall. The three robotic arms are adjusted in a double 90º position; (2) Place of trocar ports. The left trocar port should be placed in anterior axillary line 5 cm below the right costal margin, lower than that of conventional laparoscopic cholecystectomy, so as to favor the left robotic arm to retract gallbladder from various angles; (3) Tactile feedback is compensated by visual feedback. During robot-assisted procedures the surgeon cannot touch both the tissue and the instruments and lose the tactile feedback, which makes performing and judging more difficult. But, high-resolution and vivid three-dimensional operative field provided by robotic surgical system make it possible for the surgeon to observe the tiny morphological change of tissue. Thus the surgeon should utilize the high-quality visual feedback to compensate the lost tactile feedback, such as observing the deformation of tissue under pressure to judge strength[15]; (4) Conversion of the performing habits. During robot-assisted procedures, basic surgical manipulations, such as incision, separation, hemostasis, suture and so on, are converted to squeeze, relax, or rotate the egg-shaped manipulators. Due to these entirely changes of operative maneuvers, the surgeon is forced to form new habits through special training and practice.

Though the experience through the 40 Zeus robot-as-

sisted laparoscopic cholecystectomies was preliminary, we understood the advanced technical advantages of robotic surgical system, such as independent fine visualization, restored dexterity, *etc*. With the experience accumulated and performing craft enhanced, these advantages will convert to superior therapeutic efficacy, and the system will become a new therapeutic technical platform for the surgeons.

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RAPID COMMUNICATION

Three-dimensional conformal radiotherapy combined with FOLFOX4 chemotherapy for unresectable recurrent rectal cancer

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Abstract

AIM: To investigate the effect of three-dimensional conformal radiotherapy (3-DCRT) in combination with FOLFOX4 chemotherapy for unresectable recurrent rectal cancer.

METHODS: Forty-eight patients with unresectable recurrent rectal cancer were randomized and treated by 3-DCRT or 3-DCRT combined with FOLFOX4 chemotherapy between September 2001 and October 2003. For the patients without prior radiation history, the initial radiation was given to the whole pelvis by traditional methods with tumor dose of 40 Gy, followed by 3-DCRT for the recurrent lesions to the median total cumulative tumor dose of 60 Gy (range 56-66 Gy); for the post-radiation recurrent patients, 3-DCRT was directly given for the recurrent lesions to the median tumor dose of 40 Gy (36-46 Gy). For patients in the study group, two cycles chemotherapy with FOLFOX4 regimen were given concurrently with radiotherapy, with the first cycle given simultaneously with the initiation of radiation and the second cycle given in the fifth week for patients receiving conventional pelvis radiation or given in the last week of 3-DCRT for patients receiving 3-DCRT directly. Another 2-4 cycles (average 3.6 cycles) sequential FOLFOX4 regimen chemotherapy were given to the patients in the study group, beginning at 2-3 wk after chemoradiation. The outcomes of symptoms relieve, tumor response, survival and toxicity were recorded and compared between the study group and the control group.

RESULTS: For the study group and the control group, the pain-alleviation rates were 95.2% and 91.3% ($P > 0.05$); the overall response rates were 56.5% and 40.0% ($P > 0.05$); the 1-year and 2-year survival rates were 86.9%, 50.2% and 80.0%, 23.9%, with median survival time of 25 mo and 16 mo ($P < 0.05$); the 2-year distant metastasis rates were 39.1% and 56.0% ($P = 0.054$), respectively. The side effects, except peripheral neuropathy which was relatively severer in the study group, were similar in the the two groups and well tolerated.

CONCLUSION: Three-dimensional conformal radiotherapy combined with FOLFOX4 chemotherapy for unresectable recurrent rectal cancer is a feasible and effective therapeutic approach, and can reduce distant metastasis rate and improve the survival rate.

Key words: Rectal neoplasms; Radiotherapy; Chemotherapy

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INTRODUCTION

Despite all previous efforts at radical curative resection and multidisciplinary treatment, locally recurrent rectal cancer (LRRC) occurs in up to one third of patients[1-3]. A curative treatment is possible only when local recurrences represent limited disease that may be amenable to surgical re-excision[4,5]. Unfortunately, most patients with LRRC will be excluded from curative surgery on the basis of medical fitness, the presence of distant metastasis, locally unresectable disease or an unwilling to accept the considerable associated morbidity and mortality. In these patients, palliative intervention still may be required[6].

Patients with unresectable LRRC are often treated with nonsurgical palliation, including radiation therapy, chemotherapy and chemoradiation. Radiation has been confirmed as an effective method to palliate the symptoms and dose-response relationship between radiation doses and subjective response of LRRC has been revealed by some studies[7-10]. The strategy to elevate the radiation dose by adopting

new techniques, such as three-dimensional conformal radiotherapy (3-DCRT) or intensity-modified radiotherapy (IMRT), is needed to improve the local control of LRRC. It is also reasonable to combine chemotherapy into the multi-modality treatment for LRRC patients because systemic metastasis is a common problem. We designed herein a randomized, controlled study to compare the efficacy and the toxicities of exclusive 3-DCRT and 3-DCRT combined with FOLFOX4 chemotherapy for patients with unresectable LRRC.

MATERIALS AND METHODS

Patients and characteristics

Between September 2001 and October 2003, 48 patients with unresectable LRRC were randomized and treated by 3-DCRT at the Radiation Oncology Department, Sir Run Run Shaw Hospital. Twenty-five cases among them were treated by exclusive 3-DCRT and defined as the control group, while the other 23 cases received 3-DCRT combined with FOLFOX4 regimen and were defined as the study group. Table 1 shows the clinical data and pathologic characteristics with the initial operation. The diagnosis of local recurrence of rectal cancer mainly depended on imaging exam. Of 44 patients, presacral masses were detected by pelvis B-ultrasound, computerized tomography (CT) or magnetic resonance imaging, 10 cases were confirmed by biopsy pathological result. Thirty-five cases among them had solitary presacral mass, 9 cases (5 cases in the study group and 4 cases in the control group) had multiple masses or accompanied with adjacent lymph nodes metastases, but all the lesions can be dealt as a whole target. Of the other 4 cases, solitary masses in the pelvic sidewall were found by imaging exam. All the cases were consulted by radiologists and surgeons and evaluated as unresectable. Systemic examination was carried out to exclude distant metastasis. The median interval between local recurrence and initial operation was 15 (range 7-42) mo. Eighteen cases among the study group and 20 cases among the control group had received peri-operative radiotherapy with dose of 40-50 Gy. Twenty cases among the both groups had received 5-flurouracil (5-Fu)-based peri-operative chemotherapy.

Table 1 Clinical and pathologic characteristics of the patients

Variables	Control group (n = 25)	Study group (n = 23)
Age (yr): Median (range)	62 (36-70)	62 (40-72)
Sex		
Male	17	14
Female	8	9
Dukes' stage of initial lesion		
A	1	1
B	7	5
C	17	17
Tumor pathologic type		
Well and moderately differentiated adenocarcinoma	19	17
Mucinous adenocarcinoma	4	3
Signet ring cell carcinoma	2	3
Recurrent sites		
Presacral	23	21
Pelvic sidewall	2	2

No significant differences in clinical or pathologic variables between the two groups were observed.

Treatment

For the patients without prior radiation history, the initial radiation was given to the whole pelvis by traditional methods with tumor dose of 40 Gy, followed by 3-DCRT for the recurrent lesions to the median total cumulative tumor dose of 60 Gy (range 56-66 Gy); for the post-radiation recurrent patients, 3-DCRT was directly given for the recurrent lesions to the median tumor dose of 40 Gy (36-46 Gy). The entire pelvis was irradiated with 10 MV photons using AP/PA portals or PA portal and two lateral wedged portals. The schedule was once daily, 5 times a week, using 200 cGy fractions, to a final dose of 40 Gy. Belly board was used to reduce the volume of small bowel irradiated. For 3-DCRT, all patients had a CT scan in the treatment position immobilized by thermoplastic molds for treatment planning purposes. Using the CT data set, the clinical target volume defined as the gross tumor volume with 5-mm margin was delineated and confirmed by radiologists, radiation oncologists and radiotherapy physicians. An additional 5-10 mm was added for planning target volume. Radiotherapy treatment planning was performed using Pinnacle3 3-D conformal radiation treatment planning system. Three to seven fields with individualized blocks derived from beam's-eye-view were used to implement the 3-DCRT. PTV was surrounded by 90% isodose curvature. Three-dimensional CRT was delivered with 10 MV photon and conventional fractionization: once daily, 5 times a week, 200 cGy per fraction. For patients in the study group, two cycles chemotherapy with FOLFOX4 regimen were given concurrently with radiotherapy, with the first cycle given simultaneously with the initiation of radiation and the second cycle given in the fifth week for patients receiving conventional pelvis radiation or given in the last week of 3-DCRT for patients receiving 3-DCRT directly. Another 2-4 (average 3.6) cycles sequential FOLFOX4 regimen chemotherapy were given to the patients in the study group beginning at 2-3 wk after chemoradiation. FOLFOX4 regimen comprised of intravenous injection of oxaliplatin at a dose of 85 mg/m^2 on d 1, intravenous injection of leucovorin at a dose of 300 mg/m^2 and intravenous injection of 5-Fu at a dose of 400 mg/m^2 and continuous intravenous injection of 5-Fu at a dose of 600 mg/m^2 on d 1 and d 2.

Evaluation of patients

Patients were observed at 3-mo intervals for 18 mo after the completion of therapy and every 6 mo for 3 years. All patients were followed up till December 2004, with follow-up duration of 6-39 (median 23) mo, except two who were lost to follow-up and presumed dead. Assessment of pain was scored from 0 (no pain) to 10 (as bad as you can imagine) by numeric rating scale. If the pain score decreased more than a half, good pain palliation was considered. Assessments of tumor dimensions by CT scan were performed before the start of treatment and repeated

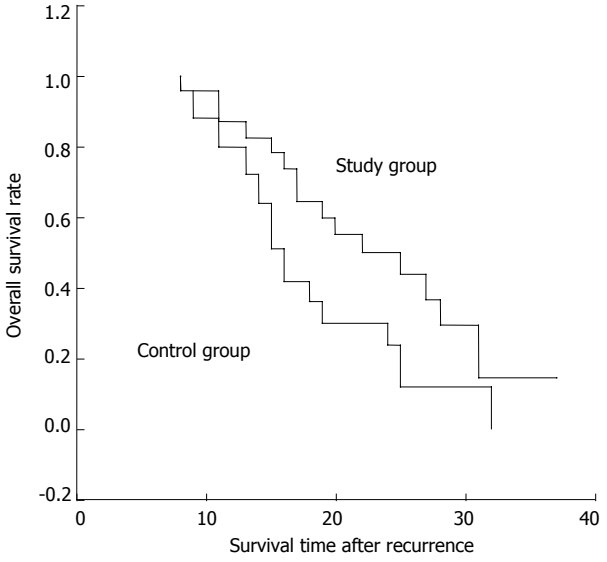

Figure 1 Overall survival curves of patients in the two groups. Five patients survived in the control group, and 8 patients survived in the study group.

1 mo after the end of 3-DCRT. Tumor objective responses were classified as complete response, partial response, stable disease and progression disease based on standard World Health Organization (WHO) criteria. The data of toxicity were ranked according to the WHO evaluation.

Statistical analysis

Survival estimates were calculated using Kaplan-Meier curves and a two-sided log-rank test was used to compare survival curves. χ^2 test was used to determine the difference of pain palliation rates, objective response rates and distant metastasis rate. P value < 0.05 was considered statistically significant.

RESULTS

Good pain palliation rate was 95.2% (20/21) in the study group and 91.3% (21/23) in the control group, with median palliation time of 13 d (range 6-58 d) and 15 d (range 8-65 d), respectively ($\chi^2=0.261$, $P=0.609$).

