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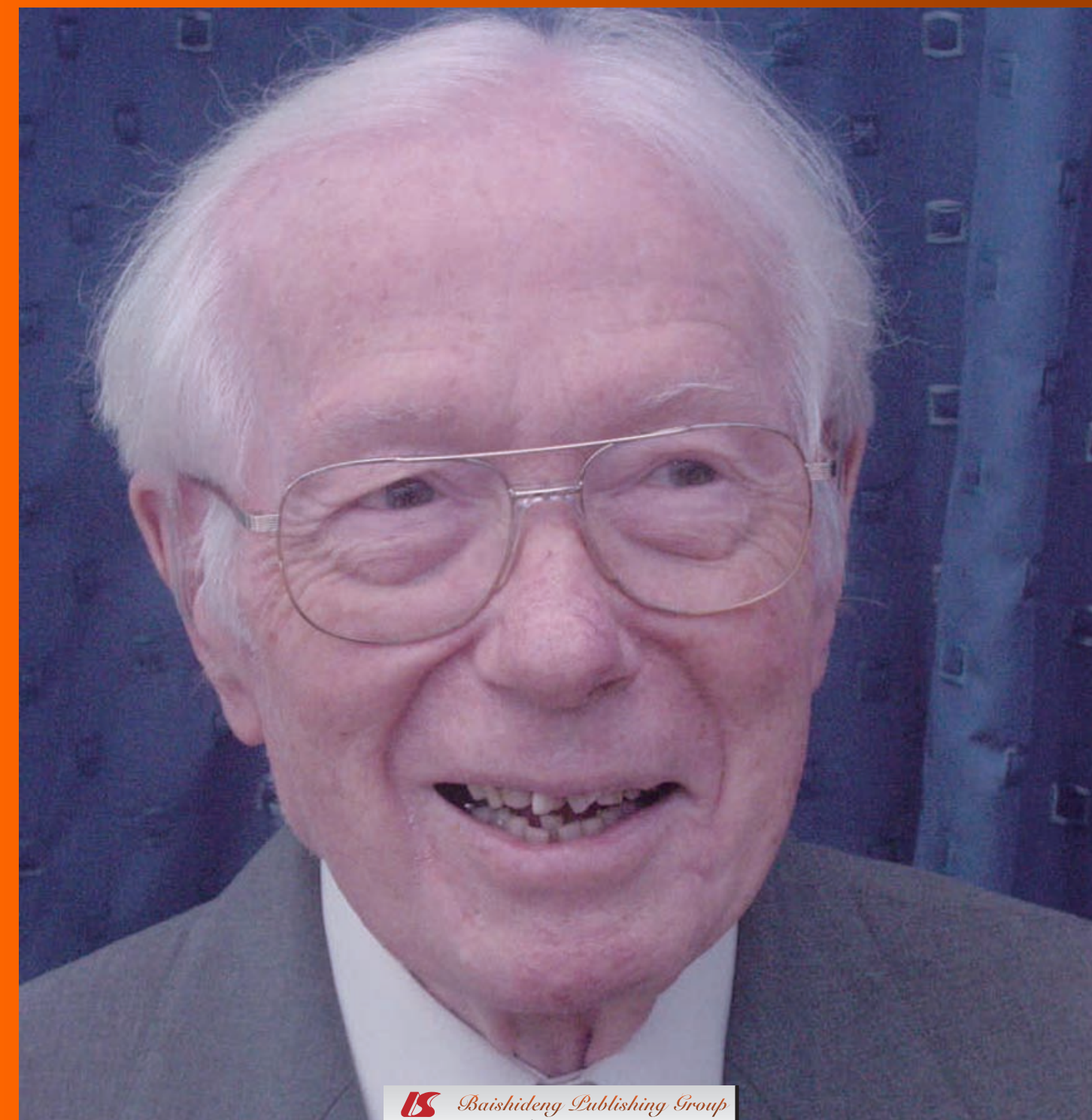


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## A tribute to Dr. Frank I Tovey on his 90th birthday

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### Abstract

This paper pays a tribute to Dr. Frank I Tovey on his 90th birthday which happens on September 1, 2011, and briefly describes the major findings in his research career and contributions as follows. The geographical prevalence of duodenal ulceration is related to staple diets. Unrefined wheat and maize, soya, certain pulses and millets are associated with a low prevalence while refined wheat, maize and rice, yams, cassava and green banana with a high prevalence. Predominant foodstuffs from low prevalence areas are ulceroprotective in rat peptic ulcer models. The protective activity lies in the lipid fraction present in these foodstuffs. The lipid fraction also promotes ulcer healing, is active both orally and intramuscularly and is ulceroprotective against non-steroidal anti-inflammatory drugs (NSAIDs). The phospholipids and phytosterols present in the lipid have been identified to be responsible for this protective activity. The combination of phospholipids and phytosterols may be of value in the prevention and treatment of duodenal ulceration and protection against the ulcerogenic effect of NSAIDs.

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**Key words:** Duodenal ulceration; Staple diets; Protective factors; Phospholipids; Phytosterols; Non-steroidal anti-inflammatory drugs; *Helicobacter pylori*

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**Figure 1** Frank I Tovey, OBE, ChM, FRCS (Eng), Honorary Senior Research Associate, Department of Surgery and Interventional Science, University College London, London W1W 7EJ, United Kingdom.

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September 1st, 2011 marks the 90th birthday of Dr. Frank I Tovey, ChM, FRCS (Eng) (Figure 1).

Dr. Tovey worked as a surgeon at the Methodist Hospital in Zhaotong, Yunnan, southwest China from 1948 to 1949 and at the Holdsworth Memorial Hospital, Mysore, South India from 1951 to 1967. He was appointed OBE in 1966 for services to surgery and leprosy in India. From 1968 to 1986, he was a Consultant Surgeon in the United Kingdom at Basingstoke District Hospital and Honorary Research Fellow in the Department of Surgery, University College Hospital, London. His present appointment is Honorary Senior Research Associate, Department of Surgery and Interventional Science at University College London.

His main research interests include reconstructive surgery in leprosy, the nutritional effects of surgery for pep-

tic ulceration both in the United Kingdom and in developing countries, the relationship between the prevalence of duodenal ulceration and staple diets worldwide, and the absence of any relationship between the prevalence of duodenal ulceration and the prevalence of *Helicobacter pylori* (*H. pylori*) infection. These interests have led to a number of publications.

His interest in duodenal ulceration arose when working in Mysore in India where duodenal ulceration was a major problem particularly in men and requiring surgery. He found that partial gastrectomy was inappropriate for people living on one large meal a day because they were not able to eat enough. This led to trials of the long-term nutritional effect of different types of vagotomy and drainage procedures, which continued after his return to the United Kingdom<sup>[1-3]</sup>. He also noted that the majority of duodenal ulcer patients came from the wetlands where rice was the staple diet and very rarely from the dry areas where millets or pulses were the staple food. This suggested a relationship between staple diets and the prevalence of duodenal ulceration and led to researches which extended over 55 years. Information was gathered from all over India and confirmed a higher prevalence in rice-eating areas particularly in the South and a lower prevalence in the unrefined wheat or millet-eating drier areas in the North. In association with Denis Burkitt and the Medical Research Council, information was obtained from many countries including Africa, China and Malaysia. This was at a time when surgery was the accepted procedure for duodenal ulceration and information about the incidence of surgical procedures thus reflected its prevalence. The evidence showed a consistent pattern. A higher prevalence was found in areas where the staple diet was principally milled rice, refined wheat or maize, yams, cassava, sweet potato or green bananas, and a lower prevalence in areas where the staple diet was based on unrefined wheat or maize, soya, certain millets or certain pulses. These diets and individual foods were investigated using several rat peptic ulcer models, and the results confirmed the ulceroprotective activity of the foods predominating in the diet in the lower duodenal ulcer prevalence areas. The experiments showed that the protective activity lay in the lipid component of these foods. The lipid fraction was protective when given orally or intramuscularly, and it also promoted ulcer healing. The activity was found to lie in the phospholipid and sterol fractions of the lipid, and their nature has been subsequently identified. This combination of phospholipids and phytosterols, may prove to be of value in giving protection against not only duodenal ulceration but also the ulcerogenic effect of non-steroidal anti-inflammatory drugs<sup>[4-17]</sup>.

The geographical study of the prevalence of duodenal ulceration also showed no relationship with the prevalence of *H. pylori* infection<sup>[18-22]</sup>.

Dr. Tovey served as a member of the *World Journal of Gastroenterology* (WJG) Editorial Board for 11 years, during which he reviewed 55 articles and published 8 articles in WJG<sup>[3,13,19-24]</sup>, making a great contribution to the improvement of the academic quality of WJG.

On behalf of all the WJG Editorial Board members

and all WJG editorial staff, I would like to wish Dr. Frank I Tovey a very happy birthday!

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## Management of Crohn's disease in smokers: Is an alternative approach necessary?

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### Abstract

Inflammatory bowel disease (IBD) is a chronic condition with a pathogenic background that involves both genetic and environmental factors. Although important progress has been made regarding the former in the last decade, scarce knowledge is available for the latter. In this sense, smoking remains the most important environmental factor in IBD. Active smoking increases the risk of developing Crohn's disease (CD). Moreover, CD patients who start or continue smoking after disease diagnosis are at risk for poorer outcomes such as higher therapeutic requirements and disease-related complications, as compared to those patients who quit smoking or who never smoked. However, the harmful effect of active smoking is not uniform in all patients or in all clinical scenarios. Interventions designed to facilitate smoking cessation may impact the course of the disease. In this article, the available evidence of the deleterious effects of smoking on CD is reviewed in detail, and alternative therapeutic approaches to CD in smokers are proposed.

### INTRODUCTION

Crohn's disease (CD) is a chronic idiopathic inflammatory bowel disease of unknown etiology. The pathogenesis of CD is multifactorial, and several factors have been implicated in its development including genetic and environmental ones<sup>[1]</sup>. Differences in incidence rates across age, time, and geographic areas suggest that environmental factors are implicated in inflammatory bowel disease (IBD), but only cigarette smoking and appendectomy have consistently been identified as risk factors, the former being the one with the greatest impact on both CD development and disease behavior. The disease is more frequent in active smokers than in non-smokers<sup>[2,3]</sup>, and smoking may alter the natural course of CD since smokers are more likely to develop complications and relapses, and to need surgery<sup>[4]</sup>.

There is evidence that interventions designed to facilitate smoking cessation may improve the course of the disease<sup>[5]</sup>. This article reviews the available data on smoking and its effects among patients with an established diagnosis of CD.



## CLINICAL COURSE AND PROGNOSIS OF CD IN SMOKERS

It is well known that CD is more common in smokers<sup>[3]</sup>. The relationship between smoking and worsening of the clinical course of the disease has also been established, though the underlying mechanisms are complex and are still subject to research. Of note is the fact that in the case of ulcerative colitis (UC), the effects of smoking are the opposite of those seen in CD. The potential mechanisms explaining such opposing behavior include the different effects of smoking upon cellular and humoral immune function in both diseases, their cytokine profiles, and bowel wall motility and permeability<sup>[6]</sup>.

Among the elements contained in tobacco smoke, it seems clear that nicotine has the greatest impact upon the clinical course of CD<sup>[7]</sup>. However, it has been recently established that tobacco glycoprotein may be responsible for promoting a Th1 cell response<sup>[8]</sup>, while nicotine maintains its relevance as the cause of anti-inflammatory action in UC<sup>[9]</sup>. Smokers show an increased production of reactive oxygen species and a lessened antioxidant capacity<sup>[10]</sup>; this, in turn, could act synergically with oxidative stress in CD<sup>[11]</sup>. Moreover, environmental factors may interact with genetic factors. In this sense, recent research has demonstrated that smoking may influence the gene expression profile of the colonic mucosa in CD patients<sup>[12]</sup>.

Smoking has been associated with a poorer prognosis of CD and a worse quality of life<sup>[13]</sup>. Holdstock *et al*<sup>[14]</sup> reported for the first time that CD patients who were active smokers had an increased number of disease relapses and more severe pain; this was also associated with an increased probability of hospital admission and intestinal resection among patients with ileal involvement. This increased relapse rate was later estimated to be two-fold higher among smokers in a Canadian prospective study involving 152 CD patients<sup>[15]</sup>.

Some epidemiological factors such as gender or disease location may influence the impact of smoking habits on CD outcomes. Most data on the impact of smoking on CD natural history come from the studies performed in the Hôpital Saint Antoine in Paris<sup>[16-18]</sup>. One of their first studies included 400 consecutive CD patients who were specifically interviewed to assess the effects of smoking upon the long-term course of the disease<sup>[16]</sup>. The need for corticosteroids and immunomodulators was greater among smokers, but no differences were found in terms of intestinal resection requirements except in those patients who started smoking after CD diagnosis. In addition, the deleterious effect of smoking was found to be dose-dependent, and more marked among women. In a later study, the same authors underscored the existence of a poorer prognosis among women<sup>[18]</sup>.

Exclusive colon involvement possibly does not imply a poorer disease course in smokers as compared to non-smokers. In another study published by the same French group that involved 622 CD patients, the risk of relapse was found to be significantly greater among patients with inactive disease and without colon involvement<sup>[17]</sup>. A mul-

ticenter survey involving 457 CD patients from 19 European countries evaluated several clinical parameters at the time of disease diagnosis. Prescription of corticosteroids or immunomodulators - as a surrogate marker of a less favorable disease course - proved greater among smokers within the first year from disease diagnosis<sup>[19]</sup>. The proportion of individuals with ileal involvement was likewise significantly greater among smokers, in agreement with the findings in our own setting<sup>[20]</sup>. In fact, these higher surgical requirements among smokers could be related to a more frequent ileal involvement<sup>[21]</sup>.

The negative effects of smoking seem to be dose-dependent, with the risk of a poor disease prognosis being particularly high among heavy smokers. Lindberg *et al*<sup>[22]</sup>, in a series of 231 CD patients, reported that heavy smokers (over 10 cigarettes/d) presented an increased risk of surgery at 5 and 10 years from diagnosis as compared to patients who never smoked (OR 1.14 and 1.24, respectively). The risk for further operations was even higher, with an OR at 10 years of 1.79. In another study, the proportion of time with intestinal inflammatory activity during follow-up was reported to be 37% among non-smokers, 46% among patients who smoked less than 10 cigarettes/d, and 48% among heavy smokers<sup>[23]</sup>. Surprisingly, a recent study has reported no unfavorable clinical course in smokers, though passive smokers did show a poorer course<sup>[24]</sup>.

Smoking has been correlated to a lesser prevalence of inflammatory (non-stricturing, non-penetrating) behavior of the disease, thus suggesting that tobacco consumption influences progression towards fistulizing or stricturing disease<sup>[22,25-27]</sup>. Available data are contradictory when assessing the risk of perianal disease, since it was included in the definition of the fistulizing CD pattern in the initial Vienna phenotypic classification of CD<sup>[28]</sup>, but not in the Montreal adaptation<sup>[29]</sup>. Of note is the fact that phenotypic characteristics may differ greatly according to the ethnical origin of the studied population. Thus, for the French Canadian population, the phenotypic pattern has been differentiated from that of other Caucasian populations, with an important trend towards aggressive fistulizing behavior<sup>[30]</sup>. In this population, the association between smoking and CD is strong enough to have possible implications. The lack of association between smoking and CD has now been established in Jewish patients in Israel. The stronger genetic tendency in CD may contribute to this discrepancy<sup>[31]</sup>.

## POST-SURGICAL RECURRENCE IN SMOKERS

Intestinal resection still remains a cornerstone in the management of CD despite the availability of biologic agents and the widespread use of immunomodulators. Recently, two independent studies reported a cumulative probability of undergoing abdominal surgery of about 40% and 60% within five and ten years from disease diagnosis, respectively, in an adult hospital-based and in a pediatric population-based cohort of CD patients<sup>[32,33]</sup>. However, disease recurrence is almost the rule after a "curative" resection and, for this reason, preventive algorithms have been repeatedly

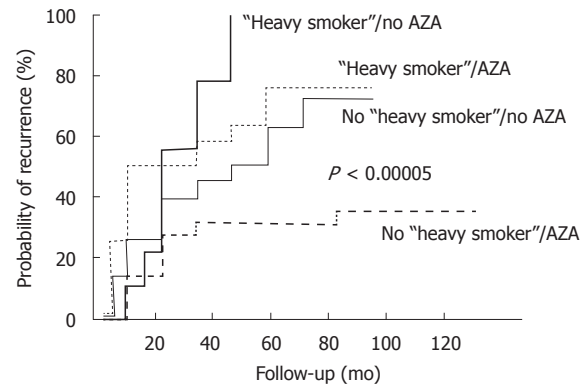


proposed<sup>[34,35]</sup>. Although it is still to be established whether all patients should start immunomodulators after surgery or not, all authors agree that smoking cessation must be strongly encouraged. In fact, many studies have found that active smoking is firmly correlated with an increased risk of CD postoperative recurrence<sup>[36]</sup>. This has been recently confirmed in two large studies that found smoking to be an independent risk factor for surgical recurrence (re-operation)<sup>[37,38]</sup>. Postoperative recurrence usually occurs among patients operated on because of ileal disease, and the harmful effect of tobacco seems to be greater in patients with ileal involvement<sup>[17]</sup>. However, the results of most of these studies are handicapped by methodological aspects. Firstly, most of them are retrospective; smoking habits may vary with time and this is not always registered in medical records. Secondly, the effects of tobacco may be influenced by some potential confounding factors such as gender<sup>[39,40]</sup>, daily cigarette dose<sup>[41]</sup>, or even ethnicity<sup>[41,42]</sup>, and these have not always been taken into account. Finally, definitions of “active smoker” or “former smoker” are heterogeneous between studies; this is especially important when evaluating smoking cessation, since the effect of giving up smoking seems to be clinically relevant from 1 year on<sup>[43]</sup>.

Cottone *et al*<sup>[44]</sup> reported the results of a retrospective study which included 182 CD patients who underwent intestinal resection, 109 of whom had an endoscopic assessment for mucosal recurrence 1 year after surgery. The authors found, for the first time, that active smoking was an independent risk factor for endoscopic, clinical, and surgical recurrence. Moreover, this deleterious effect of tobacco on clinical recurrence was dose-dependent. The major drawbacks of this study were that it did not accurately assess the severity of endoscopic lesions and that preventive treatment (if any) was not taken into account. Cortés *et al*<sup>[45]</sup> recently reported the results of the first prospective study assessing factors associated with endoscopic recurrence. The study included 152 patients participating in three prospective trials that evaluated the efficacy of diagnostic procedures or different preventive strategies for postoperative recurrence and in which endoscopic and clinical monitoring was systematically performed. Smoking and thiopurine use were the only independent predictors of significant postoperative recurrence as defined by the occurrence of clinical recurrence and/or Rutgeerts grade 3 or 4 of endoscopic recurrence (Figure 1). Once again, this risk was much more marked among heavy smokers (patients who smoked > 10 cigarettes/d). It has to be noted that, despite the suggestion that the harmful effect of tobacco in CD might be neutralized by the use of immunomodulators<sup>[16]</sup>, this does not seem to be the case in all clinical settings; as regards postoperative recurrence, two different studies have identified both azathioprine use and active smoking as independent factors associated with both endoscopic and surgical recurrence<sup>[38,45]</sup>.

## SMOKING INFLUENCING THERAPEUTIC RESPONSE

Although it has been proven that smoking increases thera-



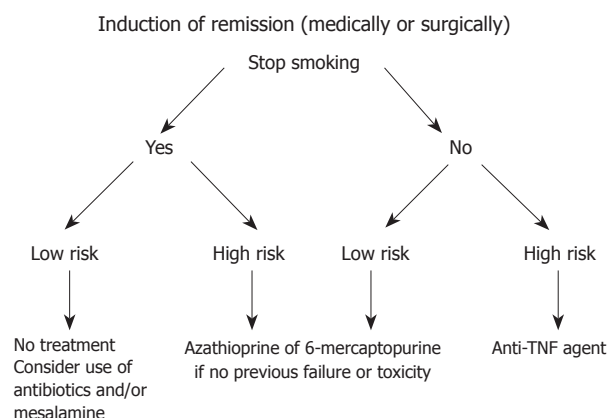
**Figure 1** Cumulative probability of relevant recurrence (grade 3 or 4 of endoscopic and/or clinical recurrence) depending on preventive use of azathioprine and active smoking. Reproduced from Cortés *et al*<sup>[45]</sup> with permission. AZA: Azathioprine.

peutic requirements in CD (steroids, immunomodulators, surgery), only a few studies addressing the impact of smoking on drug efficacy have been performed to date.

Some studies have tried to assess the influence of smoking habits on the response to infliximab. Parsi *et al*<sup>[46]</sup> aimed to identify those demographic and clinical parameters associated with the response to infliximab in 59 patients with luminal CD. Logistic regression analysis found that only smoking and the concomitant use of immunomodulators were independent predictors of response to one single infusion of the drug. Similar results were obtained in a subsequent study in 60 patients treated with a single infliximab infusion for refractory luminal CD<sup>[47]</sup>. However, a larger European study including 137 patients treated for luminal disease found that age, disease location and concomitant use of immunomodulators, but not smoking habits, were the only factors associated with clinical response in the multivariate analysis<sup>[48]</sup>. A North American study<sup>[49]</sup> performed with 122 patients who received one single infliximab infusion for refractory luminal CD did not find any predictor of response among several demographic and clinical factors, including smoking habits. Finally, an Italian multicenter study involved 382 patients who received infliximab for induction of remission in luminal CD (137 of them with a single infusion and 245 with a conventional three-infusion schedule)<sup>[50]</sup>; among several clinical parameters, only treatment with one single infusion and previous surgery were associated with a lesser probability of response in both univariate and multivariate analyses. All the above-mentioned studies also included patients with fistulizing CD, but no predictor of response (including smoking habits) was found in any of them.

In summary, despite initial data which suggested that active smoking reduced the likeliness to respond to one single infusion among patients with luminal CD, larger studies repeatedly found no association between smoking and infliximab response; however, it has to be said that most of these studies did not consider the tobacco dose.

The influence of smoking on other IBD-related drugs has been infrequently addressed. In this regard, a Spanish study evaluated for the first time the relationship between



**Figure 2** Algorithm for patient in medically or surgically induced remission. Low risk is defined as long-standing, short segment fibrostenotic disease without or with minimum active inflammation. TNF: Tumor necrosis factor.

smoking and the response to thiopurines<sup>[51]</sup>. The study included 163 IBD patients (103 CD and 60 UC) who started thiopurine therapy because of steroid dependency and who were followed up in two Catalan centers. Smoking habits at the time thiopurines were started were carefully assessed. No difference in the proportion of responders was found, in CD or in UC, suggesting that, once again, tobacco does not influence the efficacy of drug therapies in IBD. Results remained the same when several exploratory sub-analyses combining gender, disease location (colonic or ileal involvement, colonic or ileal isolated disease) and smoking habits (non-smokers, smokers, heavy smokers of > 10 cigarettes/d) were performed. Of note, CD responders who continued smoking had a higher rate of relapses during follow-up, although this did not lead to higher requirements of biological agents or surgery. Surprisingly, the authors found that treatment discontinuation because of thiopurine-related side effects was independently associated with active smoking in the multivariate analysis. This led to a reduced treatment efficacy among CD patients (as compared to UC) when evaluated by intention-to-treat analysis. Similarly, the largest survey of thiopurine-related toxicity in IBD patients reported to date, which included 3900 IBD patients treated with thiopurines from the Spanish ENEIDA Register [a nationwide register of IBD patients promoted by the Spanish Working Group in IBD (GETECCU)] found that both hepatotoxicity and acute pancreatitis were significantly more frequent in CD as compared to UC, although the smoking status information when starting thiopurines was not available<sup>[52]</sup>.

In summary, it seems clear that active smoking increases the risk of a more disabling course of CD, particularly in patients with ileal disease, women, and heavy smokers. This harmful effect might be genetically modulated, as seen in certain ethnic groups. Once those populations at risk are identified, interventional measures to ensure smoking cessation should be considered (Figure 2). If the patient fails in giving up smoking, more intensive CD treatment strategies such as earlier use of immunomodulators and/or biological agents should be taken into account in order to anticipate complications.

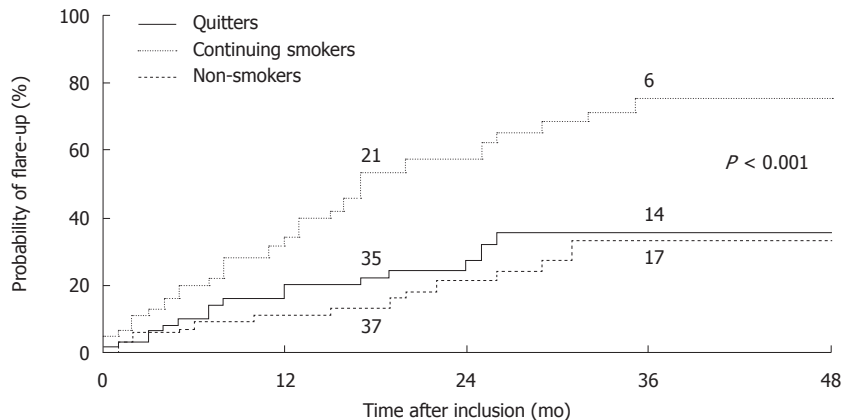
## INTERVENTIONS FOR SMOKING CESSATION: ARE THEY EFFECTIVE?

The strongest evidence of the deleterious effect of tobacco consumption upon the course of CD is precisely the beneficial consequences of smoking cessation<sup>[5]</sup>. In the cohort study published by Cosnes *et al.*<sup>[17]</sup>, patients were followed up for 12-18 mo and a lower relapse risk was observed among those patients who stopped smoking for at least 6 mo. In ex-smokers, the clinical course was similar to that seen in patients who have never smoked.

There is no doubt regarding the beneficial effects of smoking cessation upon the clinical course of CD. Such benefit might be even greater than that afforded by the use of thiopurines as maintenance therapy. The only interventional study published to date included 474 consecutive smokers with CD who were offered a smoking cessation program<sup>[18]</sup>. Patients who stopped smoking for more than one year (quitters) were included in a prospective follow-up study comparing disease course and therapeutic requirements with two control groups - continuing smokers and non-smokers - matched for age, gender, disease location and activity. Fifty-nine patients were able to quit smoking (12%). After a median follow-up of 29 mo, the risk of flare-up in quitters did not differ from that in non-smokers, and was lower than that of smokers (Figure 3). Steroid and immunomodulator requirements were similar among quitters and non-smokers, but greater among smokers. Finally, the risk of surgery was not significantly different between the three groups. Interestingly, the authors found that the physician in charge, previous intestinal surgery, high socioeconomic status and, in women, oral contraceptive use, were predictors of quitting tobacco consumption.

Despite the negative effect of smoking on health, and specifically on the clinical course of CD, smoking cessation is not an easy matter. Management of smokers is based on two complementary interventions: behavioral intervention and drug therapy. Physicians must remember the “four As” needed to correctly address this topic: (1) asking about current smoking habit; (2) advising them to stop; (3) assisting the patient by way of the available methods; and (4) arranging follow-up. It is very important to stress the importance of smoking cessation, since patients who are aware of the strongly negative impact of the habit upon the course of the disease will find it easier to stop smoking. Unfortunately, CD patients are too often unaware of the risks that smoking poses for their disease, thus indicating the need for increased patient information with regard to the effects of smoking on CD<sup>[53,54]</sup>.

Recently, an interesting study designed to increase the motivation to stop smoking involved 140 smokers without CD<sup>[55]</sup>. Individuals were informed about the characteristics of CD, and underwent a genetic study (*NOD2/CARD15* mutations) in order to classify them according to the risk of developing the disease. The results confirmed the hypothesis that increased information on the genetic burden of CD could modify the intention to quit smoking, to the extent that those individuals at higher risk showed a greater willingness to stop smoking.



**Figure 3** Relapse (flare-up) risk during follow-up of Crohn's disease in continuing smokers, ex-smokers (quitters) and patients who have never smoked. The stated *P*-value corresponds to comparison between the quitters and continuing smokers. Reproduced from Cosnes *et al*<sup>[18]</sup> with permission.

### Behavioral interventions designed to stop smoking

Behavioral interventions designed to facilitate smoking cessation include specific warnings from the general practitioner, intensive advice or counseling by specialists on disease-related risks, and supportive measures in the form of written material or telephone calls<sup>[5]</sup>.

Simple warning by physicians to stop smoking has shown some usefulness in the studies conducted by the Cochrane Tobacco Addiction Review Group<sup>[56]</sup>, though the effect is relatively poor<sup>[57]</sup>; assuming an unassisted quitting rate of 2%-3%, a brief advice intervention can increase quitting by a further 1%-3%. Direct comparison of intensive *vs* minimal advice has shown a small advantage for intensive advice [relative risk (RR) 1.37, 95% confidence interval (95% CI): 1.20-1.56]. Additional components appear to exert only a minor effect, though a small additional benefit is derived from more intensive interventions compared to very brief interventions.

Behavioral interventions are useful, especially when combined with drug therapy, and particularly over the short term. Motivational interviewing is a directive patient-centered style of counseling designed to help people to explore and resolve ambivalence about behavior change. It was developed as a treatment for alcohol abuse, but may help smokers to make a successful attempt to quit smoking<sup>[58]</sup>. Innovative effective smoking cessation interventions are required to appeal to those who are not accessing traditional cessation services. Mobile phones are widely used and are now well integrated into daily life, particularly among young adults - as most CD patients are at disease onset. Mobile phones are a potential medium for the delivery of health programs such as those designed to facilitate smoking cessation, but current evidence shows no effect of mobile phone-based smoking cessation interventions upon long-term outcome<sup>[59]</sup>. Pooled data from the Internet and mobile phone programs show statistically significant increases in both short- and long-term self-reported quitting (RR 2.03, 95% CI: 1.40-2.94). While short-term results are positive, more rigorous studies of the long-term effects of mobile phone-based smoking cessation interventions are needed.

Many European centers have outpatient clinics spe-

cifically targeted to help smoking cessation by means of a multidisciplinary approach (with nurses, psychologists, pneumologists and general practitioners), but drug therapy is almost universally used in these clinics as a complement to behavioral interventions.

### Drug therapy

Pharmacological smoking cessation aids are recommended for all smokers who are trying to quit, unless contraindicated. The available drugs include the following.

**Nicotine replacement products:** Such products offer a way to administer nicotine without smoking. They can be used in the form of patches, chewing gum, or nasal spray formulations. The aim of nicotine replacement therapy (NRT) is to temporarily replace much of the nicotine from cigarettes to reduce motivation to smoke and nicotine withdrawal symptoms, thereby easing the transition from cigarette smoking to complete abstinence. A recent Cochrane review<sup>[60]</sup> identified 132 trials on the use of NRT in people willing to quit smoking. Of these, 111 trials involving over 40 000 participants contributed to the primary comparison between any type of NRT and a placebo or non-NRT control group. The RR of abstinence for any form of NRT relative to control was 1.58 (95% CI: 1.50-1.66). The pooled RR for each type were 1.43 (95% CI: 1.33-1.53, 53 trials) for nicotine gum, 1.66 (95% CI: 1.53-1.81, 41 trials) for nicotine patch, 1.90 (95% CI: 1.36-2.67, 4 trials) for nicotine inhaler, 2 (95% CI: 1.63-2.45, 6 trials) for oral tablets/lozenges, and 2.02 (95% CI: 1.49-3.73, 4 trials) for nicotine nasal spray. The effects were largely independent of the duration of therapy, the intensity of the provided additional support, or the setting in which NRT was offered. The effect was similar in a small group of studies that aimed to assess the use of NRT obtained without a prescription. In highly dependent smokers there was a significant benefit of 4 mg gum compared with 2 mg gum, but weaker evidence of a benefit from higher doses in patch form. There was evidence that combining a nicotine patch with a rapid delivery form of NRT is more effective than a single type of NRT. Only one study directly compared NRT to another drug treat-



ment modality. In this study the smoking cessation rates with nicotine patch were lower than with the antidepressant bupropion.

**Bupropion:** While not a substitute for nicotine, this drug reduces the anxiety associated with not smoking. Bupropion is an atypical antidepressant that acts as a norepinephrine and dopamine reuptake inhibitor. Initially researched and marketed as an antidepressant, bupropion was subsequently found to be effective as a smoking cessation aid. Conversely to selective serotonin reuptake inhibitors (e.g. fluoxetine), the antidepressants bupropion and nortriptyline contribute to long-term smoking cessation. It has been suggested that the mode of action of bupropion and nortriptyline could be independent of their antidepressant effect, and that their efficacy is similar to that of nicotine replacement<sup>[61]</sup>.

**Varenicline:** This new smoking dishabituating agent acts by blocking the nicotinic receptors and induces similar effects to that of nicotine, counteracting the craving to smoke, lessening the withdrawal syndromes, and reducing the gratifying and reinforcing effects of smoking. Varenicline was developed as a nicotine receptor partial agonist from cytisine, a widely used drug in Central and Eastern Europe for smoking cessation. Nicotine receptor partial agonists may help people to stop smoking by a combination of maintaining moderate levels of dopamine to counteract withdrawal symptoms (acting as an agonist) and reducing smoking satisfaction (acting as an antagonist). The first reports of trials with varenicline were published in 2006<sup>[62]</sup>. This drug offers higher efficacy rates (smoking cessation) than those of the existing alternatives<sup>[63]</sup>. Varenicline increased by 2- and 3-fold the chances of successful long-term smoking cessation as compared with pharmacologically unassisted quitting attempts. There is a need for independent community-based trials of varenicline to test its efficacy and safety in smokers with varying co-morbidities and risk patterns. Likewise, there is a need for further trials of the efficacy of treatment extended beyond 12 wk.

In February 2008, the United States Food and Drug Administration issued a public health advisory note, reporting a possible association between varenicline and an increased risk of behavior change, agitation, depressed mood, and suicidal ideation and behavior<sup>[64]</sup>. The possible risks of serious adverse events occurring while using varenicline or bupropion should always be weighed against the significant health benefits of quitting smoking that include not only a better prognosis for CD but also a reduction in the chance of developing lung or heart disease, and cancer.

## MANAGEMENT OF SMOKING RELAPSE

Less than 10% of all patients who quit smoking without medical help are able to maintain abstinence over the long term<sup>[65]</sup>. Interventions, whether pharmacological or surgical, increase the long-term cessation rates as compared to

control interventions, though there is a permanent reduction in the general success rates due to the fact that a proportion of individuals who are initially able to stop smoking subsequently relapse over time.

At present there is insufficient evidence to support the use of any specific behavioral intervention for helping smokers who have successfully quit for a short time to avoid relapse<sup>[66]</sup>. The verdict is strongest for interventions focusing on identifying and resolving tempting situations, as most studies have been concerned with these. There is little research available regarding other behavioral approaches. Extended treatment with varenicline may prevent relapse, though extended treatment with bupropion is unlikely to have a clinically important effect. Studies of extended treatment with nicotine replacement are needed.

## DIFFERENT APPROACH TO CD PATIENTS WHO CONTINUE SMOKING

When compared to similar data for the general population, patients with CD are not found to be more refractory to smoking cessation<sup>[67]</sup>. CD patients must be informed of the importance of smoking cessation for the course of their disease, and individualized medical intervention should be established to reach this objective. This is particularly important in smoking women with CD. Nicotine patch replacement therapy during the first 6-12 wk of abstinence is appropriate in heavy smokers and in smokers with a high degree of tobacco dependency. If, despite reinforced medical advice, the patient is unable to quit smoking, referral to a specific smoking cessation clinic is recommendable. If this is not possible, then the physician should offer behavioral and drug treatment support.

Varenicline is likely to be clinically and cost-effective for smoking cessation, assuming that each user makes a single attempt to quit smoking. The key area of uncertainty concerns the long-term experience of subjects who have remained abstinent beyond 12 mo. Guidelines issued by the National Institute for Health and Clinical Excellence in July 2007 state that varenicline is recommended under its licensed indications as an option for smokers who have expressed their wish to quit smoking, and that varenicline should normally be prescribed only as part of a behavioral support program<sup>[68,69]</sup>. In the presence of depressive symptoms or other contraindications to the use of these drugs, NRT may be used.

Whenever a CD patient is not able to stop smoking, a close monitoring (clinical and/or even endoscopic) is recommended and early introduction of more intensive therapeutic strategies (immunomodulators and/or biological agents) should be considered, especially in women with ileal involvement.

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## Management of the complications of endoscopic submucosal dissection

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of post ESD complications and review published papers on the topic.

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### Abstract

Endoscopic submucosal dissection (ESD) is currently widely accepted as a standard treatment option for early gastrointestinal neoplasms in Korea. However, ESD has technical difficulties and a longer procedure time than conventional endoscopic resection. So it may have a higher risk of complications than conventional endoscopic resection techniques. We, the ESD study group of Korean Society of Gastrointestinal Endoscopy, have experienced many complications, mostly treated by endoscopic or conservative management. Here, we introduce and share our experiences for management

### INTRODUCTION

Endoscopic submucosal dissection (ESD) enables complete and *en bloc* resection of large or ulcer related superficial gastric cancer. However, the complication risk can be increased due to the long procedure time of submucosal dissection<sup>[1]</sup>. The major complications include bleeding and perforation<sup>[2]</sup>. The bleeding which occurs during ESD is almost controlled. Minor bleeding does not cause any problems to patients. So it is not considered to be a complication of ESD<sup>[1]</sup>. Risk of perforation, which is another major complication, is not reduced because of extended ESD indications. However, ESD complications can be mostly treated by endoscopic or conservative management and do not affect the prognosis of patient.



## COMPLICATIONS OF ENDOSCOPIC SUBMUCOSAL DISSECTION

### Bleeding

The bleeding rate of endoscopic mucosal resection (EMR) has been reported as from 4% to 38%. The risk of bleeding in ESD is from 13% to 38% which is slightly higher than EMR<sup>[2]</sup>. Bleeding is categorized by acute bleeding during the procedure and delayed bleeding after procedure. Acute bleeding is defined as any bleeding which occurs during the procedure while delayed bleeding does not occur during the operation; it is defined by endoscopic evaluation at least 24 h after the operation<sup>[3]</sup>.

Acute bleeding is controlled easily by endoscopic coagulation but in cases of delayed bleeding, transfusion, emergency endoscopic evaluation and even surgical procedure could be required<sup>[3]</sup>. It is manifested with hematemesis or melena. It occurs within 24 h in most cases, but it can also happen 2 wk after the procedure<sup>[1]</sup>. A considerable duration, at least 8 wk, is needed for healing of EMR related ulcers and bleeding can be detected for quite a long time<sup>[4]</sup>.

**Endoscopic hemostasis:** It is important that accurate incision or submucosal dissection between the submucosa and muscularis propria layer is performed to prevent procedure related bleeding. There are large vessels in the middle of submucosa layer and the bleeding risk can be increased with blind dissection. Targeted dissection and precoagulation are needed to prevent bleeding due to the presence of large vessels in the dissected layer<sup>[5]</sup>. There are several kinds of endoscopic hemostasis with hemoclip, electronic coagulation and argon plasma coagulation<sup>[2]</sup>. Also it is common for proton pump inhibitors (PPI) or histamine receptor (H2) blockers to be prescribed for 4 to 8 wk after ESD to prevent bleeding and encourage healing of post ESD ulcer<sup>[6]</sup>.

Hemoclips can control bleeding without any tissue damage. It is not usually recommended to use these in ESD for several reasons. It can disturb the procedure to use knives, or may obstruct the operator's field of vision and cause problems when trying to perform another endoscopic hemostasis<sup>[2]</sup>. It can be used in cases of uncontrolled massive bleeding that are not controlled with other methods. The major indication for hemoclippping is for preventing bleeding of visible vessels on post ESD ulcer (Figure 1A)<sup>[5]</sup>. It can also prevent delayed bleeding with mucosal protection from gastric acid, pepsin and mechanical damage of vessels<sup>[7]</sup>.

Electrocoagulation is useful for hemostasis of visible vessels or active bleeding during or after the procedure. The electrosurgical unit can be readily used as the knife itself during the procedure. In oozing of blood, electrocoagulation controls the bleeding immediately (Figure 1B and C). However, it is not indicated for pulsating bleeding and condensed vessels. The hemostatic forcep is used for pulsatile or nonlocalized bleeding (Figure 1D and E). Repeated electrocoagulation can cause tissue damage and perforation. Pyloric obstruction can occur

at the healing stage after excessive and repeated electrocoagulation<sup>[2]</sup>. Microvessels can be coagulated by applying an electric current via the knife tip itself (Figure 1F). For large vessels (larger than 1 mm), the site of bleeding is cauterized by an electric current using hemostatic forceps or hot biopsy forceps<sup>[5]</sup>. When the bleeding site is unclear, washing helps to find the bleeding focus, and then bleeding is controlled by coagulation using the lift technique of hemostatic forceps. If massive bleeding occurs or the site of bleeding focus is unclear, hot biopsy forceps are useful<sup>[7]</sup>.