The overall objective response rate in the study group was 56.5% (1 complete response, 12 partial responses), while that in the control group was 40.0% (0 complete response, 10 partial responses) ($\chi^2=1.283$, $P=0.257$).

Figure 1 shows overall survival of the two groups. In the study group, 1-year and 2-year overall survival rates were 86.9% and 50.2%, while those of the control group were 80.0% and 23.9% with median survival time of 25 mo and 16 mo, respectively (Log-rank = 4.01, $P = 0.045$).

Interestingly, 2-year distant metastasis rates of the two groups were 39.1% and 56.0% with median distant metastatic time of 16 (range 7-26) mo and 10 (range 3-23) mo, respectively ($\chi^2=3.715$, $P=0.054$).

Toxicities of patients were scored according to WHO scale. A detailed description of acute toxicities was given in Table 2. The main toxic reactions of the control group patients included diarrhea, rectal tenesmus, perianal area skin reaction and bone marrow suppression. The main toxicities of the study group patients were similar but relatively severer peripheral neuropathy. No toxicity-related death was observed in both the groups. In the study group, radiotherapy of 3 patients was interrupted and delayed for 2-4 d due to severe diarrhea and rectal tenesmus, and the 2nd cycle chemotherapy of 1 patient was canceled due to severe bone marrow suppression. In the control group, radiotherapy of 2 patients was interrupted and delayed for 2 and 3 d, respectively, due to severe diarrhea and rectal tenesmus. No severe late toxicity was observed in both groups in the follow-up duration.

Table 2 WHO scale for acute toxicities

Toxicity	Grade	Control group	Study group	χ^2 value
Leukopenia	I	6	10	
	II	2	8	3.41
	III	0	3	
	IV	0	0	
GI tract	I	8	3	
	II	5	10	
	III	5	6	0.66
	IV	1	2	
Proctitis	I	10	9	
	II	6	8	
	III	3	3	0.01
	IV	0	0	
Skin	I	5	3	
	II	8	9	
	III	12	11	0.00
	IV	0	0	
Peripheral neuropathy	I	0	10	
	II	0	4	1.09
	III	0	1	
Bladder	I	12	11	
	II	6	8	
	III	2	1	0.27
	IV	0	0	

$P>0.05$. P value was calculated by subgroups for toxicity grade $\geqslant 3$.

DISCUSSION

The optimal treatment for locoregionally recurrent rectal cancer after curative surgery has not yet been defined[5,11,12]. Most of the recurrent rectal cancers are not resectable and require nonsurgical approaches. Multimodality treatment would probably offer the best result[13]. In the past, patients with locally unresectable recurrent rectal cancer were assumed incurable and received mostly palliative therapy. Traditional radiotherapy and chemotherapy given with palliative goal have been confirmed good palliation result of symptoms, such as local pain and bleeding with unknown effect on survival. Noticeably, several series have found a dose-response relationship of radiotherapy for symptoms control[9,14,15]. Some of them revealed the relationship also for local control and survival rate[9]. Sanfilippo *et al*[16] reported that despite aggressive multimodality therapy, a high rate of pelvic recurrence occurred in patients with clinically staged T_4 disease, and regional disease recurred almost exclusively in the radiation field. Under such circumstances, there is a significant need to adopt new techniques, such as intraoperative radiation therapy, brachytherapy and 3-DCRT, to safely deliver tumoricidal dose of radiation in an attempt to improve the local control[17-20].

Moreover, 3-DCRT allows more accurate definition of target volume and anatomy of critical normal structures. This technique focuses radiation to specific sites of disease, thereby minimizing injury to normal tissues. Higher doses of irradiation can be delivered by this technique to produce better tumor control without increasing the probabilities of particular sequela. It is controversial that whether previously irradiated LRRC patients could receive reirradiation and whether the reirradiation is of any value. Several investigators reported that high doses of reirradiation could be delivered with acceptable risks without prohibitive long-term side effects in patients with LRRC and could result in surgical salvage and long-term survival in selected patients[10,21,22]. This may be related to the location of local recurrence. A multicenter analysis of 123 patients with recurrent rectal cancer within the pelvis revealed that recurrent tumors were mainly situated in the posterior part of the bony pelvis and patients received abdominoperineal resection had a significantly more extension of recurrent tumors in the inferior parts of the pelvis comparing to those patients received low anterior resection[23]. There are fewer organs at risk in the lower and posterior pelvis. The usual local recurrent location of rectal cancer and 3-DCRT technique make it feasible to deliver higher radiation dose comparing with conventional radiation or to reirradiate with high dose for patients suffered from LRRC. In this study, we treated all patients with 3-DCRT or 3-DCRT boost to relatively higher dose, resulting in good palliation of pain in 93.2% (41/44) patients, the objective response rate in 47.9% (23/48) patients, and well tolerated toxicities. Thus we can roughly draw a conclusion that 3-DCRT for LRRC patients is feasible and effective.

It is now generally accepted that exclusive radiotherapy plays a minor role in improvement in survival unlike its major role in palliation of symptoms and improvement of local control for rectal cancer. Combined modality treatment is the recommended standard adjuvant therapy for patients with locally advanced rectal cancer. Currently, most adjuvant therapy includes chemotherapy[24]. Traditional chemotherapy or chemoradiation focuses on 5-Fu-based regimens, which have been confirmed to be effective. In our previous study, we have reported that preoperative radiotherapy combined with full course chemotherapy (LV + 5-Fu + 5'DfuR) is effective and safe[25]. Because of the clinical appliances of oxaliplatin during the recent years, substantial progress has been made in chemotherapy of rectal cancer. Chemotherapy with oxaliplatin combined with 5-Fu, such as FOLFOX4 regimen, is more effective and has become the standard treatment for advanced stage colorectal cancer[26]. In the United States, using similar chemotherapy regimens as adjuvant therapy has been approved. The advantages of oxaliplatin, such as its mild toxicities in gastrointestinal tract and bone marrow suppression, make it feasible to combine it with radiotherapy, especially when new radiation techniques, such as 3-DCRT, are applied. Local recurrence of rectal cancer is more common in the locally advanced patients, who have received 5-Fu-based chemotherapy in primary treatment, as exhibited in this study. For these patients, chemotherapy using more effective new drugs without cross-resistance is mandatory. In this study, we attempted to adopt 3-DCRT combined with FOLFOX4 chemotherapy for unresectable LRRC. The tumor response rates were similar in the both groups, but the 1- and 2-year overall survival rates and median survival time of the study group were better than those of the control group. Further analysis of the data revealed that the distant metastatic rate and median distant metastatic time of the study group marginally surpassed those of the control group ($P = 0.054$). We postulate that for LRRC patients receiving radiation and the combination of chemotherapy with FOLFOX4 regimen can reduce distant metastatic rate, delay the occurrence of distant metastasis and then influence the overall survival rate, even majority of the patients have received full course 5-Fu-based chemotherapy. In summary, 3-DCRT combined with FOLFOX4 chemotherapy appears to be a feasible and effective treatment for unresectable LRRC. Larger-scale studies are needed to evaluate the potency of this kind of therapeutic strategy.

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RAPID COMMUNICATION

Wnt/β-catenin signaling pathway is active in pancreatic development of rat embryo

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Abstract

AIM: To elucidate the role of Wnt/β-catenin signaling pathway in pancreatic development of rat embryo.

METHODS: The mRNAs of β-catenin, APC, cyclin D1 genes were amplified by means of semiquantitative reverse transcription polymerase chain reaction (RT-PCR) from embryonic pancreas in different periods and normal pancreas of rat, respectively. Protein expression of these genes in embryonic pancreas of E14.5-E18.5 was examined by immunohistochemical method.

RESULTS: In embryonic pancreas of E14.5, the transcript amplification of β-catenin and cyclinD1 genes was detected. In embryonic pancreas of E18.5, the transcription levels of β-catenin and cyclinD1 genes became much higher than in other periods. But in adult rat pancreas the transcription of cyclinD1 gene could not be observed. Only until E18.5, the transcript amplification of mRNA of APC gene could be detected. Surprisingly, the transcription level of APC gene became much higher in adult rat pancreas than in embryonic pancreas. By means of immunohistochemical staining, identical results were obtained to the above by RP-PCR, except for β-catenin protein in adult rat pancreas.

CONCLUSION: Active Wnt/β-catenin signaling occurs in rat embryonic pancreas and is probably important for pancreatic development and organ formation.

Key words: Wnt/β-catenin signaling; Pancreas; Development; Embryo

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INTRODUCTION

The fetal pancreatic development begins at the end of fourth week of gestational period in human being. The pancreatic bud comes from entodermal epithelium in the foregut. In rat embryo the pancreatic bud occurs at 9.5 d after mating (E9.5)[1]. With pancreatic islet arising, the rat embryonic pancreas began to become a special organ including external secretory portion and internal secretory portion. E14.5 - E18.5 is a crucial period for rat pancreatic cell proliferation, differentiation and structure formation[2-3]. Wnt/β-catenin signaling is involved in many developmental processes such as proliferation, differentiation, cell fate decisions, and morphogenesis[4]. However, little is known about Wnt/β-catenin signaling during pancreas development. β-catenin serves not only as a structural component of the E-cadherin-mediated cell-cell adhesion system, but also a signaling molecule of the Wnt/β-catenin pathway. In present study, we tried to investigate the role of Wnt/β-catenin signaling pathway in rat pancreatic development by means of RT-PCR and immunohistochemical method for collecting more data on diagnosis and therapy of pancreatic diseases.

MATERIALS AND METHODS

Experimental animals and specimen preparation

Healthy SD rats (male 10 and female 20), weighing 200-250 g, were purchased from the experimental animal center in Huaxi University of Medical Sciences, Chendu, China. Every two female rats and one male rat was put into one cage after these animals were fed for a week. Assessment of the embryonic age of the fetuses was based on the plug date, defined as embryonic d 0 (E0). The embryos were harvested from female rats with E14.5, E15.5, E16.5, E17.5 and E18.5, respectively. A part of pancreases was rapidly frozen in liquid nitrogen as soon as they were dissected from embryos under microscope.

Reagents and antibodies

Kits used in this study were as follows: Total RNA extraction kit (W6701, Watson, Shanghai), reverse transcription kit (RevertAid HMinus First Strand cDNA Synthesis Kit, Fermentas), and PCR reaction kit (DRR01AM, TaKaRa, Japan).

Table 1 Primers for RT-PCR

Genes	Sequence of primer (5′→3′)	Length of production (bp)	Annealing temperature (Tm)
	F: ACAGCACCTTCAGCACTCT		
β-catenin	R: AAGTTCTTGGCTATTACGACA	168	58.2
	F: CGGAACATGCATGACTGAGAC		
APC	R: GTCACGAGGTACGACCTCAGAT	310	60
	F: CAGAAGTGCGAAGCTTAGGTCT		
cyclin D1	R: GTAGCAGGAGAAGTTGTTGG	470	58
	F: CATGTGCAAGGCCGGCTTCG		
β-actin	R: GTAGCAGGAGAAGTTGTTGG	665	60

The following antibodies were used in this study: Rabbit polyclonal antibody against β-catenin, mouse monoclonal antibody against cyclin D1, rabbit polyclonal antibody against APC (Santa Cruz, USA).