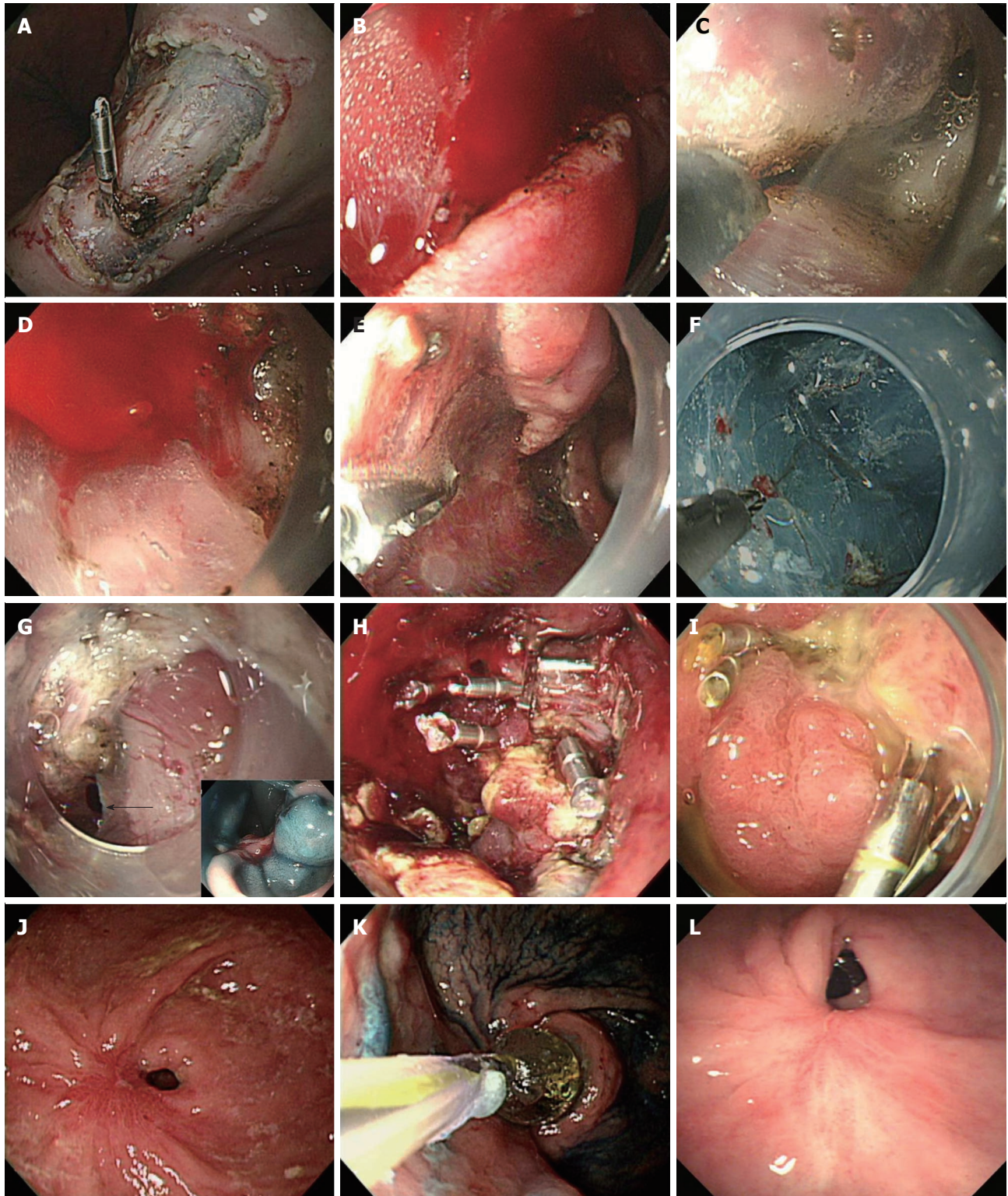
Oozy bleeding control with argon plasma coagulation (APC) during or after the operation is effective. Tissue damage with APC can be reduced by submucosal injection<sup>[8]</sup>. So as the APC method is a non-contact type, it is used to control bleeding with the front or side of the probe by forced APC mode, flow 1.8 L/min, 45 watt. The disadvantage of APC is it produces considerable smoke during coagulation and frequent suction of smoke is required. However it can not be used in pulsatile bleeding<sup>[4]</sup>.

### Perforation

Recent studies report the incidence rate about 4%, even though the rate has fallen since ESD was first used<sup>[9,10]</sup>. The rate is not decreasing any further because large and difficult lesions, which were not treated this way before, are dissected by ESD. There are no definite guidelines for treatment of perforation, even though several successful medical treatment have been reported<sup>[11]</sup>. It is important to find the perforated site and close the defect to prevent severe peritonitis with damage to other organs as soon as possible. Kaneko *et al.*<sup>[12]</sup> reported several indications for a clipping method of ESD induced perforations. It suggest that if perforation size could be covered by a metal clip, the margins of the perforation site are smooth and clean of the margin, and one can get a clear endoscopic view<sup>[12]</sup>. ESD or EMR induced perforations are suitable for treatment in this way in most cases since usually these perforations are small with a linear shape and little contamination risk due to patient's fasting state<sup>[9,11,13-15]</sup>.

In most cases, without profound peritoneal involvement and abnormal vital signs, localized peritonitis can be improved with conservative medical treatment. Mostly, microperforation can be improved with conservative treatment<sup>[16]</sup>. When perforation is suspected during the procedure, it should be sutured with metallic clips (Figure 1G, H and I, Figure 2). Antibiotics and fasting are also needed in these conditions<sup>[17]</sup>. Conservative medical treatment includes fasting, intravenous fluid therapy, Levine tube insertion for 12 to 24 h and antibiotics supply for 2 d<sup>[11,15,18-20]</sup>. Patients can take meals after 2 to 4 d with improvement of peritoneal irritation signs, intraperitoneal free air and white blood cell counts<sup>[15,19-21]</sup>. Surgical treatment can be considered in the cases of large perforation which cannot sutured with clips and presenting as aggravated peritonitis or unstable vital signs<sup>[14,22,23]</sup>. It





**Figure 1 Endoscopic views.** A: Endoscopic view of exposed vessels on post endoscopic submucosal dissection (ESD) ulcer, showing hemoclippping for prevention of delayed bleeding; B, C: Endoscopic view of oozing of blood during ESD, showing immediate electrocoagulation by IT knife itself; D, E: Endoscopic view of pulsatile bleeding during ESD (D) and showing coagulation by hemostatic forcep (E); F: Endoscopic view shows that microvessels of the submucosal layer are cauterized by flex knife; G-I: Endoscopic view of jejunal loop side of G-Jstomy showing a perforation (arrow) seen during ESD for EGC of stoma of remnant stomach (G), and the view after closure of the perforation by endoclips (H). A follow-up endoscopy showed the healed perforation 2 wk after endoscopic closure (I); J-L: Endoscopic view shows severe antral stenosis 7 wk after gastric ESD (J). Endoscopic view of balloon dilation procedure (K). A follow-up endoscopic view showed relieved stenosis without any symptoms 3 mo after balloon dilatation (L). Ulcer induced stricture was detected at 4 wk after ESD.

is an absolute indication of surgical therapy when a large amount of fluid is seen in abdominal computed tomog-

raphy which suggests intraperitoneal infection by gastric contents. But surgery can be held when perforated sites





Figure 2 Abdominal radiograph showed pneumoperitoneum.

are sutured promptly and peritoneal infection does not spread extensively<sup>[24]</sup>. Delayed perforation can develop within hours or days after ESD. It is manifested with abrupt onset of abdominal pain and fever and it can progress to extensive peritonitis. Excessive and repeated electrical coagulation makes burns on the submucosa and muscularis propria, and this condition may cause delayed perforation<sup>[19,25]</sup>. It is not clear whether peritoneal seeding of cancer cells is possible through perforation, the operator should be careful to prevent dissection induced perforation<sup>[26]</sup>.

### Other complications

**Prepyloric or pyloric stenosis:** Resection of distal antral lesions can cause pyloric stenosis, especially if lesions directly invade the pylorus or cover more than two-thirds of the luminal area. Tsunada *et al*<sup>[27]</sup> reported five cases of pyloric stenosis. One case showing severe obstruction underwent surgical resection, the others were treated by balloon dilatation. Two patients improved after balloon dilatation but the other two patients underwent surgery due to perforation after dilatation. Due to the operation risk, like these cases, this study group recommended balloon dilatation within 8 wk after resection when lesions encircle more than four-fifths of the antral luminal area. We have to consider the possibility of severe stenosis after ESD of a laterally spreading tumor. A stricture after ESD in the antrum is not easily rescued by balloon dilatation (Figure 1J, K and L) and sometimes requires surgical intervention. Therefore, we must perform ESD with precise planning of the large antral lesion and patients should be followed by endoscopic observation after the procedure<sup>[25,27]</sup>.

**Transient bacteremia:** Post ESD transient bacteremia can be improved with conservative treatment and empirical antibiotics in most cases. The American and European Society of Endoscopy recommends prophylactic antibiotics use before any endoscopic procedure. Using prophylactic antibiotics in high risk patients undergoing endoscopic variceal sclerotherapy or balloon dilatation is recommended due to the risk of endocarditis or other possible infections<sup>[28]</sup>.

**Aspiration pneumonia:** Older patients are the main risk group for aspiration pneumonia. Onozato *et al*<sup>[29]</sup> reported this complication rate in 93 older patients, aged  $\geq 75$  years who underwent ESD. Aspiration pneumonia occurred in 2 patients, fever developed in 6 patients. These 6 patients did not show obvious evidence of pneumonia in radiologic findings but fever might be considered to relate with aspiration. All of them were improved with oxygen and antibiotics treatment. The risk of aspiration pneumonia is dependent on the age of patients. It can be prevented with overtube, frequent suction and regular position change<sup>[29]</sup>.

## CONCLUSION

The complications of ESD can be prevented and improved with endoscopic and supportive management. Several essential things are needed to prevent or cure these complications such as a skilled operator, careful consideration of the patient's underlying condition and accurate preventive management.

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## Do you have what it takes for challenging endoscopic submucosal dissection cases?

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### INTRODUCTION

Recent advances in endoscopic technology, the accumulation of vast amounts of surgical data regarding the lymph node status in early gastric cancer (EGC), and increasing operator experience in the endoscopic treatment of EGC have made endoscopic submucosal dissection (ESD) a widely acknowledged standard curative therapy for EGC<sup>[1-3]</sup>. Nonetheless, not only the histology type and lesion size but also the anatomical location must be considered in deciding whether ESD is suitable for the lesion<sup>[4,5]</sup>. Generally, ESD is difficult to perform when patient cooperation is poor (because of severe belching or vomiting); when an anatomical deformity of the stomach (due to gastroesophageal reflux disease, hiatal hernia or a previous stomach operation) makes sufficient stomach inflation difficult; when the patient is in a poor condition (old age, cardiopulmonary comorbidities, patients taking anticoagulation or antiplatelet agents); and when the lesion is located at a place where endoscopic access and submucosal dissection are difficult (cardia, high body, fundus, pylorus, and duodenum). Of these factors, an anatomically difficult location is the most common factor that makes the procedure difficult even for experienced

### Abstract

Endoscopic submucosal dissection (ESD) is a widely accepted treatment for early gastric cancer (EGC), especially in Korea and Japan. The criteria for the therapeutic use of ESD for EGC have been expanded recently. However, attention should be drawn to the technical feasibility of the ESD treatment which depends on a lesion's location, size or fibrosis level, or operator's experience. In the case of a lesion with a high level of difficulty, a more experienced operator is required. Thus, the treatment for a lesion with a high level of difficulty should be performed according to the degree of the operator's experience. In this paper, the authors describe the ESD procedure for lesions with a high level of difficulty.

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endoscopists. This review is thus focused on ESD for an anatomically difficult lesion. This article is part of a series of reviews on ESD.

## COMMON ANATOMICALLY DIFFICULT LOCATIONS

### *Cardia and gastroesophageal junction*

The cardia and gastroesophageal (GE) junction are susceptible to bleeding and perforation due to their abundant submucosal vasculature and thin walls. Regarding the cardia, the approaching angle is acute, and the lumen is narrow at the GE junction (Figures 1 and 2). When performing a mucosal incision at the margin, it is easier when the endoscope is retroflexed and when the incision is initially cut from the oral to the anal side of the esophagus. While dissection of the esophageal lesion is being performed, the region of dissection must be carefully watched as the esophageal wall lacks a serosa layer and has a thin muscle layer.

An insulation-tipped electrosurgical knife (IT knife) makes cutting easy and safe. When dissecting the lesion from the stomach, it is important for the flap of the lesion made by gradual dissection to be maximally turned over by gravity. Normally, the finishing is performed under retroflexion view, but when using an IT knife, it may be easier to straighten the endoscope at the GE junction and then to make the final cut.

### *Body*

When performing ESD for a lesion located in the body of the stomach, the endoscopist has to be very cautious to prevent bleeding and perforation especially in the high body. Therefore, the endoscopist has to make sure that a sufficient visual field is secured through pre-coagulation. To guarantee a good visual field, the endoscopist has to bear in mind that the direction of the incision should not be disturbed by unpredictable bleeding in a blind fashion. For larger vessels, pre-coagulation with hemostatic forceps is needed to avoid uncontrolled bleeding during the procedure. It is also essential to understand the anatomical characteristics of this particular area for a successful and safe procedure. In the deepest muscular layer of the stomach body, especially in the anterior and posterior wall sides, lie the medial longitudinal oblique muscles, where the perforating vessels form a network. Along with this transverse vasoganglion, the attached fibrous tissue forms a so-called “myofascial layer”. The fibrotic nature and abundant vasculature of this layer explain why the cutting and dissection of the high body anterior and posterior walls are difficult. Just beneath the transverse vasoganglion (i.e. just above the muscular layer), there is a layer with less vessels and fibrotic tissue. Performing the dissection in this layer is the key point to easy and safe ESD. In dissection, cutting through the above-mentioned vessel network can cause problems due to bleeding. In this situation, the simple maneuver called “coagulation mode trimming” is useful. During this maneuver, the

endoscopist uses an IT or flex knife, which is moved back and forth to both coagulate the vessels and dissect through the transverse vasoganglion. Once the mucosa is cut to an adequate depth, the endoscopist should follow the general principles of the procedure. These include maintaining a good visual field of the submucosa, prophylactic coagulation of the visible vessels, and dissection with a curved movement along the direction of the gastric wall through the appropriate handling of the endoscope. In contrast to the anterior and posterior walls of the high body, the lesser curvature of the body has less perforating branches and fibrotic submucosa, which generally makes dissection easier. During the ESD procedure for a lesion in the lesser curvature of the body, pre-coagulation using Coagrasper hemostatic forceps is required to prevent bleeding because damage to the large blood vessels emerging from the muscle layer caused by an electrosurgical knife may induce massive bleeding.

The high body can have a normal external protrusion beside the adjacent organs, such as the liver. The muscle layer can be regionally elevated, and dissection would have to be made in a curved line to overcome the perpendicular angle between the cutting knife and the muscle layer. Without sufficient visualization of the field, the chance of perforation is high.

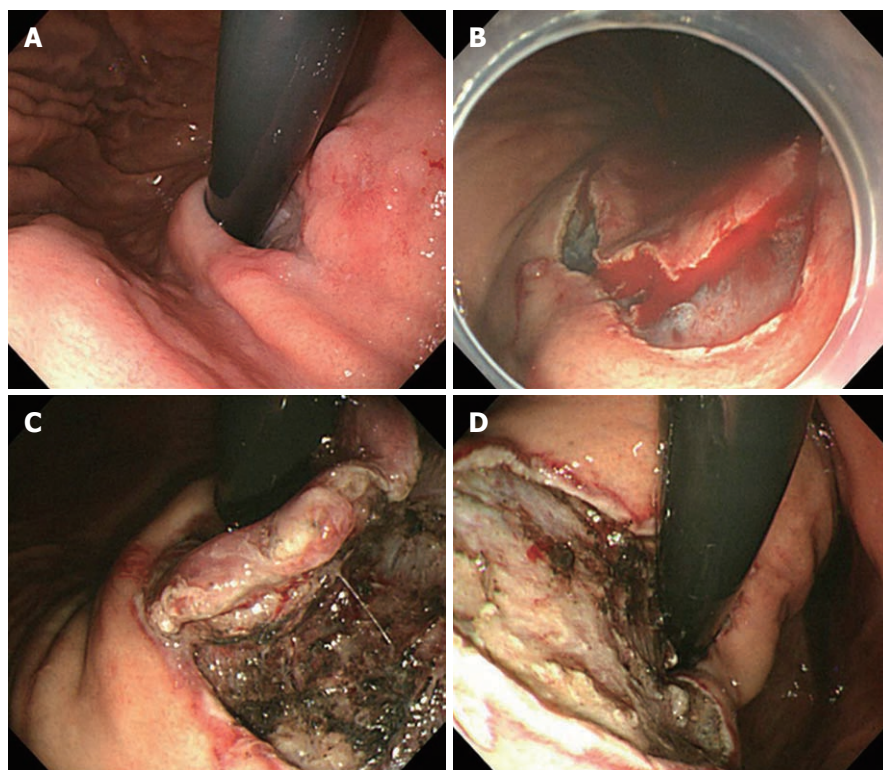
### *Fundus*

The difficulty of performing ESD for a fundal lesion lies in the limited visual field because of the stomach contents, blood retention due to gravity, movement of the lesion *via* respiration or heartbeat, and the thin muscle wall in the fundus compared to other parts of the stomach. Nevertheless, when the lesion is located obliquely in between the fundus and the anterior or posterior wall of the high body, an experienced endoscopist can dissect the lesion with an IT knife (Figure 2). For lesions that can be seen only when the endoscope is turned around, and when the lesion is located at the greater curvature side between the cardia and the fundus, ESD is generally impossible to perform. When the lesion is relatively small and cutting around its edge is possible, the removal of the lesion by snare can be an alternative treatment option.

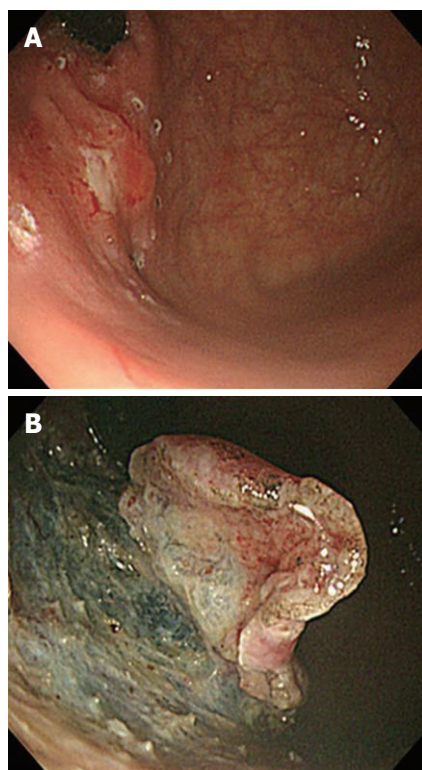
For fundal lesions, because of this highest level of difficulty, ESD is usually not recommended.

### *Pylorus*

The incidence of ESD performance for a pyloric lesion has recently been increasing, but actually reported cases are still rare. When the distal part of the lesion is located at the pyloric channel, the incision and cutting should start from the proximal part, i.e. the antrum. As the dissection progresses and when the pyloric channel is reached, the flap can be pushed into the bulb by the tip of the endoscope, which exposes the muscle and bottom layers of the lesion. With sufficient submucosal injection, the remainder of the lesion can be cut using a needle knife if the distal margin is confined to the channel ring, and if a clear margin can be assured. In the case of the



**Figure 1** Lesion at the cardia, just below the gastroesophageal junction. A: Retroflexed view of the cardia; B: A circumferential incision was made from the oral to the anal side, which is then vulnerable to bleeding; C: Submucosal dissection from the anal to the oral side; D: The lesion was completely resected.



**Figure 2** Lesion at the fundus. A: The lesion was located between the fundus and the anterior side of the high body; B: Submucosal dissection was performed from the cardia to the fundus.

pyloric lesion extended to the duodenal bulb, the endoscope has to be retroflexed gently at the duodenal bulb,

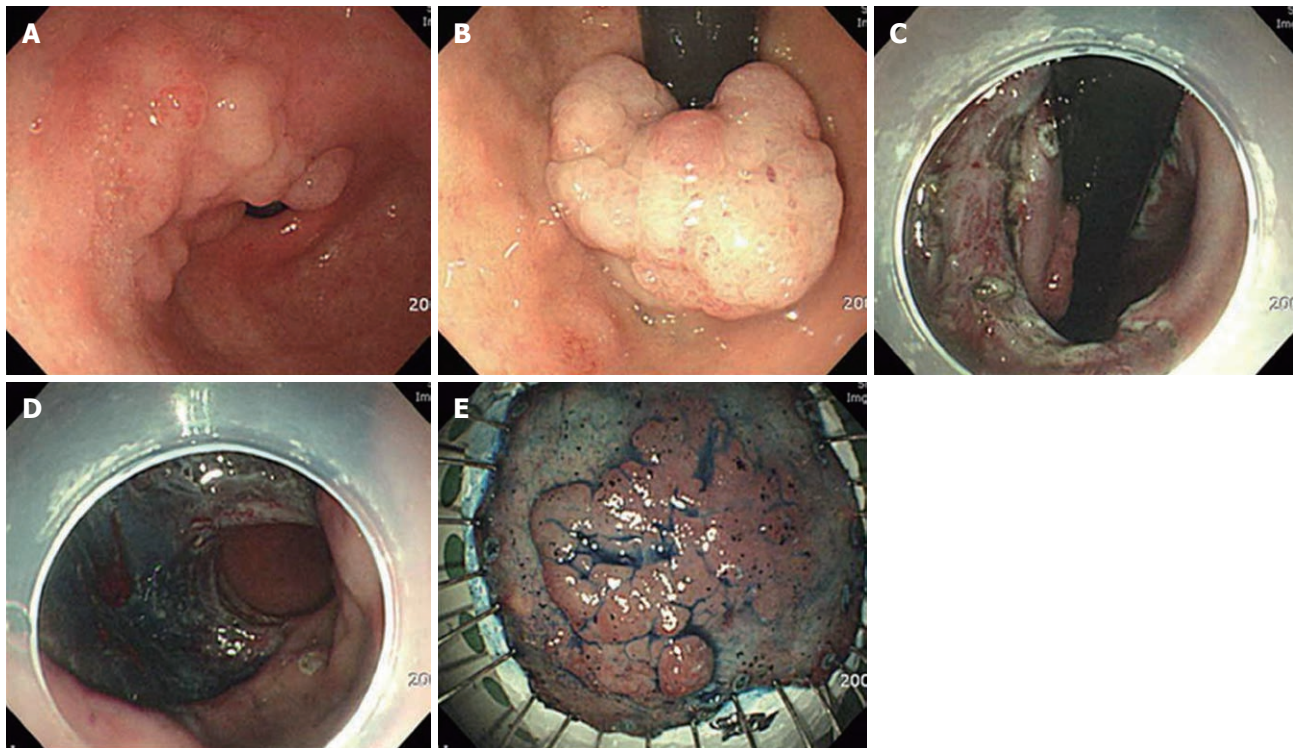
and the dissection incision should be started from the duodenal distal end (Figure 3). Through this maneuver, a safe distal margin can be secured, but when there is an anatomical deformity of the bulb, a reverse turn of the endoscope may not always be possible, and there is a risk of perforation during the dissection of the pyloric channel. Therefore, in the event of such a case, after the distal margin in the bulb is first dissected using a retroflexed endoscope, the endoscope is straightened for the dissection of the remaining proximal margin at the antrum and for the completion of the procedure.

The duodenal wall is highly susceptible to perforation if the submucosal injection is not sufficient because mucosal and muscle layers of the duodenal wall are very thin in contrast to that of the gastric wall. Thus, great care must be taken to make an incision of the mucosal layer not too deep after sufficient submucosal injection.

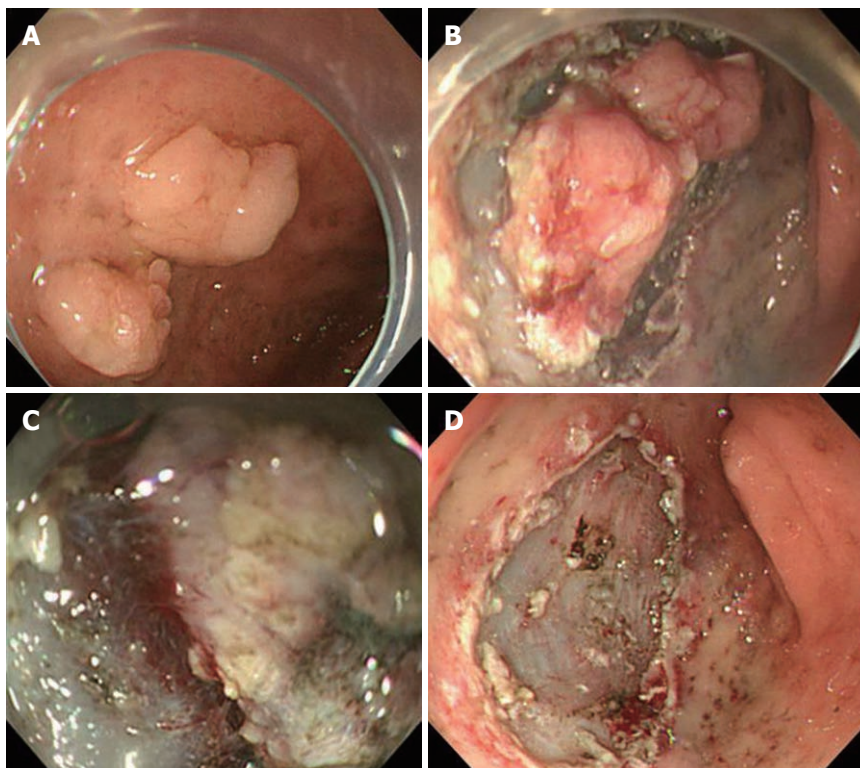
### Duodenum

Performing ESD for a duodenal lesion is much more challenging than performing it anywhere else because of the insufficient mucosal elevation and poor mucosal contraction. Moreover, the abundant vasculature in the submucosal layer, and the thin muscle layer, make the procedure very vulnerable to bleeding and perforation<sup>[6]</sup>. If the patient is uncooperative, it will be very hard to maintain the endoscope close to the lesion, especially at the second portion, because of the movement *via* respiration and belching. Therefore, it is very common for an endoscope inserted into the second portion to slip out to the bulb or the antral portion while being pulled with a





**Figure 3** Lesion at the pyloric channel extending to the duodenal bulb. A: Nodular elevated lesion involving the pyloric channel; B: Polypoid mass lesion at the duodenal bulb, retroflexed view; C: Incision and submucosal dissection were performed from the duodenal bulb to the antrum; D: The 180° circumferential dissection was completed; E: The *en bloc* resection was completed.



**Figure 4** Lesion at the duodenum. A: Two flat elevated lesions at the duodenal bulb; B: Circumferential incision; C: Submucosal dissection (the lifting of the lesion after submucosal injection was limited); D: The *en bloc* resection was completed.

needle or IT knife during incision or dissection. Thus, in such situations, the use of a hook knife is more ideal. It is

also important for the assistant to get a firm grip of the endoscope during the procedure. On account of these dif-



difficulties, the endoscopic treatment of duodenal lesions has been limited to polypectomy or mucosal resection using the endoscopic mucosal resection C or EMR sodium hyaluronate methods, and the performance of real ESD has been very rare. The thin submucosal layer, which has the unique anatomical characteristics of the duodenum, makes sufficient “cushion” formation after the submucosal injection difficult (Figure 4). Moreover, mucosal contracture after precutting does not occur as easily as in the other parts of the stomach. Therefore, submucosal dissection just above the muscle layer is an alternative solution for performing duodenal ESD.

## CONCLUSION

As regards the level of difficulty of ESD procedures, the fundus is the region with the highest level of difficulty. The fundus is the most difficult area on which to perform an ESD procedure because approach is difficult, the gastric wall is thin, and it is adjacent to the diaphragm. For the duodenum, it is impossible to perform an ESD procedure except on some portions of the bulb. Moreover, endoscopists other than highly experienced experts should avoid performing ESD procedure on the bulb. The easiest area on which to perform ESD is the antrum where even novices can perform an ESD procedure.

In this article, ESD for an anatomically difficult lesion was briefly discussed. The result of the procedure varies based on the endoscopist's skill, the size of the lesion, the type of device used, and even the coagulation mode

employed. This means that there is no such thing as “a magic bullet” for a difficult ESD. The recent development of the multibending and “R” scopes and of many other devices will hopefully help overcome the aforementioned difficulties, and further studies on this subject are needed. Training endoscopists to help them acquire the ability to manage an unplanned emergency situation during the procedure is also important.

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## Technical issues and new devices of ESD of early gastric cancer

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### Abstract

Endoscopic submucosal dissection (ESD) is a highly refined technique compared to conventional endoscopic mucosal resection. It enables complete resection of early gastric cancer (EGC) which has no possibility of lymph node metastasis. Indication for ESD of EGC generally entails early gastric cancer confined to the mucosa with well differentiated histology, though there are clinically suitable expanded criteria. As ESD requires specific skill and expertise, endoscopists need to be familiarized with basic methods and the use of special devices. The essence of the technique is to dissect the submucosal layer with direct vision and maintain the

cutting plane above the underlying proper muscle layer. Although there are some differences in the detailed technical aspect, the cardinal method of ESD is now well established and standardized. Furthermore, research and development of new ESD devices that render more efficient, safe ESD are still in progress to improve the overall result of ESD on early gastric cancer.

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**Key words:** Endoscopic submucosal dissection; Technique; Device; Early gastric cancer

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### INTRODUCTION

Endoscopic submucosal dissection (ESD) is a novel endoscopic technique that enables *en bloc* resection of large superficial gastric cancer<sup>[1]</sup>. Since this technique was introduced, there has been remarkable improvement in technique and experience regarding the safety and efficiency of ESD<sup>[2-4]</sup>. Though reliable, long term results have not been obtained, there is a large body of evidence to suggest that ESD is the therapy of choice for early gastric cancer on occasions when the risk of lymph node metastasis can be excluded<sup>[5,6]</sup>. General indications for ESD for gastric cancer were proposed on the basis of Japanese studies<sup>[7,8]</sup>. The absolute indication was non-ulcerative, well-differentiated mucosal cancer less than 2 cm in diameter. Expanded criteria could encompass non-

ulcerative well-differentiated cancer over 2 cm, ulcerative well-differentiated cancer under 3 cm and non-ulcerative, well-differentiated, submucosal invading (limited to 500  $\mu$ m below the lamina propria) cancer under 3 cm in diameter<sup>[9]</sup>.

Individual ESD technique could vary among endoscopists, but the cardinal aspect of this revolutionary technique is quite straightforward. The following section deals with a brief contemporary summary on the subject, which includes core technical issues and new devices pertaining to ESD procedure.

## THE CARDINAL TECHNIQUES OF ESD

ESD is a unique, advantageous procedure over conventional endoscopic mucosal resection in that endoscopists can determine the extent of resection through establishing an outer imaginary line around the lesion<sup>[10]</sup>. This not only helps endoscopists to control the dissection process, but also ensures complete resection of the lesion confirmed histologically after ESD. Therefore, clarifying the boundaries of the lesion by careful observation before entering the procedure is highly important. Visual enhancing methods to improve detection of the lesion, such as chromoendoscopy, narrow band imaging or magnifying endoscopy, are sometimes helpful<sup>[11-14]</sup>. Using magnified pit pattern and microvascular pattern as a reference, magnification and endoscopy with or without a narrow band imaging system can give more information about the histological differentiation, depth of invasion and clarification of the extent of the EGC<sup>[15,16]</sup>. Preoperative diagnosis by image-enhanced endoscopy may have a significant supplementary role to conventional endoscopic ultrasonography in exact localization and clarification of indication, thereby improving the overall result of the procedure (Figure 1).

### Marking

Marking around the lateral boundary of the target lesion is usually done by pointed devices at the coagulation setting of an electrosurgical unit. Devices such as the needle knife, flex knife, and hook knife are commonly used in careful contact with the mucosa, while coagulating force is fired only briefly to prevent deep thermal injury. Alternatively, argon plasma coagulation is conveniently used due to its non-contact thermal effect<sup>[17]</sup>. It is important to mark at least 5 mm apart from the outer circumferential margin of the lesion. After completion of marking, additional marking at the proximal or distal inner part of the lesion is usually required as a reference for determining the orientation of the resected specimen (Figure 1A).

### Submucosal injection

Traditionally, hyper or isotonic saline mixed with indigo-carmin and epinephrine has been used as basic injection solution. Characteristics of ideal injection materials would be to reduce submucosal blebs, have a hemostatic effect and be non-toxic to tissue. To fulfill these requirements, various injection solutions and mixtures, includ-

ing hypertonic glucose, glycerol, sodium hyaluronic acid, and fibrinogen, have been tried. Some reported that high molecular hyaluronic acid solution was superior to others, albeit at high cost<sup>[18-20]</sup>. Maintaining the angle of injection needle 45 degrees tangentially for the mucosa is advised to avoid injecting into the muscle layer. It is recommended that injection starts from the anal part the lesion first and proceeds to the proximal part to avoid interruption of the visual field. For each endoscopist's preference, partial injection and simultaneous submucosal dissection, beginning from a specific area, can be done initially instead of elevating whole circumference evenly.

### Mucosal incision (precut)

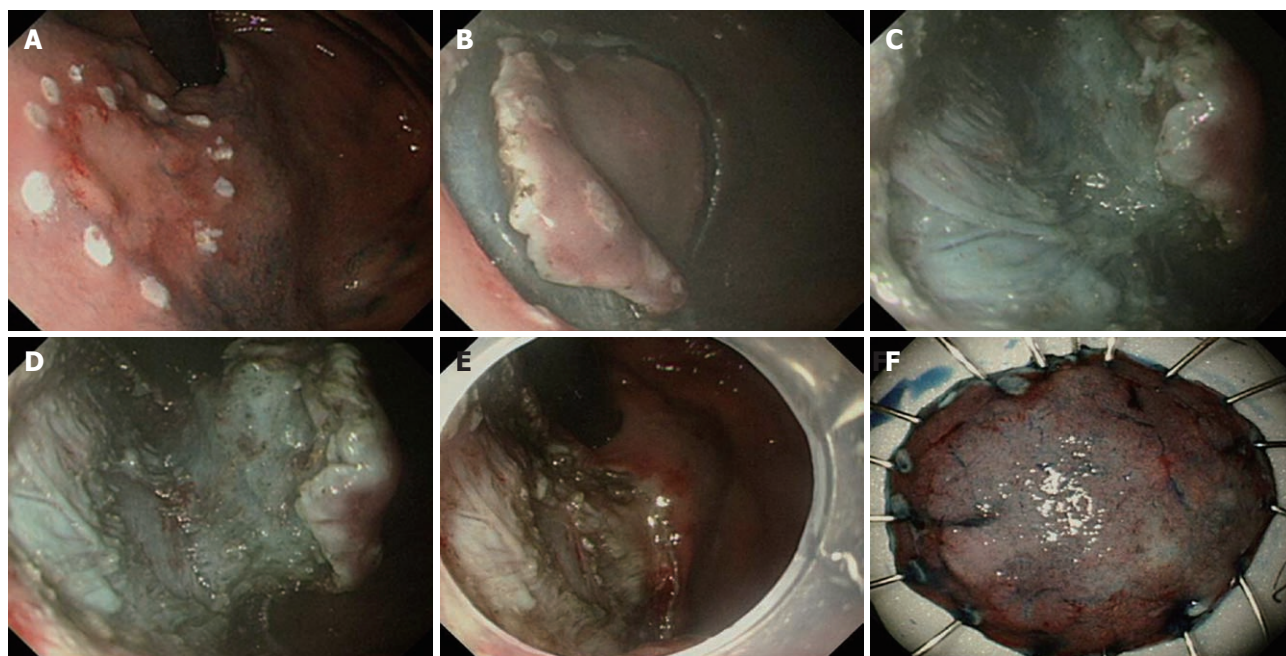
Mucosal incision usually involves circumferential cutting around the lesion prior to submucosal dissection. It is important to incise deep enough into the muscularis mucosal layer that it expose the underlying submucosal layer, because shallow incision leads to unexpected bleeding and make subsequent submucosal dissection difficult. On doing this, immediate trimming by coagulation force with knives on the initial incised spot is really helpful in completing incision and preventing bleeding from the incised area. Available devices are the needle knife, insulation-tipped (IT) knife, flex knife, hook knife, and triangular-tipped knife<sup>[21-23]</sup>. With a needle knife, a small incision hole is made prior to insertion of the IT knife into the submucosal layer and beginning circumferential incision<sup>[1]</sup>. On the other hand, needle knife, flex knife, hook knife and triangular knife can be used alone to complete the incision. The sequence and direction of the mucosal incision is dependent on the location of the lesion and selection of the devices (Figure 1B).

### Submucosal dissection

Submucosal dissection is a continuing process related to mucosal incision (precut) and also the final stage of ESD. The entire procedure, from marking to dissection, should be carefully designed prior to ESD so that each step guarantees a smooth transition into the next. Direction of gravity, location of lesion, and presence of fibrosis and ulcer should all be taken into account thoroughly in order to apply different tactics and devices for individual lesions (Figure 1C and D).

The technique of submucosal dissection relies heavily on device selection. There are two different classes of knives currently used in clinical practice. One category is pointed tip devices such as needle, flex and hook knives, which are useful for horizontal dissection and have an easy maneuvering quality in all directions. The category contains linear blade devices such as the IT knife, which has an insulated ball tip to prevent perforation and provide fulcrum during dissection. While the IT knife has some disadvantages against fibrotic lesions and features diminished horizontal cutting ability, dissection is quicker and more efficient than other devices, especially in the stomach. Careful dissection with pointed tip knives may be the only reliable option for lesions with ulcer or dense fibrosis.





**Figure 1** The cardinal steps of endoscopic submucosal dissection technique. A: Marking around early gastric cancer at fundus, marking at least 5 mm apart from the outer circumferential margin of the lesion with the argon plasma coagulation; B: Mucosal incision (precut), circumferential cutting around the lesion prior to submucosal dissection. Incision must be deep enough to expose submucosal layer fully; C, D: Submucosal dissection, early gastric cancer located in upper stomach or fundus like in this case should be dealt with great care to avoid bleeding or perforation. It is advised to always maintain a visual landmark between submucosa and underlying proper muscle layer; E: Completion of endoscopic submucosal dissection, large artificial ulcer was formed after submucosal dissection; F: Acquisition and fixation of the specimen, the specimen was fixated on the board with the pin spreading the lesion circumferentially for the preparation of the pathologic interpretation.

For successful submucosal dissection, measures to adjust to various situations, such as optimization of the operation field by repeated submucosal injection, utilization of transparent cap, adjusting air insufflations and frequent suction, are known to be essential. The cardinal aspect of dissection is maintaining an optimal dissection plane through the submucosal layer, while prudently avoiding injury to the underlying proper muscle layer. By this method, unexpected, blind dissection into the muscle layer and resultant perforation can be prevented.

Bleeding should be minimized for the clear operation field by recognition and meticulous coagulation of vessels before they are inadvertently injured by a cutting knife. Failure to do so often results in a poor field of view and a prolonged procedure time.

#### **Acquisition and preparation of specimen (Figure 1E and F)**

Dissected specimens can be dragged out of the stomach to prepare for histopathologic examination. Such specimens should be handled with care during stretching and fixation on the board. Processing the specimen should guarantee accurate analysis and correct diagnosis. Before submitting to pathology, endoscopists are obliged to determine spatial orientation and cutting direction for the preparation of pathologic specimens.

#### **Electrosurgical unit and usage of carbon dioxide (CO<sub>2</sub>)**

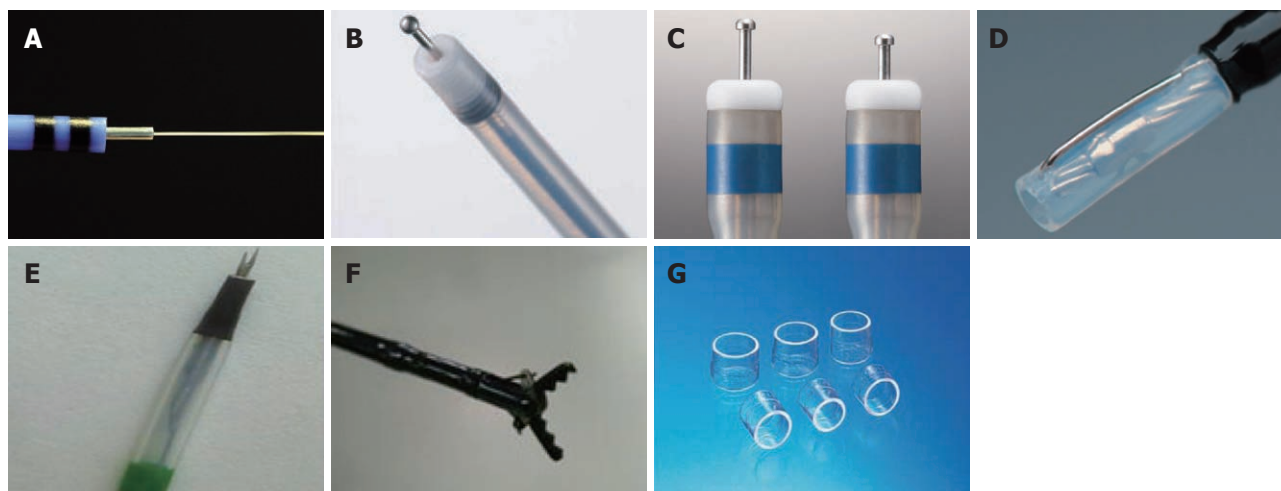
For successful ESD, the understanding and proper use of electrosurgical units is essential<sup>[24]</sup>. Earlier models of electrosurgical units were composed of a simple cutting and coagulation mode with only the output being adjust-

able. Recent models added multiple modes that could be used on different lesion characteristics. ICC 200 and VIO300D (ERBD, Germany) are equipped with sensors that pick up the changing signals from the cutting device and tissue interaction and automatically control output and maintain quality of cutting. Cutting mode was comprised of Endocut I&Q mode, dry cut, and swift coagulation, whereas coagulation mode incorporates forced, soft and sprays coagulation.