Semiquantitative RT-PCR

Semiquantitative PCR was performed to determine the levels of the mRNA transcripts encoding β-catenin, APC and cyclinD1 genes in embryonic pancreas on E14.5-E18.5. The published sequences of the primers for amplification of β-catenin, APC, cyclinD1 and the housekeeping gene β-actin are showed in Table 1. To determine the optimum number of cycles required for the amplification of these genes or β-actin, an aliquot of first strand cDNA generated from normal rat pancreas was amplified with the respective primers using an increasing number of PCR cycles (20-36). To avoid primer-dependent artifacts, the reaction mixtures were denatured at 95 ℃ for 5 min prior to the addition of the Taq polymerase. The subsequent cycling programs consisted of denaturation at 95 ℃ for 30s, annealing at 60 ℃ (Table 1) (β-actin for 30 seconds) and extension at 72 ℃ for 1 min, followed by a final extension at 72 ℃ for 8 min. A linear relationship between the band intensity of the PCR products and the number of amplification cycles performed was observed. Based upon these observations, the optimum numbers of cycles for the amplification of these genes were 28-30 cycles and of β-actin was 28 cycles. PCR reactions in which the first strand cDNA were omitted served as negative controls and cDNA generated from endometrial samples were used as positive controls for these studies. To avoid technical error, each PCR experiment has been repeated twice. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and photographed using Gel Imaging System (BioRad, USA). The intensity of the bands specific for each target gene or β-actin was quantified using the Phoretix Gel Analysis Software Version 3.01 (NonLinear Dynamics, UK). The relative mRNA levels of these genes for each sample obtained from embryonic pancreas of E14.5-E18.5 were normalized to the corresponding β-actin levels.

Immunohistochemical detection of β-catenin, APC and cyclin D1 proteins

The avidin-biotin peroxidase complex (ABC) technique was used for immunohistochemical staining. Sections were cut at 5μm thickness, deparaffinized in xylene, rehydrated and washed with water. They were treated with 3% hydrogen peroxidase for 20 min to quench endogenous peroxidase and heated in a citrate buffer solution (0.1 mol/L sodium citrate, pH 6.0) at 95℃ for 10 min. After pre-incubation with 10% normal goat serum to block non-specific binding, sections were incubated with the primary antibodies against β-catenin, APC and cyclin D1 at 4℃ overnight. Alternatively, the sections were incubated with biotinylated anti-rabbit or mouse IgG (dilution of 1 : 200) for 40 min at room temperature and with ABC (dilution of 1 : 200) for 30 min at room temperature. Between incubations, sections were washed with 0.1 mol/L PBS (pH7.4). Color was developed with diaminobenzidine tetrahydrochloride supplemented with 0.04% hydrogen peroxidase and counterstained with Mayer's hematoxylin.

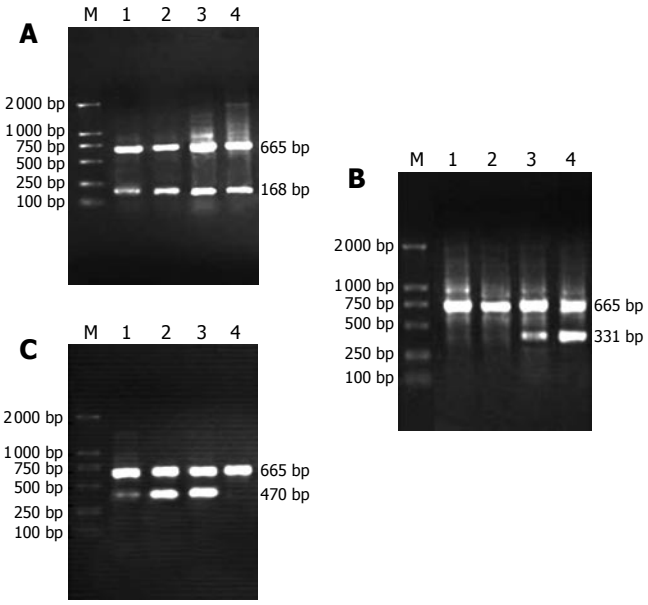

Figure 1 (**A**-**C**): Representative RT-PCR results of β-catenin, APC and cyclin D1 genes in embryonic pancreas of E14.5-E18.5 and adult rat. **A**: RT-PCR results of β-catenin gene (low) and β-actin gene (top, same in other lanes); **B**: RT-PCR results of APC gene (low); **C**: RT-PCR results of cyclinD1 gene (low). M: Molecular -weight marker, lane 1-4: amplification result of RT -PCR for β-catenin, APC and cyclin D1 genes from embryonic pancreas of E14.5, 16.5, 18.5 d and adult rat, respectively.

Statistical analysis

The results of semiquantitative RT-PCR are presented as the ratio of the mean relative absorbance of β-catenin, APC and cyclin D1 to corresponding β-actin respectively for at least three independent experiments of each sample. Statistical differences among different periods of embryo were assessed by *t* test. A $P < 0.05$ was considered significant.

RESULTS

Expression of β-catenin, APC and cyclinD1 mRNA in different pancreas

As shown in Figure 1(A-C), the amplification bands of mRNA of β-catenin and cyclinD1 genes could still be detected in rat embryonic pancreas from E14.5-E18.5. In

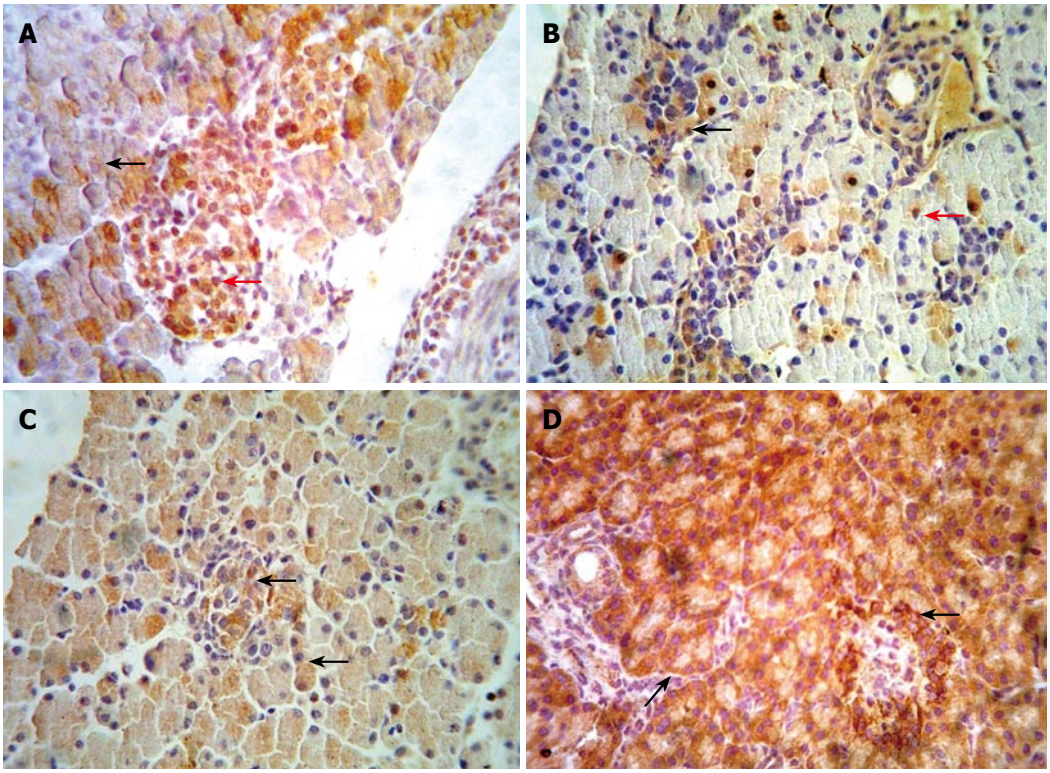

Figure 2 (**A-D**) Representative immunohistochemical results of β-catenin, APC and cyclin D1 proteins in embryonic pancreas of E14.5-E18.5 and adult rat. Immunohistochemical staining was performed using an anti-β-catenin APC and cyclin D1antibodies respectively. Slides were detected at ×400 magnification under a light microscope. **A:** showed staining of β-catenin at cytoplasm (black arrow) and nucleus (red row) in embryonic pancreas of E18.5. **B:** showed staining of cyclin D1 within cytoplasm (black arrow) and/or nucleus (red row) in embryonic pancreas of E16.5. **C:** showed staining of APC within cytoplasm (black arrow) in embryonic pancreas of E18.5. **D:** showed staining of APC within cytoplasm (black arrow) in adult rat pancreas.

adult rat pancreas the transcription of cyclinD1 gene could not be observed while the transcription of β-catenin gene could be found. Only in pancreas of E18.5, the transcript amplification of mRNA of APC gene could be detected. Surprisingly, the transcription level of APC gene was higher in adult rat pancreas than in embryonic pancreas.

Immunohistochemical staining of β-catenin, cyclinD1 and APC

We examined the β-catenin, cyclinD1 and APC protein in embryo from E14.5-E18.5 by immunohistochemical staining. Representative results are shown in Figure 2 (A-D). In the embryo of E14.5, β-catenin staining appeared at the cytoplasm of pancreatic cells. The cells with positive staining showed a diffuse distribution in embryonic pancreas. In the embryo of E18.5, we could observe full pancreas including external and internal secretion portions in morphology. At the same time, our results showed that β-catenin could be detected frequently both in external and internal secretion portions. The positive cells of cyclinD1 with diffuse distribution were observed in the pancreas of E14.5. The positive staining located in the cytoplasm and/or nuclei of pancreatic cells. In pancreas of E18.5, the expression of cyclinD1 protein was significantly higher than that in other period. However, none of positive cells expressing cyclinD1 protein was observed in adult pancreas. Expression of APC has not been observed in the early embryonic pancreas until E18.5. In contrast, the positive cells of APC staining scattered throughout the pancreas in adult rat.

DISCUSSION

At present, morbidity rate of diabetes has an ascending tendency in the world while high morbidity and mortality of pancreatic carcinoma is becoming one formidable topic for surgeon[5]. In order to treat these diseases effectively, fully understanding of the development and differentiation of embryonic pancreas, as well as the underlying molecular mechanism of pancreatic diseases is needed.

Wnt/β-catenin signaling pathway is highly conservative during embryonic development and tumorigenesis of human being and animal[6]. Recent studies have suggested that activation of Wnt/β-catenin signaling pathway may play an important role in hematonosis[7], gastroenteric tumor such as colon cancer[8], and development of embryo[9]. In this study, we have investigated the transcription of gene and expression of protein of β-catenin, APC and cyclin D1 which are three important components in Wnt/β-catenin signaling pathway.

β-catenin serves not only as a structural component of the E-cadherin-mediated cell-cell adhesion system, but also as a signaling molecule of the Wnt/β-catenin signaling pathway. The β-catenin protein is at the core of the canonical Wnt/β-catenin signaling pathway. Wnt stimulation leads to β-catenin accumulation, nuclear translocation and interaction with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to regulate genes important for embryonic development and proliferation. In a word, accumulation of ββ-catenin protein in the cytoplasm and/or nuclei of cell are popularly considered a hallmark of activation of the canonical Wnt/β-catenin signaling pathway[7,10]. At this study, we have observed the transcription of β-catenin gene by the RT-PCR and positive stain of β-catenin protein with immunohistochemical techniques in pancreas of E14.5-E18.5. Moreover the positive cells of β-catenin existed in not only the external secretion portion but also internal secretion portion. Therefore we presumed that Wnt/β-catenin signaling pathway may be very important for morphogenesis of pancreas. In pancre-

as of E18.5, the positive stain of β-catenin protein could be found in nucleus especially in pancreatic islet. We have known that pancreatic islet began to mature during late period of embryo[11]. So β-catenin may be essential for proliferation and differentiation of pancreas islet in embryo. Dessimoz *et al*[12] examined the role of β-catenin gene in development of pancreas using an animal model with the conditional gene knockout.They found a reduction in endocrine islet numbers after deleting the β-catenin gene in the epithelium of the pancreas. This result in some degree coincided with our conclusion. On contrast, Murtaugh *et al*[13] confirmed β-catenin is essential only for pancreatic acinar not for islet development. Obviously the development process of pancreas is too complicated to be understood only by Wnt/β-catenin signaling.

In the study, we investigated simultaneously cyclin D1 gene which is one of target genes of Wnt/catenin signaling pathway[14]. The changed tendency of cyclin D1 on the transcription level of mRNA and protein expression is almost in step with β-catenin in pancreas of E14.5-E18.5. It is known that cyclin D1 is a key regulator of the G1 phase of cell cycle[15]. Expressing of cyclin D1 protein in the cytoplasm and/or nuclei indicated that pancreatic cells should be in the condition of proliferation and differentiation. We therefore had come to another conclusion that cyclin D1 exactly is one of activated target genes of Wnt signaling pathway in development of embryonic pancreas.

It has been reported that APC is a negative regulatory factor as well as Axin in Wnt signaling pathway[16]. Experiments in Drosophila ultimately revealed that genetic ablation of APC indeed resulted in upregulation of β-catenin signaling[17]. In pancreas of E14.5-17.5, we could not observe the amplification band of mRNA and protein expressing of APC gene. Only in pancreas of E18.5, could we first find positive stain of APC protein as well as transcription of mRNA of APC gene. In adult rat the positive cells of APC protein spread throughout external secretion portion and internal secretion portion of pancreas. We have known that the proliferation level of pancreas is very low in adult rat particularly in diabetes[18]. This is coincident with the negative role of APC in Wnt/β-catenin signaling pathway.

In this study the cell with positive stain of β-catenin couldn't be found in adult rat pancreas although the transcription of β-catenin gene could be detected by RT-PCR. The reasons are discussed below. First the destruction complex including GSK3、Axin and APC can degradate the free β-catenin in the cytoplasm in time in adult rat pancreas[19]. Secondly the quantity of β-catenin protein which are attached to membrane with E-cadherin may be too little to be detected by immunohistochemical techniques used in present experiments[20].

Although this signaling pathway was active, we had known little about the role of Wnt /β-catenin signaling pathway in development of embryonic pancreas indeed. Which are the upperstream signals[21-22]? Which target genes beside of cyclin D1 are activated? How to crosstalk with other signaling pathway[23]? All these topics need us to investigate further.

Meanwhile, some workers have investigated the Wnt/β-catenin signaling in tumor of pancreas. They suggested the notion that Wnt/β-catenin signaling pathway had been activated in adenocarcinoma, cystocarcinoma and solid tumor of pancreas[24-26]. We will believe that the more we know the Wnt/β-catenin signaling in embryonic pancreas and tumor of pancreas, the better the therapeutic measures for patient with diabetes and tumor of pancreas clinically.

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CASE REPORT

A rare complication of a common disease: Bouveret syndrome, a case report

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Abstract

This is a case report of an 85-year old patient who presented with abdominal pain, nausea and vomiting associated with altered liver function test. The plain X-rays and CT scan showed pneumobilia with an ectopic gallstone. The patient was diagnosed with Bouveret syndrome and managed surgically. The report is followed by a discussion about Bouveret syndrome.

Key words: Bouveret syndrome; Gall bladder; Stone; Fistula; Gastric outlet obstruction

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INTRODUCTION

Gallstone ileus is a rare complication of gallstones but it accounts for almost 25% of non strangulated intestinal obstruction in patients above 65 years of age. Mortality has improved but remains at 15%-18% due to the age and co-morbidity of patients[1]. Bouveret syndrome, first described in 1896, is a subgroup of gallstone ileus in which a cholecystoduodenal fistula allows the passage of a stone which impacts in the duodenum and causes gastric outlet obstruction. It is rare with 175 cases reported in the literature up to the year 2000[2]. We present a case of Bouveret syndrome, with typical radiological findings, that was successfully managed surgically.

CASE REPORT

An 85-year old lady presented with a five-day history of right upper quadrant (RUQ) abdominal pain radiating to her back, associated with nausea and vomiting. On examination she had tenderness, guarding and rebound in the RUQ and epigastrium. The leukocyte count was 20.6 (predominantly neutrophils), the amylase 1039 and urea and creatinine were elevated. Plain abdominal X-ray showed a radio-opaque shadow in the right side of the abdomen, air in the gall bladder and biliary tree but no evidence of small bowel obstruction (Figure 1). She was assumed to have pancreatitis and a suspected cholecystoenteric fistula.

CT scan with oral contrast revealed a large amount of air in the biliary tree, a fistula between the second part of duodenum and the gall bladder and a large stone in the third part of duodenum. A small amount of contrast outlined the stone and reached the small bowel (Figure 2). This impacted stone (7.5 cm × 4 cm × 4 cm) was removed surgically from the junction of the second and third part of duodenum through a small jejunostomy after mobilization of the duodenum and the DJ flexure. Her post operative recovery was uneventful.

DISCUSSION

Cholecystoenteric fistulae occur in less than 1% of patients with gallstones. Most (60%) are cholecystoduodenal fistulae, but cholecystocolic, cholecystogastric and choledochoduodenal fistulae have been described. Large stones passing through the fistula may cause intestinal obstruction, especially in the terminal ileum[1,3]. A plain abdominal X-ray is diagnostic in about 50% of cases[3] and may demonstrate intestinal obstruction, pneumobilia, an ectopic gallstone, alteration in the position of the previously observed stone or two air fluid levels in the right upper quadrant secondary to air in the gall bladder.

Patients with Bouveret syndrome usually present with symptoms of gastric outlet obstruction, though presenting with other complications of gall stone disease or upper gastrointestinal bleeding has been reported[4]. A plain abdominal X-ray may show a dilated stomach, and CT may demonstrate pneumobilia, intestinal obstruction and an ectopic gallstone[5]. Open surgery, endoscopic removal[6] and laparoscopic or laparoscopic assisted enterolithotomy[7] have all been used successfully for stone removal. A stone in the duodenum can be difficult to access laparoscopically but may be reached endoscopically[6].

Whether the gallbladder should be disturbed is controversial but the recurrence of gallstone ileus following enterolithotomy is rare, and complications related to the persistence of a cholecystoenteric fistula are unusual[3,8].

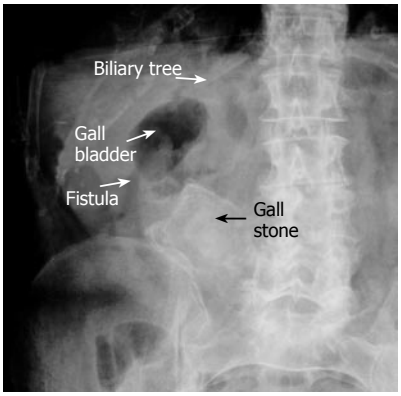

Figure 1 Plain X-ray showing air in the biliary tree and an ectopic bizarre-shaped gall stone suggesting the diagnosis of cholecystoenteric fistula.

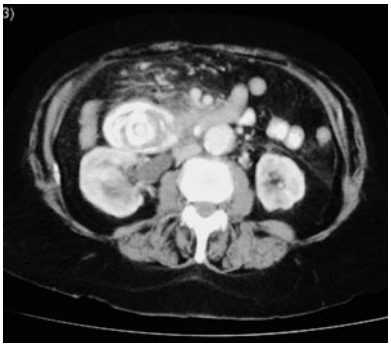

Figure 2 CT abdomen with contrast showing the gall stone in the duodenum with some contrast passing distal to the stone.

Bouveret syndrome is a rare complication of cholelithiasis with a relatively high mortality. In the patient has a pre-operative diagnosis of Bouveret syndrome, endoscopic disimpaction should be attempted. Failing this laparotomy is likely to be required but laparoscopy may allow identification of the stone's position minimizing subsequent skin incision.

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CASE REPORT

Intestinal Behcet's disease with esophageal ulcers and colonic longitudinal ulcers

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Abstract

Intestinal Behcet's disease in a 38-year-old woman was diagnosed because of the history of recurrent oral aphthous ulcers, erythema nodosum-like eruptions, genital ulcer, and endoscopic findings of esophageal and ileocolonic punched-out ulcers with colonic longitudinal ulcers. Esophageal lesions and colonic longitudinal ulcers are rarely seen in intestinal Behcet's disease. The ulcers of esophagus and ileocolon healed with 3 wk of treatment with prednisolone and mesalazine without any adverse effect. Mesalazine may decrease the total dose of prednisolone required to treat the disease.

Key words: Colonic longitudinal ulcer; Esophageal ulcer; Intestinal Behcet's disease; Mmesalazine

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INTRODUCTION

In Japan, Behcet's disease, when accompanied by intestinal involvement, is called intestinal Behcet's disease, which primarily affects the terminal ileum, caecum, or ascending colon[1]. However, esophageal aphthous ulcerations and colonic longitudinal ulcers are rare in intestinal Behcet's disease. Intestinal lesions in Crohn's disease tend to be longitudinal ulcers with a cobblestone appearance, while those in Behcet's disease are round and oval "punched-out" ulcers. Moreover, epithelioid granuloma is one of the pathological characteristics of Crohn's disease, whereas it is uncommon in intestinal Behcet's disease. Another feature of Behcet's colitis is lymphocyte venulitis, which is a disorder of vasculitis. Despite these differences, it can be difficult on occasions to make a differential diagnosis between these two diseases. We present a case of intestinal Behcet's disease with esophageal and colonic longitudinal ulcers.

CASE REPORT

The patient was a 38-year-old woman who was admitted to our department in December 2003 for epigastralgia and melena that occurred after transient fever. For the previous two days, she was in good health, except for numerous recurrences of aphthous stomatitis. Physical examinations on admission revealed the presence of multiple oral aphthous ulcers, in addition to slight tenderness in the epigastrium. Ophthalmologic and neurologic examinations showed no remarkable findings despite the presence of erythema nodosum-like eruptions in the bilateral inferior limbs and genital ulcers. Negative results were obtained by a prick test of the skin. Clinical laboratory tests showed the following: white blood cells, 7 830/μL; red blood cells, 360×10^4/μL; hemoglobin, 9.6 g/dL; hematocrit, 29.7%; platelets, 61×10^4/μL; C-reactive protein, 10.9 mg/dL; total protein, 8.6 g/dL; AST, 36 IU/L; ALT, 64 IU/L; LDH, 171 IU/L; and total bilirubin, 0.2 mg/dL. Human lymphocyte antigens were negative for B51, but positive for B52. All bacteriologic examinations of blood, urine, and cultured stool showed negative results. Upper gastrointestinal endoscopy demonstrated small oval and discrete ulcers with reddish margin scattered between the middle and lower parts of the esophagus (Figure 1A). Colonoscopy revealed multiple aphthas and erosions scattered in the terminal ileum (Figure 1B) as well as small aphthas on the ileocecal valve (data not shown). However, punched-out ulcers were not observed on ileocecal lesions. Multiple erosions and ulcers including longitudinal irregular-outlined ulcers were extant in the ascending and transverse colon (Figure 1C). Pathological examination of the endoscopic biopsy specimen showed nonspecific ulceration and no evidence of Crohn's disease could be found.