The time taken for ESD can be longer than 1 or 2 h if the lesion is large and in a difficult location. The patient often complains of abdominal distension and an urge for belching, owing to continuous air inflation of the stomach for maintaining visual field. It appears that there was less bloating and pain after procedures using CO<sub>2</sub> for gut distension compared to air<sup>[25]</sup>. CO<sub>2</sub> was found to be superior to air insufflations during balloon enteroscopy, endoscopic retrograde cholangio-pancreatography and invasive procedure such as colonic submucosal dissection<sup>[26-28]</sup>. Usage of CO<sub>2</sub> clearly has a clear advantage when perforation occurs during ESD, because rapid absorption into splanchnic blood makes patients' symptoms more tolerable and helps to stabilize vital signs.

### **BRIEF KNACK ON ACQUIRING CLEAR FIELD OF VIEW**

Unlike the esophagus or large intestine, which is a long tubular structure, the stomach is a distensible bottle-shaped organ that requires diverse approaching techniques depending on the location. For a successful pro-



**Figure 2** New devices. A: Water-jet hybrid knife allows needleless infusion and lifting of the lesion as well as cutting and coagulation at the same time without the need of changing instruments; B: Ball tipped flush knife (Flush knife-BT) features improved hemostatic efficacy and dissection speed compared with standard flush knife; C: Dual knife is a newly devised version of pre-existing flex knife, having 0.3 mm needle tip shaped like a doorknob makes the needle less likely to slip, simplifies marking and hemostasis; D: Mucosectome is made of non-conducted tip and endo-knife which is located at the side of the non-conducted tip; E: Fork knife has two interchangeable knives, a fixed flexible snare and a forked knife, which form a single working unit, and has an inlet for material injection or saline irrigation during the procedure; F: Grasping-type scissors forceps has a 0.8-mm-wide and 6-mm-long serrated cutting edge to facilitate grasping of tissue; G: Distal attachment helps keep the field of view clear throughout the procedure and can be chosen from various sized and shaped models fitted with endoscopes.

cedure, the anatomical structures and characteristics of each region should be first acknowledged so that individualized incision and dissection techniques can be applied.

As stated earlier, it is important to maintain a constant depth of incision or dissection while securing the desired operation field during the procedure by appropriately using turns of the endoscope (J-turns and U-turns), adjustments of the left/right levers, and changes of body position. In cases where fibrosis is severe, linear incision knives (such as needle, flex or hook knives) could be more advantageous, while incidence of perforation should be minimized by moving the knives elaborately by small amounts. Certain areas of stomach, such as the cardia or angularis, could be difficult to reach with a conventional endoscope. A multi-banding endoscope (GIF-2TQ260M, Olympus, Tokyo) can sometimes provide assistance in this situation, with its additional bending section enabling easier approximation to the lesion.

### New devices (Figure 2)

Knives are a basic instrument for ESD. Selection of the proper instrument influences the quality of the procedure and overall outcome. Every device has its own merits and disadvantages, with new devices usually giving specific modifications to cover up the weaknesses of earlier models.

Several knives, such as the needle, IT/IT-2 knife, hook, flex and flush knives are currently used. Constant effort has been paid to improve the dissection efficiency and safety of each knife. New devices have been devised to maximize ESD potential, while minimizing the ESD time, complication rate and patient discomfort.

The ERBE Hybrid Knife (ERBE, Tübingen, Germany) combines an ultrafine high-pressure fluid jet with an electrocautery needle, making this device an attractive

tool for performing ESD. This device allows submucosal fluid elevation with a preselected pressure and subsequent cutting or coagulation, and is used as a combination of a high-pressure water-jet and a radiofrequency surgical intervention. This allows needleless infusion and lifting of the lesion, as well as cutting and coagulation at the same time without the need to change instruments<sup>[29]</sup>. However, there is little experimental data and even less human experience with this device at the time of writing.

The ball tipped flush knife (Flush knife-BT) is the improved model of the flush knife, and was developed for the further improvement of the operability and ability of the hemostasis by the knife itself. It has a ball tip of 0.9 mm in diameter and 3 projecting parts of 1.5, 2 and 2.5 mm in length<sup>[30,31]</sup>. In one case-control study, Flush knife-BT appeared to improve hemostatic efficacy and dissection speed, compared with the standard flush knife.

The dual knife is a newly revised version of the pre-existing flex knife. Having a 0.3 mm needle tip shaped like a doorknob makes the needle less likely to slip, and simplifies marking and hemostasis. A two-step knife extrusion provides length adjustment, with no need for confirmation under endoscopic view, makes up for the weakness of the flex knife. These features enable a precise and effective cutting ability while reducing the burning effect and perforation.

Mucosectome is made of a non-conducted tip and endo-knife. Its blade is located at the side of the non-conducted tip and the tip is rotatable, so the blade can face the lumen and the non-conducted portion of the tip can face the wall of the hollow viscus<sup>[32]</sup>.

The fork knife has two interchangeable knives; a fixed flexible snare and a forked knife, which forms a single working unit, and has an inlet for material injection.

tion or saline irrigation during the procedure. The knives can be changed during ESD by using two switches, the fork knob and core knob, located on the center of the instrument<sup>[33]</sup>.

Grasping-type scissors forceps have a 0.8-mm-wide and 6-mm-long serrated cutting edge to facilitate grasping tissue. The outer side of the forceps is insulated so that electrosurgical current energy is concentrated at the blade to avoid burning the surrounding tissue. The forceps are also able to rotate to the desired orientation<sup>[34]</sup>.

One of the most important devices in ESD is the distal attachment, which leads to safe and fast ESD. It can be fitted at the tip of the scope, making it possible to position the knives at the submucosal layer *en face*<sup>[1]</sup>. The distal attachment helps keep the field of view clear throughout the procedure and can be chosen from various sized and shaped models fitted with endoscopes. The most widely used hood is a disposable, transparent hood (D-201, Olympus, Japan). It is soft so that endoscopists can compress the submucosal layer without muscle injury and still get a good view. The small caliber tip transparent hood was reported to be useful in getting a higher complete resection rate and preventing perforation<sup>[35]</sup>. To facilitate the evacuation of blood and water that can be retained on the inner part of the hood and hinder field of the vision, hoods equipped with a irrigation port or side hole have been devised.

## CONCLUSION

The technique of ESD for gastric cancer, though there can be slight differences between endoscopists, is relying on the basic concept of lifting the lesion and dissecting the submucosal layer under direct vision. Complications such as bleeding and perforation should be minimized for the invasive nature of the procedure. There are several knives and devices currently available for various purposes. However, the conclusion for which is superior is difficult to be judge because each set of devices has a unique advantage under specific circumstances. The development of new devices has focused on improving dissecting ability and shortening the procedural time while keeping safety in mind. It seems to be clear that current ESD techniques have certain limitations for the full application in all indications regarding early gastric cancer. Therefore, there should be a constant effort to refine and improve ESD devices to come up with ways to improve the ESD technique for early gastric cancer.

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## Outcome after endoscopic submucosal dissection for early gastric cancer in Korea

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### Abstract

Endoscopic treatment, such as endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), has been established as one of the treatment options for selected cases with early gastric cancer (EGC). Most studies on this topic have been carried out by researchers in Japan. Recently, the experience in EMR/ESD for EGC outside Japan is increasingly reported. In Korea, gastric cancer is the most common malignant disease, and the second leading cause of cancer death. Currently, EMR for EGC is widely performed in many centers in Korea. Early results with a short-term follow-up period are very promising in Korea. The

complete resection rate of EMR was 37.8%-94.3%, and that of ESD was 77.4%-93.1%. In this review, we will provide an overview of the outcomes of endoscopic treatments in Korea.

**Key words:** Early gastric cancer; Endoscopic mucosal resection; Endoscopic submucosal dissection; Outcome

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### INTRODUCTION

Gastric cancer is the most common malignancy and the second leading cause of cancer death in Korea. The detection rate of early gastric cancer (EGC), however, has been steadily increasing in Korea. One of the main reasons for this trend is the widespread use of endoscopy as a screening tool for gastric cancer-especially in individuals without symptoms<sup>[1,2]</sup>.

Endoscopic mucosal resection (EMR) and/or endoscopic submucosal dissection (ESD) is currently accepted as a standard treatment for selected cases with EGC<sup>[3,4]</sup>. Early data suggest that EMR/ESD provides a survival rate of more than 90% comparable to that of surgery if the technique is applied for the appropriate indication<sup>[5,6]</sup>. Most studies on this topic were performed in Japan, where the incidence of EGC is very high (for review, see these articles<sup>[4,7]</sup>). The philosophy and technique of endoscopy is quite different between Eastern and Western endoscopists<sup>[8]</sup>. Even among Eastern coun-

tries, the idea of the use of endoscopy for gastric cancer is quite different between Korean doctors and Japanese doctors. Therefore, extrapolation of Japanese data to other groups of patients may lead to suboptimal results.

The experience of EMR/ESD for EGC outside Japan has been increasingly reported<sup>[9-16]</sup>. The gold standard study design for evaluation of the efficacy of endoscopic treatment of EGC is a long-term, large-scale, randomized controlled trial. However, the excellent prognosis after surgical treatment of EGC, especially in cases indicated for endoscopic resection, makes randomized controlled trials unethical. Therefore, the best feasible evidence of the efficacy of EMR/ESD comes from long-term clinical follow-up data. In this review, we will provide an overview of the updated outcomes of EMR/ESD for EGC in Korea (Table 1).

### Endoscopic treatment of EGC before the introduction of ESD

In Korea, EMR for EGC was first reported in 1996<sup>[9]</sup>, followed by a number of clinical studies. A typical example with relatively long-term follow-up data was a study performed by Youn *et al.*<sup>[17]</sup> from Yonsei University Medical Center in 2006. Between April 1996 and March 2005, 147 patients were treated by EMR. The overall rate of complete resection was 84.6% (126/149), while a complete resection rate of 93.5% was achieved in mucosal cancers (115/123). The success of complete resection was significantly affected by endoscopic gross type (depressed lesion), the degree of differentiation, and the depth of invasion, independently. There were only 5 cases (4.0%) of local recurrence during the follow-up period. There was no disease-related or treatment-related mortality.

Following endoscopic resection of EGC, the development of additional gastric cancer is a significant problem. Investigators from Yonsei University studied the factors related to the multiple synchronous and/or metachronous gastric cancers in EMR/ESD patients<sup>[18]</sup>. After endoscopic treatment of EGC(s), they followed 235 patients for 24 mo or longer. Twenty-three patients (9.8%) were found to have additional gastric cancer within 1 year. Twenty metachronous cancers (8.5%), which were defined as cancers detected after 1 year of treatment, were also found. Interestingly, initial histology of the resected specimen was related to the development of additional cancer; undifferentiated histology of the primary lesion was related to synchronous and metachronous gastric cancer ( $P < 0.001$  and  $P = 0.002$ , respectively)<sup>[18]</sup>. This is very interesting data which should be considered in the discussion of expanding the indications of EMR/ESD.

### Techniques and results of ESD for EGC

In Korea, the most commonly used endoscopic treatment modality for EGC has been changed from endoscopic mucosal resection with precutting (EMR-P) to ESD (Figure 1)<sup>[19]</sup>. The techniques of ESD used in Korea and Japan are quite similar. In brief, ESD is usually performed under conscious sedation or slightly deeper sedation using either midazolam or propofol. Cardiores-

piratory function is continuously monitored during the procedure. After identifying the target lesion, marking dots were made circumferentially at about 5 mm lateral to the margin of the lesion using a needle knife or an argon plasma coagulation probe. After marking, a submucosal injection of various solutions, such as normal saline and epinephrine mixture or glycerol mixture, is performed around the lesion to make a submucosal cushion. An initial short incision of the mucosa was made with the needle knife to allow the submucosal insertion of the tip of the insulation-tipped (IT)-knife or other knives. Circumferential mucosal cutting is performed outside the marking dots to separate the lesion from the surrounding non-neoplastic mucosa. After the circumferential cutting, an additional submucosal injection is carried out. Finally, direct dissection of the submucosal layer is performed using one of the various knives. When needed, an electrocautery snare may be used at the final step. During the ESD procedure, endoscopic hemostasis is performed with a needle knife or specialized hemostatic forceps.

Jung *et al.*<sup>[20]</sup> from Asan Medical Center reported early results of ESD in their institution. From 2005 to 2006, ESDs for 264 cases of EGC were performed. The median size of the tumor was 19 mm, and the median size of the resected specimen was 50 mm. The rate of complications was 14.0% (bleeding 9.8% and perforation 3.8%). The complete resection rate was 87.9% (232/264)<sup>[20]</sup>. Recently, researchers at Asan Medical Center presented their updated results of ESD for EGC as an abstract<sup>[21]</sup>. In their institution, EMR or ESD was performed on 1340 EGCs in 1187 patients from July 1994 to January 2009. The complete resection rate was 96.6% and was 86.9% in the absolute indication group and in the extended indication group ( $P < 0.001$ ). The local recurrence rate was similar<sup>[21]</sup>.

Min *et al.*<sup>[22]</sup> from Samsung Medical Center reported their experience of EMR-P and ESD with short-term follow-up data. From 2003 to 2006, 346 consecutive patients with EGC were treated by either EMR-P (103 patients) or ESD (243 patients) and their clinical outcomes were compared. In the ESD group, the rate of en bloc plus R0 resection was significantly higher than the EMR-P group (88.9% *vs* 75.7%,  $P = 0.002$ ). For small EGC (diameter  $< 20$  mm), however, the en bloc plus R0 resection rate for EMR-P was comparable to ESD. The complication rate was slightly higher in the ESD group, but there was no statistical significance. In the case of R0 resection of intramucosal differentiated cancer, neither group showed local recurrence during the median 29 and 17 mo of follow-up<sup>[22]</sup>.

Jang *et al.*<sup>[23]</sup> from Dong-A Medical Center reported their follow-up data after ESD. A total of 198 patients with EGC were treated with ESD from 2004 to 2007. In EGC patients, en bloc resection was achieved in 89.7% (177/198), and the complete resection rate was 87.9% (174/198). During the median follow-up period of 30 mo, local recurrence was found in 10 patients (5.1%). Tumor size  $> 20$  mm was significantly associated with



**Table 1** Clinical outcome of endoscopic mucosal resection or endoscopic submucosal dissection for early gastric cancer in Korea (selected) (%)

Author	Year	<i>n</i>	Methods	Complete resection	Local recurrence	Bleeding	Perforation
Lee	1996	19	Strip biopsy	37.8	28.6	-	-
Cheon	2000	28	Strip biopsy	64.3	3.6	-	-
Kim	2000	20	EMR-L	85.0	5.9	0.0	0.0
Seong	2002	35	Strip biopsy	94.3	6.1	-	-
Hyun	2003	45	Strip biopsy	55.6	0.0	24.4	0.0
Kim	2005	109	Strip biopsy, EMR-C, EMR-P	67.9	1.4	8.3	2.8
Youn	2006	149	Strip biopsy, EMR-C, EMR-L, ESD	84.6	4.0	22.8	1.3
Kim	2007	514	Strip biopsy, EMR-C, EMR-L, EMR-P, ESD, polypectomy	77.6	6.0	13.8	0.6
Jung	2007	360	EMR-P	82.8	-	10.6	1.1
Jung	2007	264	ESD	87.9	-	9.8	3.8
Kang	2008	456	ESD	80.3	0.0	-	-
Park	2008	434	ESD	77.4	1.8	8.1	2.3
Min	2009	103	EMR-P	75.7 <sup>1</sup>	0.0	3.9	1.9
Chung	2009	534	ESD	87.7 <sup>1</sup>	-	15.6	1.2
Jang	2009	198	ESD	87.9	5.1	7.4	2.9
Lee	2010	806	ESD	93.1	0.4	4.2	3.0

<sup>1</sup>Complete *en bloc* resection rate; EMR-C: Endoscopic mucosal resection using a transparent cap; EMR-P: Endoscopic mucosal resection with precutting; EMR-L: Endoscopic mucosal resection with band ligation; ESD: Endoscopic submucosal dissection.

local recurrence. The 3-year cancer-free survival rate was 94.9%. Among 10 patients with local recurrence, 6 were successfully treated with a second ESD, and 4 were treated surgically after a failed attempt at ESD. Six metachronous cancers were also found, which were treated with ESD. As a whole, the 3-year cancer-free survival rate was 94.9%<sup>[23]</sup>.

Kim *et al*<sup>[24]</sup> from Soonchunhyang University reported their experience using a novel device, the Fork knife (Endo FS). Although the authors did not report the long-term follow-up data after ESD, the *en-bloc* resection rate was 95.8% (254/265) using the Fork knife, and was comparable with that of 93.1% (67/72) using a more popular Flex knife. Complete ESD without tumor cell invasion of the resected margin was obtained in 81.1% (215/265). The mean procedure time was shorter in the Fork knife group compared to the Flex knife group (59.6 min *vs* 76.7 min,  $P < 0.05$ ). The authors concluded that the Fork knife is useful for clinical practice and has the advantage of reducing the procedure time<sup>[24]</sup>.

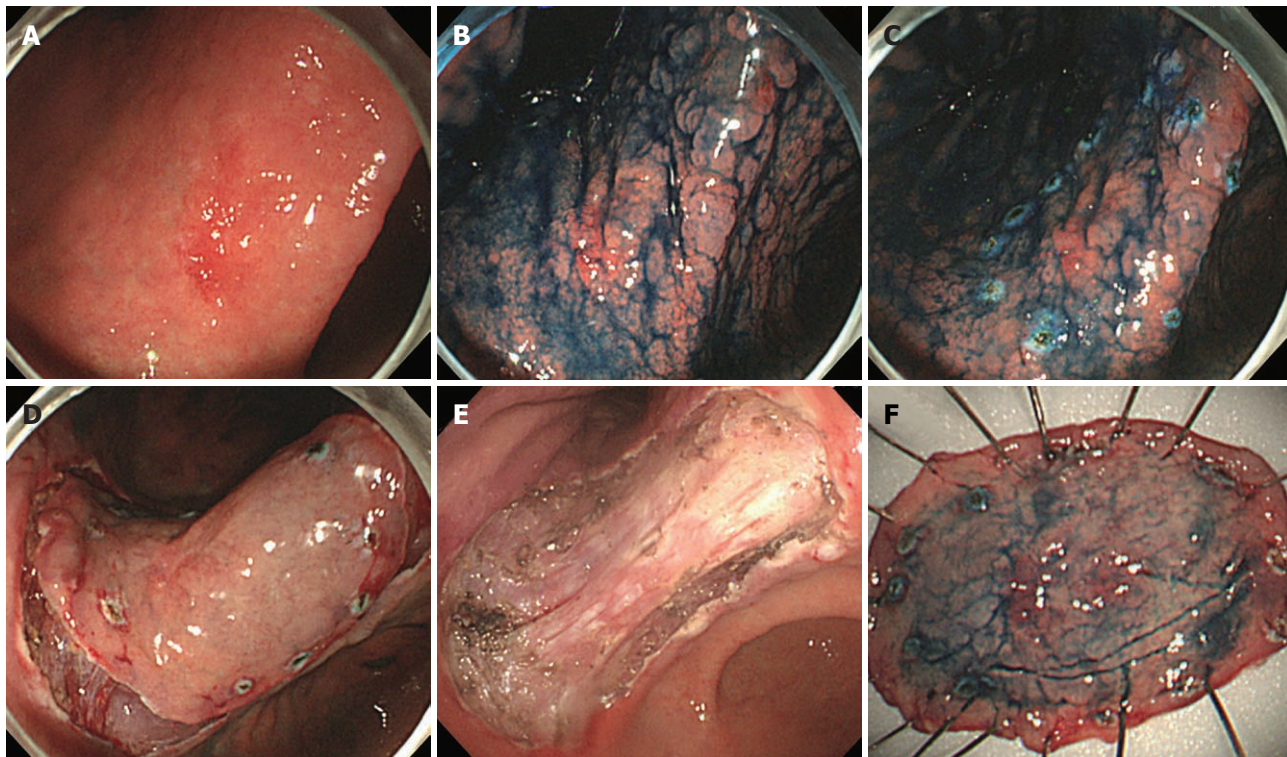
Recently, Lee *et al*<sup>[16]</sup> from Samsung Medical Center reported their updated data of ESD for EGC<sup>[16]</sup>. Before March 2009, 806 lesions of EGC in 780 patients were treated with ESD at their institution. They divided their cases into two groups: a conventional indication group ( $n = 595$ ) and an expanded indication group ( $n = 211$ ). The complete resection rate was 97.3% and 81.0% in the conventional indication group and in the expanded indication group, respectively. The conventional indication group and expanded indication group did not differ with regard to the rates of local recurrence (0.7% *vs* 0%), or metachronous recurrence (3.6% *vs* 3.3%). The rate of perforation was higher in the expanded indication group than in the conventional indication group (6.6% *vs* 2.4%,  $P < 0.001$ ). When they followed 458 patients for a median of 26 mo, there were no cancer-related deaths in the two groups. Two cases (0.4%) with local recurrence and 16 cases (3.5%) with metachronous recurrence were

observed. Disease-free survival rate was not different between the two groups<sup>[16]</sup>.

### Korean multicenter studies

Two multicenter retrospective studies on the clinical efficacy of endoscopic treatment of EGC have recently been published. The first study was published in 2007 by Kim and other members of the Korean EMR Study Group (changed to the Korean ESD Study Group in 2009)<sup>[25]</sup>. Data were collected retrospectively using the on-line database registry system. From 2000 to 2002, 514 EGCs in 506 patients were treated by various techniques in 13 institutions. EMR-P was the most commonly used technique (52.3%). ESD was used in only 6.6%. The resection was regarded as incomplete if histopathologic examination revealed a positive resection margin, submucosal invasion, positive lymphovascular invasion, or undifferentiated histologic diagnosis. The rate of complete resection was 77.6%. For completely resected mucosal cancers ( $n = 399$ ), the median duration of follow-up was 23.5 mo (range 5-70 mo). In this group, local recurrence was detected in 24 cases (6.0%) with a median interval between EMR and recurrence of 17.9 mo (range 3.5-51.7 mo). There were 3 cases with perforation and 71 cases with bleeding. No deaths were related to recurrence of gastric cancer during the overall median follow-up period of 39 mo<sup>[25]</sup>.

After the first multicenter study, ESD was widely used in different hospitals in Korea. The Korean ESD study group has carried out a second multicenter retrospective study on the safety and effectiveness of ESD<sup>[26]</sup>. From January 2006 to June 2007, 1000 EGCs in 952 patients (502 men, 450 women; mean age 62.1 years, range 43-90 years) were treated using ESD at 6 Korean ESD study group-related university hospitals in Korea. The rates of *en bloc* resection and complete *en bloc* resection were 95.3% and 87.7%, respectively. The rates of significant bleeding and perforation were 0.6% and 1.2%,



**Figure 1** Endoscopic submucosal dissection procedure for early gastric cancer. A: 1.5 cm × 1.2 cm sized hyperemic slightly elevated early gastric cancer was seen at the lesser curvature side of the lower body just above the gastric angle. Previous forceps biopsy results showed moderately differentiated adenocarcinoma; B: Indigo carmine dye was sprayed onto the lesion to define the lateral margin more clearly. Gastric mucosa around the cancer lesion showed severe metaplastic change; C: Using the tip of the needle knife, marking dots were made circumferentially at about 5 mm to 10 mm lateral to the estimated margin of the lesion; D: After submucosal injection of saline mixed with epinephrine and indigo carmine, a circumferential mucosal cutting was performed outside the marking dots to separate the lesion from the surrounding non-cancerous mucosa; E: After additional submucosal injection, direct dissection of the submucosal tissue was performed using an IT-knife and endoscopic hemostasis was carried out. A large artificial ulcer was made; F: The resected specimen with a central cancerous lesion. In the pathologic examination, a 1.8 cm × 1.1 cm sized moderately differentiated tubular adenocarcinoma limited in the mucosal layer was identified. The resection margin was free of cancer, and there was no lymphovascular invasion.

respectively. The mean procedure time was  $47.8 \pm 38.3$  min. However, multicenter long-term follow-up data after ESD have not yet been reported<sup>[26]</sup>.

## DISCUSSION

Endoscopic treatment of EGC was developed in Japan. However, experience in endoscopic treatment has now been reported in many other countries. As shown in this review, ESD for EGC has become quite a common procedure in Korea. In 2009, a multicenter study of ESD was reported in Taiwan<sup>[27]</sup>. In China, early experience of ESD has been reported<sup>[28]</sup>. Even in Western countries where early EGC scheduled for endoscopic treatment is uncommon, small studies evaluating the usefulness of ESD have been published<sup>[11,29,30]</sup>. Because of the advantages of ESD in terms of complete resection rate and curative resection rate, we expect that more cases of EGC will be treated by ESD not only in Korea but also in many other countries.

The complete resection rate of endoscopic treatment for EGC depends on the inclusion criteria and the definition of complete resection, so head to head comparisons are difficult. As shown in a recent meta-analysis<sup>[31]</sup>, the complete resection rate of ESD is generally higher than

EMR. In Korea, the complete resection rate of EMR was 37.8-94.3%, and that of ESD was 77.4-93.1% (Table 1). This is quite an important achievement, because the selection criteria for endoscopic treatment of EGC have been expanded. The size of the endoscopically treated lesions these days is larger, and technical developments have made the complete resection rate better. Although the procedure time is longer, and the complication rate is higher in ESD, most complications can be treated medically without surgery.

Early results of endoscopic treatment of EGC in Korea have been very promising. However, the duration of follow-up is rather short, which makes conclusive comments difficult. Before expanding indications for endoscopic treatment, we need to examine the reported and unreported data very carefully. In this regard, a nationwide registry of endoscopically treated EGC cases seems to be mandatory.

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## Special diaphragm-like strictures of small bowel unrelated to non-steroidal anti-inflammatory drugs

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### Abstract

**AIM:** To summarize clinical, endoscopic, radiologic and pathologic features of special diaphragm-like strictures found in small bowel, with no patient use of non-steroidal anti-inflammatory drugs (NSAIDs).

**METHODS:** From January 2000 to December 2009, 5 cases (2 men and 3 women, with a mean age of 41.6 years) were diagnosed as having diaphragm-like strictures of small bowel on imaging, operation and pathology. All the patients denied the use of NSAIDs. The clinical, endoscopic, radiologic and pathologic findings in these 5 patients were retrospectively reviewed

from the hospital database. Images of capsule endoscopy (CE) and small bowel follow-through (SBFT) obtained in 3 and 3 patients, respectively, and images of double-balloon enteroscopy and computed tomography enterography (CTE) obtained in all 5 patients were available for review.

**RESULTS:** All patients presented with long-term (2-16 years) symptoms of gastrointestinal bleeding and varying degrees of anemia. There was only one stricture in four cases and three lesions in one case, and all the lesions were located in the middle or distal segment of ileum. Circumferential stricture was shown in the small bowel in three cases in the CE image, but the capsule was retained in the small bowel of 2 patients. Routine abdomen computed tomography scan showed no other abnormal results except gallstones in one patient. The lesions were shown as circumferential strictures accompanied by dilated small bowel loops in the small bowel on the images of CTE (in all 5 cases), SBFT (in 2 cases) and double-balloon enteroscopy (in all cases). On microscopy, a chronic inflammatory infiltrate and circumferential diaphragm were found in all lesions.

**CONCLUSION:** Diaphragm-like strictures of small bowel might be a special consequence of unclear damaging insults to the intestine, having similar clinical, endoscopic, radiologic and pathologic features.

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**Key words:** Small bowel; Gastrointestinal bleeding; Diaphragm; Stricture; Endoscopy; Computed tomography; Enterography

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## INTRODUCTION

Many medications, diseases and processes may cause insult to the small bowel and result in strictures of the bowel cavity, such as potassium chloride tablets, surgical anastomoses, radiation, ischemia, Crohn's disease, tuberculosis, eosinophilic enteritis, lymphoma, *etc.*

Diaphragm disease was first defined by Lang *et al*<sup>[1]</sup> in 1988, who described the pathologic findings of non-specific small-bowel disease in patients taking non-steroidal anti-inflammatory drugs (NSAIDs). The mucosal disease caused by these drugs in the small bowel is termed NSAID enteropathy. The abnormalities of NSAID enteropathy include inflammation, erosion, fibrosis, stricture, perforation, and formation of diaphragm disease. The most frequent manifestations are iron-deficient anemia, acute hemorrhage, perforation and obstruction of the small bowel. Many studies have reported cases with multiple diaphragm-like strictures in the whole gastrointestinal tract that are associated with the chronic use of NSAIDs<sup>[2-6]</sup>. In contrast, from the references we can find, only one reported case with small bowel diaphragm disease is not associated with the use of NSAIDs<sup>[7]</sup>. Multiple diaphragm-like strictures emerged in the ileum and jejunum at different times in this patient and he underwent three surgical operations.

Here, we report on a group of 5 patients who presented symptoms of gastrointestinal bleeding and characteristics of diaphragm-like strictures of small bowel that were not attributable to the utilization of NSAIDs. The purpose of this study was to summarize the clinical, radiologic, endoscopic and pathologic features of these special diaphragm-like strictures of small bowel.

## MATERIALS AND METHODS

### Ethics

This work has been carried out after receiving the approval from our institutional review board. All patients were not individually asked for consent to be included in this study, but each patient in the study did agree to the retrospective use of their medical records and images for research purposes during treatment at our hospital.

### Patient data

From cross-referenced records in the Departments of Gastroenterology, Radiology and Pathology at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, from January 2000 to December 2009, 5 patients were identified with clinically confirmed

diaphragm-like strictures of small bowel. All the 5 patients denied the use or prescription of NSAIDs, which was confirmed by their family members and medical history records. These 5 patients included two men and three women. Clinical data from the patients including sex, age, hemoglobin level, white blood cell (WBC) count, C-reactive protein (CRP) level and major onset symptoms were obtained from the hospital database and most information is shown in Table 1. All cases underwent removal of the lesions by laparoscopically assisted enterectomy.

### Endoscopic procedure

Capsule endoscopy (CE) was performed in 3 patients (cases 1, 2 and 5). The small bowel capsule, manufactured by Given Imaging (Yoqneam, Israel) measures 11 mm × 26 mm and weighs 3.7 g. The camera in the capsule moves through the gastrointestinal (GI) tract *via* peristalsis and transmits 2 images per second to a data recorder located on the waist of the patient.

All 5 patients underwent double-balloon endoscopy (Fujinon, En-450P5/20, Fujinon Inc, Saitama, Japan). When the location of the lesion could be predicted in advance by the color of the feces or other examination findings, an insertion approach close to the lesion was selected, either oral or anal. When the location could not be predicted, the anal approach was selected first, in principle. However, whether the responsible lesion was specified or not by the first insertion approach, insertion with the other approach was performed later to observe the entire small bowel.

All the images were reviewed by two gastroenterologists who had no knowledge of the final radiologic, endoscopic, or pathologic findings. The endoscopic evaluation included number, location, size, shape, color and texture.

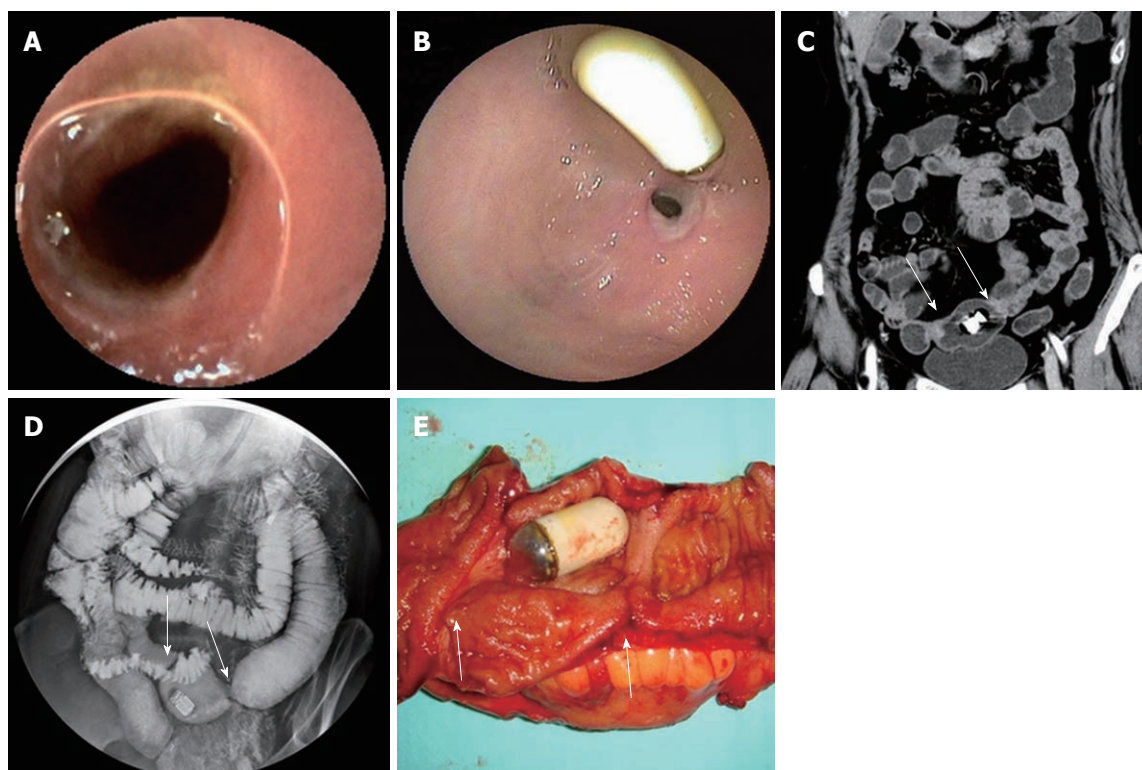
### Radiologic examination

Routine abdominal computed tomography (CT) scan was performed in one patient. CT enterography (CTE) was performed in all five patients. For CTE examination, all patients underwent an intestinal preparation according to the following plan: the day before a light diet free of fruit and vegetables; 500 mL of a mixture of Sennosides and tea at 5:00 pm, another 500 mL followed at 6:00 pm. All patients accepted oral administration of 2.5% mannitol solution (1500-2000 mL) over 30-45 min. Thirty minutes after oral administration, CT scans were performed on a multi-slice multidetector computed tomography (MDCT) scanner [two patients had scans performed on a 16-slice MDCT scanner (LightSpeed-16, GE Medical Systems, Milwaukee, WI) and the other three patients were on a 64-slice MDCT scanner (LightSpeed Volume CT, GE Medical Systems, Milwaukee, WI)]. Section thickness ranged between 5 mm and 7.5 mm. Intramuscular injection of 20 mg anisodamine was administered 10 min before CT scan. After unenhanced CT scan, all patients received an intravenous injection of 1.5 mL/kg of iohexol (Omnipaque300; Amersham, Shanghai, China) at a rate

Table 1 Clinical data of study patients

Case	Sex	Age at onset (yr)	Age at diagnosis (yr)	Past history	Main symptoms		Physical examination	HGB (g/dL)	WBC count ( $\times 10^9/L$ )
					GI bleeding	Abdominal pain			
1	F	30	33	Gallbladder stone	P	P	Normal	9.0	3.2
2	F	48	64	No	P	P	Normal	5.8-9.6	4.1
3	M	24	26	Appendectomy	P	N	Normal	10.9	5.3
4	F	40	44	Hysteromyoma	P	P	Normal	9.2	5.7
5	M	32	41	No	P	N	Normal	9.7	5.0

F: Female; M: Male; P: Positive; N: Negative; GI: Gastrointestinal; HGB: Hemoglobin; WBC: White blood cell.



**Figure 1** Sixty-four-year-old woman presenting with 16 years of intermittent black stools. A: Circumferential stricture seen on capsule endoscopy image; B: Double-balloon enteroscopy image shows an unusual diaphragm-like stricture in the ileum and the retained capsule; C: Computed tomography enterography image (oblique MPR) showing two diaphragm-like strictures and capsule retention in a dilated small bowel loop in the ileum (arrows); D: Diaphragm-like strictures and capsule retention in a dilated small bowel loop shown by small bowel follow-through (arrows); E: Longitudinal section of specimen containing laterigrade diaphragms and a retained capsule endoscope in the middle, and a laterigrade ulcer can be seen in one of the diaphragms (arrows).

of 3 mL/s. Contrast-enhanced CT images were acquired in arterial phase (25-30 s) and venous phase (60-65 s). Multiplanar reconstructions and maximum intensity projection were performed at the workstation (ADW4.2 and ADW4.4).

In addition, small-bowel follow-through (SBFT) was performed in 3 patients (cases 2, 4 and 5). Selective mesenteric angiography examination and bowel isotope scans using  $^{99m}\text{Tc}$ -labeled red blood cells ( $^{99m}\text{Tc}$ -RBC) were also performed for case 2.

The radiologic images were reviewed in consensus by two radiologists who had no knowledge of the final endoscopic, radiologic, or pathologic results. At the workstation, the CT images from each patient were reviewed to analyze the following criteria: (1) location of lesion; (2) number of lesions; (3) thickness of bowel wall; (4) lumen cavity; (5) mesenteric vessels; and (6) lymph nodes.

At non-enhanced CT, attenuation in the lesions was classified as hypodense, isodense, or hyperdense compared with the normal bowel wall. After contrast enhancement, the degree of enhancement was classified into no enhancement, mild (10-20 Hu), moderate (20-50 Hu), or marked ( $> 50$  Hu) enhancement. These findings were used to characterize the imaging and the gross pathological features of the lesions.

#### Pathological technique

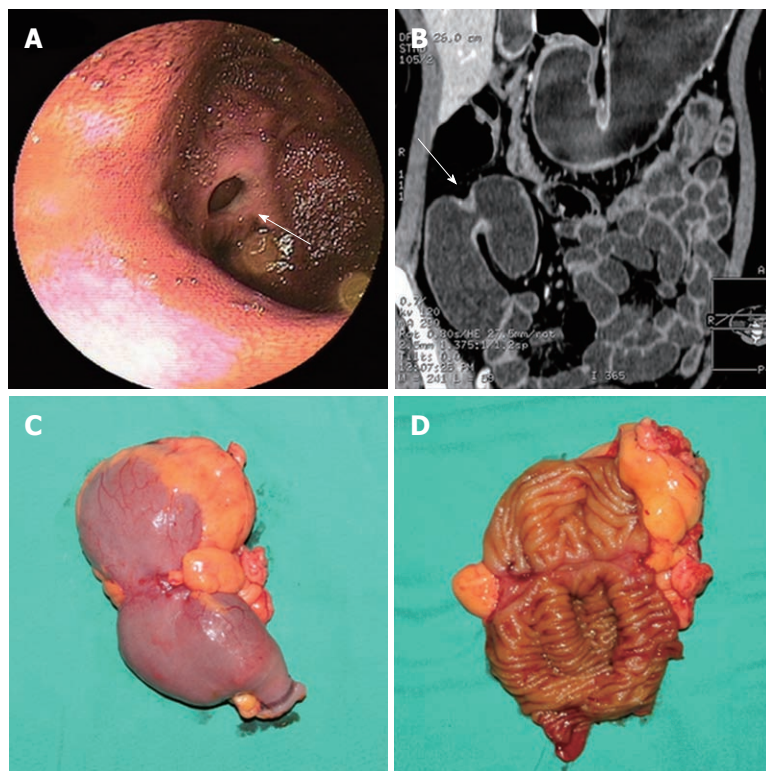
All the specimens were fixed in 10% neutral-buffered formaldehyde solution and were embedded in paraffin wax. Hematoxylin and eosin staining was performed in all the pathologic specimens. All the pathologic specimens were reviewed retrospectively by two pathologists. The macroscopic appearances of each resected segment were analyzed with photomicrographs; the analysis in-



Table 2 Data of enteroscopic findings

Case	n	Location (distance to ileocecal valve) (cm)	Type of lesion	Stricture		Edema in mucosa	Pass-through of scope
				Mild to moderate	Severe		
1	1	80	Ulcer	1	0	P	P
2	3	100-115	Ulcer and erosion	1	2	P	P
3	1	100	Ulcer	1	0	P	P
4	1	80	Ulcer	1	0	P	P
5	1	150	Erosion	0	1	P	P

P: Positive; N: Negative.