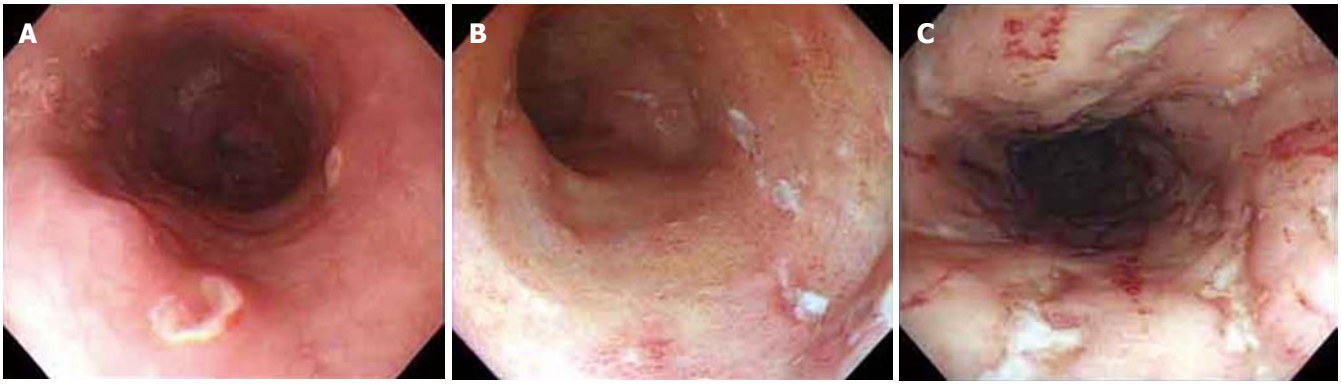

Figure 1 Endoscopic view of discrete ulcers with reddish margin in the middle part of the esophagus (**A**), multiple aphthas and erosions scattered in the terminal ileum (**B**), and multiple erosions and ulcers including longitudinal irregular-outlined ulcers extant in the transverse colon (**C**).

Table 1 Reported cases of Behcet's disease with colonic longitudinal ulcers

n	Age	Gender	Symptoms				Type	Colonic longitudinal ulcer		Other ulcers	Treatment	Outcome
			Oral	Eye	Skin	Genital		Location	Histology			
1	29	Female	+	+	+	+	Complete	Transverse Descending	Vasculitis		Operation Steroid	Healing
2	70	Female	+		+	+	Incomplete	Ascending Transverse Descending	Nonspecific colitis	Ileum	Colchicine Salazopyrin	Died
3	37	Female	+		+	+	Incomplete	Ascending Transverse	Granuloma	Ileum	Steroid 5-Aminosalicylate	Healing
4[1]	39	Female	+		+	+	Incomplete	Ascending Transverse	Nonspecific colitis	Ileum	Healing Mesalazine	Healing

[1]Case reported here.

In this patient, ulcers were detected in the esophagus, terminal ileum, and ascending and transverse colon, in addition to erythema nodosum-like eruptions in the bilateral inferior limbs and genital ulcers. Based on these findings and the history of recurrent oral aphthous ulcers, the patient was diagnosed as having incomplete type intestinal Behcet's disease.[1] The treatment for this patient was initiated by oral administration of prednisolone (50 g) and mesalazine (1 500 mg daily). Consequently, her symptoms disappeared rapidly, and the results of all clinical laboratory tests were normalized. Upper gastrointestinal endoscopy and colonoscopy performed 3 wk after admission revealed the disappearance of ulcers. Mesalazine was maintained at the same dose, but the dose of prednisolone was gradually decreased to 2.5 mg daily, which was maintained thereafter. However, remission was not obtained until 8 mo.

DISCUSSION

Behcet's disease is characterized by repeated eye, skin and visible mucosal lesions. The prevalence of this disease differs widely among races; the rate is 0.3/100 000 in the United States, but 1/10 000 in Japan[2]. Abdominal complaints also differ among races. Shimizu *et al* reported abdominal pain in 75% of patients with Behcet's disease[3], as noted in the present patient showing epigastralgia.

In Japan, diagnostic criteria for Behcet's disease have been established by the Behcet's Disease Research Committee[1]. Based on these criteria, a diagnosis was made in the present case of incomplete type intestinal Behcet' s disease manifests mainly in the terminal ileum, cecum, and ascending colon, although esophageal lesions are rare and colonic longitudinal ulcers are very rare rather than esophageal lesions. To the best of our knowledge, there are only three existing reports describing intestinal Behcet's disease with longitudinal ulcers (Table 1)[4-6]. Although Lee presumed the cause of the longitudinal ulcers to be multifocal vasculitis, in our case vasculitis was not significant. However, we cannot conclude that the patient had Crohn's disease, because microscopic characteristics of Crohn's disease - that is, chronic inflammation involving all layers of intestinal wall or granulomas - were absent.

In addition, only nine cases including our case of intestinal Behcet's disease with both esophageal and ileocolonic ulcers were reported in English literature[7-13]. One case was of the complete type; 6, the incomplete type; and 2, the suspected type. Three patients had strictures; and 2 had perforation. The treatment of intestinal Behcet's disease is controversial. Surgical treatment was not effective in 1 of the 9 patients (Table 2). However, the esophageal ulcers healed in 6 of 7 patients with medical treatment, including corticosteroids or acid suppressive drugs. Corticosteroids, the major therapeutic agent in this disease, were effective in 3 of the 4 patients. However, they can have serious adverse effects, and their use may be

Table 2 Reported cases of Behcet's disease with esophageal and ileocolonic ulcers

n	Age	Gender	Symptoms				Type	Esophageal ulcer		Other ulcers	Treatment	Outcome
			Oral	Eye	Skin	Genital		Number	Complication			
1	52	Female	+	+	+		Incomplete	1	Perforation	Stomach, Duodenum Jejunum, Ileum	?	?
2	21	Female	+			+	Suspected	3		Colon	Antacid Transfer factor	Healing
3	16	Female	+	+		+	Incomplete	1	Perforation	Colon	Steroid	Healing
4	12	Female	+	+	+	+	Complete	1	Stenosis	Ileum	Steroid Operaton	No healing
5	50	Male	+		+	+	Incomplete	1	Stenosis	Ileum, Colon	?	?
6	52	Male	+	+		+	Incomplete	Diffuse	Stenosis	Ileum	Healing	Healing
7	19	Female	+				Suspected	A few		Ileum	Healing Mesalazine	Healing
8	33	Male	+			+	Incomplete	1		Ileum	Healing	Healing
9[1]	39	Female	+		+	+	Incomplete	8		Ileum, Colon	Healing Mesalazine	Healing

[1]Case reported here.

associated with colonic perforation.

Sonta *et al* have suggested that mesalazine is effective for treatment of intestinal Behcet's disease[13]. It may decrease the total dose of a corticosteroid required to treat the disease. The esophageal and ileocolonic ulcers in the present case healed 3 wk of treatment with prednisolone and mesalazine without any adverse effect. However, it was reported that the recurrence rate even with medical treatment was 90% in patients with Behcet's disease[14]. The present case has not relapsed for 8 mo with treatment of 2.5 mg prednisolone and 1 500 mg mesalazine. Thus, the post-treatment course of this patient should be followed up carefully.

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CASE REPORT

Remission of primary low-grade gastric lymphomas of the mucosa-associated lymphoid tissue type in immunocompromised pediatric patients

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Abstract

We report the remission of primary gastric lymphoma of the mucosa-associated lymphoid tissue (MALT) type in two immunocompromised pediatric patients. Patient 1, a 14-year-old boy in an immunocompromised state of unknown cause, complained of repeated abdominal pain. Examinations revealed gastric MALT with local invasion and lymph node involvement. Serum anti-*Helicobacter pylori* (*H pylori*) antibody was positive. *H pylori* eradication was abandoned due to its adverse effects. The MALT lesion spontaneously regressed over the next 24 months without any treatment for lymphoma. Patient 2, a 6-year-old boy, underwent cord blood transplantation for the treatment of adrenoleukodystrophy. He was administered immunosuppressants for graft-versus-host disease after transplantation. Nausea and hematochezia appeared and further examinations revealed gastric MALT with *H pylori* gastritis. Treatment consisting of medication for the *H pylori* infection alone eradicated the *H pylori* and completely resolved the patient's MALT lesion, as well. Patients 1 and 2 were followed up over periods of 10 years and 3 years, respectively, without any signs of relapse. In conclusion, gastric lymphoma of the MALT type can be cured by conservative treatment even in immunocompromised pediatric patients.

Key words: Pediatric gastric lymphoma; Mucosa-associated lymphoid tissue; Immunocompromised states

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INTRODUCTION

The stomach is one of the most common sites of extranodal malignant lymphomas. Since the first report by Isaacson and Wright in 1983, the concept of low-grade gastric B-cell lymphomas of the mucosa-associated lymphoid tissue (MALT) type has become widely accepted[1]. Studies from the 1990s have established that the development of low-grade gastric lymphoma of the MALT type is strongly associated with chronic gastritis caused by Helicobacter pylori[2-4]. Low-grade gastric lymphoma of the MALT type occurs commonly in middle and old age but rarely in children[5,6]. The first long-term follow-up of a pediatric patient with *H pylori*-associated gastric lymphoma of the MALT type was reported in 1995[7]. The present report describes the development of primary gastric lymphomas of the MALT type in two pediatric patients in immunocompromised states followed by tumor regression with conservative treatment.

CASE REPORT

Patient 1

A 14-year-old boy complained of repeated abdominal pain. Upper gastrointestinal endoscopy revealed a tumor-like lesion in the stomach and the patient was referred to our hospital. The patient had a history of autoimmune hemolytic anemia at the age of 1 year and measles infection at 2 years. Thereafter, he developed systemic lymphadenopathy and hyper-gamma-globulinemia. He had been administered oral prednine from the age of 5 years onward based on a putative diagnosis of immunoblastic lymphadenopathy.

A tender mass, hen-egg in size, was discovered in the epigastrium upon admission. Endoscopy revealed a polypoid lesion in the posterior wall of the lower corpus of the stomach. The mass was diagnosed as a low-grade gastric lymphoma of the MALT type based on examination of a biopsy specimen (Figure 1). The

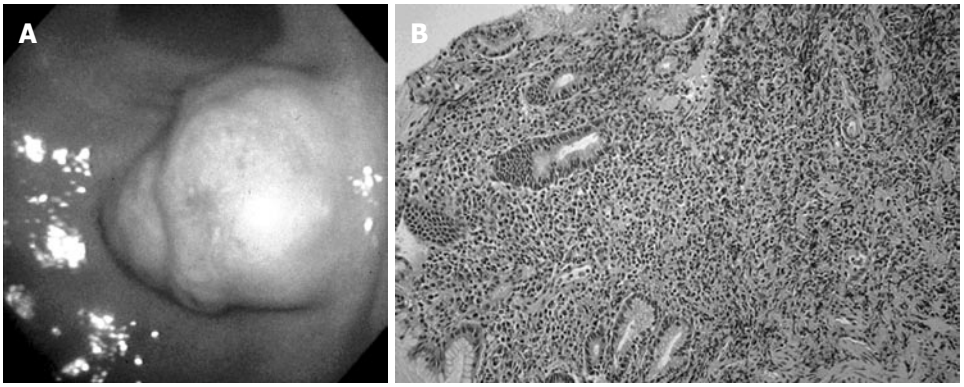

Figure 1 Mucosa-associated lymphoid tissue lymphoma in Patient 1. **A:** A polypoid lesion on the posterior wall of the lower corpus of the stomach, suggesting the existence of lymphoproliferative disease; **B:** The histological features show characteristic appearance of low-grade gastric lymphomas of the mucosa-associated lymphoid tissue type with a diffuse infiltrate of centrocyte-like cells and the formation of lymphoepithelial lesions (H & E, original magnification x10).

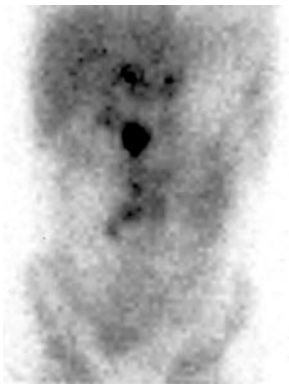

Figure 2 Gallium scintigraphy in Patient 1 shows an abnormal accumulation of the nuclide in the para-aortic lymph nodes.

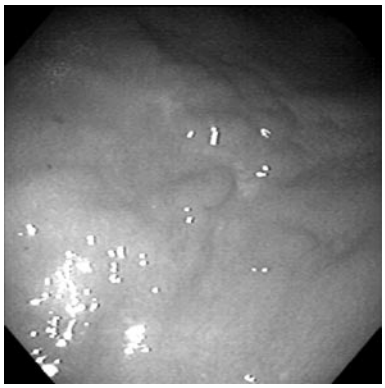

Figure 3 Twenty-four months later, an area of partly whitish rugged mucosa appeared at the site previously occupied by the mucosa-associated lymphoid tissue lymphoma in Patient 1.

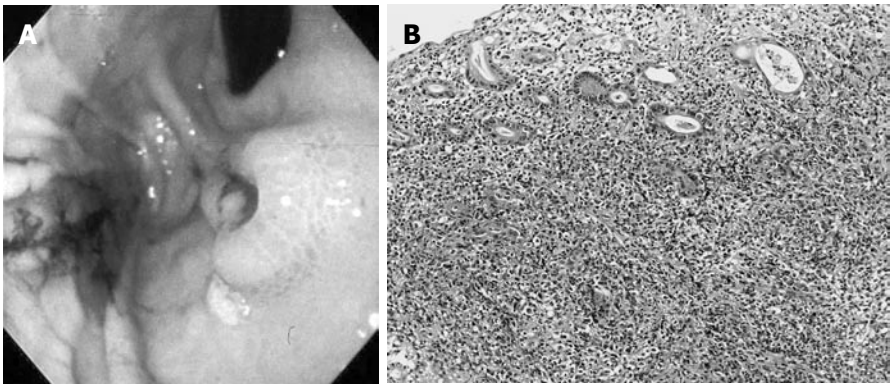

Figure 4 Mucosa-associated lymphoid tissue lymphoma in Patient 2. **A:** An ulcerated lesion on the anterior wall of the upper corpus before eradication; **B:** The histological features show the appearance of low-grade gastric lymphomas of the mucosa-associated lymphoid tissue type similar to that found in Patient 1 (H & E, original magnification x10).

biopsy also confirmed an invasion to the duodenum. Both computed tomography and Gallium scintigraphy strongly suggested metastases to the para-aortic lymph nodes (Figure 2).

Fearing that a complete, en bloc removal of such an advanced tumor would be too invasive in an immunocompromised patient, the authors opted for a watch-and-wait strategy. The patient's serum was positive for anti-*H pylori* antibody, but *H pylori* eradication had to be abandoned due to its adverse effects. Thankfully, the MALT lesion spontaneously regressed over the next 24 mo without any treatment for lymphoma (Figure 3). As the MALT lesion grew less serious, an abdominal pain gradually disappeared. The patient has been followed up for 10 years and continues to do well with no signs of relapse as of this writing.

Patient 2

A 6-year-old boy was referred to our department with a complaint of nausea with hematochezia. He had been diagnosed with adrenoleukodystrophy at the age of 5 years and underwent cord blood transplantation for the treatment of the condition at that time. After the transplantation he was administered oral immunosuppressants, including methylprednisolone and FK506, for graft-versus-host disease.

Upper gastrointestinal endoscopy revealed an ulcerated lesion in the anterior wall of the upper corpus of the stomach. The mass was diagnosed as a low-grade gastric lymphoma of the MALT type associated with *H pylori* gastritis, based on an examination of a biopsy specimen (Figure 4). Neither local invasion nor lymph node involvement was present on diagnostic modalities. When the dosages of the immunosuppressants were tapered he was additionally treated for the *H pylori* infection using a proton pump inhibitor combined with clarithromycin and amoxicillin according to international guidelines[8]. The treatment not only eradicated the *H pylori*, but brought

about a complete remission of the MALT lesion as well (Figure 5). The patient has been followed up for 3 years and continues to do well without any signs of relapse as of this writing.

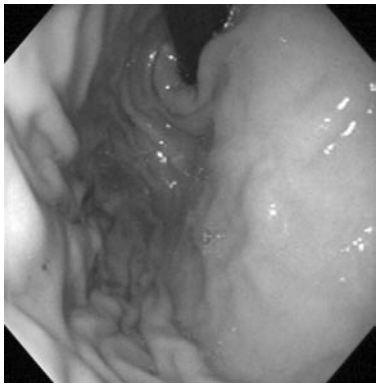

Figure 5 Two months after eradication therapy in Patient 2, an area of glossy whitish mucosa appeared at the site previously occupied by the low-grade gastric lymphoma of the mucosa-associated lymphoid tissue type.

DISCUSSION

Primary low-grade gastric B-cell lymphoma of the MALT type is generally defined as an extranodal lymphoma characterized by an infiltration of the mucosa by centrocyte-like B-cells with formation of lymphoepithelial lesions[9]. Gastric lymphoma of the MALT type is thought to be derived from chronic gastritis caused by *H pylori*[2-4]. *H pylori* infection has been documented in up to 90% of patients with low-grade gastric lymphoma of the MALT type. At first, it has been believed that the MALT acquired in response to *H pylori* infection provides the background on which unidentified factors act and then lead to the development of lymphoma. However, the immune response of B-cell lymphomas of MALT type to *H pylori* antigen is proved to occur only with the presence of T cells on the culture[10,11]. Furthermore, the cellular proliferation of low-grade gastric B-cell lymphomas of MALT type to *H pylori* seems to be dependent on *H pylori*-specific T cells and their products, rather than the bacteria themselves[11]. The inhibitive action against such a mechanism would break down in immunocompromized state so that B-cell lymphomas of MALT type may develop even in *H pylori* infected children.

H pylori eradication is now established to be effective in reducing primary gastric lymphomas of the MALT type and is well accepted as an initial therapy for patients with localized low-grade gastric lymphomas. Remission rates in the recent literature range from 60% to 80%, though recurrence can be expected in some 5% of cases[12-15]. As described above, authors addressed the contributions of the persistent exposure to *H pylori* antigen and the presence of *H pylori*-specific T cells in the pathogenesis of lymphomas of MALT type. With the eradication of *H pylori*, chronic inflammation decreases and the density of submucosal lymphocytes dramatically declines[7]. Thus, the cessation of exposure to *H pylori* antigen due to either the spontaneous cure of *H pylori* or the result of the eradication might progress the tumor regression through the immunologic response.

The major negative predictive factor of tumor response to anti-*H pylori* treatment in patients with primary low-grade gastric lymphoma of the MALT type is involvement of the regional lymph nodes. The depth of the tumor infiltration in the gastric wall correlates with the involvement of the regional lymph nodes and with the degree of tumor malignancy[16-18].

Primary gastric lymphoma of the MALT type has been documented in liver, heart, and kidney transplant recipients[19-22]. The development of these post-transplantation lymphomas of the MALT type appears to be closely related to the abnormal response of the dysregulated host immune system. Post-transplantation lymphoma of the MALT type is also associated with *H pylori* infection and is usually of an Epstein-Barr Virus-negative, B-cell origin. A combination of treatments to minimize immunosuppression and eradicate *H pylori* frees most patients from disease[21-23].

Primary gastric malignant tumors are very rare in children, as are most lymphomas. The majority of primary gastric lymphomas are high-grade non-Hodgkin's lymphomas of B-cell origin. To our knowledge, the literature has not reported significant numbers of primary gastric lymphomas of the MALT type in children[7,24-26]. The clinicopathological features in pediatric cases are considered to be similar to those observed in adults.

In this report we have described the complete remission of primary gastric lymphomas of the MALT type in two pediatric patients under an immunocompromised state. In patient 1, an advanced gastric lymphoma of the MALT type with duodenal invasion and lymph node involvement regressed spontaneously without any treatment. This experience prompts us to question whether regional lymph node involvement is a negative predictive factor of the tumor response, as described above. Anti-*H pylori* treatment with minimization of immunosuppression was satisfactory in our second patient. To the best of our knowledge, patient 2 is the first reported case of primary gastric lymphoma of the MALT type occurring in a post-cord blood transplantation setting.

In conclusion, the authors propose that low-grade gastric lymphoma of the MALT type can be cured by conservative treatment even in immunocompromised pediatric patients. A watch-and-wait strategy combined with *H pylori* eradication and close follow-up should be an option before attempting more aggressive treatments, even in cases with advanced disease.

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CASE REPORT

A case of idiopathic colonic varices: A rare cause of hematochezia misconceived as tumor

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Abstract

Colonic varices are a very rare cause of lower gastrointestinal bleeding. Fewer than 100 cases of colonic varices, and 30 cases of idiopathic colonic varices (ICV) have been reported in the English literature. Among these 30 cases of ICV, 19 cases were diagnosed by angiography, and 7 operated cases were diagnosed later as ileocecal vein deficit, hemangioma, and idiopathic in 1, 1, 5 cases, respectively. We report the case of a 24-year-old man who suffered from multiple episodes of hematochezia of varying degree at the age of 11 years. He had severe anemia with hemoglobin of 21 g/L. On colonoscopy, tortuously dilated submucosal vein and friable ulceration covered with dark necrotic tissues especially at the rectosigmoid region were seen from the rectum up to the distal descending colon. It initially appeared to be carcinoma with varices. Mesenteric angiographic study suggested a colonic hemangioma. Low anterior resection was done due to medically intractable and recurrent hematochezia. Other bowel and mesenteric vascular structures appeared normal. Microscopic examination revealed normal colonic mucosa with dilated veins throughout the submucosa and serosa without representing new vessel growth. Taken all of these findings together, the patient was diagnosed as ICV. His postoperative course was uneventful.

Key words: Idiopathic colonic varices; Hematochezia; Colon cancer; Hemangioma

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INTRODUCTION

Colonic varices, a very rare cause of lower gastrointestinal bleeding, are usually secondary to portal hypertension. It was reported that the incidence of colonic varices is 0.07% (2 of 2912 cases)[1]. One case had liver failure, and the other case had chronic pancreatitis. There are no autopsy data about idiopathic colonic varices. Fewer than 100 cases of colonic varices have been reported[1], and 23 reports containing 30 patients with idiopathic colonic varices (ICV) have been reported in the English literature[2-23]. Diagnosis of idiopathic colonic varices is most accurately achieved by mesenteric angiography[3-19]. But in some cases, operation could reveal secondary causes or changed pathologic diagnosis[18,19,24]. We report a patient whose diagnosis of idiopathic colonic varices was confirmed by the segmental colonic resection.

CASE REPORT

A 24-year-old man was admitted to hospital with exertional dyspnea and weakness. He had a 13-year history of multiple episodes of hematochezia of varying degree. At age 22, he visited our hospital due to dizziness. He denied alcohol consumption, or any family history of gastrointestinal bleeding. He had severe anemia with hemoglobin of 27 g/L. After 2-unit blood transfusion, he refused further investigations or management.

On admission, he was pale with mild tachycardia but had no abnormality on physical examination. Laboratory studies showed 4.93×10^9/WBC, 21 g/L hemoglobin, 0.09% hematocrit, 256×10^9/L platelets, and 22.2 μmol/L iron. Coagulation studies, liver function tests, and viral hepatitis serology were normal. Colonoscopy revealed considerable bluish and tortuously dilated submucosal veins which were seen from the rectum up to the distal descending colon, and friable ulceration covered with dark necrotic tissues

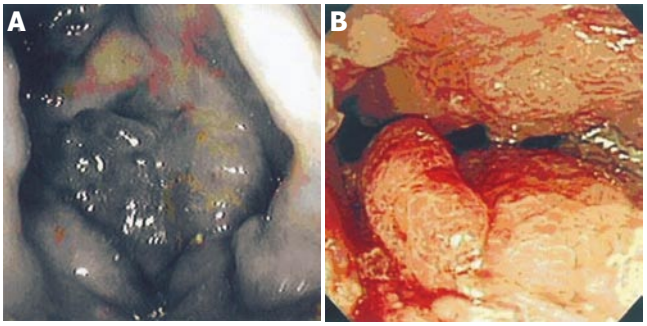

Figure 1 Colonoscopy view of colonic varices at the rectosigmoid region.