**Figure 2** Thirty-one-year-old woman presenting with a 3-year history of recurrent episodes of abdominal pain, incomplete intestinal obstruction and intermittent black stools. A: Double-balloon enteroscopy image showing circumferential diaphragm-like stricture in the ileum (arrow); B: Computed tomography enterography image (oblique multiplanar reconstruction) showing mild bowel expansion in the ileum with a diaphragm-like stricture in the middle (arrow); C: A diaphragm-like stricture in the middle can be seen in the iliac specimen; D: Longitudinal section of specimen containing a laterigrade ulcer.

cluded number, location, size, shape depth and edge.

## RESULTS

### Clinical information

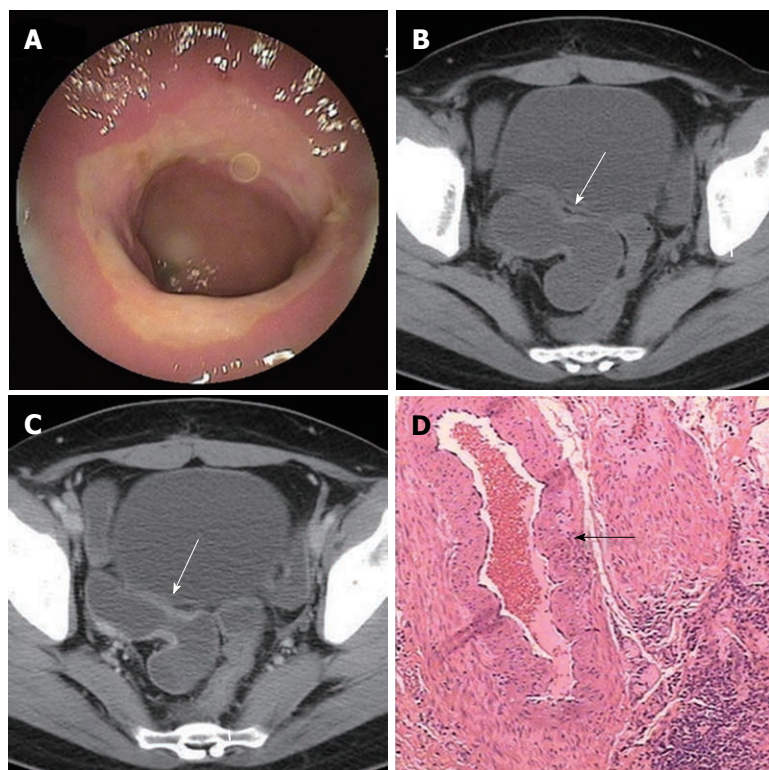
All the 5 patients presented long-term (2-16 years) symptoms of gastrointestinal bleeding (hematochezia appeared in case 4 and intermittent black stools occurred in the other four patients) and varying degrees of anemia, accompanied by obscure abdominal pain in three patients (cases 1, 2 and 4) (Table 1) and incomplete intestinal obstruction in one case. No significant changes were detected on physical examination, gastroscopy and colonoscopy. Three patients (cases 1, 2 and 3) were suspected of having small bowel Crohn's disease and received mesalazine medication. However, this medication was ineffective in these cases. In addition, administration of hemostatic agents and iron supplementation eliminated bleeding and improved the condition of all patients. Three lesions were detected in case 2 and the remaining 4 cases had one lesion in the ileum. WBC count

was not elevated and the proportion of eosinophils did not increase in all cases. One patient had a CRP level test with normal result. All cases had removal of the lesions by laparoscopically assisted enterectomy and remained well at 1 to 3.5 years follow-up with no signs of gastrointestinal bleeding.

### Endoscopic findings

On the images of capsule endoscopy, a mild circumferential stricture in the ileum was shown in two lesions which the capsule could pass through (Figure 1A). In another two cases, the capsule was stopped by markedly circumferential stricture of the lumen.

All the 7 strictures in five patients were found in the double-balloon enteroscopy (DBE) examination undergone by all five patients. All lesions appeared in the middle or distal segment of ileum which was about 80 cm to 150 cm away from the ileocecal valve and the scope could pass through. Circumferential ulcers or erosions with clear margin on the surface of stenoses were found in all lesions. All ulcers were covered by faint white mucous exu-



**Figure 3** Forty-four-year-old woman presenting with a 4-year history of recurrent episodes of hematochezia and abdominal pain. A: Double-balloon enteroscopy image showing circumferential stricture in the ileum; B: Computed tomography (CT) plain scan image showing mild bowel wall thickening and circumferential stricture of the lumen in the ileum (arrow); C: Contrast-enhanced CT scan image showing that contrast enhancement of thickened bowel wall is homogenous and moderate (arrow); D: Blood vessel with thickened wall and expanded lumen shown in the submucosa (hematoxylin and eosin, orig. mag  $\times 40$ ).

dates. Congestion and edema in the neighboring mucosa and dilated small bowel loop adjacent to the stricture were also observed in all lesions (Table 2, Figure 1B, 2A and 3A).

### Radiologic findings

Routine abdominal CT scan was performed in only one patient and showed no other abnormal results except gallstones. The main features of diaphragm-like strictures of small bowel seen at CTE include thickening of bowel wall, circular stricture in the middle or distal segment of the ileum and dilated small bowel loop adjacent to the stricture. These features were observed in three patients. In case 2, only two out of three lesions were detected by CTE. A minor stricture could not be shown. In case 3, CTE could only show the thickened bowel wall, the significant dilated small bowel loop and several enlarged lymph nodes. However, the stricture was not shown in CT images. Bowel wall thickening appeared as mild, symmetrical isodensity with respect to the normal bowel wall with homogenous and moderate contrast enhancement. The thickened wall ranged from 2.5 mm to 5.0 mm in thickness and reached 7.0 mm in one lesion (case 2) which turned the lumen into a tight stricture and caused retention of the capsule. The diameter of the lumen in the dilated small bowel loop ranged from 2.7 cm to 5.0 cm in 4 patients and reached 7.0 cm in one case (Table 3, Figure 1C, 2B, 3B and C). There was no abnormality in the mesenteric vessels.

SBFT was performed in 3 patients (cases 2, 4 and 5). Two strictures in case 2 and 1 stricture in case 5 with dilated bowel loop were observed (Figure 1D). The

pass-through of barium was blocked and stenoses of the lumen were still at the fixed location at a later time point observation after compression. Capsules floated in the dilated small bowel loops and could not move to the bottom with the downward movement of barium. Selective mesenteric angiography was performed in case 2 and showed no positive findings. One bowel isotope scan using  $^{99m}\text{Tc}$ -RBC revealed intestinal bleeding in the distal ileum.

### Pathologic findings

On gross examination, circumferential strictures were found in all the lesions of the resected small intestine in all five patients. These strictures were perpendicular to the long axis of the intestine. The lesions appeared as laterigrade diaphragms with laterigrade pittings in the mucosa, 0.2 cm to 0.5 cm in width, with edema in the neighboring mucosa in the longitudinal section of the specimens in four lesions from four patients (cases 1, 2, 3 and 4) (Figure 1E, 2C and D). They were approximately 0.5 cm in width in case 5 and in two lesions of case 2. Edema in the neighboring mesentery and enlargement of several lymph nodes were found in case 3. No abnormality was shown in the adjacent mesentery in the other cases. On microscopy, a chronic inflammatory infiltrate was found in all five subjects. Depth of ulcer reached to the muscularis propria in local areas in cases 1 and 2. The ulcer was limited to the submucosa in cases 3 and 4. Villous adenomatous hyperplasia in the mucosal layer was found in case 5 and in two lesions of case 2. Moderate local inflammatory cell infiltration was found in the submucosal layer of cases 1, 5 and two lesions of

Table 3 Data of computed tomography findings

Case	Location	n	Stenosis	Lumen expansion	Bowel wall			Mesenteric vessels	Lymph node enlargement
					Thickness (mm)	Attenuation	Enhancement		
1	Ileum	1	P	P	2.5	Isodense	Moderate	Normal	N
2	Ileum	2	P	P	7	Isodense	Moderate	Normal	N
3	Ileum	1	N	P	3	Isodense	Moderate	Normal	P
4	Ileum	1	P	P	2.5	Isodense	Moderate	Normal	N
5	Ileum	1	P	P	5	Isodense	Moderate	Normal	N

P: Positive; N: Negative.

Table 4 Data of histologic findings

Case	Size <sup>1</sup> (cm)	Type	Depth <sup>1</sup>	Inflammatory infiltrate	Fibrosis	Mucosal atrophy	Thickening of muscularis mucosa	Edema in submucosa
1	2.0 × 0.5	Ulcer	Muscular layer	Moderate	Mild	N	Mild	N
2 <sup>2</sup>	2.0 × 0.2	Ulcer	Muscular layer	Severe	Mild	N	Moderate	N
3	3.0 × 0.2	Ulcer	Submucosa	Severe	Moderate	N	N	P
4	3.0 × 0.5	Ulcer	Submucosa	Severe	Moderate	N	Moderate	P
5	2.0 × 0.5	Erosion	Mucosa	Moderate	None	N	N	N

<sup>1</sup>Means the size and depth of ulcer or erosion of specimen; <sup>2</sup>Information of the most narrow lesion. P: Positive; N: Negative.

case 2. Inflammatory cell infiltration was obvious and reached to the serosa in cases 3, 4 and one lesion of case 2. Fibrosis in the submucosal layer was mild in cases 1 and 2 and moderate in cases 3 and 4 (Table 4). The rupture of the muscularis mucosa under the ulcer was also found in case 3. Specifically, proliferation of blood vessel with thick wall and expanded lumen appeared in the submucosa and distorted muscularis propria in two cases (cases 3 and 4) (Figure 3D). Granulomatous lesion was not found in any patient. No cytomegalic inclusion associated with cytomegalovirus (CMV) infection was found in any lesion.

## DISCUSSION

This retrospective study showed the clinical, endoscopic, radiologic and pathologic features of distinctive small bowel diaphragm-like strictures. All patients presented with long-term (2-16 years) symptoms of gastrointestinal bleeding and varying degrees of anemia. All cases were not associated with the use of NSAIDs and had similar clinical, endoscopic, radiologic and pathologic features.

The cause of these special diaphragm-like strictures remains uncertain. Diaphragm disease induced by NSAIDs characterized by inflammatory strictures of the small intestine has previously been recognized as an uncommon complication of NSAID enteropathy<sup>[1]</sup>. The abnormalities of NSAID enteropathy include inflammation, erosion, fibrosis, stricture, perforation, and formation of diaphragm disease. Diaphragm disease most frequently affects the ileum. It can also affect the jejunum and colon, as well as stomach and duodenum. There are usually multiple diaphragms. The depth of ulcer is restricted to the submucosal layer and it never extends to the proper muscular layer<sup>[1-6]</sup>. Improvement in clinical

findings (signs and symptoms) and/or endoscopic findings appears on cessation of NSAID utilization, except for diaphragm disease. Diaphragm disease coupled with the use of NSAIDs is a pathognomic feature of NSAID enteropathy because of its non-specific histological findings<sup>[1-6]</sup>. Regarding clinical manifestations, the imaging findings in this study group share some common features with those observed in NSAID enteropathy, namely, concentric stenosis and circular ulcers. Diaphragm-like strictures in our study group are, however, different to those induced by NSAIDs with regard to many aspects such as the location, number, fibrosis and the disease process (Table 5).

Santolaria *et al*<sup>[7]</sup> reported a male patient with diaphragm disease who had a 25-year history of relapsing abdominal pain and edema and who did not have long-term use of NSAIDs. However, the symptoms and history of this case were different to those in our group. Shimizu *et al*<sup>[8]</sup> reported a case with diaphragm-like stricture of the small intestine related to CMV infection. Multiple erosions and small ulcers in the ileum and a circumferential diaphragm-like stricture were seen in this patient, with increased C-reactive protein level, and a cytomegalic inclusion was found in the strictured lesion on biopsy. CMV infection usually occurs in immunosuppressed patients. In our group, immune response of all patients was normal and no cytomegalic inclusion was found in pathology findings. Pasha *et al*<sup>[9]</sup> reported a case with eosinophilic gastroenteritis (EGE) mimicking diaphragm disease of the small bowel. Multiple ulcerated stenoses were present and capsule retention occurred in this case. Mucosal eosinophilia (> 20/HPF) can be found in EGE cases, usually accompanied by increased level of peripheral blood eosinophil count, signs which were not found in our group. Specifically, dilated blood



**Table 5** Differences between diaphragm disease of non-steroidal anti-inflammatory drug-enteropathy and diaphragm-like strictures in study group

	History	Location	<i>n</i>	Fibrosis	Lesions in other area of bowel	Disease process
Diaphragm disease	Long term NSAID use	Whole GI tract, most frequently in ileum	Multiple	Obvious	Exist, can be inflammation, erosion, fibrosis, stricture and perforation	Improvement in clinical findings by cessation of NSAID utilization
Diaphragm-like strictures	No NSAID use	Middle or distal segment of ileum	Usually single, no more than three	Mild or moderate	No	Non-self-limiting

NSAID: Non-steroidal anti-inflammatory drug; GI: Gastrointestinal.

vessel with thickened wall appeared in the submucosa in two cases in our group. This was different to angiodysplasia or hemangioma, in which the dilated blood vessel usually has a thin wall. Moreover, the manifestations of CT angiography in arterial phase and DBE also excluded the existence of vascular malformation. The history and pathologic examination of the lesions also excluded other potential causes of intestinal strictures, including use of potassium chloride tablets, surgical anastomoses, radiation, ischemia, Crohn’s disease, tuberculosis, and lymphoma.

Diaphragm-like strictures can also be found in the small bowel in congenital cases in adults, though this is rare. Congenital atresias, diaphragm-like strictures or stenoses are well documented occurrences in the stomach, duodenum and small bowel. Small intestinal atresia/stenosis most frequently affects the duodenum, followed by the jejunum, and least often the ileum, and can affect multiple sites of the intestine<sup>[10-16]</sup>. Intestinal obstruction is described as the main symptom. Cases that occur in the ileum and lead to bleeding of the small bowel have not been found in the literatures. All seven diaphragm-like strictures of the five cases were found in the middle or distal segment of the ileum in our group. It may be possible that congenital diaphragm-like stenoses may firstly have existed in the ileum, inflammation and ulcers then occurring after a long-term limitation of intestinal motility in the stenoses and friction between food and stenoses. This could explain why there was only one lesion in most cases, the stricture was obvious even if there was no marked fibrosis in the lesion, and the symptoms disappeared after operation. However, more evidence is needed to test this hypothesis.

Diagnosis of special diaphragm-like strictures of small bowel in non-NSAID patients may be difficult. No significant findings were detected on physical examination. Routine abdominal CT scan in general could not show any abnormal results. CE, DBE, CTE and SBFT may be helpful in making a diagnosis and may facilitate preoperative evaluation of the lesions. CE has been mainly used to evaluate patients with obscure GI bleeding<sup>[17]</sup>. There have also been many reports demonstrating diaphragm-like strictures in CE examinations<sup>[18-20]</sup>. Sometimes, diaphragms may be misinterpreted as exaggerated plicae circulares. CE carries a risk of obstruction in patients with tight stenoses. Therefore, it should

not be used if a stricture is present. DBE may show circumferential diaphragm-like strictures with ulcers or erosions. DBE has been used to successfully diagnose diaphragm disease in patients with GI bleeding and ileus in many literature reports<sup>[21-23]</sup>. DBE can also be used for treatment of the diaphragm disease<sup>[22,23]</sup>. As there are no specific pathological changes, endoscopic biopsy could not help much in the diagnosis. Three patients in our study group were initially suspected of having small bowel Crohn’s disease. CTE can show thickening of bowel wall, dilated small intestinal loops and circular or diaphragm-like strictures. CTE can also show the adjacent mesentery, mesenteric vessels and lymph nodes, which could help to exclude other potential causes of intestinal strictures<sup>[24-27]</sup>. Adequate distension of the entire small intestine is crucial to display the lesions. It must be mentioned here that it is necessary to combine CTE with other inspections such as SBFT or DBE to make a correct diagnosis. This is due to the fact that the images of CT are static and may misinterpret the lesion as plicae circulares especially when the bowel lumen is not distended completely. SBFT can show diaphragm-like strictures with dilated small bowel loop in the adjacent segment. The diaphragm lesions are thin and do not distort the bowel wall<sup>[28]</sup>. The greatest advantage of SBFT is its dynamic view of the small intestine motility. The diaphragms which are not shown distinctly might be misinterpreted as exaggerated plicae circulares<sup>[3]</sup>. Mesenteric angiography may have no positive findings, which implies that the symptom of recurrent gastrointestinal bleeding is not caused by mucosal vascular abnormalities or vasculitis, *etc.* Bowel isotope scan may be not informative and may only reveal the intestinal bleeding. Though there are many imaging examinations to help make a diagnosis, in some cases surgical intervention might be necessary to make the definitive diagnosis.

Our study had several limitations. For example, the sample size is small due to the rarity of the disease. It is meaningless to conduct statistical analysis for this small number of cases. In addition, the cases were a select surgical series which had radiological and/or endoscopic presurgical work-up. Obviously, non-surgical cases and those patients who did not have imaging or endoscopic work-up would have been excluded.

Although the number of subjects was small, our results indicate that special diaphragm-like strictures

characterized by ulcers and circular stenosis can also occur in the small intestine in patients without the use of NSAIDs and might be a special consequence of unclear damaging insults to the intestine. They have similar clinical, endoscopic, radiologic and pathologic features. The diaphragm-like strictures tend to be intractable because this is a non-self-limiting condition with little tendency toward mucosal healing. Currently, enterectomy of the diseased bowel segment is the only useful therapy. Clinicians should be aware that small-bowel diaphragm-like strictures might be a cause of chronic small bowel bleeding in patients receiving no NSAID therapy.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Diaphragm disease induced by non-steroidal anti-inflammatory drugs (NSAIDs), as inflammatory strictures of the small intestine, has previously been recognized as an uncommon complication in patients taking NSAIDs. The most frequent manifestations are iron-deficiency anemia, acute small-bowel hemorrhage and perforation, and obstruction of the small bowel. However, diaphragm-like strictures in the small bowel can also not be associated with the use of NSAIDs. Here, the authors illustrate a group of five patients presenting with bleeding of small bowel and in whom diaphragm-like strictures in small bowel were present that were not attributed to NSAID use.

### Research frontiers

Bleeding within the small bowel is often difficult to diagnose. Currently, the first diagnostic procedure in patients with small bowel hemorrhage is capsule endoscopy (CE) or double-balloon enteroscopy (DBE). CE has been mainly used to evaluate patients with obscure gastrointestinal (GI) bleeding, but carries a risk of obstruction in patients with tight stenoses such as diaphragm-like strictures. DBE can show circumferential diaphragm-like strictures with ulcers or erosions and can also be used for treatment of the diaphragm disease. Contrast-enhanced computed tomography (CT) scanning is establishing itself as a rapid, noninvasive, and accurate diagnostic method in gastrointestinal bleeding. Arterial phase CT scanning is an excellent diagnostic tool for fast and accurate detection and localization of GI hemorrhage. The combination of a variety of inspection methods can help to confirm the diagnosis.

### Innovations and breakthroughs

The unique aspect of this study is that it is the largest series of intestinal diaphragm-like strictures which are not associated with NSAID use. To the best of our knowledge, there are still no descriptions about the imaging features of this disease in the English literature. The results indicate that special diaphragm-like strictures characterized by ulcers and circular stenosis can also occur in the small intestine in patients without the use of NSAIDs and might be a special consequence of unclear damaging insults to the intestine. They have similar clinical, endoscopic, radiologic and pathologic features and tend to be intractable for this is a non-self-limiting condition with little tendency toward mucosal healing. Findings at endoscopic and radiologic imaging of this disease may help to make a diagnosis and facilitate preoperative evaluation of the lesions.

### Applications

Small-bowel diaphragm-like strictures characterized by ulcers and circular stenosis might be a cause of chronic small bowel bleeding in patients without NSAID therapy and might be a special consequence of unclear damaging insults to the intestine. They have similar clinical, endoscopic, radiologic and pathologic features. The diaphragm-like strictures tend to be intractable because it is a non-self-limiting condition with little tendency toward mucosal healing. Currently, enterectomy of the diseased bowel segment is the only useful therapy.

## Terminology

Diaphragm disease is inflammatory strictures of the small intestine and is an uncommon complication of non-specific small-bowel disease, caused by mucosal and submucosal fibrosis and thickening in patients taking NSAIDs. Computed tomography enteroclysis (CTE) is to perform contrast-enhanced CT scanning and image post-processing after small intestine distension by administering a high volume of contrast medium into the small intestine orally or via a nasojejunal catheter. CTE can display the cavity and wall of small intestine, parenteral lymph nodes, mesentery, mesenteric vessels and the adjacent strictures, etc.

## Peer review

In this study, the authors summarized the characteristics of diaphragm-like strictures of the small bowel without use of NSAIDs. Their report contained 5 cases and described the clinical, endoscopic, radiographic and pathologic features. Although there are many papers that have described diaphragm disease of the small bowel associated with NSAIDs, diaphragm disease unrelated to NSAIDs rarely exists in clinical settings. It is necessary to accumulate many more clinical cases to reveal the clinical significance of this disease phenotype. Although this report is preliminary as it stands, it might see the light in this field.

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## Ginsenoside Rg3 inhibit hepatocellular carcinoma growth *via* intrinsic apoptotic pathway

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was detected by 5, 5', 6' 6' - tetrachloro-1, 1', 3, 3' - tetraethylbenzimidazolylcarbocyanine iodide. Forty liver tumor-bearing C57Bl6 mice were divided randomly into 4 groups for intra-tumor injection of saline, ginsenoside Rg3, cyclophosphamide (CTX) and ginsenoside Rg3 + CTX combination.

**RESULTS:** The survival time was followed up to 102 d. The mice in the Rg3 + CTX group showed significant increased survival time compared with those in the control group ( $P < 0.05$ ). Rg3 could inhibit HCC cell proliferation and induce cell apoptosis *in vitro* in the concentration and time dependent manner. It also induced mitochondria membrane potential to decrease. Caspase-3 activation can be blocked by the inhibitor z-DEVD-FMK. Bax was up-regulated while Bcl-2 and Bcl-XL were down-regulated after Rg3 treatment.

**CONCLUSION:** Our data suggested that Rg3 alone or combined with CTX inhibited tumor growth *in vivo* and prolonged mouse survival time by inducing HCC cell apoptosis *via* intrinsic pathway by expression alterations of Bcl-2 family proteins.

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### Abstract

**AIM:** To investigate the anti-tumor function of ginsenoside Rg3 on hepatocellular carcinoma (HCC) *in vitro* and *in vivo*, and its mechanism.

**METHODS:** Hep1-6 and HepG2 cells were treated by Rg3 in different concentrations (0, 50, 100 and 200  $\mu\text{g/mL}$ ) *in vitro*. After incubation for 0, 6, 12, 24 and 48 h, cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Apoptosis was identified by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling. Caspase-3 activity was measured by chromophore p-nitroanilide and flow cytometry. Bcl-2 family proteins were ascertained by Western-blotting. Mitochondria membrane potential

**Key words:** Ginsenoside Rg3; Apoptosis; Hepatocellular Carcinoma; Bcl-2 family proteins; Cyclophosphamide

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common fatal human malignancy worldwide<sup>[1]</sup>. HCC is highly resistant to chemotherapeutic drugs and there is no single effective chemical against it. Two or three agents are often combined to enhance the efficacy of chemical agents. Chemotherapy causes serious toxic effects<sup>[2]</sup>. Thus, there is an urgent need to develop novel treatment modalities.

*Ginseng* is a traditional herbal medicine well known for its wide spectrum of pharmacological effects<sup>[3]</sup>. Recently researchers have found ginsenoside Rg3 can inhibit growth of several cancer cell lines<sup>[4-9]</sup>; however, the mechanism is not fully understood so far. In this study, two liver cancer cell lines, Hep1-6 and HepG2 cells were treated with ginsenoside Rg3 *in vitro* to explore the possible molecular mechanism. Ginsenoside Rg3 was also injected into tumor-bearing mice to investigate the anti-tumor effect in a long-term way.

## MATERIALS AND METHODS

### Ginsenoside Rg3

Ginsenoside Rg3 was purchased from Fusheng Pharmaceutical Ltd. Rg3 was dissolved in dimethyl sulfoxide (DMSO) and filtered by 0.2  $\mu\text{m}$  membrane. It was diluted by cell culture media to various final concentrations (0, 50, 100, 200  $\mu\text{g}/\text{mL}$ ).

### Cell lines and cell culture

Hep1-6 and HepG2 cells were purchased from the Institute of Biochemistry and Cell Biology, Academy of Science (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium and Eagle's Minimum Essential Medium (ATCC, Manassas, VA, United States) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 4 mmol/L 1-Glutamine (Cellgro) and 2% penicillin-streptomycin solution (Cellgro). The cells were incubated at 37 °C in a mixture of 5% CO<sub>2</sub> and 95% air.

### HCC animal model

Forty female C57BL/6 mice (4 wk, 16g  $\pm$  3 g, purchased from Shanghai Experimental Animal Center of the Chinese Academy, Shanghai, China) were divided randomly into 4 groups of 10 mice in each group: control (saline), ginsenoside Rg3, cyclophosphamide (CTX) and Rg3 + CTX combination. After being transplanted with  $1 \times 10^6$  Hep1-6 cells in 50  $\mu\text{L}$  PBS on the flank, the mice were given an intra-tumor injection of ginsenoside Rg3 (3.0 mg/kg) and CTX (20.0 mg/kg) or Rg3 + CTX for 10 d following inoculation of Hep1-6 cells. The negative control was saline injection (1.5 mg/kg). Mice were euthanized according to IACUC proposals when the tumor was larger than 20 mm in diameter. The survival days were recorded. Mouse weight and tumor weight were measured.

After treatment, the survival study began. The animal technician, who was blind to the study, monitored the mouse weight and tumor size every day. When the diam-

eter of tumor was larger than 2 cm on the tumor-bearing mouse, or the mouse weight loss was more than 20% on the tumor free mouse, the mouse was euthanized by cervical dislocation according to the animal experiment protocol and the date was determined as endpoint of survival dates.

### Cell viability analysis

The viability of Hep1-6 and HepG2 cells treated with and without Rg3 was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells in logarithmic growth phase were seeded in 96-well plates. Rg3 was added to the medium to different final concentrations: 0, 50, 100 and 200  $\mu\text{g}/\text{mL}$ . After 0, 6, 12, 24 and 48 h incubation, 20  $\mu\text{L}$  medium containing 5 mg/mL MTT was added to each well. After another 3 h incubation, DMSO (100  $\mu\text{L}$ ) was added to dissolve the formazan crystals. Light absorbance at 540 nm was measured. To determine the percentage of surviving cells, absorbance values of indicated concentrations were normalized to the values obtained from the cells without Rg3 treatment. Each assay was performed in 3 replicates.

### Apoptosis detection

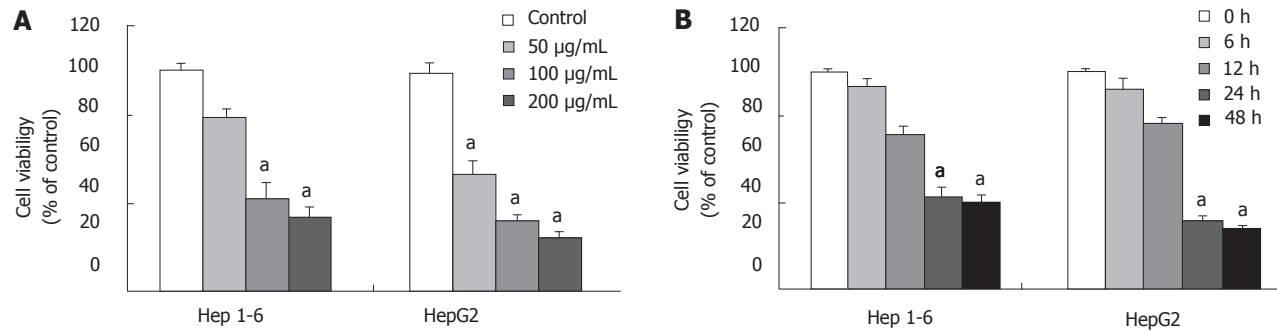
The HCC cells were incubated on the 8-well chamber slides (Nalge Nunc Corp, IL, United States) in medium with 0, 50, 100, 200  $\mu\text{g}/\text{mL}$  Rg3. After 0, 6, 12, 24 and 48 h cell chambers were removed and the slides were fixed for hematoxylin and eosin (HE) stain and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) fluorescent detection kit (Chemicon, United States). All the nuclei were stained blue by 2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI) while the apoptotic cells were stained as red fluorescent by apoptotic probe. The apoptotic cells were counted and statistically analyzed by the software Image J.

### Caspase 3 activity assay

Caspase 3 activity was tested by colorimetric assay kit (Genscript, NJ, United States, Cat. No. L00289). The HCC cells were treated by Rg3 in different concentrations (0, 50, 100, 200  $\mu\text{g}/\text{mL}$ ) for 24 h. Then the cells were lysed for detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The result was quantified as the  $A$  value at 405 nm. The relative increase of caspase-3 activity was determined by comparing the absorbance of pNA from Rg3 treated HCC cells to non-treated control.

### Z-DEVD-FMK inhibitory assay

Cells were pretreated for 1 h with 20 mmol/L z-DEVD-FMK (R&D, Catalog Number: FMK004) prior to Rg3 treatment. The cells were then treated with Rg3 in different concentrations (0, 50, 100, 200  $\mu\text{g}/\text{mL}$ ) for 24 h. The cells were lysed for caspase activity measurement. Then z-DEVD-FMK was added to measure the caspase activity. The inhibitory rate was calculated by comparing caspase activity with/without z-DEVD-FMK.



**Figure 1 Ginsenoside Rg3 inhibits cell viability of human and murine liver cancer cells.** A: Concentration-dependent inhibitory effects of Ginsenoside Rg3 on cell viability in Hep1-6 and HepG2 cell lines. Cells were treated with Rg3 at 0, 50, 100, 200 µg/mL in 10% fetal bovine serum-supplemented medium for 24 h; cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay ( $n = 6$  for Hep1-6,  $n = 6$  for HepG2). B: Time-dependent inhibitory effects of Rg3 on cell viability in Hep1-6 and HepG2 cell lines. Cells were treated with Rg3 100 µg/mL for 0, 6, 12, 24, 48 h. One-way ANOVA was performed to test the concentration and time-dependent effects. <sup>a</sup> $P < 0.05$  vs untreated controls.

### Western blot analysis

After being treated with 0, 50, 100, 200 µg/mL Rg3 for 24 h, Hep1-6 and HepG2 cells were washed with ice-cold PBS twice and lysed on ice. Mitochondrial fraction and cytosolic fraction were extracted by Cytosol/Mitochondria Fractionation Kit (Calbiochem, United States). Extracted proteins were separated by 12% SDS-PAGE and transferred onto PVDF membrane. The membrane was incubated with primary antibodies: procaspase 8, cytochrome c, Bcl-2, Bax, Bad, Bcl-XL (Santa Cruz Biotechnology Inc. dilution: 1:200) and beta-actin (Cell Signaling Technology, dilution: 1:500) in blocking buffer for 1 h at room temperature followed by incubation with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc. dilution: 1:500). The protein expression was detected by X-ray film.

### Measurement of transitions in mitochondrial transmembrane potential

Hep1-6 and Hep G2 cells were grown in 4-well cover glass chambers (Nalge Nunc) and treated with Rg3 100 µg/mL containing DMEM supplemented with 5% FBS. After incubation for 24 h, cells were stained with 5 µg/mL of 5, 5', 6, 6' - tetrachloro-1, 1', 3, 3' - tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a widely used dye for measuring membrane potential of mitochondria. Cells were irradiated at an excitation wavelength of 488 nm, and the irradiated field was photographed using a confocal microscope equipped with an emission filter of 533 nm (100 magnification, Leica). Depolarized mitochondrial membranes were detected by the presence of a diffuse green fluorescence in cells.

### Flow cytometry

After treatment with Rg3 100 µg/mL or saline for 24 h, 105 Hep1-6 and Hep G2 cells were suspended in 50 µL HBSS containing propidium iodide (PI) and fluorescein isothiocyanate (FITC) caspase-3 (Bioss LTD, Beijing, China) to identify apoptosis and necrosis, respectively. Fluorescent dyes were diluted to 1 µg/mL in HBSS containing 1% FBS. Incubations were carried out for 30 min on ice.

After staining, the cells were washed twice in HBSS/1% FBS and then analyzed by a flow cytometry (LSR II, BD).

### Tumor histopathology

When the tumor was as large as 20 mm in diameter, the animal was euthanized and the tumor was dissected and fixed in 40 g/L neutral formaldehyde. After 24 h it was embedded in paraffin, cut into 3 µm sections, stained with HE, and examined under light microscopy.

### Statistical analysis

The present data are expressed as mean  $\pm$  SD. For statistical comparison of values, a Student's *t* test was used. *P* values less than 0.05 were deemed to indicate statistical significance. The Kaplan-Meier method is used to analyze the cumulative survival and draw the survival curve by software SPSS 11.0. Log rank statistic and significance were also presented by SPSS.

## RESULTS

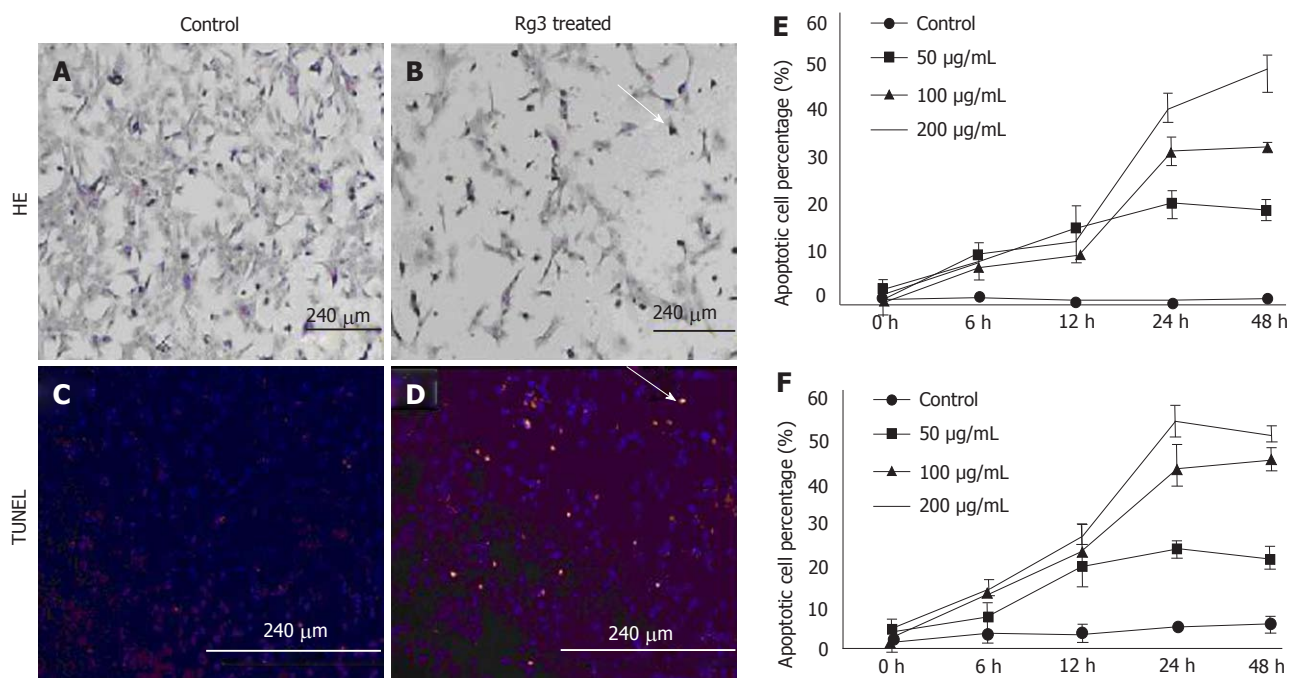
### Rg3 represses liver cancer cell viability in a dose- and time-dependent way

MTT assay was done to examine if Rg3 could affect liver cancer cell growth in culture. Hep1-6 and HepG2 cells were treated with increasing concentrations of Rg3 (0, 50, 100, 200 µg/mL) for 24 h. The viable Hep1-6 and HepG2 cells consistently decreased with the higher concentrations of Rg3 as shown in Figure 1A. When Hep1-6 cells were treated by 100 and 200 µg/mL Rg3, the cell viability was significantly decreased ( $P < 0.005$  vs control). HepG2 cells had significantly decreased viability when they were treated by 50, 100 and 200 µg/mL Rg3. When both cell lines were treated by 100 µg/mL Rg3 for 0, 6, 12, 24, 48 h, the cell viability declined significantly over 24 h ( $P < 0.005$  vs control, Figure 1B).

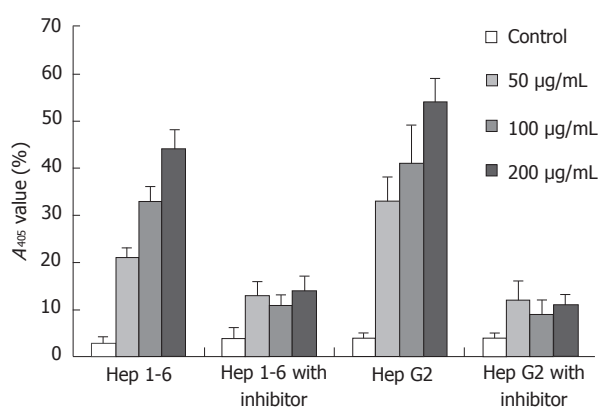
### Rg3 induced liver cancer cells apoptosis in vitro

To determine if Rg3 causes apoptosis in Hep1-6 and HepG2 cells, DNA degradation and cleavage were detected in Rg3-treated liver cancer cells. When the HCC cells





**Figure 2** Ginsenoside Rg3 caused hepatocellular carcinoma morphological changes of apoptotic cells. A, B: Cell apoptosis morphology was observed by hematoxylin and eosin (HE) stain. After 50 µg/mL Ginsenoside Rg3 (Rg3) incubation for 12 h, the Hep1-6 cells (B) indicate less survival cells compared to control group (A); C, D: DNA fragmentation *in situ* was detected by transferase-mediated dUTP-biotin nick end labeling. The control cells was stained blue (C) and the Rg3 treated group present apoptotic cells stained red (D); E, F: The apoptotic cells showed reduced volume and condensed chromatin. In both Hep1-6 and HepG2, the apoptotic induction effect is dose and time-dependent. Rg3: Ginsenoside Rg3; HE: Hematoxylin and eosin; TUNEL: Transferase-mediated dUTP-biotin nick end labeling.



**Figure 3** The caspase activity was measured by the chromophore p-nitroanilide. Hep1-6 and HepG2 cells were pretreated with or without 20 mm z-DEVD-FMK for 1 h, and then cultured with 0, 50, 100, 200 µg/mL ginsenoside Rg3 for 24 h. The caspase activity was measured by the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA by a plate reader  $A_{405}$  nm.

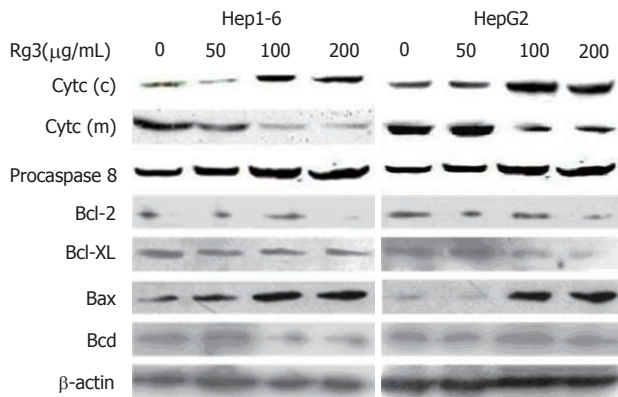
were treated with 100 µg/mL Rg3 for 24 h, both Hep1-6 and HepG2 cells displayed typical apoptotic morphology, including reduced volume and condensed chromatin (Figure 2A) compared to the control cells without Rg3 treatment (Figure 2B). To further specify apoptotic cell death, we stained nuclei with DAPI, a DNA-specific fluorescent dye. We also detected *in situ* DNA fragmentation in Hep1-6 and HepG2 cells by using TUNEL method. All the Hep1-6 cell nuclei were stained blue by DAPI while the apoptotic cells were stained red by apoptotic probe (Figure 2D). Hep1-6 cells treated by Rg3 showed a higher

percentage of apoptotic cells (Figure 2D) compared to the control group (Figure 2C). The apoptotic Hep1-6 and HepG2 cells were counted and statistically analyzed by the software Image J. Rg3-induced apoptosis occurred in Hep1-6 and HepG2 cells when treated by 50-200 µg/mL Rg3 for 12-24 h (Figures 2E and F).