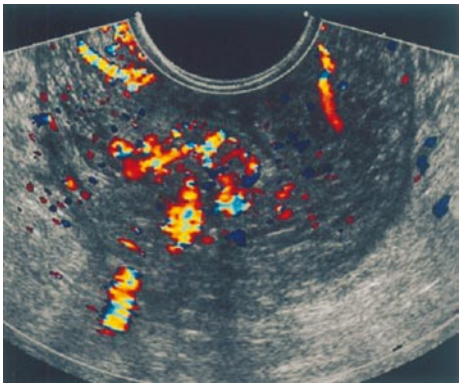

Figure 3 Trans-rectal Doppler sonography of colonic varices.

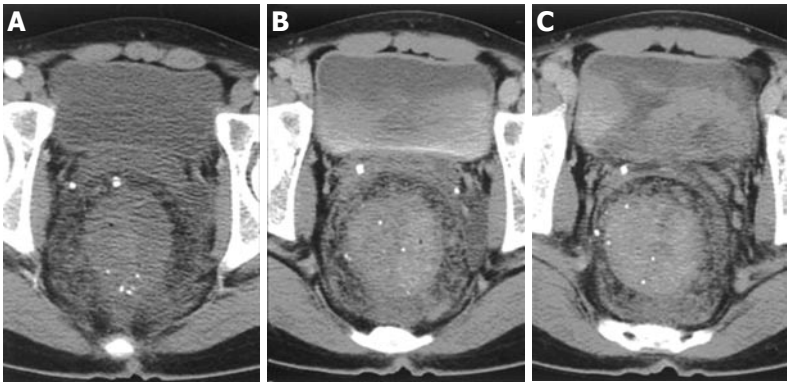

Figure 2 Abdomen-pelvis triphasic computed tomography showing an arterial phase image of mucosal enhacement (**A**) venous (**B**) and delayed(**C**) phase images of delayed diffuse wall enhancement.

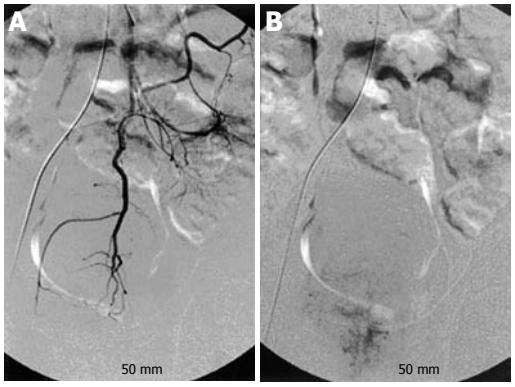

Figure 4 Selective angiography of superior and inferior mesenteric artery showing normal arterial phase (**A**) and venous pooling in rectum in the delayed phase (**B**).

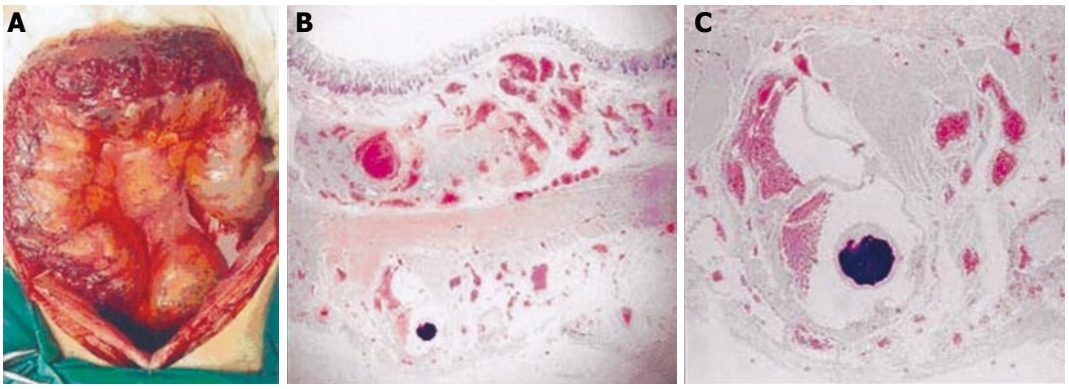

Figure 5 Dilated subserosal veins seen in operation (**A**), great enlarged vessels observed in submucosal and serosal layer at low power, HE×10 (**B**), and distortion of vascular wall found in enlarged vessels at high power, HE×40 (**C**).

especially at the rectosigmoid region (Figure 1). It seemed to be carcinoma with varices. Gastroduodenoscopic examination showed unremarkable finding, without evidence of varices or arteriovenous malformations. Abdominal ultrasonography also revealed no abnormal finding. An abdomen-pelvis triphasic computed tomography showed thickened bowel wall, intramural calcification and delayed enhancement from the rectum up to the descending colon (Figure 2). Transrectal Doppler sonography also demonstrated diffuse wall thickening with internal hypervascular structure (Figure 3). Selective angiography of the superior and inferior mesenteric artery showed delayed venous pooling in rectum but no other abnormal vascular structure (Figure 4). The angiographic diagnosis was a colonic hemangioma.

Low anterior resection was done due to medically intractable and recurrent hematochezia. During surgery, tortuously dilated subserosal and mesenteric vessels were seen at the rectum, sigmoid colon and distal descending colon (Figure 5A). Other bowel and mesenteric vascular structures appeared to be normal. Microscopic examination revealed normal colonic mucosa with dilated veins throughout the submucosa and serosa without new vessel formation (Figures 5B and 5C). In addition, thrombi were frequently observed in the endovascular lumen, and dystrophic calcification was also often found in the wall of vessels. He remained well showing no further signs of gastrointestinal hemorrhage at the time when he was reported in this paper.

DISCUSSION

Colonic varices are a very rare cause of lower gastrointestinal bleeding. Portal hypertension is the most common cause of colonic varices, which are usually located in the rectosigmoid region and the cecum[16]. Other less common causes of colonic varices are congestive heart failure, mesenteric vein thrombosis, pancreatitis with splenic vein thrombosis and adhesions, and mesenteric vein compression[1, 2].

The varix is a descriptive term of enlarged and convoluted vein, artery or lymphatic vessel, which does not indicate the etiology of them. Establishing the diagnosis of ICV amid various factors remains difficult. It needs to exclude secondary causes by laboratory and imaging studies, such as liver function test, hepatitis serology, and liver sonography or computed tomography. Diagnosis of ICV usually requires angiography with visualization of the enlarged vessels showing prolonged observation of the venous phase but no portal/mesenteric vein obstruction[3, 5]. Gross examination and biopsy under laparoscope or exploration can definitely confirm ICV.

We fully reviewed 30 cases reported as ICV. Hemangioma and vascular malformation are described as ICV in some of these reports[2,3,18,19]. But hemangioma has obviously different pathologic findings, and vascular malformation has plainable etiology. So, in our opinion, hemangioma and vascular malformation can be ruled out from ICV. According to these, 21 reports containing 25 patients remain to be idiopathic[4-17,20-23]. In more than half of the cases, the total colon is affected[6-9,14-16,20-22], and in segment-involved cases, the lesions are distributed equally between right and left colon. About 30% of these have familial tendency[8-11,14-16,20,21] and more than half of these manifest the onset of hematochezia before the third decade[9-13,15-17,22], suggesting that ICV may be congenital in origin. But the number of the cases is too small to conjecture the inheritance pattern of ICV. Angiography has been performed in only 19 cases[3-19]. Before angiography, two of these cases, suspected to be ICV, were diagnosed as congenital anomaly of the portocaval system[2] and hemangioma[3]. 7 cases which were surgically confirmed, were diagnosed as congenital failure of ileocecal vein[18], hemangioma[19], and ICV[15-17,19,20] in 1, 1, 5 cases, respectively.

ICV is usually accompanied with recurrent and massive rectal bleeding. It is presumed that bleeding from colonic varices is the result either of abrasion from hard stool in the distal colon, or of pressure ischemia and sloughing of the overlying mucosa in the cecum[16].

The barium contrast enema may be helpful, but is often misinterpreted as air bubble, fecal material, polyposis, carcinoma, or as normal[12]. Therefore, it is unreliable. Colonoscopy is more sensitive, but collapse of varices by excessive air inflation can make the physician to mistake ICV as normal condition. ICV can mimic polyp, cancer, and ulcerative colitis. Biopsy may induce massive hemorrhage[15].

Scintigraphic studies have not been found to be adequate in localizing the segment of colon associated with bleeding varices but can detect hemorrhage with greater sensitivity than angiography[25].

Mesenteric angiography is a most accurate radiologic diagnostic tool, but secondary colonic varices could be misinterpreted as an ICV on angiography. Defreyne *et al*[24] have explained that angiographical diagnosis is in disagreement with the histopathological diagnosis of an arteriovenous malformation probably because the blood passage through the small fistulas is too slow. The same principle can be applied to our case. In about 30% cases, operation revealed secondary causes or changed histopathologic diagnosis as previously described[15-21], suggesting that angiography is an incomplete tool to confirm ICV. Laparoscopy may be a more accurate and definitive diagnostic tool than histology, because histological tissue sampling is very dangerous if not in operation.

The prognosis of ICV seems to be good at all ages compared with cirrhotic varices, which may be related to low pressure in the varices as well as the absence of significant hepatocellular disease[5]. Conservative therapy is sufficient for most ICV patients. Patients with intractable and persistent bleeding may require partial colectomy for involved area.

In conclusion, it is very difficult to diagnose ICV as in our case. The most important thing is to role out secondary causes of varices. In the suspected case, the physician should consider the clinical, radiologic, and surgical information collectively for the proper diagnosis. This case report describes a life-threatening bleeding episode of ICV, mimicking carcinoma in colonoscopy and hemangioma in angiography. ICV can be confirmed by surgical histopathogy, and successfully treated with partial colectomy on involved area.

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CASE REPORT

A rare case: Spontaneous cutaneous fistula of infected splenic hydatid cyst

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Abstract

Hydatid disease is caused by the larval stage of the genus *Echinococcus*. Live hydatid cysts can rupture into physiologic channels, free body cavities or adjacent organs. Although hydatid disease can develop anywhere in the human body, the liver is the most frequently involved organ, followed by the lungs. Cysts of the spleen are unusual. There are only five case reports of spontaneous cutaneous fistulization of liver hydatid cysts in the literature. But there isn't any report about cutaneous fistula caused by splenic hydatid cyst. We report a first case of spontaneous cutaneous fistula of infected splenic hydatid cyst.

A 43-year-old man was admitted to our Emergency Service with abdominal pain and fluid drainage from the abdominal wall. He has been suffering from a reddish swelling on the abdominal wall skin for four months. After a white membrane had been protruded out from his abdominal wall, he was admitted to our Emergency Service. On physical examination, a white membrane was seen to protrude out from the 2 cm x 1 cm skin defect on the left superolateral site of the umblicus. Large, complex, cystic and solid mass of 9.5 cm-diameter was located in the spleen on ultrasonographic examination. At operation, partial cystectomy and drainage was performed. After the operation, he was given a dosage of 10 mg/kg per day of albendazole, divided into three doses. He was discharged on the postoperative 10th d. It should be kept in mind that splenic hydatid cysts can cause such a rare complication.

Key words: Hydatid cyst; Cutaneous fistula; Spleen

INTRODUCTION

Hydatid disease is caused by the larval stage of the genus *Echinococcus*. Echinococcosis has a worldwide distribution because of increasing migration and a growing incidence of world travel. It is endemic in many Mediterranean countries, the middle and Far East, South America, Australia, New Zealand and East Africa[1].

The most common form, cystic hydatid disease, is caused by *Echinococcus granulosus*, whereas the alveolar type is caused by *E. multiocularis*. Approximately 70 percent of hydatid cysts are located in the liver and there are multiple cysts in one-quarter to one-third of these cases[2].