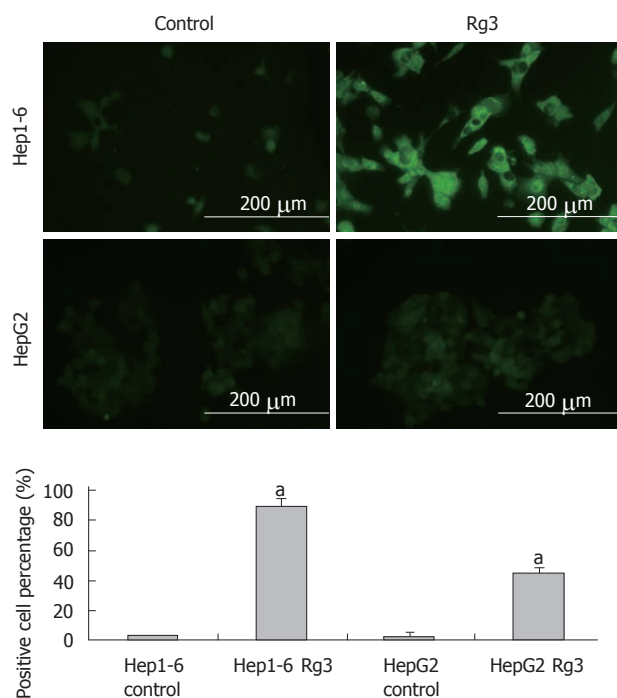
#### Induction of apoptosis by Rg3 depends on mitochondria-mediated caspase cascade

To determine whether Rg3-induced apoptosis in Hep1-6 and HepG2 cells was mediated *via* caspase cascade, caspase-3 activity was measured by pNA. The relative increase of caspase-3 activity was determined by comparing the absorbance of pNA from Rg3 treated HCC cells to the non-treated control. In both Hep1-6 and HepG2, caspase-3 was activated by Rg3 treatment in a dose dependent manner (the means and SDs in Hep1-6 cells were 5% ± 1%, 22% ± 4%, 32% ± 3%, 43% ± 5% when Rg3 concentration were 0, 50, 100, 200 µg/mL, respectively; the HepG2 cells were 4% ± 0.5%, 32% ± 6%, 41% ± 7%, 54% ± 4%) (Figure 3). However, when both cell lines were pretreated with 20 mmol/L z-DEVD-FMK for 1 h, the caspase-3 activity was decreased significantly (4% ± 1%, 13% ± 3%, 11% ± 1%, 14% ± 4% when Rg3 concentration were 0, 50, 100, 200 µg/mL, respectively; the HepG2 cells were 4% ± 0.5%, 11% ± 1%, 11% ± 3%, 10.5% ± 1%), suggesting Rg3 induce apoptosis *via* caspase-3 dependent apoptotic pathway in Hep1-6 and HepG2 cells. The caspase-3 activity is still elevated even in the presence of z-DEVD-FMK (Figure 3).

Because the activation of caspase-3 could be preceded



**Figure 4** Effects of ginsenoside Rg3 on the cytochrome c release, caspase 8 cleavage and Bcl2-family. Hep1-6 and HepG2 cells were treated with 0, 50, 100, 200  $\mu\text{g/mL}$  ginsenoside Rg3 (Rg3) for 24 h, and western-blot was used to detect the pro-caspase-8, cytochrome c in cytosolic fractions (c) and mitochondria fractions (m), Bcl-2, Bcl-XL, Bax, and Bcl.  $\beta$ -actin is the protein loading control. Pro-caspase-8 remains static with or without Rg3 treatment. Cytochrome c decreased in the mitochondrial fraction and increased in the cytosolic fraction. Bcl-2 and Bcl-XL were down-regulated while Bax was up-regulated. Bcl remains unchanged in the cells with and without Rg3 treatment. Rg3: Ginsenoside Rg3.



**Figure 5** Hep1-6 and HepG2 cells were treated with Rg3 100  $\mu\text{g/mL}$  or saline for 24 h then cells were stained with 5, 5', 6, 6' - tetrachloro-1, 1', 3, 3' - tetraethylbenzimidazolylcarbocyanine iodide dye. Depolarized mitochondrial membranes were detected by the presence of a diffuse green fluorescence. Ginsenoside Rg3 treated groups had a significantly higher percentage of green fluorescent cells: Hep1-6 (87%  $\pm$  6% vs control 2%  $\pm$  1%) and HepG2 (46%  $\pm$  4% vs control 3%  $\pm$  2%). Rg3: Ginsenoside Rg3.  $^aP < 0.05$  vs control group.

by either caspase-8 *via* the death receptor pathway or caspase-9 *via* the mitochondria pathway, we tested pro-caspase-8 and cytochrome c to determine which pathway is dominant in Rg3-induced apoptosis. As illustrated in Figure 4, cleavage of caspase-8 was not evident, but cytochrome c decreased in the mitochondrial fraction and

increased in the cytosolic fraction, which suggested that Rg3 mainly induced cytochrome c release.

### Rg3 altered apoptotic related gene expression

To further investigate the molecular mechanism of mitochondria pathway activation, Bcl-2 family protein expression in Hep1-6 and HepG2 cells was detected by western-blot after they were treated by Rg3 in different concentration (0, 50, 100, 200  $\mu\text{g/mL}$ ) for 24 h. As shown in Figure 4, Bcl-2 and Bcl-XL, the anti-apoptotic members of Bcl-2 family, were reduced by Rg3 treatment. Bax, the pro-apoptotic member, was increased. Bcl was unchanged in the cells with and without Rg3 treatment.

In order to determine whether the increase in mitochondrial Bax was associated with altered mitochondrial transmembrane potential, we measured changes in JC-1. JC-1 is a lipophilic cationic dye that can selectively enter into mitochondria and reversibly change color as illustrated in Figure 5; depolarized mitochondrial membranes were detected by the presence of a diffuse green fluorescence in cells. The green fluorescence shift indicates loss of mitochondrial function, which suggested that Rg3 activated the mitochondrial pathway by decreasing mitochondrial trans-membrane potential.

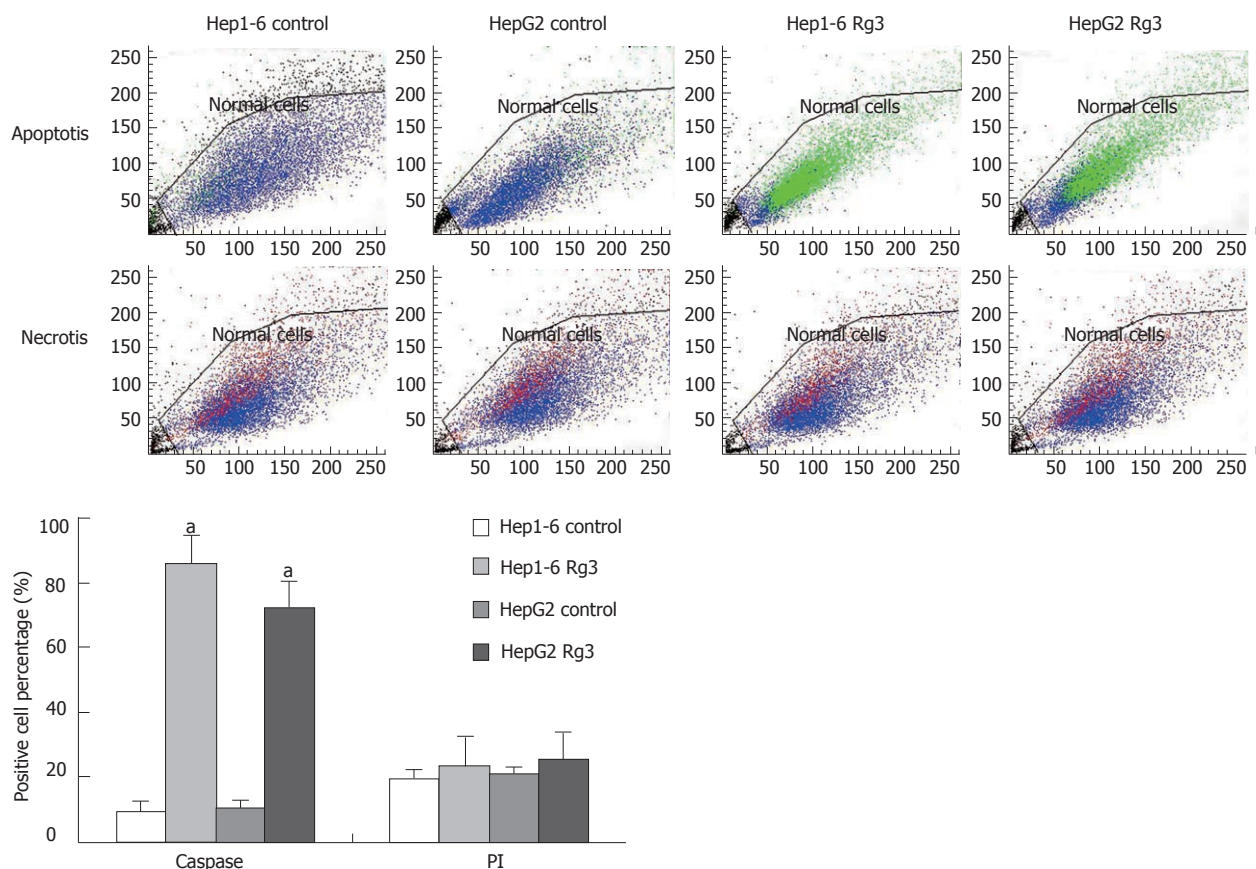
To further characterize the observed apoptotic phenotype, we carried out double staining of caspase-3-FITC and PI in two cell lines treated with saline and Rg3. Caspase-3-FITC can be detected in apoptosis. PI enters the cell in late apoptosis or necrosis. Viable cells were negative for both caspase-3-FITC and PI; early apoptotic cells were positive for caspase-3-FITC and negative for PI; late apoptotic or necrotic cells displayed both positive caspase-3-FITC and PI; non-viable cells which underwent necrosis were positive for PI and negative for caspase-3-FITC (Figure 6). After Rg3 treatment for 24 h, the percentage of early apoptotic cells induced by Rg3 in Hep1-6 and HepG2 were 85%  $\pm$  9%, 71%  $\pm$  8%, respectively. Their controls were 9%  $\pm$  3%, 11%  $\pm$  2%, respectively (Figure 6).

### Rg3 improved HCC tumor bearing animals' survival time

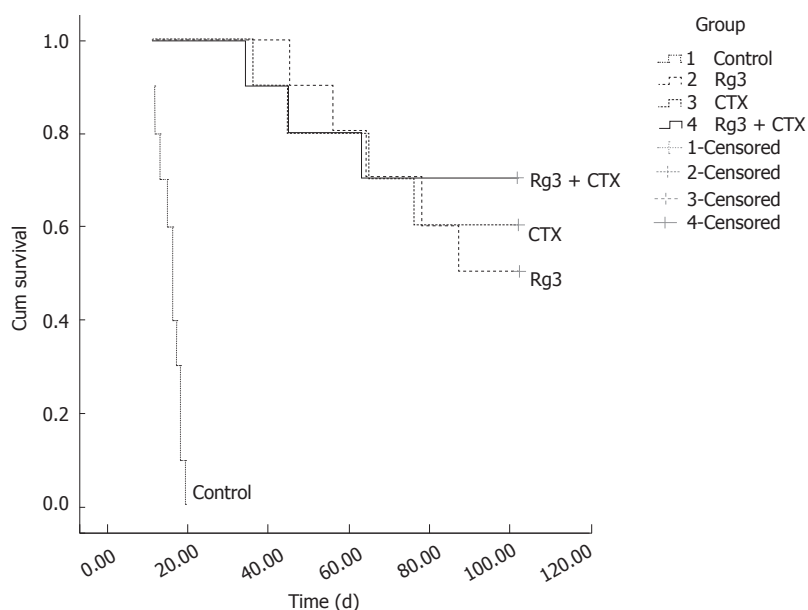
The survival study was carried out up to one hundred and two days after the last intra-tumor injection. The number of living mice in the ginsenoside Rg3 group, CTX group, combined treatment and saline control group are summarized in Figure 7. Mice in the control group were euthanized within 20 d when tumors were larger than 20 mm in diameter. The tumor of the mice in the Rg3 treated group reached 20 mm in diameter within 102 d. There were no significant abnormalities in mental state, activities, or response to stimulus. The survival time of mice in the ginsenoside Rg3 group, CTX group and combined treatment group was significantly longer than that in the control group ( $P < 0.001$ ), which demonstrated that ginsenoside Rg3 inhibited the tumor growth and prolonged survival time of tumor-bearing mice.

### Tumor growth and pathology

Tumors reached 20 mm in diameter on day 14 ( $\pm$  6.3), day 87 ( $\pm$  9), day 93 ( $\pm$  11) and day 95 ( $\pm$  7) in the control

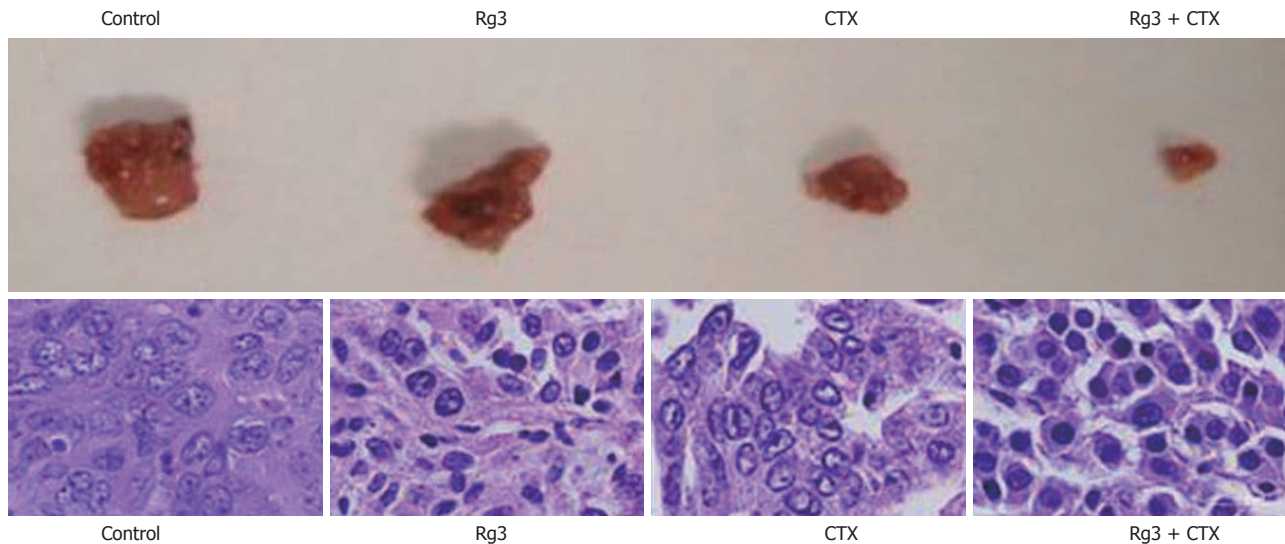


**Figure 6** Flow cytometry after caspase-3-fluorescein isothiocyanate/propidium iodide staining. After being treated by ginsenoside Rg3 (Rg3) 100  $\mu$ g/mL or saline for 24 h, Hep1-6 and HepG2 cells were stained by caspase-3-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Viable cells are shown as blue and early apoptotic cells are green (caspase-3-FITC). The non-viable necrotic cells are red (PI). Rg3 treated groups had a significantly higher percentage of caspase-3 positive cells: Hep1-6 (85%  $\pm$  9% vs 9%  $\pm$  3%) and HepG2 (71%  $\pm$  8% vs 11%  $\pm$  2%). There are no statistical difference in PI staining between Rg3 treated group and control group: Hep1-6 (23%  $\pm$  3% vs 19%  $\pm$  2%) and HepG2 (25%  $\pm$  4% vs 21%  $\pm$  3%). Rg3: Ginsenoside Rg3. <sup>a</sup> $P$  < 0.05 vs control group.



**Figure 7** Survival time and survival rate of mice bearing hep1-6 tumor. The forty mice were divided into 4 groups with ten in each group, and inoculated with  $1 \times 10^6$  Hep1-6 cells in each mouse. The mice were given an intratumoral injection of ginsenoside Rg3 (Rg3) (3.0 mg/kg) and cyclophosphamide (CTX) (20.0 mg/kg) or saline (1.5 mg/kg) for 10 d following inoculation of Hep1-6 cells. The animal survival study was followed up to 102 d, to observe and compare their survival time and survival rate. The Rg3 and CTX combination group, Rg3 group, and CTX group were statistically significant compared with the saline injected control group. Rg3: Ginsenoside Rg3; CTX: cyclophosphamide.





**Figure 8 Tumor grosses dissection and histology.** Tumors were dissected immediately after the mice were euthanized. Tumor blood vessels and their wall were abundant in the control group. The Ginsenoside Rg3 (Rg3) and cyclophosphamide (CTX) group showed smaller tumor size. The CTX group had the necrosis in the center. Rg3 + CTX had the smallest volume without necrosis. Samples were stained with hematoxylin and eosin in succession. In the control group, moderately differentiated hepatocellular carcinoma cells were surrounded by thick fibrous capsule. The nuclei were large. In the Rg3 treated group, tumor cells were irregular and condensed. In the CTX treated group, the tumor cells lost connective tissue or blood supply. In Rg3 + CTX group, there was obvious chromatin condensation in the hepatocyte. Rg3: Ginsenoside Rg3; CTX: Cyclophosphamide.

**Table 1 Comparison of body weight, tumor weight, and the ratio of tumor weight/body weight in the different groups**

Groups	Control	Rg3 alone	CTX	Rg3 + CTX
Body weight (g)	18.3 ± 1.8	19.1 ± 2.3	18.1 ± 1.6	21.9 ± 1.4
Tumor weight (g)	1.7 ± 0.5	0.9 ± 0.2 <sup>a</sup>	0.5 ± 0.3 <sup>a</sup>	0.3 ± 0.1 <sup>a</sup>
Tumor weight/ Body weight (%)	0.09 ± 0.004	0.05 ± 0.003	0.03 ± 0.004	0.01 ± 0.002 <sup>a</sup>

Values were pressed by Mean ± SD, <sup>a</sup>*P* < 0.05 in Student's *t* test *vs* the control group. Rg3: Ginsenoside Rg3; CTX: Cyclophosphamide.

group, Rg3 group, CTX group and Rg3 + CTX group, respectively. The Rg3, CTX and Rg3 + CTX treatment resulted in a delayed tumor growth compared with the control group (*P* < 0.01). Tumors observed in the control group, Rg3, CTX and Rg3 + CTX treated groups were dissected and sent for HE stain. Dissected tumors are shown in Figure 8. Tumor weights at the time of sacrifice are present in Table 1. The inhibitory effects of Rg3 + CTX on tumor growth were comparable and significant *vs* control (*P* < 0.05).

Ultra structure and nuclear change were revealed by HE. The tumors in the mice of the control group showed aggressive growth and a regular nest shape with a rich blood supply. Tumor cells featured clear and regular nuclei with prominent nucleoli. The cytoplasm was characteristically pink and clear. In Rg3 alone treated tumors, the nuclei dramatically shrink. CTX treated tumors lost the cord-like supporting structure on which tumor cells extend. In Rg3 + CTX treated tumors, individual cells elongated and condensed, nuclear to plasma ratio decreased with obvious chromatin condensation in the hepatocytes.

## DISCUSSION

*Ginseng*, the root of *panax ginseng*, has been widely used in Asian medicine for more than 2000 years. *Ginseng* contains many active components such as ginsenosides, polysaccharides, peptides, fatty acids and mineral oils<sup>[2]</sup>. Among these components, ginsenosides were found most responsible for the pharmacological and immunological activities such as tonic, immunomodulatory, anti-mutagenic, adaptogenic, anti-aging activities, function and immune improvement<sup>[3]</sup>. Recently Rg3 has been suggested to inhibit cancer cell growth, invasion and metastasis, e.g. lung carcinoma<sup>[4]</sup>, prostate cancer<sup>[5]</sup>, colorectal cancer<sup>[6]</sup>, ovarian cancer<sup>[7,8]</sup> and breast cancer<sup>[9]</sup>. Our present study in liver cancer cell lines demonstrated that ginsenoside Rg3 can also inhibit Hep1-6 and HepG2 growth. TUNEL and HE stain suggest Rg3 can induce apoptosis in a concentration and time dependent manner.

Understanding of the mechanism of Rg3-induced apoptosis will shed some light on the intracellular function of Rg3 in HCC cells. Caspases are a family of proteases regulating apoptosis<sup>[10,11]</sup> which includes upstream initiator caspases, such as caspase-8 and 10, and downstream executor caspases, such as caspase-3<sup>[12,13]</sup>. In our study, we examined the involvement of caspase-3 and found that Rg3 could activate caspase-3 in a concentration dependent way. Confirming caspase-3 is essential for Rg3-induced HCC cell apoptosis, HCC cells were pretreated by an irreversible pan-caspase inhibitor, z-DEVD-FMK, and then caspase-3 activation was blocked, suggesting Rg3-induced apoptosis is caspase-3 dependent.

There are two possible pathways that can lead to caspase-3 activation<sup>[14]</sup>, either through caspase-8 *via* the death receptor pathway or caspase-9 *via* the mitochondria path-

way<sup>[15,16]</sup>. Thus we tested pro-caspase-8 and cytochrome c to determine which pathway is dominant in Rg3-induced apoptosis. As illustrated in Figure 4, cleavage of caspase-8 was not evident, but cytochrome c decreased in the mitochondrial fraction and increased in the cytosolic fraction, which suggested that Rg3 induced cytochrome c release from the intermembrane space of mitochondria. Our results suggested that mitochondria probably acted as the main switch of Rg3-induced apoptosis in Hep1-6 and HepG2 cells.

The BCL-2 family regulates the apoptotic mitochondrial pathway<sup>[17,18]</sup> and can be divided into two types: anti-apoptotic proteins and pro-apoptotic proteins<sup>[19]</sup>. Many agents for cancer chemotherapy target the balance of pro- and anti-apoptotic proteins<sup>[20,21]</sup>. Bcl-2 and Bcl-XL are pro-survival proteins of the BCL-2 family, and BAX is an apoptotic protein. Our results showed that Rg3 down-regulated Bcl-2 and Bcl-XL, but up-regulated BAX. As an overall result, Rg3 altered the Bcl-2 family protein expression by shifting the balance towards cell death.

There are primarily two major events involved in apoptosis *via* the mitochondrial pathway. The first event is a change in mitochondrial membrane permeability, which leads to decreased mitochondrial membrane potential. Our data demonstrated Rg3 reduced mitochondrial membrane potential as indicated by JC-1 staining. The second event in the mitochondria-induced apoptotic pathway is the release of cytochrome c from the intermembrane space of the mitochondria into the cytosol<sup>[16]</sup>. As shown by western blot, Rg3 increased the release of cytochrome c in the cytosol.

In summary, we demonstrate that Rg3 induces HCC cell apoptosis *via* the mitochondrial pathway: (1) Rg3 induces HCC cell apoptosis by triggering Bax translocation to the mitochondria; (2) Rg3-treated HCC cells causes the release of cytochrome c into the cytosol from the mitochondria; and (3) over expression of Bcl-2 attenuated Rg3-induced apoptosis, while down-regulating Bcl-2 expression also enhances cell apoptosis.

Results on cell lines often represent a distorted and incomplete picture of the *in situ* physiopathology of cancer where the tumor microenvironment and neovascularization play a critical role in tumor growth and progression, thus we expanded our study to matched primary tumors using xenograft models. Hep1-6 cells were transplanted into mice. Animal survival time was prolonged by Rg3, CTX and Rg3 + CTX treatment. Tumor formation was delayed and its growth was significantly slowed down by Rg3, CTX and Rg3 + CTX treatment. Furthermore, Rg3 + CTX resulted in a significantly smaller ratio of tumor weight/ body weight. The combination of low-dose CTX and Rg3 suppresses growth of experimental tumors more effectively than CTX therapy or Rg3 alone. The possible reason for this is that the occurrence of side effects was also considerably lower. Therefore, the combination of ginsenoside Rg3 and CTX has a better effect on antitumor than ginsenoside Rg3 or CTX alone.

In conclusion, in this study, Rg3 treatment inhibited

Hep1-6 and HepG2 growth by inducing apoptosis *via* the intrinsic apoptotic pathway. Ginsenoside Rg3 alone suppressed the growth of Hep1-6 tumor and combination with CTX was more effective than conventional CTX alone. Therefore, ginsenoside Rg3 is able to block the caspase-dependent signaling cascade and is valuable for developing new pharmaceutical means that will decrease the side effect of chemotherapy and increase the survival rate.

## COMMENTS

### Background

Liver tumor is the fifth most fatal human malignancy worldwide. It is highly resistant to chemotherapeutic drugs. Two or three agents are often combined to enhance the efficacy of chemical therapy. Chemotherapy causes serious toxic effects. Thus, there is an urgent need to develop novel treatment modalities. *Ginseng* is a traditional herbal medicine and its anti-tumor effect was recently discovered. This study investigates the anti-tumor effect of ginsenoside Rg3 on liver tumors and also explores its molecular mechanism.

### Research frontiers

*Ginseng* is a popular herbal medicine in China and Korea. Thousands of years of clinical practice have proven its wide spectrum of pharmacological effects, but the herb extract was not quantitative and was hard to repeat. The mechanism was also unclear. This study selected *Ginseng* Rg3, a standard quantitative chemical by which the experiment could be repeated. This study focuses on apoptosis- the focus of tumor therapy, indicating the molecular mechanism of how Rg3 triggers the tumor cells to clear themselves from the normal cells.

### Innovations and breakthroughs

This study found the antitumor effect of *Ginseng* Rg3 alone is not as effective as the combination of Rg3 and cyclophosphamide (CTX). Together, they could inhibit liver tumor cell proliferation, induce cell apoptosis and prolong mouse survival time. Its molecular mechanism is by inducing hepatocellular carcinoma cell apoptosis *via* the intrinsic pathway by alternating Bcl-2 family proteins and activating Caspase-3.

### Applications

This study provides the experimental data for clinical application of Rg3 combined with CTX to treat liver tumor. The study screened the proper drug dosage and optimal functional time.

### Terminology

Rg3 is a chemical compound isolated from the traditional Chinese herb *ginseng*. Apoptosis is also called programmed cell death or cell suicide. It is different from another form of cell death called necrosis, in which uncontrolled cell death leads to lysis of cells. Apoptosis is a process in which cells play an active role in their own deaths.

### Peer review

The current paper falls within the scope of the journal, its research objectives are clearly stated, study design and methodology are clearly described and the conclusions are based on the results.

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## Bimodal visualization of colorectal uptake of nanoparticles in dimethylhydrazine-treated mice

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### Abstract

**AIM:** To investigate colorectal uptake of solid lipid nanoparticles (SLNs) in mice receiving different doses of 1,2-dimethylhydrazine (DMH) using magnetic resonance (MR) and laser-scanning confocal fluorescence microscope (LSCFM) imaging.

**METHODS:** Eight mice were sacrificed in a pilot study to establish the experimental protocol and to visualize colorectal uptake of SLNs in normal mice. Gadopentetate dimeglumine and fluorescein isothiocyanate (FITC)-loaded SLN (Gd-FITC-SLN) enemas were performed on mice receiving DMH for 10 wk (group 1,  $n = 9$ ) or 16 wk (group 2,  $n = 7$ ) and FITC-SLN enema was

performed on 4 DMH-treated mice (group 3). Pre- and post-enema MR examinations were made to visualize the air-inflated distal colorectum. Histological and LSCFM examinations were performed to verify colorectal malignancy and to track the distribution of SLNs.

**RESULTS:** Homogeneous enhancement and dense fluorescence (FITC) deposition in colorectal wall were observed in normal mice and 1 DMH-treated mouse (group 1) on fluid attenuated inversion recovery (FLAIR) and LSCFM images, respectively. Heterogeneous mural enhancement was found in 6 mice (4 in group 1; 2 in group 2). No visible mural enhancement was observed in the other mice. LSCFM imaging revealed linear fluorescence deposition along the colorectal mucosa in all groups. Nine intraluminal masses and one prolapsed mass were detected by MR imaging with different enhancement modes and pathologies. Interstitial FITC deposition was identified where obvious enhancement was observed in FLAIR images. Bladder imaging agent accumulations were observed in 11 of 16 DMH-treated mice of groups 1 and 2.

**CONCLUSION:** There are significant differences in colorectal uptake and distribution of SLNs between normal and DMH-treated mice, which may provide a new mechanism of contrast for MR colonography.

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**Key words:** Solid lipid nanoparticles; Colorectal cancer; Magnetic resonance colonography

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Wu T, Zheng WL, Zhang SZ, Sun JH, Yuan H. Bimodal visualization of colorectal uptake of nanoparticles in dimethylhydrazine-treated mice. *World J Gastroenterol* 2011; 17(31): 3614-3622 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i31/3614.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i31.3614>

## INTRODUCTION

Colorectal imaging examinations consist of double-contrast barium enema (DCBE), colonoscopy, computed tomography (CT) colonography and magnetic resonance (MR) colonography. The four methods have their advantages and disadvantages. Colonoscopy, however, is the dominant technique for colorectal examination due to its high diagnostic sensitivity and capability for immediate intervention and histological evaluation. Despite the merits and continuous technical innovation, colonoscopy, as an invasive test, is not well accepted in a screening setting and cannot be performed on certain conditions such as inflammatory or malignant bowel stenosis. MR colonography, introduced in the last decade, has demonstrated encouraging initial results in the detection of polyps greater than 1 cm in diameter. Unlike CT colonography, MR colonography is a “pure noninvasive” test because it is ion-free<sup>[1-5]</sup>.

High contrast between the bowel wall and lumen, implemented by either “dark-lumen” or “bright-lumen” technique, is essential for successful MR colonography. The contrast mechanisms depend on the combination of ultrafast MR sequences and an appropriate rectal enema recipe<sup>[3-7]</sup>. In most cases, a low uptake of bowel contrast agents means low toxicity. To the best of our knowledge, no radiological research aiming at visualizing the uptake of bowel contrast agents has been described.

Recently, however, intestinal uptake of particulate matter in the micro- and nanometer range has been a hot topic in pharmacological research. Studies on oral delivery of insulin, vaccine and a set of hydrophobic drugs using various nano-vehicles are under way<sup>[8-13]</sup>. Solid lipid nanoparticles (SLN) are colloidal carriers for controlled drug delivery introduced after the development of emulsions, liposomes, polymer-based microparticles and nanoparticles. SLN combines the advantages of polymeric nanoparticles and oil/water fat emulsions for drug delivery, such as good tolerability, high oral bioavailability and feasibility, for large-scale production<sup>[14]</sup>.

The aim of this study is to exemplify the feasibility of using colorectal uptake of SLNs as an extra source of contrast in colonography. To model non-familial colorectal carcinoma (CRC) in rodents, 1,2-dimethylhydrazine (DMH), a specific colon carcinogen, was administered to the mice to produce CRC and impair the bowel wall. Colorectal uptake of SLNs in mice receiving different doses of DMH was investigated using MR and laser-scanning confocal fluorescence microscope (LSCFM) imaging.

## MATERIALS AND METHODS

### Synthesis of SLNs

Fluorescein isothiocyanate-labeled octadecylamine (ODA-FITC) and gadopentetate dimeglumine (Gd-DTPA) loaded SLNs (Gd-FITC-SLN) were synthesized by “solvent diffusion method in a nano-reactor system,” as described previously<sup>[15]</sup>. Briefly, Gd-DTPA (25 mg) and Tween 80 (18 mg) were dissolved in water (1 mL) to

prepare the “aqueous phase”. The water in an oil mini-emulsion was obtained by mixing, stirring and ultrasonic treatment of the “aqueous phase” and the “oil phase,” which consisted of Span 80 (200 mg) and n-Hexane (10 mL). A mixture of 45 mg monostearin and 5 mg ODA-FITC, dissolved in 1 mL ethanol in a 60 °C water bath, was quickly dispersed into the mini-emulsion under mechanical agitating at 400 for 5 min. The dispersion was centrifuged for 15 min at 20 000 r/min to precipitate SLNs, which were subsequently washed twice with n-hexane and re-dispersed in Poloxamer 188. The resultant SLNs, dispersed to equal milligrams of manicol, were freeze-dried and kept away from light at 4 °C. Both Gd-DTPA and ODA-FITC were omitted to produce blank SLN; only one of the imaging agents, Gd-DTPA or ODA-FITC, was added to synthesize Gd-SLN or FITC-SLN. The physicochemical properties of the SLNs were characterized as documented previously<sup>[11,15]</sup>.

### Pilot study

All animal experiments were approved by the institutional animal care and use committee and performed in accordance with the committee’s regulations. The mice were deprived of food and allowed to drink 5% glucose saline 24 h before the examination to clean the gastrointestinal tract. An intra-peritoneal injection of pentobarbital (50 mg/kg body weight) was performed before any surgical manipulation. Eight male Kunming mice (22-25 g) were sacrificed in the pilot study. An operative procedure was established to limit enema within the distal colorectum.

MR pulse sequence (SE T2WI and FLAIR) and microscopic fluorescence imaging techniques were evaluated. The concentration of enema agents, including Gd-DTPA solution, Gd-SLN, FITC-SLN and Gd-FITC-SLN suspensions, were adjusted according to MR and fluorescence image findings. Qualified data from the pilot study were included into the study results.

### Animal model and groups

Subcutaneous injection of DMH (20 mg/kg body weight) was performed wkly on 5-wk-old Kunming mice for 10 ( $n = 15$ ) and 16 wk ( $n = 15$ ) to induce colorectal tumors. Ten mice were excluded from the study due to DMH- and anesthesia-related mortality ( $n = 7$ ) and operation failures ( $n = 3$ ).

Gd-FITC-SLN (40 mg/mL) enema was performed on 9 mice receiving DMH for 10 wk (group 1) and 7 mice receiving DMH for 16 wk (group 2). FITC-SLN (40 mg/mL) enemas were performed on 4 mice (group 3) receiving DMH for 10 ( $n = 2$ ) and 16 wk ( $n = 2$ ).

### Operative procedure

After anesthesia, an abdominal incision was made into the peritoneal cavity, and the sigmoid colon was ligated. The peritoneal cavity was then closed by two layers of continuous sutures. Subsequently, the distal colorectum was slightly inflated by infusing about 0.3 mL room air *via* the anal orifice and gently ligating tissues around to prevent air leakage. Thus, the mouse was ready for the

pre-enema MR examination. After the pre-enema MR test, an SLN enema was performed for 20 min by infusing 0.3-0.4 mL of the dispersion into the rectal lumen and ligating tissues around the anus. In-enema MR imaging was performed during the enema process. After the enema was performed and the anal ligate was removed, the enema agents were cleared by warm saline coloclisis. The distal colorectal lumen was then inflated by air again for the post-enema MR examinations, performed 25 and 60 min after the SLN enema was started. The mice were warmed by placing a hot water bag aside during the experiment.

### MR imaging and analysis

Image acquisition was performed with a 1.5 T clinical MR device (Signa 1.5 T; GE Medical Systems, Milwaukee, Wis). A 5-cm custom-built coil was used for signal emission and reception. Animals were examined in the supine position. Transverse FLAIR MR images from sigmoid colon to the anus were acquired using the following parameters: repetition time, 2000 ms; echo time, 11.1 ms; inversion time, 750 ms; section thickness, 2 mm; intersection gap, 0 mm; field of view, 6-8 cm; matrix,  $320 \times 192$ ; number of signals acquired, one. Transverse T2WI imaging (repetition time, 3860 ms; echo time, 106.0 ms) with the same section thickness and image size was also performed. Multi-planar FLAIR and T2WI imaging were performed continuously if colorectal masses had been detected in initial imaging.

The MR images of the colorectal wall and masses were at first interpreted in consensus by two radiologists with 20 and 10 years of experience, respectively. Colorectal masses were located by measuring the mass to anus distance. Then, quantitative analysis was performed based on the recommended procedure<sup>[16]</sup>. First, identical axial FLAIR slices before and after enema were selected for region of interest (ROI) definition. Second, a curved ROI encompassing the colorectal wall or an irregular ROI encompassing the intraluminal mass, a round ROI on the back or pelvic muscle and an oval ROI along the phase encoding direction encircling air were defined; the signal intensity (SI) values were recorded (Image J, version 1.38; National Institutes of Health, Bethesda, MD). Third, the SI difference-to-noise ratios (SDNRs) for the colorectal wall or tumors were calculated using the following formula:  $SDNR = (SI_t - SI_m) / SDN$ , where  $SI_t$  is the mean SI value of target (the colorectal wall or intraluminal mass);  $SI_m$ , the mean SI of the muscle; and  $SDN$ , the standard deviation of the background noise (air).

### Histopathologic and fluorescent evaluation

Animals were euthanized by an overdose of pentobarbital immediately after MR examination. The colorectum was harvested. The macroscopic morphology of the bowel as well as the location and size of the masses within the ligated distal colorectum were recorded. The colorectal wall and masses were then sampled, frozen with liquid nitrogen, and cut into 5-7  $\mu$ m slices with a

microtome for LSCFM (Leica TCS-SP5, Wetzlar, Germany) evaluation and HE slice preparation. Diamidino-phenyl-indole (DAPI 1:15 000 dilution, Sigma, St. Louis, MO, United States) staining was performed on slices of one normal mouse to visualize the nuclei of intestinal cells. FITC carried by SLNs was excited at 488 nm and detected at 500-535 nm wavelengths. DAPI was excited at 405 nm and detected at 430-550 nm. The remaining tissues were sampled and immersed in 10% buffered formalin to prepare the standard hematoxylin and eosin (HE)-stained slices.

### Statistical analysis

SDNR data of colorectal wall, intraluminal mass size and other observations were expressed as means  $\pm$  standard deviations. Statistical analysis was performed with software (SPSS for Windows, release 16.0; SPSS, Chicago). One-way analysis of variance with least significant difference tests was applied for multiple comparisons of pre- and post-enema SDNRs of the colorectal wall (groups 1-3) and SDNRs of intraluminal masses (groups 2 and 3).  $P$  value  $< 0.01$  was considered a significant difference.

## RESULTS

### Characterization of SLNs

SLNs exhibited bimodal particle sizes ranging from 50 to 300 nm and zeta potentials ranging from  $-29.3 \pm 3.4$  to  $-39.1 \pm 2.0$  mV. The particle size increased slightly as ODA-FITC was loaded. Entrapment efficiency for Gd-DTPA in Gd-SLNs or in Gd-FITC-SLNs was 55.8% or 55.0%, respectively. Loading capacity of Gd-DTPA in Gd-SLNs or Gd-FITC-SLNs was about 50%. Hence, 40 mg Gd-FITC-SLN or Gd-SLN freeze-dried powder dispersed in 1 mL water, as used in the current study, contains about 10 mg Gd-DTPA and 20 mg Mannitol. MR images of SLN dispersions and pure water are shown in Figure 1.

### Pilot study findings

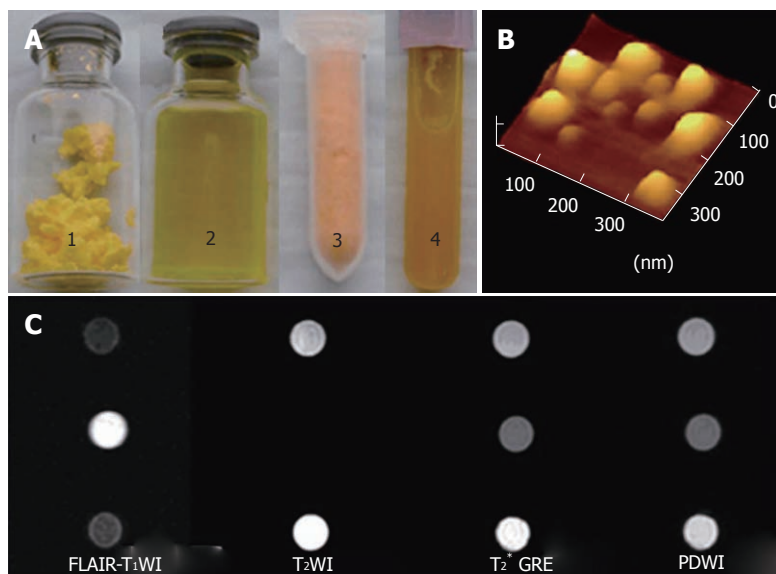
Four mice were sacrificed to establish the experimental protocol. The colorectal wall was detectable on FLAIR (low-SI) and T2WI (iso- to high-SI) images. However, bowel layers could not be differentiated. While LSCFM (Leica TCS-SP5, Germany) provided fine fluorescent images, the fluorescence microscope (Zeiss Axioskop 2, Carl Zeiss, Marburg, Germany) seemed applicable in tracking the distribution of FITC-loaded SLNs.

Homogeneous mural enhancement on post-enema FLAIR images was observed after Gd-SLN ( $n = 1$ ) and Gd-FITC-SLN ( $n = 2$ , 20/22 slices) retention enema. Dense FITC deposition was observed in fluorescence imaging after FITC-SLN ( $n = 1$ ) and Gd-FITC-SLN enema (Figure 2). No positive MR or fluorescence image finding was observed after Gd-DTPA solution ( $n = 1$ ) enema.

### MR and LSCFM image features of colorectal wall and pathologic correlation

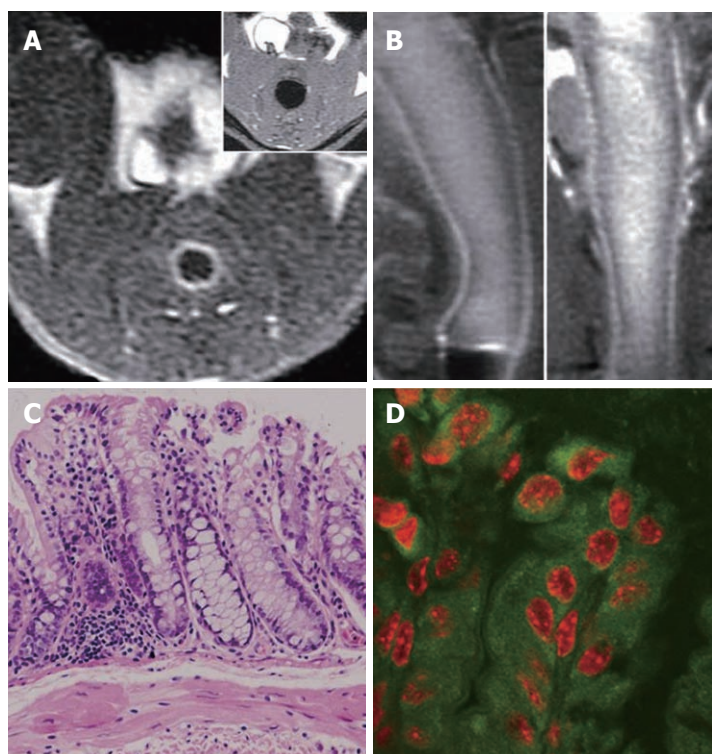
MR data of only 20 of the 30 DMH-treated mice were





**Figure 1** Characterization of solid lipid nanoparticles.

A: Gadopentetate dimeglumine and fluorescein isothiocyanate-loaded solid lipid nanoparticles (Gd-FITC-SLNs) freeze-dried powder (1) and dispersion (2); Fluorescein isothiocyanate solid lipid nanoparticles (FITC-SLNs) freeze-dried powder (3) and dispersion (4); B: Atomic force microscopy images of blank solid lipid nanoparticles; C: Magnetic resonance (MR) images of FITC-SLN dispersions (top). Gd-FITC-SLN suspension (middle). Water (bottom) obtained with fluid attenuated inversion recovery (FLAIR) (left), T<sub>2</sub>WI (middle left), T<sub>2</sub>\* GRE (middle right) and PDWI (right). FLAIR was obtained with the following parameters: 2000/11.1/750/2, TR/TE/TI/NEX; T<sub>2</sub>WI: 3860/106/2 (TR/TE/NEX); T<sub>2</sub>\* GRE: 550/14/2/200 (TR/TE/NEX/Flip); PDWI: 3220/12/1 (TR/TE/NEX). Both sequences used a 256 × 160 matrix, a 140 mm FOV, and 4-mm-thick sections.



**Figure 2** Magnetic resonance and fluorescent images of colorectal wall in normal mouse and bright rim sign in 1,2-dimethylhydrazine treated mouse. A: Axial fluid attenuated inversion recovery (FLAIR) image 5 min after gadopentetate dimeglumine and fluorescein isothiocyanate (FITC)-loaded solid lipid nanoparticle enema, showing the homogeneous mural enhancement; B, C: No abnormality was found in hematoxylin and eosin slices of the mouse; D: Laser-scanning confocal fluorescence microscope image of the post-enema colorectal wall of normal mouse with diamidino-phenyl-indole (DAPI) stain, showing the dense cytoplasmic FITC deposition and red DAPI-stained nucleus.

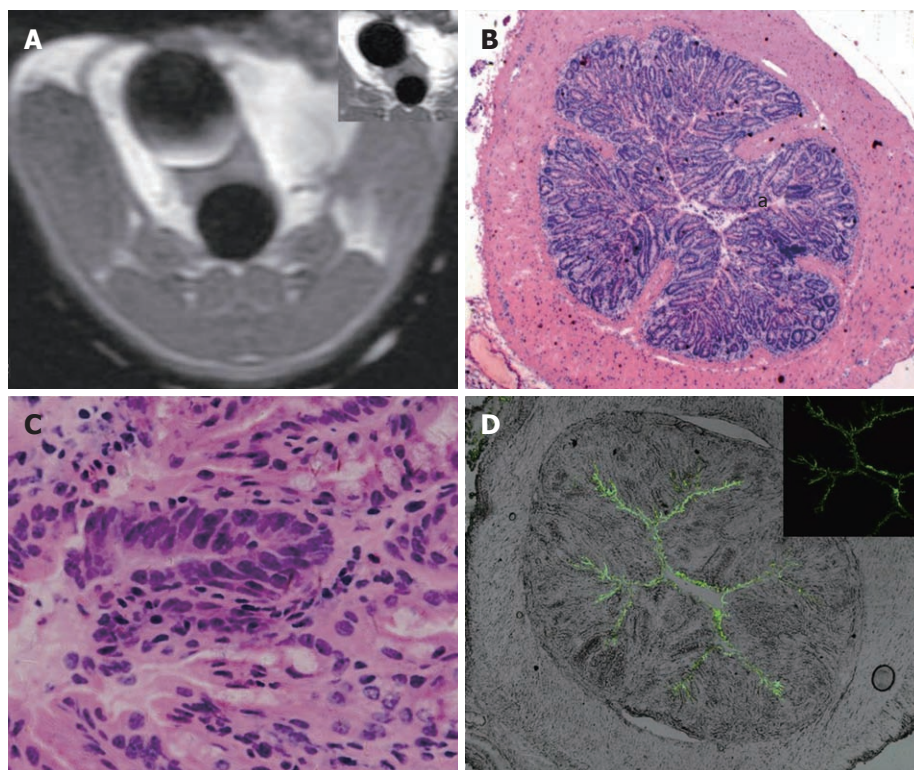
available due to DMH- and anesthesia-related mortality ( $n = 7$ ) and operation failures ( $n = 3$ ). In group 1, homogeneous enhancement and dense FITC deposition were observed in 1 mouse (8/10 slices) with no histological abnormality; the enhanced colorectal wall manifested as a bright rim sign on in-enema FLAIR images (Figure 2B). Heterogeneous enhancement was observed in 4 of the other 8 mice (26/48 slices). No visible mural enhancement was identified in the other 4 mice; mild dysplasia was identified in HE slices of the 8 mice with linear FITC deposition observed along the intestinal lumen in LSCFM images (Figure 3). In group 2, heterogeneous enhancement was shown in 2 of the 7 mice (6/16 slices); no other mural enhancement was observed. In group 3,

no enhancement was found on FLAIR images after the FITC-SLN enema. Obvious dysplasia and intraepithelial neoplasia (low to high grade) were found in HE slices of mice receiving DMH for 16 wk (groups 2 and 3) with linear FITC deposition along the intestinal lumen observed in LSCFM images. Non-enhanced colorectal walls manifested as low signal rings around the “bright lumen” in in-enema FLAIR images, which was observed in 4 of the 7 mice in group 2 (Figure 4B).

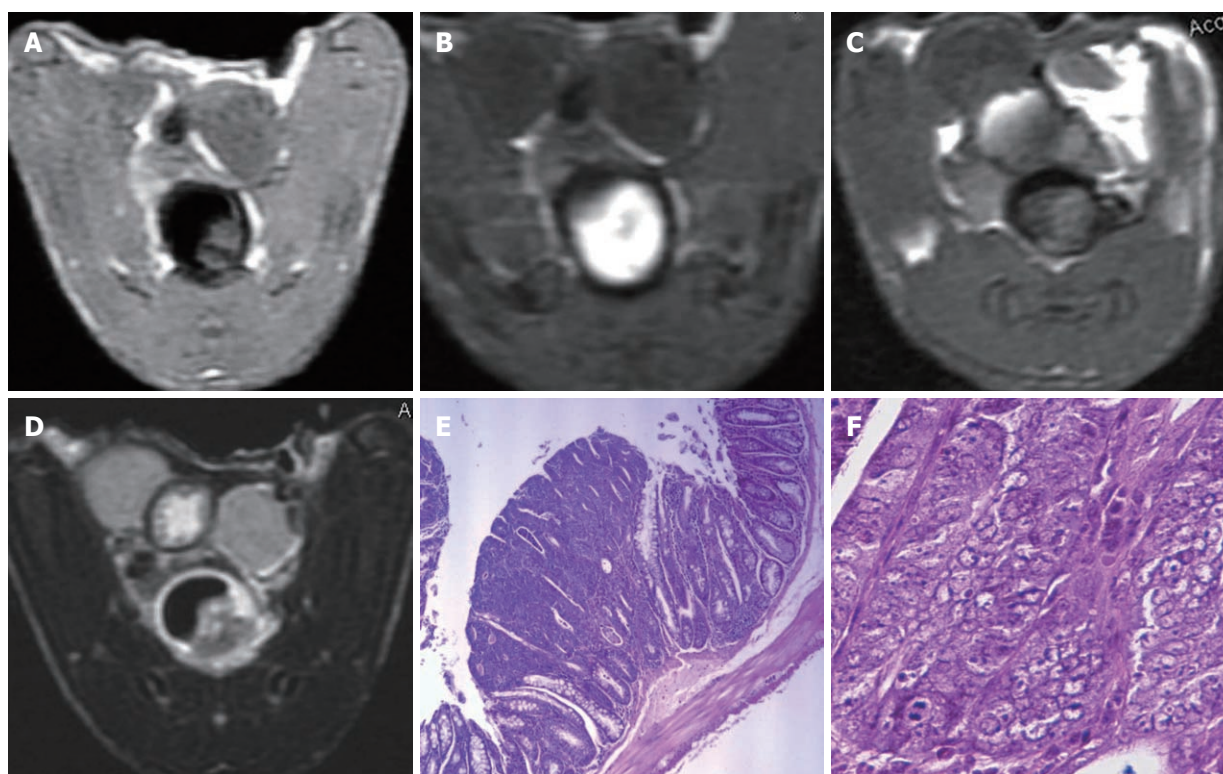
#### MR and LSCFM findings of colorectal mass in DMH groups and pathologic correlation

MR detected 9 intraluminal masses (short axis  $2.06 \pm 0.98$  mm) and 1 prolapsed mass (well-differentiated squamous





**Figure 3** Magnetic resonance and fluorescent images of 1,2-dimethylhydrazine impaired colorectal wall. A: Axial fluid attenuated inversion recovery (FLAIR) image 5 min after gadopentetate dimeglumine (Gd-DTPA) and fluorescein isothiocyanate (FITC)-loaded solid lipid nanoparticle enema. No mural enhancement was identified. Note the bladder Gd-DTPA deposition (top right: pre-enema FLAIR image); B, C: Hematoxylin and eosin image of the same slice; nuclear atypia identified; B:  $\times 40$ ; C:  $\times 400$ . D: Laser-scanning confocal fluorescence microscope image of the post-enema colorectal wall, showing the linear extracellular FITC deposition along the mucosa.

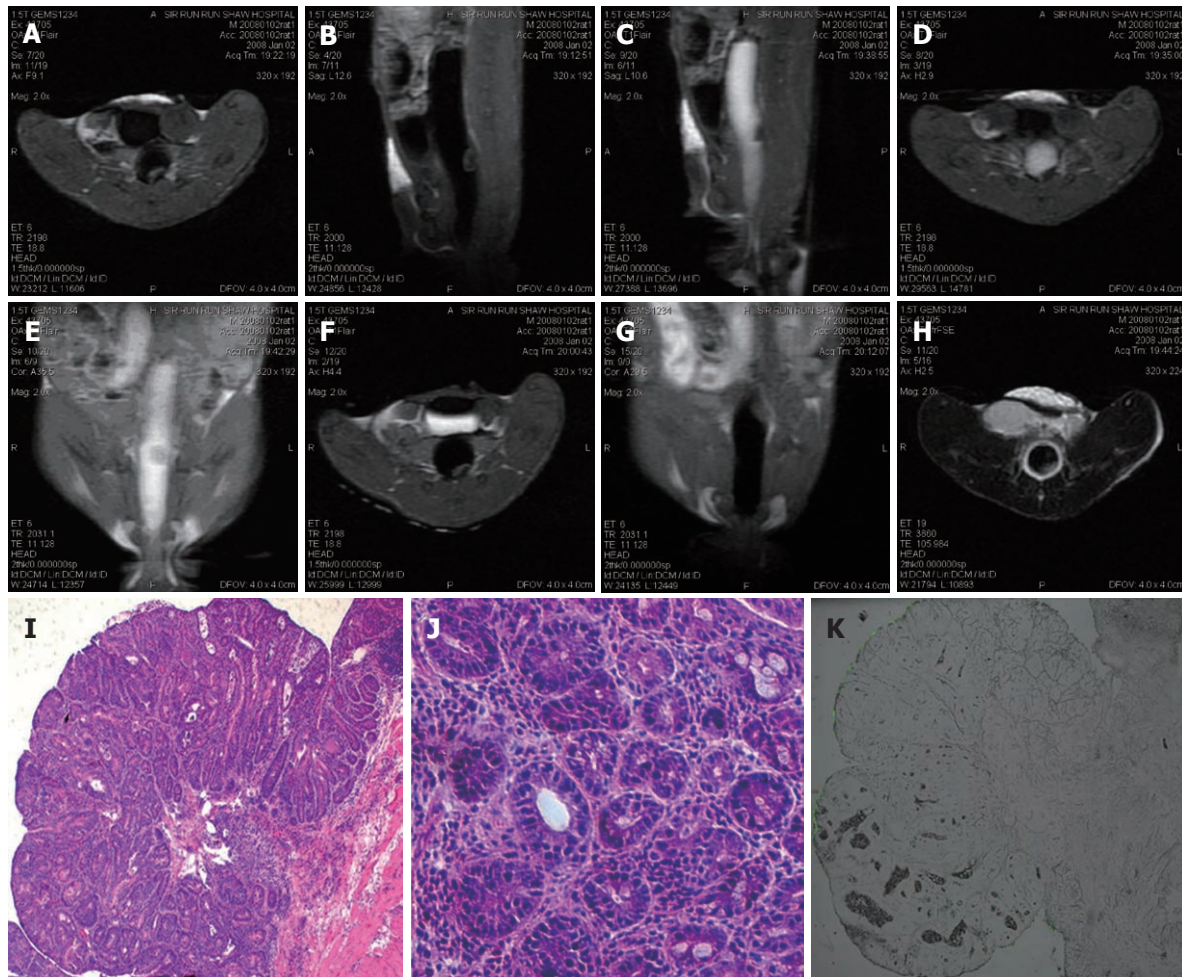


**Figure 4** Magnetic resonance and histological images of adenocarcinoma. A: An irregular-shaped mass in the pre-enema fluid attenuated inversion recovery (FLAIR) image; B: Low signal ring around the bright lumen (halo sing) in the in-enema FLAIR image; C: Tumor enhancement and bladder imaging agents accumulation both occurred in the post-enema FLAIR image; D: T2 weighted post-enema image, heterogeneous signal intensity within the tumor; E, F: Hematoxylin and eosin images of the tumor, adenocarcinoma cells identified.

carcinoma) in groups 2 and 3. Eight of the 9 intraluminal masses were adenomas with different levels of malignancy; one of them was histologically proven as adeno-

carcinoma. The intraluminal masses manifested as filling deficits on Gd-FITC-SLN inflated bright-lumen FLAIR images. No visible enhancement was found in post-enema





**Figure 5** Magnetic resonance and fluorescent images of a non-enhanced adenoma. A, B: Axial and sagittal pre-enema fluid attenuated inversion recovery (FLAIR) images, note the inner low signal of the mass; C-E: The mass manifests as a filling deficit in the gadopentetate dimeglumine (Gd-DTPA) and fluorescein isothiocyanate-loaded solid lipid nanoparticle (Gd-FITC-SLN) enema inflated FLAIR images; F, G: No enhancement identified after the enema. Note the bladder Gd-DTPA deposition; H: Axial T2 weighted image after the Gd-FITC-SLN enema. Iso-signal observed in the adenoma; high signal observed for the bowel wall; I-K: Hematoxylin and eosin and laser-scanning confocal fluorescence microscope images of the adenoma.

FLAIR images in one narrow-based adenoma with minimum FITC deposition along the edge and within the mass (Figure 5). Various degrees of enhancement were found in the other masses with interstitial FITC depositions in LSCFM images (Figure 6).

### Other MR and pathohistologic findings

Colorectum stiffness was observed in 3 of 9 mice (groups 2 and 3) receiving DMH for 16 wk. Macroscopic evaluation of the animal's distal colorectum revealed an extra adenoma measuring  $2 \times 3$  mm in size in the sigmoid colon, which was missed in MR examination. No false-positive MR findings were observed by postmortem evaluation. Bladder imaging agent accumulation was accidentally found in 11 of the 16 DMH-treated mice (groups 1 and 2) 3–28 ( $11.2 \pm 9.6$ ) min after the Gd-FITC-SLN enema was started (Figures 3–6).

### Statistical results

Pre- and post-enema SDNR values of colorectal wall in each group and intraluminal masses in DMH-treated mice

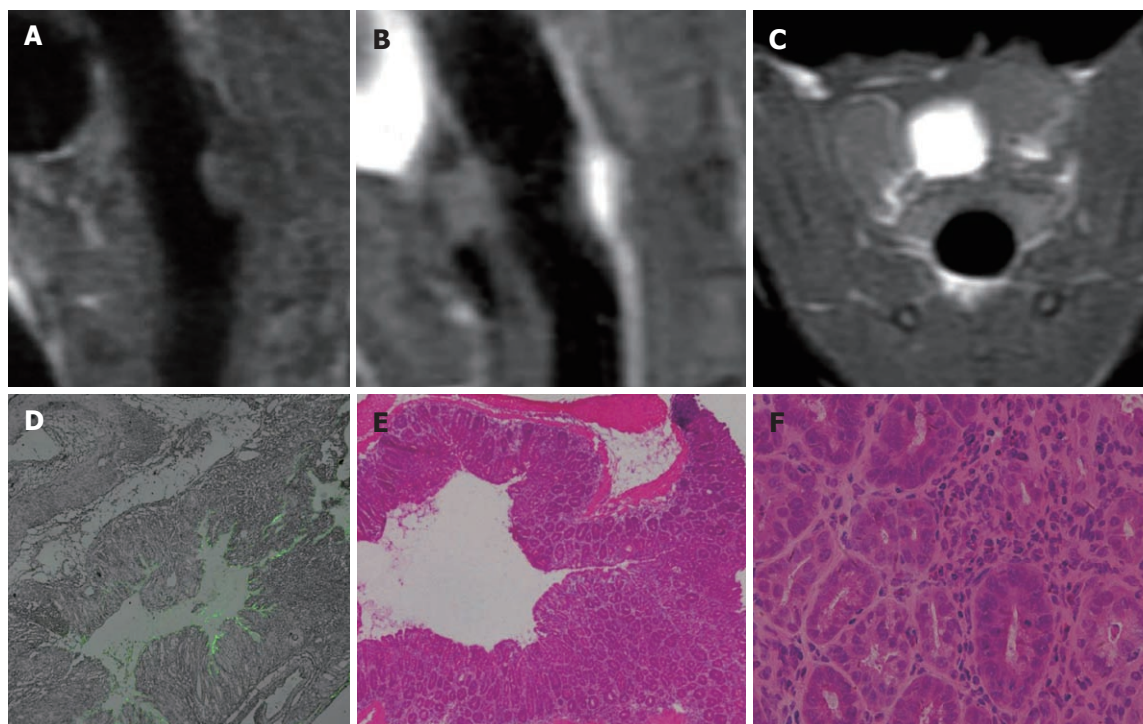
were plotted against time (Figure 7) with brief annotation.

## DISCUSSION

We used MR and LSCFM to study the colorectal uptake of SLNs. The bimodal imaging used elsewhere for tumor angiogenesis and lymphatic vessel imaging<sup>[17,18]</sup> can mutually confirm the information both macroscopically and microscopically. We used FLAIR for colorectal imaging. An early study reported that FLAIR sequences are more sensitive to low gadolinium concentrations than T1-weighted sequences<sup>[19]</sup>. Another study showed that post-contrast FLAIR imaging may improve the lesion depiction when a higher lesion SI exists on the T2-weighted images<sup>[20]</sup>. A set of methods for quantitative MR imaging analysis were evaluated, and a standardized method was proposed<sup>[16]</sup>. We followed the suggested procedure in terms of ROI definition and SDNR calculation.

We reviewed the studies on intestinal particulate substance uptake, colon-specific drug delivery and DMH-induced intestinal and renal impairment in order to explain





**Figure 6** Peri-tumor interstitial fluorescein isothiocyanate deposition. A: A broad-based mass (high-level adenoma) in the pre-enema fluid attenuated inversion recovery (FLAIR) image; B, C: Tumor and peri-tumor mural enhancement, together with bladder imaging agent accumulation, in the post-enema FLAIR image; D: Interstitial linear fluorescein isothiocyanate deposition in the peri-tumor colorectal wall; E, F: Hematoxylin and eosin images at the same slice, obvious nuclear atypia identified.

the current results. It was proven, by lymph and plasma analysis, that more than 70% of the absorbed SLN was transported into systematic circulation *via* lymph, which is a major SLN transport pathway in the gastrointestinal tract<sup>[11]</sup>. Recent studies have further verified that the oral bioavailability of poorly water soluble contents (insulin, nitrendipine, tobramycin) increased significantly when encapsulated in the inner lipid matrix of SLNs<sup>[21,22]</sup>.

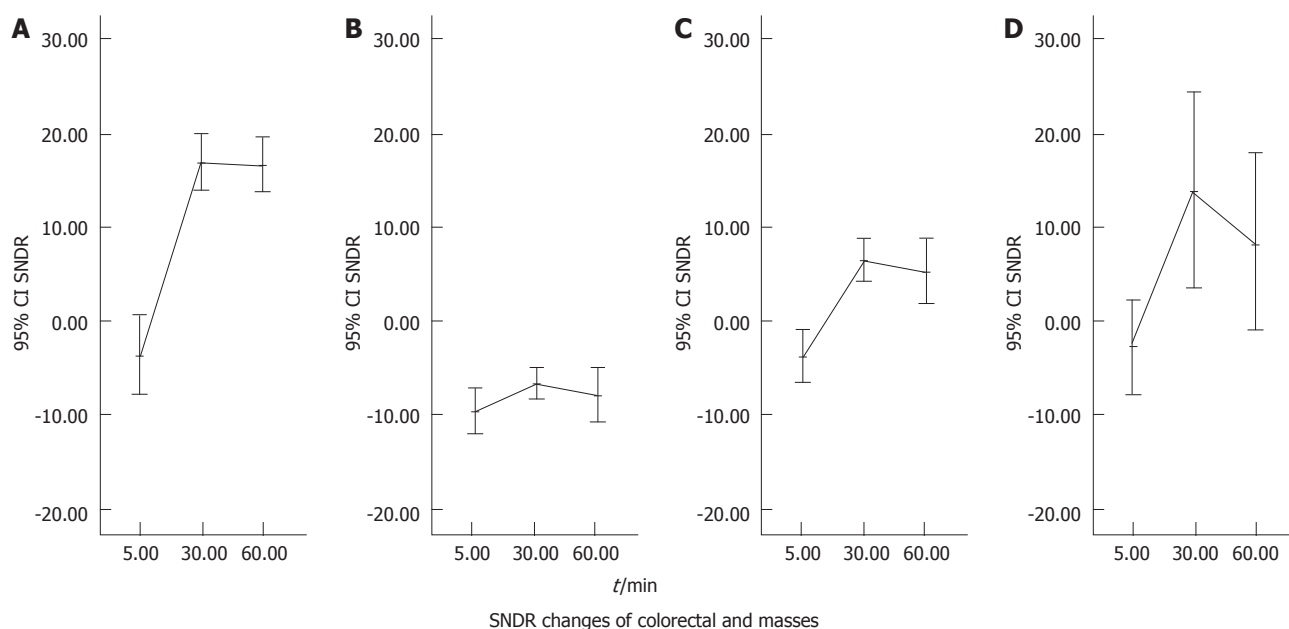
The intestinal uptake of inert particles of the micrometer and nanometer range has been intensively studied in pharmaceutical research. The particles, usually used as oral drug delivery systems, include SLN (50-1000 nm), chitosan microspheres (2.1-12.5  $\mu\text{m}$ ), latex (2  $\mu\text{m}$ ), dendrimer and polymers, etc. The major conclusions of the studies<sup>[10,23-27]</sup> are summarized as follows. First, inert particulate uptake takes place along the entire length of the small and large intestines. Second, the process occurs not only *via* the M cells in the Peyer's patches and the isolated follicles of the gut-associated lymphoid tissue, but also *via* the normal intestinal enterocytes. Third, factors affecting the uptake include particle size, surface charge and surface modification. Larger (micrometer range) and surface modified particles may be retained for longer periods in the Peyer's patches, while smaller particles are transported to the thoracic duct. Fourth, an *in vitro* study using a Caco-2 cell model showed that 2 Gy X-irradiation increased particle (2  $\mu\text{m}$  latex particles) uptake and translocation through the epithelium.

We hypothesize, based on the current results and earlier studies, that two different routes exist for the rec-

tally administered SLN particles to enter the systematic circulation. In normal mice, the SLNs are mainly taken up by enterocytes and transferred to the lymphatic vessels and finally transported to the systematic circulation from the thoracic duct. In DMH-treated mice, however, the dominant route is from the carcinogen impaired colorectal mucosa, *via* the submucosal capillary network, to the mesenteric vein and then to the liver. As observed in this study, colorectal uptake and drainage of SLNs are an intracellular process; the transportation of SLNs in DMH-treated mice is a pathological process that occurs through an extracellular or interstitial route.

DMH and its metabolite azoxymethane (AOM) are specific colon carcinogens to model non-familial CRC in rodents<sup>[28]</sup>. Previous studies documented that DMH is also a renal carcinogen in mice<sup>[29-31]</sup>. The bladder gadolinium accumulation observed in this study may result from renal and intestinal epithelial impairment caused by DMH and its metabolites and the diuresis effect of manicol contained in SLN freeze-dried powder (20 mg /mL).

We believe that the bright rim sign in post-enema FLAIR images and corresponding cytoplasmic FITC deposition in normal and group 1 mice is a manifestation of normal intestinal uptake function. Likewise, the halo sign observed in group 2 mice and linear extracellular FITC deposition is attributed to the impairment of intestinal epithelial barrier function. As functional changes always precede morphological lesions, further experiments are necessary to exemplify the early diagnostic potential by clarifying the mechanism at both cellular and



**Figure 7** The signal intensity difference-to-noise ratios changes of colorectal wall and masses before and after the gadopentetate dimeglumine and fluorescein isothiocyanate-loaded solid lipid nanoparticle enema. A: The signal intensity difference-to-noise ratios (SDNRs) of colorectal wall in normal mice increased sharply after 20 min of the gadopentetate dimeglumine and fluorescein isothiocyanate-loaded solid lipid nanoparticle (Gd-FITC-SLN) enema ( $P < 0.01$ ) and remain at a high level in the following 30 min with a minimum decrease ( $P > 0.05$ ); B: The SDNRs of colorectal wall in group 1 [1,2-dimethylhydrazine (DMH) treated for 10 wk] increased significantly, with about one half of the amplitude compared with that of the normal mice, after the Gd-FITC-SLN enema; a visible decrease with no statistical significance ( $P > 0.05$ ) of SDNRs occurred in the following 30 min; C: The SDNRs of colorectal wall in group 2 (DMH treated for 16 wk) increased and decreased non-significantly in post-enema images ( $P > 0.01$ ); D: The SDNRs of intraluminal masses in DMH treated mice increased significantly ( $P < 0.01$ ) and decreased non-significantly in the post-enema images ( $P > 0.01$ ). SDNR: The Signal Intensity Difference-to-noise ratios.

molecular levels.

There were limitations in our study. First, layers of murine colorectal wall could not be differentiated on MR images due to the small animal size. We noticed that layers of the bowel and tumor invasion in the excised human colon cancer specimen were clearly depicted in one study<sup>[32]</sup>. Optimal delineation of layers of colorectal wall may likely be achieved if a porcine model was adopted. Second, sharp contrast in post-enema MR images (partial enhancement) existed but was rare, which may be explained by the diffuse impairment caused by DMH and its metabolite azoxymethane (AOM), delivered to the distal colorectum *via* the biliary system. Adoption of another colorectal tumor model, focally administered AOM, may improve the post-enema contrast between normal and cancerous tissues. Third, the long acquisition times of FLAIR pulse sequence may not be suitable for clinical MR colonography. More researches are, therefore, needed to optimize the technique in a clinical context.

## COMMENTS

### Background

High contrast between the bowel wall and lumen is essential for successful magnetic resonance (MR) colonography, which has been intensively studied in recent years. However, there has been no research aiming at MR visualizing the colorectal uptake of contrast medium.

### Research frontiers

In this study, colorectal uptake of solid lipid nanoparticles (SLNs) in normal and dimethylhydrazine (DMH)-treated mice was visualized by MR and laser-scanning confocal fluorescence microscopic imaging.

### Innovations and breakthroughs

Significant differences in colorectal uptake and distribution of SLNs were revealed in normal and DMH-treated mice, which may provide new mechanisms of contrast for MR colonography.

### Applications

Direct and *in vivo* imaging of colorectal uptake of nanoparticles could be translated into radiological and pharmaceutical applications. Further work is needed to explore the potential value of current findings for personalized therapy and radiographic follow-up.

### Terminology

SLNs are colloidal drug delivery systems with mean particle diameters ranging from 50 up to 1000 nm. SLNs combine the advantages of polymeric nanoparticles and fat emulsions for drug delivery administration, such as good tolerability, high oral bioavailability and large-scale production by high pressure homogenization. Magnetic resonance imaging is a cross-sectional imaging technique that does not utilize radiation and provides excellent tissue differentiation. MR colonography, based on the use of ultrafast MR sequences and relevant bowel contrast agents, is a less invasive colon imaging tool compared with optic colonoscopy.

### Peer review

The authors concluded that the uptake of SLNs into the colon wall was significant difference between normal and 1, 2-DMH, specific colon carcinogens, treated mice. This paper has very interesting results, but the objective is not clear.

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## Combined inhibitors of angiogenesis and histone deacetylase: Efficacy in rat hepatoma

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### Abstract

**AIM:** To evaluate the antitumoral effect of combined inhibitors of angiogenesis and histone deacetylases in an experimental rat hepatoma model.

**METHODS:** MH7777A hepatoma cells were injected into the liver of male Buffalo rats. After 7 d treatment with the vascular endothelial growth factor receptor antagonist PTK787/ZK222584 (PTK/ZK), the histone deacetylase inhibitor MS-275, tamoxifen (TAM) and/or retinoic acid was initiated ( $n \geq 8$  animals/group). Natural tumor development was shown in untreated control groups (control 1 with  $n = 12$ , control 2 with  $n = 8$ ). The control groups were initiated at different time points to demonstrate the stability of the hepatoma model. For documentation of possible side effects, we documented any change in body weight, loss of fur and diarrhea. After 21 d treatment, the rats were euthanized. Main target parameters were tumor size and metastasis rate. Additionally, immunohistochemistry for the proliferating cell nuclear antigen (PCNA) and TdT-

mediated dUTP-biotin nick end labeling (TUNEL) assay were performed.

**RESULTS:** The control groups developed large tumor nodules with extrahepatic tumor burden in the lung and abdominal organs (control 1:  $6.18 \text{ cm}^3 \pm 4.14 \text{ cm}^3$  and control 2:  $8.0 \text{ cm}^3 \pm 4.44 \text{ cm}^3$  28 d after tumor cell injection). The tumor volume did not differ significantly in the control groups ( $P = 0.13$ ). As single agents MS-275 and PTK/ZK reduced tumor volume by  $58.6\% \pm 2.6\%$  and  $48.7\% \pm 3.2\%$  vs control group 1, which was significant only for MS-275 ( $P = 0.025$ ). The combination of MS-275 and PTK/ZK induced a nearly complete and highly significant tumor shrinkage by  $90.3\% \pm 1\%$  ( $P = 0.005$ ). Addition of TAM showed no further efficacy, while quadruple therapy with retinoic acid increased antitumoral efficacy (tumor reduction by  $93 \pm 1\%$ ) and side effects. PCNA positive cells were not significantly reduced by the single agents, while dual therapy (MS-275 and PTK/ZK) and quadruple therapy reduced the PCNA-positive cell fraction significantly by 9.1 and 20.6% vs control 1 ( $P < 0.05$ ). The number of TUNEL-positive cells, markers for ongoing apoptosis, was increased significantly by the single agents (control 1: 6.9%, PTK/ZK: 11.4%, MS-275: 12.2% with  $P < 0.05$  vs control 1). The fraction of TUNEL-positive cells was upregulated highly significantly by dual therapy (18.4%) and quadruple therapy (24.8%,  $P < 0.01$  vs control 1). For the proliferating (PCNA positive) and apoptotic cell fraction, quadruple therapy was significantly superior to dual therapy ( $P = 0.01$ ).

**CONCLUSION:** Combined PTK/ZK and MS-275 were highly effective in this hepatoma model. Quadruple therapy enhanced the effects microscopically, but not macroscopically. These results should be investigated further.

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**Key words:** PTK787; ZK222584; MS-275; Hepatocellu-

## lar carcinoma; Histone deacetylase inhibitor

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumor entities in Asia and Africa, but not in western countries<sup>[1,2]</sup>. However, the increasing rate of hepatitis C virus infection and alcohol abuse has led to a rising incidence of HCC in industrial countries<sup>[1,2]</sup>.

Life-prolonging HCC therapy is based on surgical tumor elimination (liver transplant, resection) or interventional local ablation<sup>[3,4]</sup>. For large tumor volume or metastasis, therapeutic options are limited, because HCC is resistant to systemic chemotherapy<sup>[3,4]</sup>. A wide range of experimental compounds, such as vitamin D or tamoxifen (TAM) were not effective in placebo-controlled trials<sup>[1,3,4]</sup>. Therefore, not single, but combination treatment should be evaluated. We have shown that combined application of TAM and cis-retinoic acid (CRA) induces moderate antitumoral effects in a rat model, while single agents are ineffective<sup>[5,6]</sup>. Current investigations have put combination molecular targeted therapy into focus<sup>[7-9]</sup>. Sorafenib is a molecular targeted agent with antiproliferative as well as antiangiogenic activity. It is the first effective compound in HCC patients, which proves the concept of combination treatment. However, the antitumoral and life-prolonging effects of sorafenib are very limited<sup>[3,7-9]</sup>. Nevertheless, the strategy of combined molecular targeted agents has to be further investigated.

In most HCCs, a high grade of vascularity has been demonstrated. Folkman *et al.*<sup>[10-12]</sup> have demonstrated that any tumor larger than a few millimeters in diameter induces angiogenesis for self-supplementation. Furthermore, connection with the host's vessel system and degradation of the extracellular matrix leads to metastasis<sup>[11]</sup>. Therefore, inhibitors of angiogenesis have been shown to reduce hepatoma volume *in vivo* and *in vitro*<sup>[9-12]</sup>. In recent years, highly effective synthetic angiogenesis inhibitors, such as PTK787/ZK222584 (PTK/ZK), have been developed for cancer therapy. These are currently being evaluated in clinical trials or approved for therapy of advanced colorectal cancer<sup>[12,13]</sup>.

Histone deacetylase (HDAC) inhibitors suppress the post-translational deacetylation of histone proteins. In consequence, these histone proteins are hyperacetylated and the DNA structure is loosened, which mediates enhanced binding of transcription factors to certain gene

loci and higher gene expression<sup>[14]</sup>. HDAC inhibitors have been shown to induce growth inhibitory genes and proapoptotic factors *in vivo* and *in vitro*<sup>[7,14,15]</sup>. MS-275 is a benzamide with activity against HDAC class 1 and 2. Ongoing phase I - III trials have displayed no adverse effects, therefore, further clinical development is ongoing<sup>[16,17]</sup>.

In this experimental setting, we evaluated a combination of the angiogenesis inhibitor PTK/ZK and the HDAC inhibitor MS-275<sup>[12,16]</sup> in a syngeneic rat model of hepatoma. PTK/ZK is an aminophthalocine and a potent inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinases, which impairs VEGF-induced responses and tumor growth after oral administration (Vatalanib®)<sup>[12,16,18]</sup>. VEGF receptors mediate an increase in vascular permeability, angiogenesis and lymphogenesis. Mainly, the expression of VEGF receptor 1 is enhanced in tumor tissue<sup>[16,18]</sup>. PTK/ZK inhibits VEGF receptors 1-4 with highest potency towards receptors 1 and 2. Additionally, we added TAM and CRA, which have been shown to act *via* intracellular receptors and to be effective combination partners in this HCC model<sup>[5]</sup>.

## MATERIALS AND METHODS

### Reagents and cell culture

For *in vivo* experiments MS-275 was suspended in methanol, while 9-cis retinoic acid (cRA), TAM and PTK787/ZK222584 (Vatalanib®, PTK/ZK) were dissolved in DMSO. Any of these stock solutions were diluted at least 20-fold with Aqua injectabile (Baxter, Deerfield, IL, United States) to a final maximum concentration of 5% DMSO or 0.5% methanol before injection. MS-275 and PTK/ZK were kindly donated by Schering AG (Berlin, Germany).

### Morris hepatoma of the rat

Male Buffalo rats (200-350 g) from Charles River Laboratories (Schweinfurt, Germany) were kept as pairs in polycarbonate cages (Eurostandard III H; Techniplast, Berlin, Germany). Room temperature was kept at 27 °C and room humidity maintained at 30%. The rats were fed a standardized gluten-poor diet (Altromin, Frankfurt, Germany) and water. Animal maintenance and experimental procedures were approved by the government of middle Franconia and carried out according to "The 1996 Guide for the Care and Use of Laboratory Animals" as published in ILAR<sup>[19]</sup>.

For tumor induction, MH777A cells (DSMZ, Braunschweig, Germany) were grown in Dulbecco's Modified Eagle's Medium (Biochrom, Berlin, Germany) that contained 10% fetal calf serum (Gibco BRL, Karlsruhe, Germany), penicillin (100 U/L), streptomycin (10 mg/L), insulin and dexamethasone at 37 °C under 5% CO<sub>2</sub> (5-10 passages). The cells were trypsinized, suspended in PBS (Biochrom) at a concentration of 10<sup>6</sup> cells/100 µL. Buffalo rats were anesthetized using ethyl ether. After a median laparotomy (2 cm), the liver was embedded into wet sterile compresses. One hundred microliters of the cell suspension was injected into the subcapsular space

of the left liver lobe and leakage of tumor cells was prevented by compression and a hemostatic (Tabotamp; Ethicon, Johnson and Johnson, Norderstedt, Germany). The animals received metamizol for analgesia (7 d). They were controlled for diarrhea, loss of hair, food intake and unusual behavior daily, and body weight was measured weekly. On postoperative day 7 (tumor size 5–7 mm diameter), treatment with single or combined drugs was started. The drugs were administered at the recommended dose of 50 mg/kg per day i.p. for PTK/ZK, 3 mg/kg per day i.p. for MS-275, 10 mg/kg per day i.p. for TAM and 6 mg/kg per day i.p. for CRA. After 21 d treatment (day 28 after tumor implantation), the rats were euthanized with ether anesthesia. At least eight animals were evaluated per group.

### Macroscopic evaluation

The liver was removed and the tumor volume calculated using the formula (largest diameter  $a \times$  smallest diameter  $b^2$ )/2 as recommended in the literature<sup>[6]</sup>. The following organs were inspected for tumor nodules: lungs, spleen, kidneys, peritoneum and diaphragm. The primary tumor and both lungs were fixed in 5% buffered formalin.

### Microscopic analysis

TdT-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were analyzed using the *in situ* Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, formalin-fixed tissues were permeabilized with proteinase K (30 min, 37 °C) and peroxidase blocked in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub>. Fluorescent nucleotides mixed with terminal deoxynucleotide transferase were added for 60 min at 37 °C, followed by incubation with converter-peroxidase (POD) conjugated anti-fluorescein antibody (provided in the kit) for 30 min at 37 °C. Slides were developed using diaminobenzidine (DAB) substrate for 10 min and counterstained using methylene green (7.5%, 7 min at room temperature).