Live hydatid cysts can rupture into physiologic channels, free body cavities or adjacent organs[1]. There are only five case reports of spontaneous cutaneous fistulization of liver hydatid cysts in the literature[3-7]. But there isn't any report about cutaneous fistula caused by splenic hydatid cyst.

We report a first case of spontaneous cutaneous fistula of infected splenic hydatid cyst.

CASE REPORT

A 43-year-old man was admitted to our Emergency Service with abdominal pain and fluid drainage from the abdominal wall. He has been suffering from a reddish swelling on the abdominal wall skin for four months. He stated that some hemopurulent drainage was occured from this swelling two months before. He didn't go to any doctor during this period. The drainage was stopped spontaneously in two days. He was no other complaints after that. Two days before, hemopurulent drainage and pain were started again. After a white membrane had been protruded out from his abdominal wall, he was admitted to our Emergency Service. On physical examination, there was a 2 cm x 1 cm skin defect on the left superolateral site of the umblicus (Figure 1). This defect was at a distance of 3 cm to the umblicus. A white membrane was protruded out from this defect. This membrane looked like the germinative memb-

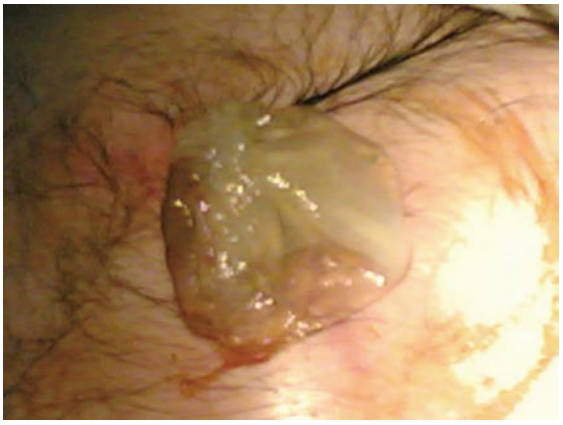

Figure 1 Germinative membrane protruded out from the 2 cm x 1 cm skin defect on the left superolateral site of the umblicus.

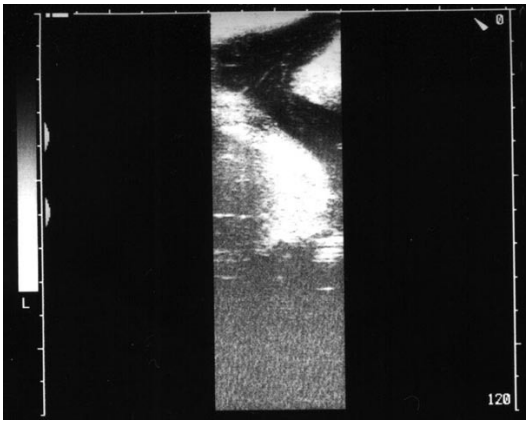

Figure 2 Fistula tract on the anterior abdominal wall.

rane of hydatid cyst. Laboratory findings were as follows; hemoglobin: 11.7 g/dL, white blood count: 18 800/mm^3, platelet: 632 000/mm^3, alkaline phosphatase: 270 U/L, gamma glutamyl trans-peptidase: 51 U/L. Other laboratory findings were normal. Ultrasonography (USG) was the modality of choice for this patient. Large, complex, cystic and solid mass of 9.5 cm-diameter was located in the spleen. Mass showed a complex internal character with multiple floating membranes, echogenic foci due to hydatid sand and heterogenous-echogenic matrix which are characteristic imaging findings for hydatid disease. Mass passed through the anterior abdominal wall from 5 cm-diameter peritoneal defect. Fistula tract on the anterior abdominal wall and cutaneous orifice were also seen (Figures 2 and 3).

At operation, 300 mL purulent material was drained from the subcutaneous tissue after skin incision. The germinative membrane in the fascial defect was taken in the abdomen. Approximately 1.5 liters of infected hydatid cyst material was aspirated from the cyst cavity. On exploration, the cyst was found to be localized in the splenic parenchyma. There were multiple adhesions between the cyst and spleen, liver, stomach and abdominal wall. There was a 5 cm-diameter fascial defect on the lateral side of the left rectus muscle. This was the defect from where the germinative membrane was protruded out of the abdomen. Adhesions were dissected and partial cystectomy was performed. There were splenic fragments in the cyst cavity. The cyst cavity was cleaned and 10% povidone iodine was applied to the cyst wall. Two drains were left in the abdomen; one in the cyst cavity and the other in the left paracolic region. The perforated skin area was debridated but not sutured. After the operation, he was given a dosage of 10 mg/kg/d of albendazole, divided into three doses. He was discharged on the postoperative 10th d. He came to first control three mo after the operation. USG and computerized tomography (CT) examinations together with serologic tests were normal.

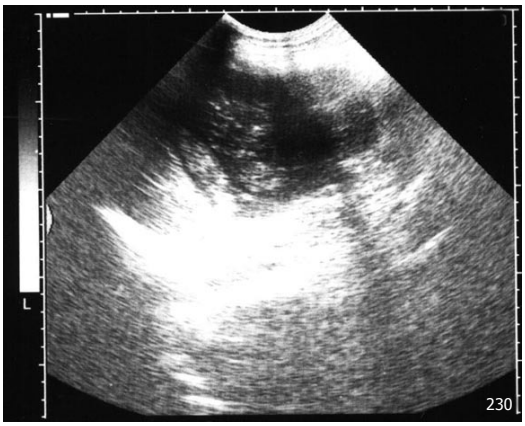

Figure 3 Large, complex, cystic and solid mass located in the spleen.

DISCUSSION

We present the first case of spontaneous cutaneous fistula of infected splenic hydatid disease. The clinical features of hydatid disease depend on the site, size, stage of development, whether the cyst is dead or alive and whether there is a complication or not[1]. Patients with simple or uncomplicated multivesicular cysts are usually asymptomatic. The clinical symptoms are related to pressure on adjacent organs or the presence of complications. Abdominal pain and tenderness are the most common complaints[2].

The hydatid disease can remain symptom-free for years or cause serious complications resulting in death. The main complications are rupture into the peritoneal cavity, infection, compression of the biliary tree, intrabiliary rupture, anaphylaxis, and secondary hydatosis[8].

Intrabiliary rupture represents the most common complication and occurs in 5 to 10 % of cases[2]. T-tube drainage, choledochoduodenostomy and transduodenal sphincteroplasty are effective procedures in the management of intrabiliary ruptured hydatid disease[9-11]. Suppuration, the second most common complication, is caused by bacteria from the biliary tract. Intraperitoneal rupture results in the showering of hydatid fluid, brood capsules, and scolices into the peritoneum; leading to systemic anaphylactic reactions or the development of new hydatid cyst[2,12,13].

Spontaneous cutaneous fistulization is a very rare complication of liver hydatid cysts. There are only five case reports in the literature[3-7]. Two of them are cysto-hepato-bronchial fistula[3,5]. In these cases, liver hydatid cysts were

fistulized simultaneously and spontaneously to the skin and in the bronchia. The other cases were cutaneous fistula of liver hydatid cysts[4,6,7].

A viable hydatid cyst is a space-occupying lesion with a tendency to grow. In confined areas, such as the central nervous system, small cysts cause serious symptoms. In less restricted areas the symptoms depend on the site and size of the cyst. Symptoms may result from direct pressure or distortion of neighboring structures or viscera. The cyst grows in the direction of the least resistance. Another consequence of cyst enlargement is that it can rupture. Live hydatid cysts can rupture into physiologic channels, free body cavites or adjacent organs. The other factor responsible for fistulization of hydatid disease is inflammation. Infection and continued expansion of the cyst causes pressure erosion and adhesion to the adjacent structures. In time, with increasing intracystic pressure, the cyst ruptures. Inflammation leads to necrosis and causes fistulization[1]. In our case, inflammation is probably the main factor of cutaneous fistulization.

Although hydatid disease can develop anywhere in the human body, the liver is the most frequently involved organ (52%-77%), followed by the lungs (10%-40%)[8]. Cysts of the spleen are unusual. Parasitic cysts are usually due to echinococcal involvement, while non-parasitic cysts can be categorized as dermoid, epidermoid, epithelial, and pseudocysts[2]. The spleen is infrequently involved in hydatid disease. Ozdogan *et al*[14] reported that the spleen was involved in 2.5% of all abdominal hydatidosis cases. They suggested that although splenectomy was the conventional treatment, partial cystectomy and omentopexy could be another choice for the treatment of splenic hydatosis.

Splenic abscess is an uncommon cause of splenic abdominal sepsis. Splenectomy is the operation of choice, but some patients have been treated with splenectomy and drainage when there were gross adhesions or the condition of the patient did not permit splenectomy[2]. In our case, we performed partial cystectomy and drainage. Because of dense adhesions and abscess formation, we didn't perform splenectomy. There was no recurrence in abdominal cavity, including spleen, at USG and CT imaging performed 3 mo after the operation. If recurrence occur, we will perform total splenectomy as a second operation.

Benzimidazole carbamates (mebendazole and albendazole) are antihelmintic drugs that kill the parasite by impairing its glucose uptake. Albendazole is the drug of choice because of its better absorption and better clinical results in comparison with mebendazole. Continuous daily treatment for a 3-mo period has better results[13]. We proposed our patient a dosage of 10 mg/kg per day albendazole for 3 mo and a 3-mo period controls.

We reported a first case of spontaneous cutaneous fistula of infected splenic hydatid cyst. It should be kept in mind that splenic hydatid cysts can cause such a rare complication.

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Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
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ILTS 12th Annual International Congress
3–6 May 2006
Milan
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www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
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Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
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icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

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convention@edinburgh.org
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Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
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1 **Das KM,** Farag SA. Current medical therapy of inflammatory bowel disease. *World J Gastroenterol* 2000; 6: 483-489 [PMID: 11819634]

2 **Pan BR**, Hodgson HJF, Kalsi J. Hyperglobulinemia in chronic liver disease: Relationships between *in vitro* immunoglobulin synthesis, short lived suppressor cell activity and serum immunoglobulin levels. *Clin Exp Immunol* 1984; 55: 546-551 [PMID: 6231144]

3 **Lin GZ,** Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287

Books and other monographs (list all authors)

4 **Sherlock S,** Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

5 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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6 **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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1	4 days	4 d	In figures, tables and numerical narration
2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0,1,2 yr	In figures and tables
12	0,1,2 year	0,1,2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (*V/V*)	500 mL/L	
20	50% (m/*V*)	500 g/L	
21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_4	
25	4rd edition	4^{th} edition	
26	15 year experience	15- year experience	
27	18.5 kDa	18.5 ku,18 500u or M_r18 500	
28	25 $g.kg^{-1}/d^{-1}$	25 g/(kg·d) or 25 g/kg per day	
29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	13 000 rpm	13 000 *g*	High speed; *g* should be in italic and suitable conversion.
35	1000 *g*	1 000 r/min	Low speed. *g* cannot be used.
36	Gene bank	GenBank	International classified genetic materials collection bank
37	Ten L	Ten liters	At the beginning of a sentence
38	Ten mL	Ten milliliters	At the beginning of a sentence
39	umol	μmol	
40	30 sec	30 s	
41	1 g/dl	10 g/L	10-fold conversion
42	OD_{260}	A_{260}	"OD" has been abandoned.
43	One g/L	One microgram per liter	At the beginning of a sentence
44	*A*260 nm	A_{260} nm	*A* should be in italic.
	$^bP<0.05$	$^aP<0.05$	In Table, no note is needed if there is no significance instatistics: $^aP<0.05$, $^bP<0.01$ (no note if $P>0.05$). If there is a second set of *P* value in the same table, $^cP<0.05$ and $^dP<0.01$ are used for a third set: $^eP<0.05$, $^fP<0.01$.
45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=67.4	Notices in or under a table
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
70	*iv*	iv	intravenous injection
71	Wang et al	Wang *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	