Proliferating cell nuclear antigen (PCNA)-positive cells in formalin-fixed tissue were detected after blocking of endogenous biotin with chicken egg and 1.5% fat milk for 15 min at room temperature. Mouse PCNA antibody (Novo Laboratories, Newcastle, United Kingdom) was diluted 1:50 in Tris buffer and added for 2 h, followed by 30 min incubation with the biotinylated second antibody. Color was developed with streptavidin-alkaline phosphatase complex (DAKO, Mannheim, Germany) and FAST Red (Sigma, Frankfurt, Germany).

The stained sections were examined using a light microscope (Axiophot, Nikon coolpix 99; Zeiss, Jena, Germany) and the CellExplorer 2001 software (BioSciTec, Frankfurt/Main, Germany). For quantification of TUNEL- and PCNA-positive cells, 10 high power fields per slide were investigated at 400  $\times$  magnification. Four of eight animals were analyzed per experimental group. All cell nuclei were related to the specifically stained cells to obtain the percentage of positive cells per slide.

For qualitative validation of the anti-angiogenic activity cryofixed sections (6  $\mu$ m, lysine-coated slides) were blocked (buffer containing 2% BSA, 0.2% low fat milk, 2% mouse serum and PBS) and incubated with a rabbit anti-von Willebrand factor (vWF) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States; 1:200 dilution) for 1 h at 37 °C. After several washing steps and addition of a biotinylated second antibody (30 min, room temperature), color was developed with streptavidin-peroxidase complex (DAKO) and DAB. Counterstaining was done using standard haemalaun.

### Statistical analysis

Statistical analysis was performed using SPSS for Windows version 16.0. Significance was calculated using the *t* test or Wilcoxon test for paired samples (if not otherwise stated *vs* control 1).  $P < 0.05$  was regarded as significant, and  $P < 0.01$  as highly significant.

## RESULTS

### In vivo studies

In untreated animals (control 1,  $n = 12$ ) the tumor volume was  $6.18 \text{ cm}^3 \pm 4.14 \text{ cm}^3$  after 28 d. In a second untreated control group (control 2,  $n = 8$ ), which was started at an independent time point, tumor volume was  $8.0 \text{ cm}^3 \pm 4.44 \text{ cm}^3$ . This was not significantly different to control 1 ( $P = 0.13$ ) and may confirm the reproducibility of the animal model.

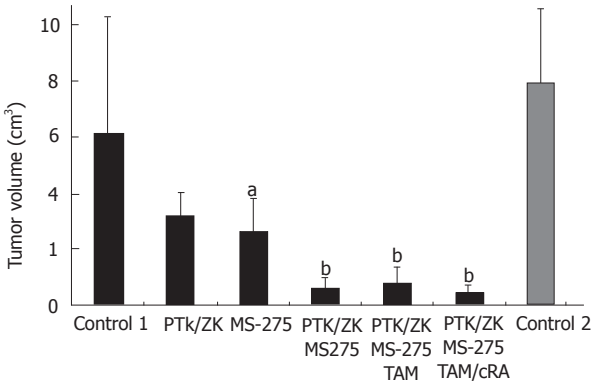
Twenty-eight days after tumor implantation 80%–90% of the animals suffered from diffuse lung metastases and tumor spread to kidneys, spleen and peritoneum. Eighty percent of the animals developed ascites. Bleeding from the eyes and the nose showed impaired coagulation. The body weight was increased by  $17\% \pm 15\%$  (due to ascites and tumor burden).

As stated before, treatment with TAM or CRA as single agents showed no significant antitumoral efficacy, while a combination of both showed moderate tumor reduction (14%, 8.5% and 60% for TAM, CRA and TAM/CRA)<sup>[7]</sup>.

Even the single agents PTK/ZK and MS-275 induced some antitumoral effects and reduced tumor volume by 50 and 60% (Figure 1). However, PTK/ZK failed to show significant antitumoral efficacy ( $P = 0.212$ ), while MS-275 showed significant tumor growth reduction even as a single agent ( $P = 0.025$ ). Dual therapy with PTK/ZK and MS-275 reduced tumor growth by  $> 90\%$  in a highly significant manner ( $P = 0.005$ , Figures 1 and 2). PTK/ZK + MS-275 + TAM showed no additional effect on tumor volume, while the quadruple therapy enhanced the efficacy slightly, but not significantly ( $P = 0.007$  and  $0.002$  for triple and quadruple therapy *vs* control 1;  $P = 0.49$  and  $P = 0.039$  *vs* dual therapy, Figure 1).

Monotherapy did not change the extent of extrahepatic tumor burden, while combination therapy reduced rate of metastases significantly (Table 1). Again, quadruple therapy failed to enhance the effects of combined





**Figure 1 Macroscopic tumor growth.** The results are given as absolute values. <sup>a</sup>*P* = 0.025, MS-275 vs control 1; <sup>b</sup>*P* = 0.005, ZK/PTK/MS vs control 1. PTK/ZK: PTK787/ZK222584 (Vatalanib<sup>®</sup>); TAM: Tamoxifen; cRA: 9-*cis*-retinoic acid.

PTK/ZK + MS-275 (data not shown).

Compared to placebo treatment, monotherapy increased the rate of diarrhea and loss of fur. Combination therapy intensified the number of these side effects significantly (Table 1). Subgroup analysis showed fewer side effects for dual therapy compared to triple and quadruple therapy. However, this particular result was not significant due to the low number of animals/group (data not shown).

Any effective treatment (monotherapy, dual therapy and quadruple therapy) induced loss of weight, which can be explained by the increased rate of diarrhea and the reduced amount of ascites in these treatment groups (Table 1). No animal had to be euthanized due to the number or severity of side effects.

**Microscopic results**

After treatment with PTK/ZK alone and in combination, vessel density decreased qualitatively, which was exemplified by staining with vWF antibody in a subgroup of animals (data not shown). Quantification of microvessel density was not performed, because the antiangiogenic efficacy of PTK/ZK has been well described<sup>[12,19]</sup>.

In hematoxylin and eosin (HE) and immunohistochemically stained tissue, any effective treatment went along with an increase in areas of necrosis (Figure 3). Depending on the extent of the necrotic areas, we detected 200-500 cells/field. The number of TUNEL-positive cells as a marker for ongoing apoptosis increased significantly after monotherapy (*P* = 0.04 and 0.02 for PTK/ZK and MS-275 *vs* control 1). As expected, the number of apoptotic cells increased even more markedly after dual therapy (highly significant *vs* control 1 with *P* = 0.002). The results of quadruple therapy were significantly higher even if compared to dual therapy (*P* = 0.01 for quadruple therapy *vs* dual therapy; Table 2). The signal for proliferating cells (PCNA positive) remained stable for monotherapy, but decreased significantly for dual therapy (*P* = 0.04 *vs* controls). Again, quadruple therapy showed a significant difference compared to the combination of PTK/ZK + MS-275 (*P* = 0.01 for quadruple *vs* dual therapy) (Table 2).

Table 1 Extrahepatic tumor burden and side effects in untreated and treated tumor-bearing rats (%)				
	Control group (n = 12)	Monotherapy (n = 17)	Combination therapy (n = 24)	P value <sup>2</sup>
Tumor burden - pulmonary	83.3	82.4	70.8	0.586
Tumor burden - abdominal	91.7	100.0	66.7	0.013
Ascites	83.3	29.4	0.0	< 0.001
Behavior <sup>1</sup>	41.7	0.0	25.0	0.019
Loss of fur	16.7	41.2	66.7	0.015
Loss of weight (> 10% body weight)	0.0	47.1	79.2	< 0.001
Diarrhea	16.7	29.4	62.5	0.015

<sup>1</sup>Reduced food consumption, isolation, stereotypic movements, apathia; <sup>2</sup>*P* values for combined *vs* single therapy.

Table 2 Histological analysis of the tissue					
	Control 1	PTK/ZK	MS-275	PTK/ZK + MS-275	PTK/ZK + MS-275 + TAM + cRA
PCNA pos. cells	44.2 ± 12.4	42.7 ± 9.4	43.7 ± 5.5	35.1 ± 3.6 <sup>a</sup>	23.6 ± 3.8 <sup>a,c</sup>
TUNEL pos. cells	6.9 ± 4.4	11.8 ± 6.6 <sup>a</sup>	12.2 ± 7.7 <sup>a</sup>	18.4 ± 5.5 <sup>c</sup>	24.8 ± 10 <sup>b,c</sup>

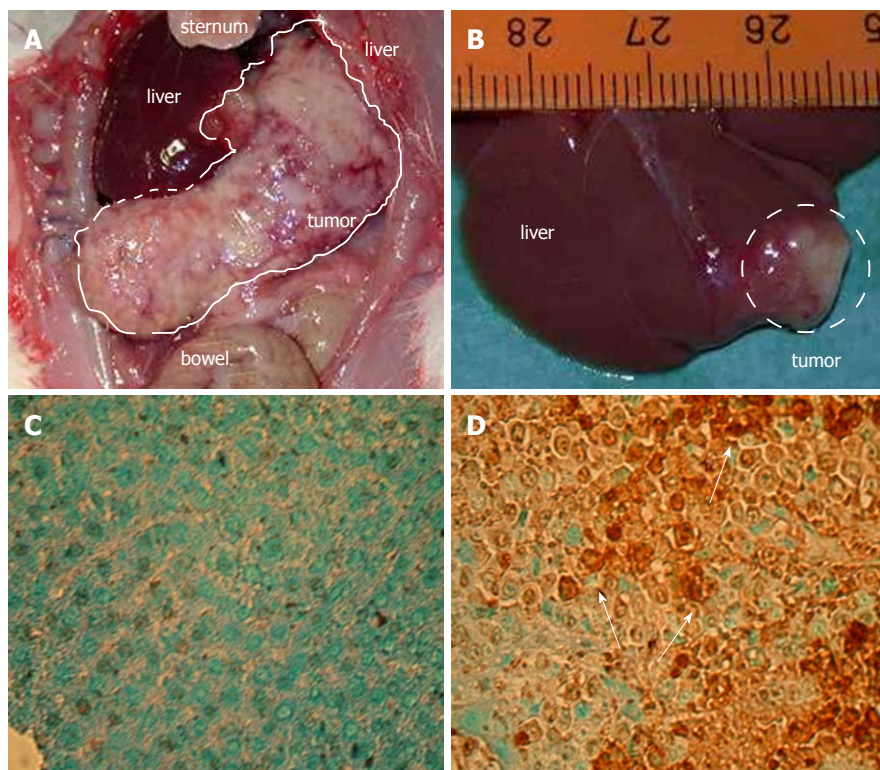
<sup>a</sup>*P* < 0.05 *vs* control 1; <sup>b</sup>*P* < 0.01 *vs* control 1; <sup>c</sup>*P* = 0.01 dual therapy *vs* quadruple therapy for TdT-mediated dUTP-biotin nick end labeling and proliferating cell nuclear antigen staining. PTK/ZK: PTK787/ZK222584; TAM: Tamoxifen; cRA: 9-*cis*-retinoic acid; PCNA: Proliferating cell nuclear antigen; TUNEL: TdT-mediated dUTP-biotin nick end labeling.

**DISCUSSION**

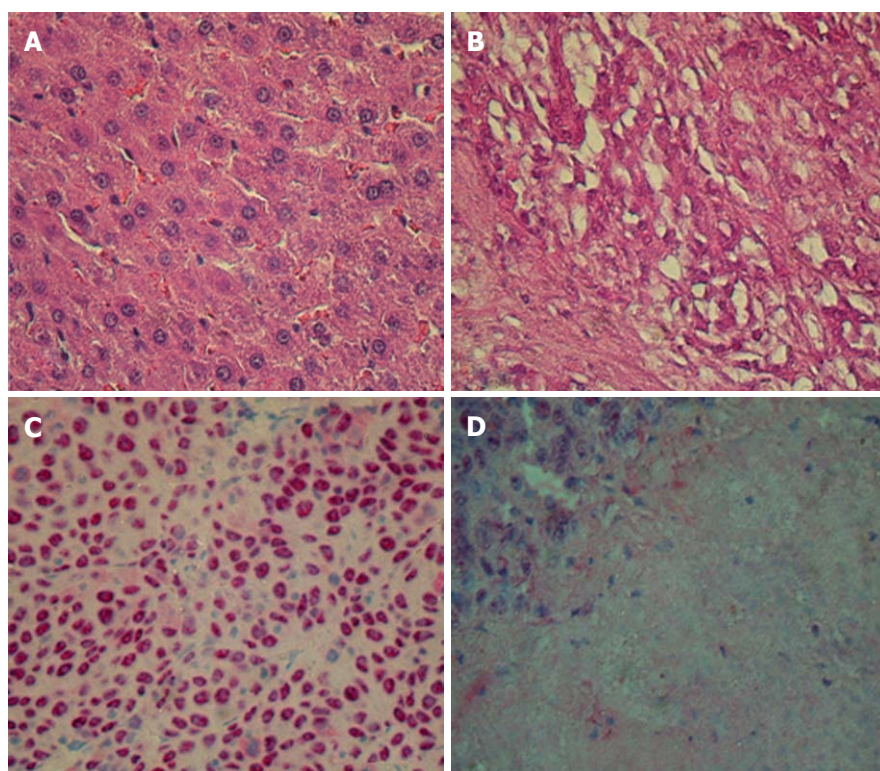
Extensive screening procedures and better imaging techniques have increased the mean survival time of HCC patients<sup>[2-4]</sup>. The curative or life-prolonging therapeutic options are based on tumor ablation *via* liver transplantation, resection or local instillation of heat or ethanol<sup>[3,4]</sup>. To date, sorafenib is the only systemic treatment option. Sorafenib acts *via* antiproliferative and antiangiogenic effects and is the proof of concept for systemic treatment in HCC. However, the achieved life-prolonging effect of a few months is a very limited benefit, and a wide range of side effects is induced<sup>[7-9]</sup>. The majority of patients have advanced-stage tumors and are beyond effective therapy when diagnosed<sup>[3,4]</sup>.

Basic science studies have shown that HCC develops distinct molecular changes and patterns. It has been hypothesized that a couple of mutations leads to a change from cirrhotic nodules to invasive carcinoma. Mutations are observed for the tumor suppressor gene p53 or for members of signal transduction pathways such as insulin receptor substrate-1 or β-catenin<sup>[2,20-22]</sup>. Different subtypes of HCC have been classified. Each subtype displays distinguishable genetic alterations and characteristics in clinical behavior<sup>[20-22]</sup>.

This may explain why systemic treatment with a single agent fails to be effective for HCC, and why some compounds display their antitumoral efficacy only in specific subgroups of HCCs. Therefore, combination therapy



**Figure 2** Macroscopic tumor growth and TdT-mediated dUTP-biotin nick end labeling assay. A: Control 1, large tumor volume. B: PTK787/ZK222584 (PTK/ZK) + MS-275, small tumor volume. C: Control 1, (mediated dUTP-biotin nick end labeling) TUNEL assay for apoptotic cells (dark brown). D: PTK/ZK + MS-275: TUNEL assay for apoptotic cells (dark brown).



**Figure 3** Hematoxylin-eosin and proliferating cell nuclear antigen staining. A: Control 1, hematoxylin-eosin staining. B: Quadruple therapy, disintegrating cells, necrosis. C: Control 1, proliferating cell nuclear antigen (PCNA) staining for proliferating cells. D: Quadruple therapy, PCNA staining, reduced number of cells, and large necrotic area.

should represent a possible treatment option as shown in other malignancies, such as colorectal cancer. We have shown that HDAC inhibitors combined with retinoids or conventional chemotherapeutic agents induce apoptosis and decrease growth of hepatoma cells in an additive manner<sup>[5,23]</sup>. Furthermore, we have confirmed that retinoids or TAM as monotherapy have no effect *in vivo*, while the combined agents are moderately effective<sup>[7]</sup>.

In the current setting, we evaluated combination therapy in a syngeneic rat hepatoma model. HCC is known to be highly vascularized and to produce a wide range of proangiogenic factors<sup>[4,10]</sup>. In an experiment by Yao *et al*<sup>[24]</sup>, 70% of resected HCC nodules showed increased expression of VEGF, which correlates with metastasis rate and poor prognosis. Therefore, we chose PTK/ZK, which selectively inhibits the tyrosine kinase domains of VEGF



receptors, platelet-derived growth factor receptors and c-KIT<sup>[12,18,24]</sup>. PTK/ZK is an accepted antiangiogenic partner in the treatment of colorectal cancer. In the preceding clinical evaluation only minor adverse effects occurred, such as headache, vertigo and arterial hypertension<sup>[12]</sup>.

Here, we observed a remarkable but nonsignificant effect with reduction of tumor burden. As expected, no animal was cured by monotherapy with PTK/ZK. Inhibition of angiogenesis does not reduce the tumor mass completely. Small aggregations of malignant cells can exist without a vessel system<sup>[11]</sup>, therefore, inhibition of angiogenesis can never represent a monotherapeutic option.

As a combination partner we chose MS-275, an HDAC inhibitor. HDAC inhibitors are known to change gene expression *via* hyperacetylation of histones, which are transcription-regulatory intranuclear proteins. Subsequently, upregulation of genes induces growth arrest and cell differentiation and maturation (e.g. p21, transforming growth factor  $\beta$  and gelsolin)<sup>[5,14-17]</sup>. Therefore, HDAC inhibitors may be of value in antitumoral therapy, as shown *in vitro* and *in vivo*<sup>[14]</sup>. MS-275 has shown acceptable results in phase I trials and has proceeded to phase II evaluation<sup>[16,25]</sup>.

In the current experimental setting, the single agent MS-275 showed significant antitumoral effects. Combination with PTK/ZK induced an excellent reduction of tumor volume in this aggressive tumor model, which was even highly significant when compared to the effects of the single agents. Histological evaluation showed necrotic areas as a sign of tumor destruction. An unproved explanation could be an increase of toxic radicals and reduced oxygen supplementation. PCNA staining and TUNEL assay confirmed the superiority of dual therapy. This supports the hypothesis that combination therapy exceeds the efficacy of monotherapy significantly and should be further evaluated.

Since HDAC inhibitors are known to interfere with intracellular retinoid and estrogen receptors and to enhance their antiproliferative effects at least *in vitro*<sup>[4,5,21]</sup>, we decided to evaluate triple and quadruple therapy. The combination of PTK/ZK + MS-275 + TAM did not increase the macroscopic antitumoral effect compared to dual therapy.

Quadruple therapy (PTK/ZK + MS-275 + TAM + CRA) induced a slight, but nonsignificant benefit in tumor volume, while the results for PCNA staining and TUNEL assay were enhanced significantly. Additionally, HE staining revealed large necrotic areas in these tumor samples (after quadruple therapy). Similar results have been reported for VEGF and epidermal growth factor inhibition in other tumor entities (e.g. colorectal cancer), which did not reduce the absolute tumor volume, but increased the areas of necrosis within the tumor<sup>[11,12]</sup>. Unfortunately a 3D analysis of the necrotic tumor regions in untreated controls *vs* animals with single or combined treatment was not done in this study. We can only postulate a similar mechanism and recommend dynamic imaging (magnetic resonance imaging or computed tomogra-

phy) for the estimation of necrotic *vs* vital tumor regions in future studies. The same goes for the effects on angiogenesis: due to the proven antiangiogenic effect of PTK/ZK, we did not quantify the microvessel density. However, effects of certain histone deacetylases on the extracellular matrix have recently been shown<sup>[26]</sup>. Therefore, the changes in microvessel density after combination therapy compared to those with PTK/ZK monotherapy would be particularly interesting, and could explain the enhanced effects of combination therapy.

Analysis of the side effects showed diarrhea and loss of fur in animals treated with single agents, which was intensified by combined therapy. Subgroup analysis did not reach significance, but showed fewer side effects for dual therapy compared to triple and quadruple therapy. The observed loss of weight may be explained by diarrhea and the reduced amount of ascites after tumor treatment. Altogether, no single or combined treatment induced unacceptable side effects.

The relatively small number of animals in this study did not allow evaluation of the increased side effect profile *vs* the additional benefit of triple and quadruple therapy. Investigations with a higher number of animals and a longer treatment period are necessary to assess the benefit of this quadruple therapy *vs* dual therapy.

In summary, we showed that combination therapy is superior to monotherapy. At least in this rat model for HCC, PTK/ZK and MS-275 were highly effective, which justifies further investigation. The antitumoral effects were seen by macroscopic evaluation of tumor volume and evaluation of proliferation and apoptotic cells, which was especially marked in relation to decreasing tumor mass. The effects of triple and quadruple therapy need to be analyzed in further experiments. In the next step, the efficacy of dual therapy should be evaluated in different genetic, well-defined hepatoma models, which could possibly provide insight into the triggered pathways. If dual therapy (PTK/ZK and MS-275) is successful in this additional experimental setting, clinical development seems feasible.

## COMMENTS

### Background

The incidence of hepatocellular carcinoma (HCC) is increasing. Curative and life-prolonging therapeutic options for early tumor stages are resection, transplantation and interventional treatment. Sorafenib is the first systemic treatment option. However, the life-prolonging effect of sorafenib is limited to a few months. Effective systemic treatment for far-advanced hepatoma is still lacking.

### Research frontiers

Tumor cells show signs of dedifferentiation, reduced apoptosis, and an increase in proliferation rate. They induce angiogenesis and changes in the extracellular fibers. Biomodulators are directed against these tumor-cell-specific patterns. Histone deacetylase (HDAC) inhibitors change the expression of proliferation and apoptosis-inducing factors, and lead to normalization of protein expression in tumor cells. Vascular endothelial growth factor (VEGF) receptor antagonists reduce tumor-cell-induced neoangiogenesis and destruction of the extracellular matrix. The antitumoral efficacy of these biomodulators, such as HDAC inhibitors and VEGF receptor blockers, can be evaluated *in vitro* and *in vivo*. The authors showed that combination therapy was far superior to monotherapy *in vitro* (pro-



liferation rate, induction of apoptosis). However, to date, there are not sufficient data to prove this principle *in vivo*. We used a syngeneic rat model. Morris hepatoma cells were implanted into the liver. The endpoint was macroscopic tumor growth and microscopic changes in proliferation, apoptosis and chemotaxis.

### Innovations and breakthroughs

The authors evaluated monotherapy and combination therapy with four different agents: the HDAC inhibitor MS-275, the VEGF receptor blocker PTK787/ZK222584 (PTK/ZK), tamoxifen and retinoic acid. We reported significant antitumoral efficacy. Combined treatment was superior to the single agents. The side effect profile was acceptable even after combination therapy.

### Applications

Combination therapy should be compared to the gold standard sorafenib in an *in vivo* model. The agents have been well described, and the next step could be a phase I trial.

### Peer review

In this study, the authors examined the effects of combination therapy using PTK/ZK and MS-275 in a rat HCC model, and showed that combined therapy was highly effective. This study is significant because development of systemic chemotherapy for advanced HCC is an important subject.

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## Ketamine and midazolam sedation for pediatric gastrointestinal endoscopy in the Arab world

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### Abstract

**AIM:** To evaluate the safety and effectiveness of intravenous ketamine-midazolam sedation during pediatric endoscopy in the Arab world.

**METHODS:** A retrospective cohort study of all pediatric endoscopic procedures performed between 2002-2008 at the shared endoscopy suite of King Abdullah University Hospital, Jordan University of Science & Technology, Jordan was conducted. All children were > 1 year old and weighed > 10 kg with American Society of Anesthesiologists class 1 or 2. Analysis was performed in terms of sedation-related complications (desaturation, respiratory distress, apnea, bradycar-

dia, cardiac arrest, emergence reactions), adequacy of sedation, need for sedation reversal, or failure to complete the procedure.

**RESULTS:** A total of 301 patients (including 160 males) with a mean age of 9.26 years (range, 1-18 years) were included. All were premedicated with atropine; and 79.4% (239/301) had effective and uneventful sedation. And 248 (82.4%) of the 301 patients received a mean dose of 0.16 mg/kg (range, 0.07-0.39) midazolam and 1.06 mg/kg (range, 0.31-2.67) ketamine, respectively within the recommended dosage guidelines. Recommended maximum midazolam dose was exceeded in 17.6% patients [34 female (F):19 male (M),  $P = 0.003$ ] and ketamine in 2.7% (3 M:5 F). Maximum midazolam dose was more likely to be exceeded than ketamine ( $P < 0.001$ ). Desaturation occurred in 37 (12.3%) patients, and was reversible by supplemental oxygen in all except 4 who continue to have desaturation despite supplemental oxygen. Four (1.3%) patients had respiratory distress and 6 (2%) were difficult to sedate and required a 3rd sedative; 12 (4%) required reversal and 7 (2.3%) failed to complete the procedure. None developed apnea, bradycardia, arrest, or emergence reactions.

**CONCLUSION:** Ketamine-midazolam sedation appears safe and effective for diagnostic pediatric gastrointestinal endoscopy in the Arab world for children aged > 1 year and weighing > 10 kg without co-morbidities.

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**Key words:** Pediatric endoscopy; Sedation; Ketamine; Arab

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## INTRODUCTION

Although progress is being made in improving medical services in modern third world countries, limited financial resources are a critical concern. This environment necessitates effective but cost conscious approaches to medical care. Sedation for medical procedures is a potential area for intervention. For example, ketamine-based intravenous sedation has proven effective in suboptimal circumstances, such as the wartime battlefield, avoiding the need for general anesthesia<sup>[1-2]</sup>.

Endoscopic procedures are frequently required for the diagnosis and treatment of gastrointestinal diseases in children. Since such procedures can cause considerable anxiety and distress, many children find the procedures worse than disease itself.

The goal of sedation is to provide a patient who is only lightly sedated, cooperative on demand, free from anxiety and amnesic after the procedure<sup>[3]</sup>. It must have a rapid onset, short duration of action, and should be safely administered by a non-anesthesiologist without significantly increased risk of potential complications<sup>[4]</sup>. Unfortunately, there is no ideal sedation protocol for gastrointestinal (GI) endoscopy that is agreed upon by pediatric gastroenterologists as confirmed by a recent survey by the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN)<sup>[5]</sup> and by another survey by the Francophone Pediatric Hepatology, Gastroenterology and Nutrition Group<sup>[6]</sup>. It appears that there is a wide variety of sedation techniques used by practicing pediatric gastroenterologists.

Although ketamine-based sedation provides many of the desired effects of an ideal sedative, it is not widely used in pediatric gastrointestinal endoscopy. There is limited published research regarding its efficacy and safety, particularly in developing countries. The aim of this study is to assess the safety and efficacy of ketamine + midazolam sedation for GI endoscopy in pediatric patients in Jordan.

## MATERIALS AND METHODS

A retrospective cohort study of all pediatric endoscopic procedures done under intravenous sedation by a combination of ketamine and midazolam over a period of six years (August 1st, 2002 - July 31st, 2008) done at the endoscopy suite of King Abdullah University Hospital (affiliated with Jordan University of Science and Technology) in Jordan was conducted.

All procedures were performed in the endoscopy suite which is shared with adult gastroenterologists and pulmonologists and a part of a general university hospi-

tal. All procedures were performed by a single pediatric gastroenterologist. A registered nurse provided constant patient monitoring (before, during and after the administration of sedatives) including continuous cardiac monitoring, respiratory rate, pulse oximetry and blood pressure monitoring. During the study period, the same gastroenterologist performed all the procedures, calculated and administered the sedative medications. The operating physician and nursing staff assessed the adequacy of sedation and documented all the above. The operating physician is Pediatric Advance Life Support certified.

Pre-sedation risk assessment included a detailed history and complete physical examination, review of current medications and drug allergies as well as an assessment of the cardiopulmonary status. All patients in the study were at American Society of Anesthesiologists (ASA) class 1 or 2. Procedures in patients with a higher ASA class were excluded from the study and performed under general anesthesia<sup>[7,8]</sup>. General anesthesia was used in the following groups of patients: (1) children below the age of one year or weighing less than 10 kg; (2) patients underwent the therapeutic endoscopy (e.g. esophageal dilation, variceal treatment and polypectomy) under general anesthesia for their safety, the need to be motionless during the procedure as these procedures are typically associated with more pain and discomfort; (3) patients with known neurologic disorders (seizures), developmental delay (cerebral palsy), and psychiatric disorders (phobia); (4) increased intracranial pressure (hydrocephalus); and (5) patients known to have abnormal anatomy of the upper airways (Pierre Robin sequence).

All patients were premedicated with atropine at a dose of 0.01 mg/kg - 0.02 mg/kg (a minimum dose of 0.1 mg to avoid paradoxical bradycardia, and a maximum dose of 0.4 mg). Standard dosage guidelines for sedatives are: midazolam 0.05 mg/kg - 0.20 mg/kg and ketamine 0.5 mg/kg - 2.0 mg/kg used at each dose<sup>[4,8]</sup>. Midazolam was always administered first.

In our study, three types of endoscopic procedures were used: upper (U), lower (L), and combined upper and lower (U&L). If an upper and lower endoscopy was performed in the same sedation session, they were counted as a single sedation session. If the same procedure was repeated at a later time, it was counted as a separate sedation session.

Analysis of the data included demographic details (age, gender) weight, procedure (s) performed, doses of each medication/kg body weight, effectiveness of sedation, need for other sedatives, side effects and complications.

Sedation-related complications were defined as a drop in oxygen saturation to equal or less than 94%, respiratory distress (stridor or wheezes), apnea, bradycardia, cardiac arrest and emergence reactions. The operating gastroenterologist and nursing staff assessed the adequacy of sedation; this was defined as lack of agitation, ability to complete the procedure comfortably, and no need to add other sedatives. The need of an antidote for reversal medications was also included.



**Table 1** Types of endoscopic procedures

	<i>n</i> (%)	Males	Females	Age range (yr)	Mean age (yr)
Upper (U)	218 (72.4)	106	112	1-18	8.86
Lower (L)	16 (5.3)	14	2	1-16	9.09
U & L	67 (22.3)	40	27	2-18	10.89
Total	301	160	141		

**Table 2** Midazolam and ketamine doses

	Midazolam doses			Ketamine doses		
	All patients	Males	Females	All patients	Males	Females
Min dose (mg/kg)	0.07	0.07	0.07	0.31	0.31	0.46
Max dose (mg/kg)	0.39	0.32	0.39	2.67	2.00	2.67
Mean dose (mg/kg)	0.16	0.15	0.16	1.06	1.03	1.08

**Table 3** Sedative doses

	Midazolam doses (mg/kg)			Ketamine doses (mg/kg)		
	Min	Max	Average	Min	Max	Average
Upper (U)	0.07	0.39	0.15	0.33	2.67	1.02
Lower (L)	0.07	0.32	0.17	0.60	1.84	1.12
U & L	0.08	0.27	0.18	0.31	2.11	1.17

**Table 4** Distribution of patients receiving doses exceeding recommended max dose

Procedure	Midazolam		Ketamine	
	<i>n</i> (%)	% per specific procedure	<i>n</i> (%)	% per specific procedure
Upper (U)	30 (57)	14	4 (50)	1.8
Lower (L)	5 (9)	31	0 (0)	0
U & L	18 (34)	27	4 (50)	6
Total	53 (17.6)		8 (2.7)	

**Table 5** Sedation failure

Sedative-related complications	<i>n</i> (%)
Desaturation < 94% in RA	37 (12.3)
Desaturation < 94% on supplemental O <sub>2</sub>	4 (1.3)
Respiratory distress	4 (1.3)
Apnea	0 (0.0)
Bradycardia	0 (0.0)
Cardiac arrest	0 (0.0)
Emergence reaction	0 (0.0)
Difficult to sedate/third medication	6 (2.0)
Need for reversal medications	12 (4.0)
Need for overnight stay	1 (0.3)
Failure to complete the procedure	7 (2.3)

RA: Ruba abdelhadi.

Failure of sedation was defined as: (1) The occurrence of sedative-related complications: (a) Oxygen desaturation < 94%; (b) Respiratory distress wheezes or

stridor; (c) Apnea; (d) Bradycardia; (e) Cardiac arrest; and (f) Emergence reactions; (2) Difficult to sedate, as judged by the physician or nursing staff, requiring a third sedative medication; (3) The need for reversal medications; (4) Need for overnight stay because of sedation-related issues; and (5) Failure to complete the procedure.

## RESULTS

A total of 560 procedures were performed over the study period (August 1st, 2002 - July 31st, 2008), 12 patients were excluded because of incomplete medical records. Of the 548, 247 were performed under general anesthesia, and 301 were done utilizing ketamine + midazolam. All 301 patients included in the study who had conscious sedation received combined midazolam and ketamine in addition to atropine. The sedatives were given in small boluses and titrated to achieve the desired effect. Not infrequently, patients required extra doses during the procedure; this was especially noted in longer procedures. For dosage calculations, we used the cumulative dose.

There were 160 males and 141 females (1.13:1), age ranged from 1 to 18 years, with a mean age of 9.26 years in males and 10 years in females. Among the three types of endoscopic procedures, upper endoscopy was the most frequently performed procedure (218 patients or 72.4%) followed by combined upper and lower (67 patients or 22.3%). Details are shown in Table 1.

The average dose of midazolam used in all procedures was 0.16 mg/kg, (range, 0.07 mg/kg - 0.39 mg/kg), while the average dose of ketamine was 1.06 mg/kg (range, 0.31 mg/kg - 2.67 mg/kg) (Table 2). There was no statistically significant difference in the average dose used between males and females for either of the two medications.

Analysis of sedative dosage according to the type of procedure is shown in Table 3. In general, patients require similar doses of sedatives regardless of the type of procedure.

Most patients received a dose within the recommended dosage guidelines of both medications (248 patients or 82.4%). The maximum dose for either medication was exceeded in 53 patients (17.6%) (Table 4).

The recommended maximum dose of midazolam was exceeded in 53 (17.6%) patients (19 M;34 F), which was more likely to be exceeded in females ( $P = 0.003$ ). The recommended maximum dose of ketamine was exceeded in only eight patients (2.6% of all patients) (3 M;5 F). The dose of midazolam was more likely to be exceeded than ketamine ( $P < 0.001$ ).

Maximum dose of midazolam was significantly exceeded in combined upper and lower endoscopic procedures when compared to upper endoscopies (27% *vs* 14%;  $P = 0.02$ ). Maximum midazolam dose was also exceeded more in lower endoscopic procedures when compared to upper endoscopies (31% *vs* 14%), but the limited number of lower endoscopic procedures precluded sta-

**Table 6** Effects of midazolam and ketamine dosing on development of desaturations

Procedure	Midazolam average dose (mg/kg)			Ketamine average dose (mg/kg)		
	Patients with desaturation	Patients without desaturation	P value	Patients with desaturation	Patients without desaturation	P value
Upper (U)	0.26	0.14	< 0.001	2.2	0.86	< 0.001
Lower (L)	NA	0.17	NA	NA	1.12	NA
U & L	0.24	0.168	< 0.001	2	1.01	< 0.001
n	37	264	NA	37	264	NA

NA: Not available.

tistical significance ( $P = 0.126$ ). The maximum ketamine dose was also exceeded more in combined upper and lower endoscopies (6% *vs* 1.8%), but was not statistically significant ( $P = 0.170$ ).

### Sedation failure

Two hundred and thirty-nine patients (79.4%) had effective and uneventful sedation. Sedation failure is summarized in Table 5.

**Sedative-related complications:** (1) Desaturation < 94% in room air occurred in 37 (12.3%) patients, (26 U, 11 U&L, none of L). The average doses of both medications were higher than the maximum dose. The patients received a higher dose of both medications in comparison with the patients who did not develop desaturation; the difference was statistically significant (Table 6). Oxygen by nasal cannula was administered to these patients, and normal saturation was achieved in 33/37 (89%). In four patients, the oxygen saturation did not improve and the procedure was terminated and rescheduled under general anesthesia later; and (2) respiratory distress (stridor or wheezes) developed in four patients (1.3%) after termination of procedure. All four were U, one recovered spontaneously while the other three required Albuterol (Ventolin) nebulizer treatment. None of the patients who developed respiratory distress exceeded the recommended dose of either medication, and none of them developed desaturation. No apnea, bradycardia, cardiac arrest or emergence reactions occurred in any patient.

**Difficult to sedate:** As judged by the physician or nursing staff, six patients (2%) (3 M, 3 F) required a third sedative medication and meperidine was given. Five were U&L, and one was L. The maximum dose of midazolam was exceeded in four but none of them exceeded the maximum dose of ketamine.

**Need for reversal medications:** During recovery, 12 (4.0%) patients were judged to be excessively sedated and required reversal of benzodiazepines using flumazenil (3 U, 8 U&L, 1 L). None of them had desaturation or respiratory distress. The average dose of midazolam in those patients was 0.23 mg/kg (range, 0.18 mg/kg - 0.27 mg/kg). The average dose of ketamine in those patients was 1.4 mg/kg (range, 0.80 mg/kg - 2.11 mg/kg).

**Need for overnight stay:** One.

**Failure to complete the procedure:** Seven (2.3%) patients failed to complete the procedure. Four patients had desaturation despite oxygen supplementation and all were U. The other three did not complete the procedure because of lack of cooperation of the patient.

## DISCUSSION

Intravenous ketamine + midazolam sedation for gastrointestinal endoscopy is safe and effective in most patients. Routine use of general anesthesia for endoscopic procedures is not necessary, which increases cost and is often not readily available in some developing countries. According to a recent NASPGHAN survey, 23% of the respondents described the difficulties and inconvenience in the process of scheduling a procedure in the operating room<sup>[5]</sup>. Only half (55%) reported their endoscopy suites with general anesthesia equipment. In developing countries, cost is a detrimental factor. To the best of our knowledge, this is the first study that documents the safety and effectiveness of ketamine + midazolam sedation for pediatric gastrointestinal endoscopy in an Arab country.

Ketamine is a non-barbiturate dissociative agent with a rapid onset of action (peak intravenous concentrations occur within one minute) that induces profound sedation, analgesia and amnesia, with a short duration of action (15-30 min) which is adequate for routine diagnostic endoscopy, allowing fast recovery<sup>[9,10]</sup>. It induces functional dissociation between the limbic and the cortical systems. This cataleptic state impairs sensory recognition of painful stimuli and memory inducing a state referred to as "dissociative anesthesia"<sup>[9]</sup>. Protective airway reflexes are maintained during sedation with ketamine, with minimal cardiovascular and respiratory side effects. This paramount advantage over other categories of sedatives (narcotics) lies in maintaining airway reflexes with minimal cardiovascular and respiratory side effects<sup>[11]</sup>.

The high therapeutic index of ketamine makes it useful in children with less predictable response to sedatives<sup>[12]</sup>. This might explain the low incidence of sedative-related complications in our study.

While midazolam has been used in procedural sedation in children extensively, ketamine has not. Mid-

azolam provides sedation and amnesia but it lacks any analgesic effect. The analgesic properties of small-dose ketamine have been rediscovered. Available data strongly suggest that the preemptive administration of ketamine can have profound effects on postoperative analgesic requirements with minimal risk and side effects<sup>[13,14]</sup>. The use of ketamine for procedural (endoscopic and other procedures) sedation is increasing in the developed countries<sup>[15]</sup>. There are only a handful of studies that looked at ketamine's value in the endoscopic sedation in children<sup>[13,16]</sup> but none of them in developing countries.

A prospective randomized study by Varadarajulu *et al*<sup>[17]</sup> evaluated the use of ketamine for endoscopic procedures and concluded that ketamine is a useful adjunct to conscious sedation in patients who are difficult to sedate. Its use results in better quality and depth of sedation with shorter recovery than in patients sedated using benzodiazepines and meperidine alone. This was confirmed in our study as patients who did not respond to midazolam + ketamine did not benefit from adding meperidine.

Endoscopy was completed in 97.7% (294/301) of our study patients, confirming that midazolam-ketamine is an effective sedation for such procedure. A third medication was mainly needed in the combined procedures (U&L); the ketamine dose could be increased rather than adding an additional sedative. It may be more prudent to maximize ketamine dose before adding a third medication. For example, five of the six patients who were difficult to sedate by the endoscopist and nursing staff, remained inadequately sedated even after adding a third sedative.

Respiratory distress (stridor or wheezes) was rare (1.3%). This low rate of respiratory distress could be explained by the fact that both ketamine and atropine have a bronchodilator effect which adds another advantage in the pediatric age group where reactive airway disease is common.

Like any other sedatives, the sedative response to ketamine is not uniform and may be unpredictable; hence it is prudent to increase a small dose slowly, titrating to the desired effect (typically horizontal nystagmus). Dosing of ketamine has a wide safety margin. Reported unintentional administration of overdoses (up to ten times that of recommended dosage) has been followed by prolonged but complete recovery<sup>[1,12,18,19]</sup>.

Bradycardia is a known side effect of sedatives and may be further augmented by vagal stimulation during upper endoscopy. The lack of bradycardia in our patients could be related to the fact that all patients received atropine prior to the procedure<sup>[20]</sup>.

One of the major drawbacks of ketamine is the occurrence of emergence reactions (psychological manifestations vary in severity between pleasant dream-like state, vivid imaginary, hallucinations and emergent delirium). None of our patients developed any of these reactions. The explanation for that is probably multifactorial. First, emergence reactions are more common in adults than in children. Second, this phenomenon is more common in

patients known to have psychiatric or neurological disorders. In our study, those were excluded and their endoscopies were done under general anesthesia. Third, these reactions are usually more pronounced if ketamine is used alone, in large doses and if rapidly administered<sup>[21]</sup>. All our patients received a combination of midazolam and ketamine; both medications were given slowly and in small boluses. They were observed for the development of emergence reactions for at least 2 h after the procedure. Our findings are supported by a recent study by Gilger *et al*<sup>[9]</sup>. In that study, the authors concluded that emergence phenomena were more common in those not receiving ketamine, and suggested that true ketamine-associated emergence phenomena are either rare or that midazolam does reduce the frequency of emergence reactions<sup>[9]</sup>.

The biggest worry of the endoscopist administering ketamine is the lack of an antidote<sup>[12]</sup>. One reassuring fact about ketamine is its short duration of action of 15-30 min<sup>[1]</sup>. If a patient develops an unexpected adverse reaction, he can be managed by supportive care until the drug effect wears off. This was supported by our findings among the 301 patients; only one child required an overnight stay for observation due to over-sedation. In this particular patient, the recommended doses of midazolam and ketamine were exceeded and required meperidine.

Midazolam + ketamine is not routinely used in children at KAUH outside the operating room. This "procedural sedation phobia" by non-anesthesiologists is noted among other medical institutions in other parts of the world as there is still significant resistance to pediatric sedation techniques used outside the operating room by non-anesthesiologists, as reported in a recent study by Krauss *et al*<sup>[15]</sup>. The lack of familiarity with ketamine significantly affects the comfort level of the physicians and nursing staff and brings more hesitation to use it outside the operating room by non-anesthesiologists.

In conclusion, ketamine + midazolam is a safe and effective sedative regimen for diagnostic pediatric GI endoscopy in the Arab world for children over the age of one year and weighing more than 10 kg without comorbidities. Side effects of hypoxia and respiratory distress are uncommon. None of our patients developed a serious complication (apnea, bradycardia, cardiac arrest, or emergence psychosis). Pre-sedation risk assessment and proper patient evaluation and selection are of paramount importance and cannot be over-emphasized to minimize potential complications.

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## COMMENTS

### Background

Endoscopic procedures are frequently required for the diagnosis and treatment



of gastrointestinal diseases in children. Such procedures can cause considerable anxiety and distress, and many children find the procedures worse than disease itself. Ketamine-based sedation is not widely used in pediatric gastrointestinal endoscopy. There is limited published research regarding its efficacy and safety, particularly in developing countries.

### Research frontiers

Ketamine-based intravenous sedation has been studied and proven to be effective when used in the emergency room for children requiring painful interventions.

### Innovations and breakthroughs

This research focuses on the endoscopic sedation in a pediatric population using ketamine-based sedation avoiding the need for general anesthesia.

### Applications

About 80% of children had effective and uneventful sedation using intravenous midazolam and ketamine. Side effects were uncommon and reversible, and there was no mortality associated with this type of sedation. This should encourage more clinicians to use this type of sedation, thus avoiding general anesthesia for pediatric endoscopic procedures.

### Peer review

This research confirms the safety and efficacy of ketamine-based sedation for endoscopic procedures in a third world country.

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## IL28B polymorphisms associated with therapy response in Chilean chronic hepatitis C patients

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### Abstract

**AIM:** To analyze the association of three IL28B single nucleotide polymorphisms with response to therapy in Chilean patients infected with hepatitis C virus (HCV).

**METHODS:** We studied two groups of patients with chronic HCV infection (genotype 1), under standard combined treatment with pegylated interferon plus ribavirin. One group consisted of 50 patients with sustained virological response, whereas the second group consisted of 49 null responders. In order to analyze the IL28B single nucleotide polymorphisms rs12979860, rs12980275 and rs8099917, samples were used for polymerase chain reaction amplification, and the genotyping was performed by restriction fragment length

polymorphism.

**RESULTS:** The IL28B rs12979860 CC, rs12980275 AA and rs8099917 TT genotypes were much more frequently found in patients with sustained virological response compared to null responders (38%, 44% and 50% vs 2%, 8.2% and 8.2%, respectively). These differences were highly significant in all three cases ( $P < 0.0001$ ).

**CONCLUSION:** The three IL28B polymorphisms studied are strongly associated with sustained virological response to therapy in Chilean patients with chronic HCV (genotype 1).

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**Key words:** IL28B; Hepatitis C virus; Chile; Pegylated interferon; Ribavirin

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### INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is a global health problem that affects more than 170 million people worldwide, with 3-4 million new cases each year<sup>[1]</sup>. Most (70%-80%) HCV infections persist, and about 30% of individuals with a persistent infection develop chronic liver diseases, including cirrhosis, and hepatocellular carcinoma<sup>[2]</sup>.

The most effective current standard therapy for chronic HCV infection consists of subcutaneous injections of long-acting pegylated interferon- $\alpha$  (PEG-IFN) plus oral treatment with ribavirin (RIB). This therapy, however, yields a sustained virological response (SVR) in only 40%-50% of patients who are infected with HCV genotype 1, the most common viral genotype<sup>[3]</sup>. In Chile, HCV genotype 1 is also the most prevalent<sup>[4]</sup>. Thus, since a significant number of patients will fail to respond, or will experience significant side-effects, the identification of host and viral determinants predicting virologic response is of major interest.

Recently, three independent research groups have reported the results of separate genome-wide association studies (GWAS), supporting the association of SVR in HCV genotype 1 with single nucleotide polymorphisms (SNPs) near the gene region IL28B encoding interferon lambda 3. In the first study, performed with European-American, African-American, and Hispanic individuals, the rs12979860 SNP was most strongly associated with SVR, which is located 3 kilobases upstream of the *IL28B* gene. The minor allele (T) was associated with a lower rate of SVR (26% in those with genotype TT and 79% in those with genotype CC)<sup>[5]</sup>. In the second study, carried out with 293 Australian patients, a significant association between the SNP rs8099917 and SVR was found. This was further validated by an independent cohort of 555 European individuals. From 392 patients who achieved SVR, 247 (63%) were homozygotes for the allele T, which was significantly higher than genotype GG (SVR of 3.8%)<sup>[6]</sup>. Similar findings were also reported in a Japanese study. Results of a GWAS showed a significant association between treatment response with two SNPs (rs12980275 and rs8099917), both located in the IL28B gene region, with the latter being the same SNP found by Australian researchers. In this case, for the SNP rs8099917, the G allele was associated with a significantly lower SVR (0% for genotype GG and 78% for genotype TT). For the SNP rs12980275, homozygotes for the allele A had a SVR rate of 85%, which was significantly higher than genotype GG<sup>[7]</sup>.

The aim of this study was to investigate the association between these three IL28B polymorphisms and the virological response in treatment-naïve Chilean patients infected with HCV genotype 1, which is the most prevalent viral isolate within Latin-American populations.

## MATERIALS AND METHODS

### Patient samples

The present study involved serum samples collected (January 2002-July 2010) at the Clinical Hospital University of Chile (Santiago, Chile) from 99 Chilean patients with chronic HCV infection. Patients who received at least 80% of the recommended dose of PEG-IFN $\alpha$ 2a and RIB were considered assessable for response to treatment. We included 50 patients that achieved SVR (defined as an

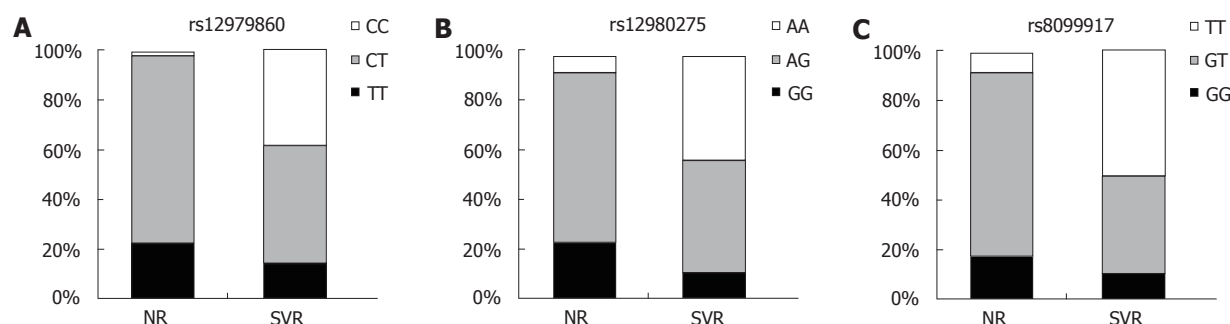
undetectable HCV RNA in serum more than 24 wk after treatment termination), and 49 null responder patients (NR) (defined as those who did not achieve an early virological response, < 2 log<sub>10</sub> decrease in viral load at week 12 of treatment). Among these patients, no co-infections with human immunodeficiency virus or hepatitis B virus were included. Both groups of patients were similar in terms of basal viral load, age, gender, and clinical degree of the liver disease. This study was approved by the ethics committee of the Clinical Hospital University of Chile (protocol number 394/10).

### Genotyping of SNPs rs12979860, rs12980275, rs8099917, and RFLP analyses

Genomic DNA was prepared from peripheral blood lymphocytes. The rs12979860, rs12980275, and rs8099917 SNPs genotyping was carried out by polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP). For rs12979860, oligonucleotide primers were: 5'- AGG GCC CCT AAC CTC TGC ACA GTC T -3' (sense), and 5'- GCT GAG GGA CCG CTA CGT AAG TCA CC -3' (antisense). For rs12980275, primer sequences were: 5'- GAG AGC AAG AGG AGG GAA GGA A -3' (sense), and 5'- GTG TGC CAT TAG CCA GTC AGA T -3' (antisense). For rs8099917, oligonucleotide primers were: 5'- TTC ACC ATC CTC CTC TCA TCC CTC AT -3' (sense) and 5'- TCC TAA ATT GAC GGG CCA TCT GTT TC -3' (antisense). PCR reaction conditions (30  $\mu$ L) were: initial denaturation at 94 °C for 10 min, followed by 40 cycles of: denaturation at 94 °C for 1 min, annealing at 58 °C for 40 s, and extension at 72 °C for 1 min. The PCR product for rs12979860, rs12980275 and rs8099917 was of 403, 441 and 401 base pairs, respectively.

In order to perform RFLP assay for the rs12979860 genotype, 20  $\mu$ L of amplicons were digested with 5U of *Bst* I restriction endonuclease (New England Biolabs, MA, United States) at 60 °C for 2 h. *Bst* I digestion of allele CC yields fragments of 184, 105, 89 and 25 base pairs, whereas DNA containing the allele TT polymorphism yields fragments of 184, 130 and 89 base pairs. For the RFLP assay for the rs12980275 genotype, 20  $\mu$ L of amplicons were digested with 5U of *Bst* I restriction endonuclease (New England Biolabs, MA, United States) at 55 °C for 2 h. *Bst* I digestion of allele AA yields fragments of 121 and 320 base pairs, whereas DNA containing the allele GG polymorphism yields fragments of 121, 30 and 290 base pairs. For the RFLP assay for the rs8099917 genotype, 20  $\mu$ L of amplicons were digested with 1U of *Mae* III restriction endonuclease (Roche Molecular Systems, Branchburg, NJ, United States) at 55 °C for 2 h. *Mae* III digestion of allele TT yields fragments of 105, 110 and 186 base pairs, whereas DNA containing the allele GG polymorphism yields fragments of 105, 110, 39 and 147 base pairs. Restriction digestion products for each were separated on agarose gels stained with ethidium bromide for visualization on a UV transilluminator.





**Figure 1** Distribution of *IL28B* single nucleotide polymorphisms by response to combined therapy with pegylated interferon- $\alpha$  plus oral treatment with ribavirin, in Chilean patients with chronic hepatitis C (genotype 1). A: rs12979860 genotype; B: rs12980275 genotype; C: rs8099917 genotype. SVR: Sustained virological response ( $n = 50$ ); NR: Null responders ( $n = 49$ ).

### Statistical analysis

Genotypic frequencies were obtained by direct counting, and statistical analysis was performed by the  $\chi^2$  test [calculated on  $2 \times 2$  contingency tables, assuming a recessive model (CC *vs* CT + TT for rs12979860; AA *vs* AG + GG for rs12980275; TT *vs* GT + GG for rs8099917)]. *P* values less than 0.05 were considered statistically significant.

## RESULTS

In the current study, results from all three recently known *IL28B* polymorphisms influencing the therapy response against HCV, rs12979860, rs12980275 and rs8099917, were available for all Chilean patients with SVR and NR, as shown in Figure 1. For the rs12979860 genotype (Figure 1A), the homozygous CC was found in 19 of 50 patients with SVR, *vs* 1 of 49 in NR patients ( $P < 0.0001$ ). The proportion of patients with the rs12979860 CC, CT and TT genotypes was 38%, 48% and 14%, respectively, in those with SVR. In NR patients, this proportion was 2%, 76% and 22%, respectively. For the rs12980275 genotype, the homozygous AA was found in 22 of 50 cases with SVR, *vs* 4 of 49 in patients NR ( $P < 0.0001$ ). The proportion of patients with the rs12980275 AA, AG and GG genotypes was 44%, 46% and 10% in those with SVR. In NR patients, this proportion was 8.2%, 69.4% and 22.4%, respectively, as indicated in Figure 1B. For the rs8099917 genotype, as shown in Figure 1C, the homozygous TT was found in 25 of 50 patients with SVR, *vs* 4 of 49 in patients NR ( $P < 0.0001$ ). The proportion of patients with rs8099917 TT, GT and GG genotypes was 50%, 40% and 10% in those with SVR. In patients NR, this proportion was 8.2%, 75.5% and 16.3%, respectively.

## DISCUSSION

Throughout the results shown herein, we have confirmed that the three recently identified genetic polymorphisms in the interferon  $\lambda 3$  gene region are strongly associated with the response to treatment with PEG-IFN/RIB in Chilean patients infected with HCV genotype 1. More-

over, our current study also represents the first analysis of these SNPs from Latin-American regions, where the genotype 1 of HCV is the most prevalent.

The significant genetic results on common *IL28B* polymorphisms with respect to treatment response in individuals with chronic hepatitis C infection may open the possibility of a personalized medicine for the treatment of this progressive disease. Further studies are now required to determine whether patients infected with genotype 1 of HCV, and bearing a favorable SNP, will benefit or not from a shorter treatment duration with the current therapy scheme. This might reduce the cost and side effects associated with longer term treatment<sup>[8]</sup>. The way in which SNP responder genotypes influence the outcomes of anti-viral strategies including those based upon protease and polymerase inhibition, requires immediate investigation. Understanding the clinical implications of these findings will be a major research goal for the immediate future.

## COMMENTS

### Background

The current standard therapy for chronic hepatitis C virus (HCV) infection genotype 1 consists of pegylated interferon alfa plus ribavirin for a period of 48 wk. This regimen, however, yields a sustained virological response in only 40%-50% of patients. Because a significant number of patients will fail to respond or will have significant side effects, it is of major interest for both patient care and economic approach to predict non response.

### Research frontiers

Recently, several independent research groups have reported results of genome-wide association studies, supporting the association of sustained virological response in HCV genotype 1 with single nucleotide polymorphisms (rs12979860, rs12980275 and rs8099917) near the gene region *IL28B*, encoding interferon lambda 3.

### Innovations and breakthroughs

This study represents the first analysis of these well-known *IL28B* polymorphisms in patients with chronic hepatitis C infection from Latin-American regions, where genotype 1 is the most commonly found. The report shows that the three *IL28B* polymorphisms are associated with the sustained virological response in Chilean patients treated with standard therapy.

### Applications

This study may contribute to better treatment strategies of hepatitis C. Genotyping of these *IL28B* polymorphisms will aid clinical decisions, improve current standard of care, and potentially lead to the integration of other agents in the future, providing an opportunity for clinicians to individualize treatment regimens

for hepatitis C patients.

### Peer review

The authors investigated the association between genetic IL28B polymorphisms, which encode interferon lambda 3, with the response to the treatment against hepatitis C with standard combined therapy: pegylated interferon- $\alpha$  plus ribavirin. They described that the rs12979860 CC, rs12980275 AA and rs8099917 TT genotypes were much more frequently found in patients with sustained virological response compared to null responder patients. This study may contribute to better treatment strategies for hepatitis C.

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## A nested case-control study of maternal-neonatal transmission of hepatitis B virus in a Chinese population

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**Author contributions:** Chen LZ designed research and wrote the paper; Zhou WQ, Zhao SS and Liu ZY performed research and analyzed data; Wen SW revised and participated in the editing of the manuscript; all authors read and approved the final manuscript.

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positive mother was matched by hospital at birth (same), gender (same), and date of birth (within 1 mo). A face-to-face interview was conducted to collect clinical and epidemiological data. Conditional logistic regression analysis was used to estimate the independent effects of various determinants on maternal-neonatal transmission of HBV.

**RESULTS:** A total of 141 HBsAg-positive infants and 141 individually matched HBsAg-negative infants were included in the final analysis. Maternal first-degree family history of HBV infection, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas systematic treatment and HBV immunoglobulin injections for mothers with HBV infection were protective factors for maternal-neonatal transmission of HBV, after adjustment for potential confounding factors.

**CONCLUSION:** For HBsAg-positive mothers, systematic treatment, HBV immunoglobulin administration, and controlling intrahepatic cholestasis and pregnancy complications may reduce the incidence of perinatal transmission of HBV.

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**Key words:** HBsAg-positive; Hepatitis B virus; Perinatal transmission; Nested case-control study

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### Abstract

**AIM:** To examine the determinants of maternal-neonatal transmission of hepatitis B virus (HBV).

**METHODS:** A nested case-control study was conducted in Changsha, Hunan, People's Republic of China from January 1, 2005 to September 31, 2006. To avoid potential maternal blood contamination, we collected vein blood of newborns immediately after birth and before initial hepatitis B vaccination to determine the HBV infection status of the newborn. For each HBsAg-positive infant, one HBsAg-negative infant born to an HBsAg-



## INTRODUCTION

China has a high incidence of hepatitis B infection. The positive rate of serum hepatitis B surface antigen (HBsAg) is about 10%-15%, and accumulated hepatitis B virus (HBV) infection rate is 60%-70% in China<sup>[1-3]</sup>. The reported positive rate of serum HBsAg in Chinese pregnant women varies from 5.9% to 21.3%<sup>[4,5]</sup>. Perinatal transmission is the most important vertical transmission route of chronic infection by HBV. About one third of HBV infections are through perinatal transmission, and mostly occur in asymptomatic carriers<sup>[6]</sup>. There are three ways to realize perinatal transmission of HBV: (1) **intrauterine transmission**; (2) **labor transmission**; and (3) **postnatal transmission**. Intrauterine transmission, including infections that are blood-borne and cell, spread mainly *via* the placenta<sup>[7]</sup>. This may be because of uplink vaginal infections and other infections. Wang proposed that HBV can be integrated into placental tissue leading to the infection<sup>[8]</sup>. The mechanisms of intrauterine transmission of HBV are not fully understood. Current theories include infection through the placenta, placental leakage, peripheral blood mononuclear cells, and paternal transmission. In general, pregnant women who are HBV-DNA positive are at increased risk of perinatal transmission of HBV<sup>[9-11]</sup>. Among HBsAg-positive pregnant women, newborn infection rate in the United States is lower than 15%, while it is higher than 40% in China and Japan<sup>[12,13]</sup>. If there is TORCH infection, this may result in placental cracks, or placental barrier damage, and therefore the risk of neonatal HBV infection is increased. HIV infection will also increase the risk of HBV infection<sup>[14-16]</sup>. There is no effective prevention of intrauterine transmission. It remains controversial whether injection of three to four doses of hepatitis B immune globulin (HBIG) can prevent vertical transmission<sup>[17,18]</sup>. Labor transmission occurs mainly through the HBV contaminated maternal blood, amniotic fluid, and vaginal secretions, which are either swallowed by the fetus or get into the fetal blood circulation by placental rupture<sup>[19]</sup>. As little as  $10^{-8}$  HBV per mL of contaminated maternal blood entering a fetal body can result in fetal infection<sup>[20,21]</sup>. A small proportion of perinatal transmission is attributable to postpartum transmission, through HBV contaminated maternal material such as breast milk and saliva. If mothers are positive for HBsAg, HBeAg, and anti-HBc, HBV-DNA can be detected from almost all mothers' breast milk, but if only HBsAg is positive, HBV-DNA can be detected in only 46% of the subjects<sup>[22,23]</sup>.

Since 1992, HBV vaccination for newborn infants has been implemented in China. The vaccination rate in urban areas has reached 90%, and the HBsAg-positive rate in these areas has been reduced to below 1%<sup>[24]</sup>. However, joint neonatal HBV vaccine and HBIG still have an immunization failure rate of 20%-30% in infants born to HBsAg-positive mothers<sup>[4,25]</sup>. Wu *et al* found that neonatal T cell function has not yet been fully developed, and newborns have immune tolerance to HBsAg. It is easier for them to become chronic carriers, and the younger the age infected, the higher probability of

becoming chronic carriers<sup>[26]</sup>. It is important to identify the determinants of perinatal transmission of HBV in this era of immunization. Moreover, previous studies in this field have largely relied on cord blood samples to determine HBV infection status of the newborn. False positives may have occurred in the diagnosis of neonatal HBV infection in these studies where cord blood sample was used because contamination from maternal blood cannot be avoided; therefore, the validity of the study findings is compromised. The objective of this study was to assess the determinants of perinatal HBV transmission in a group of Chinese pregnant women with HBV infection, using vein blood of newborns immediately after birth and before initial hepatitis B vaccination to determine the HBV infection status of the newborn.

## MATERIALS AND METHODS

This study was conducted in Xiangya Hospital and Xiangya Second Hospital of the Central South University, Yiyang Municipal Hospital, and Yiyang Maternal and Infant Hospital in Hunan, China. This study has been approved by REB of the Central South University.

All consenting HBsAg-positive pregnant women in the participating hospitals with a singleton live-born infant during the period of January 1, 2005 to September 31, 2006 were recruited into the study. Mothers with serious mental illness were excluded.

All HBsAg-positive newborns were selected as cases of the study. For each HBsAg-positive newborn, an HBsAg-negative newborn matched for hospital at birth (same) and gender (same) and date of birth (within 1 mo) was selected as the control. A questionnaire designed specifically for this study was used to collect clinical and epidemiological data, using face-to-face interview with the mother during postpartum hospital stay after childbirth.

Elbow blood of pregnant women prior to delivery and vein blood of newborns immediately after birth and before initial hepatitis B vaccination was taken for laboratory investigations. ELISA was used to detect HBsAg; Test Kits were purchased from the Shanghai Kehua Bio-engineering Technology Company, Limited. All laboratory processes were strictly followed according to the instructions provided by the company. A HITACHI 7600-automatic biochemical analyzer was used to test liver functions for HBsAg-positive pregnant women.

We first compared the baseline maternal and infant characteristics between cases and controls. Then we estimated the odds ratios (ORs) and 95% confidence intervals (CIs) of maternal-neonatal transmission of HBV. Conditional logistic regression analysis was used to estimate the independent effects of various determinants on maternal-neonatal transmission of HBV, adjusting simultaneously for several potential confounding factors. Independent variables included in the logistic regression model were maternal education, family income, maternal first-degree family history of HBV infection, liver function, systematic treatment of patients with liver function abnormality, hypertension in pregnancy, intrahepatic cholestasis, premature

Table 1 Comparison of baseline characteristics between cases and controls (Hunan, China, 2005-2006)

Research factor	Cases number (%)	Controls number (%)
Education of mother		
< College	81 (57.45)	57 (40.43)
> College	60 (42.55)	84 (59.57)
Income (yuan/mo)		
< 1500	90 (63.83)	92 (65.25)
> 1500	51 (36.17)	49 (34.75)
First-degree family history		
No	69 (48.94)	107 (75.89)
Yes	72 (51.06)	34 (24.11)
Liver function		
Normal	99 (70.21)	117 (82.98)
Abnormal	42 (29.79)	24 (17.02)
Systematic treatment		
No	119 (84.40)	94 (66.67)
Yes	22 (15.60)	47 (33.33)
EHP		
No	127 (90.07)	133 (94.33)
Yes	14 (9.93)	8 (5.67)
Intrahepatic cholestasis		
No	101 (71.63)	121 (85.82)
Yes	40 (28.37)	20 (14.18)
Premature rupture of membranes		
No	99 (70.21)	119 (84.40)
Yes	42 (29.79)	22 (15.60)
Anti-hepatitis B immunoglobulin injection		
No	100 (70.92)	68 (48.23)
Yes	41 (29.08)	73 (51.77)
Fetal distress		
No	68 (48.23)	91 (64.54)
Yes	73 (51.77)	50 (35.46)

EHP: Edema hypertension proteinuria syndrome.

rupture of membranes, maternal administration of HBIG, and fetal distress. Definition of systematic treatment in this study followed the Chinese national guideline for chronic hepatitis B prevention and treatment, which included using drugs to reduce enzyme levels, to protect the liver, and to enhance immune function in mothers with HBV infection and liver function abnormality<sup>[27]</sup>. All analyses were performed using Statistical Analysis System, Version 9.1 (SAS Institute Inc., Cary, North Carolina, United States).

RESULTS

A total of 590 HBsAg-positive mothers were recruited into the study, of which 151 HBsAg-positive newborns were defined as cases. Ten cases were excluded because no suitable controls could be identified. A total of 141 HBsAg-positive newborns and 141 individually matched HBsAg-negative newborns were included in the final analysis.

Compared with HBsAg-negative newborns, HBsAg-positive newborns tended to be born to mothers with lower education level, or with abnormal liver function, or with intrahepatic cholestasis, or with premature rupture of membranes, or who less frequently received systematic treatment for abnormalities of liver function, or who were

Table 2 Determinants of perinatal transmission of hepatitis B virus (Hunan, China, 2005-2006)

Research factor	OR (95% CI)	
	Single factors analysis	Adjust
Education of mother		
< College	Reference	Reference
> College	0.50 (0.31-0.81)	1.17 (0.56-2.45)
Income (yuan/mo)		
< 1500	Reference	Reference
> 1500	1.06 (0.65-1.73)	1.16 (0.72-1.87)
First-degree family history		
No	Reference	Reference
Yes	3.28 (1.98-5.46)	2.84 (1.47-5.48)
Liver function		
Normal	Reference	Reference
Abnormal	2.07 (1.17-3.65)	1.11 (0.48-2.55)
Systematic treatment		
No	Reference	Reference
Yes	0.36 (0.21-0.66)	0.36 (0.17-0.76)
EHP		
No	Reference	Reference
Yes	1.83 (0.74-4.52)	0.88 (0.28-2.75)
Intrahepatic cholestasis		
No	Reference	Reference
Yes	2.40 (1.32-4.36)	2.71 (1.01-7.27)
Premature rupture of membranes		
No	Reference	Reference
Yes	2.29 (1.28-4.10)	2.25 (1.08-4.68)
Anti-hepatitis B immunoglobulin		
No	Reference	Reference
Yes	0.38 (0.23-0.62)	0.27 (0.12-0.59)
Fetal distress		
No	Reference	Reference
Yes	1.95 (1.21-3.15)	1.70 (0.93-3.10)

OR: Odds ratios; CI: Confidence intervals; EHP: Edema hypertension proteinuria syndrome.

less likely to receive HBIG (Table 1). HBsAg-positive newborns were also more likely to develop fetal distress or to be born from mothers with first-degree family history of HBV (Table 1).

The result of the conditional logistic regression analysis showed that maternal first-degree family history of HBV, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas antiviral treatment for maternal HBV and maternal administration of HBIG were protective factors for maternal-neonatal transmission of HBV, after adjustment for potential confounding factors (Table 2).

DISCUSSION

Our nested case-control study, based on 141 pairs of HBsAg-positive and HBsAg-negative infants born to mothers with HBV infection in China, found that maternal first-degree family history of HBV, intrahepatic cholestasis, and premature rupture of membranes were associated with an increased risk of maternal-neonatal

transmission of HBV, whereas systematic treatment for mothers with HBV and maternal HBIG injection at late gestation were associated with decreased risk, after simultaneous adjustment for several potential confounding factors. The main strength of our study is that we used vein blood obtained from the newborns for laboratory tests of markers of HBV infection. Previous studies have largely relied on cord blood samples to determine HBV infection in the newborn. Because contamination from maternal blood cannot be avoided when cord blood samples are used, false positives can occur in the diagnosis of neonatal HBV infection, which therefore will compromise the validity of the study findings.

Our study showed that maternal first-degree family history of HBV was an independent risk factor of perinatal HBV transmission<sup>[28]</sup>. This may be caused by gene polymorphisms which result in familial aggregation of HBsAg carriers. In order to reduce perinatal HBV transmission, enhanced surveillance and additional interventions may be needed for newborns born to mothers with a first-degree family history of HBV. Intrahepatic cholestasis was an independent risk factor of perinatal transmission of HBV. This finding makes biological sense. When there is an intrahepatic cholestasis in pregnancy, bile salt deposition can cause pathological changes in placental villi, weakening the protective effect of the immune system or causing abnormal immune response<sup>[29]</sup>, which may lead to increased risk of perinatal transmission of HBV. Premature rupture of membranes was associated with increased risk of perinatal transmission of HBV, which was similar to the findings of the study by Yue *et al.*<sup>[7]</sup>. HBV infection of the fetus may happen through HBV contaminated vaginal secretions by premature rupture of membranes. Our results show that systematic treatment of HBsAg-positive mothers whose liver function was abnormal protected their offspring from HBV infection (OR = 0.36), suggesting that active and systematic treatment can improve and stabilize liver function, leading to reduction in perinatal transmission of HBV. Firstly, the risk of perinatal HBV transmission increases as the mother's viral load increases<sup>[30]</sup>; treatments such as lamivudine can reduce HBV load and thus transmission from mothers to their infants<sup>[16,31]</sup>. Secondly, improving and stabilizing maternal liver function can also reduce the risk of perinatal HBV transmission<sup>[32]</sup>.

Previous studies have found that HBIG can combine with HBsAg, forming antigen-antibody complexes, and promptly mobilizing the immune system to remove HBV<sup>[33]</sup>. Our study showed that prenatal injection of HBIG had a strong protective effect on perinatal transmission of HBV (OR = 0.38). The Chinese chronic hepatitis B prevention guidelines published in 2005 do not advocate the use of HBIG for pregnant women in advanced stages of pregnancy to prevent mother-to-infant transmission of HBV<sup>[27]</sup>. This is contrary to what happens in France, where after 6 mo of pregnancy every pregnant woman must be tested for HBsAg, and HBIG injection is mandated for all HBsAg-positive pregnant women<sup>[34]</sup>.

In summary, our study found that maternal first-

degree family history of HBV, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas systematic treatment for pregnant women with HBV infection and maternal HBIG administration were protective factors. Except for maternal first-degree family history of HBV, other factors are modifiable, suggesting that there are large areas for improvement in terms of reducing maternal-neonatal transmission of HBV.

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## COMMENTS

### Background

Hepatitis B is endemic in China and other parts of Asia. Most people in the region become infected with HBV during childhood and perinatal transmission is the most common route of HBV transmission.

### Research frontiers

Maternal screening programs and universal vaccination in infants with active and passive immunoprophylaxis have reduced perinatal HBV transmission rates dramatically. However, perinatal transmission may still be occurring despite the use of effective active and passive immunoprophylaxis. More studies are needed to assess the potential risk reduction associated with treatment of high maternal-neonatal transmission during pregnancy.

### Innovations and breakthroughs

Previous studies have largely relied on cord blood samples to determine HBV infection in the newborn in which contamination from maternal blood cannot be avoided and false positives can occur. The authors' study used vein blood obtained from the newborns for laboratory tests of markers of HBV infection, and found maternal first-degree family history of HBV infection, intrahepatic cholestasis, and premature rupture of membranes were risk factors for perinatal transmission of HBV, whereas systematic treatment and HBV immunoglobulin injection for mothers with HBV infection were protective factors for maternal-neonatal transmission of HBV.

### Applications

According to the findings, the authors suggest that clinicians consider risk factors and protective factors when a pregnant woman's HBsAg test is positive in order to prevent maternal-neonatal transmission of hepatitis B virus.

### Terminology

The nested case-control study design is used here. In the nested case-control study, cases of a disease that occur in a defined cohort are identified and, for each, a specified number of matched controls is selected from among those in the cohort who have not developed the disease by the time of disease occurrence in the case.

### Peer review

The authors' investigation was to identify the determinants of perinatal transmission of HBV in the era of immunization using venous blood of newborns immediately after birth and before initial hepatitis B vaccination to determine HBV infection status. The study design and methods seem appropriate.

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## Potentially predictive microRNAs of gastric cancer with metastasis to lymph node

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### Abstract

**AIM:** To detect the expression of 60 microRNAs (miRNAs) in gastric cancer tissues and find new predictive biomarkers of gastric cancer with metastasis.

**METHODS:** The expressions of 60 candidate miRNAs in 30 gastric cancer tissues and paired normal tissues were detected by stem-loop real-time reverse transcription-polymerase chain reaction. After primary screening of miRNAs expression, 5 selected miRNAs were further testified in another 22 paired gastric tissues. Based on the expression level of miRNAs and the status of metastasis to lymph node (LN), receiver-operating-characteristic (ROC) curve were used to evaluate their ability in predicting the status of metastasis to LN.

**RESULTS:** Thirty-eight miRNAs expressions in gastric cancer tissues were significantly different from those in paired normal tissues ( $P < 0.01$ ). Among them, 31 miRNAs were found to be up-expressed in cancer tissues and 1 miRNAs were down-expressed  $\geq 1.5$  fold vs paired normal gastric tissue. Five microRNAs (miR-125a-3p, miR-133b, miR-143, miR-195 and miR-212) were differently expressed between different metastatic groups in 30 gastric cancer biopsies ( $P < 0.05$ ). Partial correlation analysis showed that hsa-mir-212 and hsa-mir-195 were correlated with the status of metastasis to LN in spite of age, gender, tumor location, tumor size, depth of invasion and cell differentiation. ROC analysis indicated that miR-212 and miR-195 have better sensitivities (84.6% and 69.2%, respectively) and specificities (both 100%) in distinguishing biopsies with metastasis to LN from biopsies without metastasis to LN.

**CONCLUSION:** miR-212 and miR-195 could be independent biomarkers in predicting the gastric cancer with metastasis to LN.

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**Key words:** MicroRNA; miR-212; MiR-195; Gastric cancer; Metastasis to lymph node

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Wu WY, Xue XY, Chen ZJ, Han SL, Huang YP, Zhang LF, Zhu GB, Shen X. Potentially predictive microRNAs of gastric cancer with metastasis to lymph node. *World J Gastroenterol* 2011; 17(31): 3645-3651 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i31/3645.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i31.3645>

## INTRODUCTION

Gastric cancer is the fourth common malignancy and the second cause of death world widely<sup>[1]</sup>. Although radical gastrectomy with systemic lymph node (LN) dissection has saved many gastric cancer patients, the 5-year survivals are still far satisfactory<sup>[2]</sup>. Many evidences from large sample studies have shown that LN metastasis, the same to depth of invasion, histological differentiation, distant metastasis and tumor node metastasis stage, is one of the prognostic factors<sup>[3-6]</sup>. Currently we could get a little information of the status of LN metastasis before the operation while get most information from the histopathological diagnosis after the operation. Whether we could find a new way to predict LN metastasis became a new problem that faced us.

Accumulating evidences demonstrates that gastric cancer is a multigene-related disease with abnormal multi-step developing progress of associated oncogenes and tumor suppressor, including various genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators and cell adhesion molecules take part in LN metastasis<sup>[7]</sup>. Thousands of genes have been reported involved in the process of gastric cancer, such as p53, muc1, cea, E-cadherin, p16 and CD44<sup>[8-11]</sup>. Large-scale molecular techniques as DNA microarrays have been widely utilized in investigation of the molecular complexity of gastric cancer and prognostic classification based on gene expression profile<sup>[12,13]</sup>. Recently, development of MicroRNAs (miRNAs) technique may complement and enhance our current understanding of the development and progression of gastric cancer and may evolve our way to predict the status of LN metastasis before the surgical operation.

miRNAs are an abundant class of endogenous non-coding RNAs (about 18~24 nt) that regulate the stability and expression efficiency of target mRNAs at the post-transcription level. By entirely or partially base-pairing to the 3'-untranslated region of target mRNAs, the miRNAs induce translation repression or degradation of the mRNAs<sup>[14]</sup>, which plays a crucial role during various biological progresses, such as cell development, proliferation, differentiation and apoptosis<sup>[15]</sup>. Hypothetically, miRNAs are contributors to oncogenesis, functioning as tumor suppressors or oncogenes<sup>[16]</sup>. miRNAs can influence cancer development in many ways, such as the regulation of cell proliferation, cell transformation, cell death, and so forth. Recently studies have shown that some miRNAs were aberrantly expressed in many cancer tissues compared with paired normal tissues, such as the significant down-regulation of mir-143 and mir-145 in colon cancer<sup>[17]</sup> and up-regulation of mir-21 in breast cancer<sup>[18]</sup>, thus underscoring the tremendous diagnostic and therapeutic potential of miRNAs in cancer. Furthermore, some miRNAs were closely associated with the prognosis of some specific cancers, for example, up-regulated mir-155 is linked to poor survival in pancreatic cancer<sup>[19]</sup>. A large-scale study of miRNAs in gastric cancer showed that two miRNAs (mir-143 and mir-145)

were down-regulated<sup>[17,20]</sup> and one miRNA mir-27a correlated with LN metastasis<sup>[21]</sup>, which implied that miRNAs were crucial markers for diagnosis and prognosis of cancer<sup>[22]</sup>. However, whether miRNA could predict the status of gastric cancer with metastasis to LN has not been identified.

In the present study, we first selected 60 miRNAs, which had been reported to participate in the regulation of cell growth, cell proliferation, cell differentiation, cell apoptosis, tumor cell invasion, migration in other cancers (The function of candidate miRNAs is shown in the appendix), as candidate, then, detected the expressions of 60 miRNAs in 30 paired gastric cancer tissues to select target miRNAs which were differently expressed in different status of metastasis to LN. The candidate miRNAs which expressions correlated with metastasis to LN were further testified in another 22 paired gastric tissues, and the correlations between miRNAs expression and metastasis to LN were analyzed.

## MATERIALS AND METHODS

### Patients and biopsies

Paired specimens of gastric cancer tissues and corresponding normal gastric tissue (5 cm from cancer lesion without pathologically proven tumor cells), were obtained from patients with gastric cancer who underwent surgical resection at the First Affiliated Hospital of Wenzhou Medical College (Zhejiang Province, China) from December 2008 to April 2009. All the tissues were snap-frozen and stored in liquid nitrogen until total RNA was extracted. The histopathological diagnosis of gastric cancer was made by Department of Pathology, the First Affiliated Hospital of Wenzhou Medical College according to the criteria of the World Health Organization. All the patients did not received radiation therapy or chemotherapy before the surgical operation. Patients' characteristics of clinical-pathologic features were listed in Table 1.

Informed written consent was obtained from each patient and the study was approved by the Human Research Ethics Committee from the First Affiliated Hospital of Wenzhou Medical College.

### RNA isolation

Total RNA was extracted from gastric cancer tissues and corresponding normal gastric tissues using Trizol Reagent (Invitrogen Life Technologies, United States) according to the manufacturer's instructions with some modifications. Briefly, the extracted RNAs re-suspended in isopropanol were incubated at -20°C for at least 2 h (instead of 5 min at room temperature) to enhance precipitation efficiency of low-molecular-weight RNAs. Following a wash with 80% ethanol, RNA was re-suspended in diethylpyrocarbonate (DEPC)-treated water and stored at -80°C. The concentration and purity of total RNA were qualified by the ultraviolet spectrophotometer at 260 nm and 280 nm. Only the RNA samples with ratio of  $A_{260}/A_{280} > 1.8$  were used for the experiment.



**Table 1** Clinical-pathological features of 30 biopsies

Clinicopathological variables	n
Gender	
Male	16
Female	14
Age	
< 60 yr	14
≥ 60 yr	16
Tumor location	
Upper third	7
Middle third	16
Lower third	7
Tumor size	
< 5 cm	12
≥ 5 cm	18
Histological type	
Well and moderately differentiated	11
Poorly differentiated	19
<sup>1</sup> Depth of invasion	
T1	4
T2	8
T3	18
Lymph node involvement	
N0	15
N1	8
N2	7
<sup>1</sup> Tumor node metastasis stage	
Stage I	7
Stage II	8
Stage III	11
Stage IV	4

<sup>1</sup>According to the tumor node metastasis staging of Union for International Cancer Control.

### Quantification of gastric specimen's miRNAs expression

Stem-loop real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used according to Chen *et al.*<sup>[23]</sup>, Tang *et al.*<sup>[24]</sup> and Xue *et al.*<sup>[25]</sup>, and miRNA hsa-mir-let7a as internal control in this study<sup>[26]</sup>. Briefly, 4 μg total RNA was reverse transcribed to synthesize cDNA. The 20 μL reverse transcription reaction system includes 4 μL RT Buffer (Toyobo), 0.5 μL RT ACE (Toyobo), 0.5 μL RNase inhibitor (Toyobo), 1 μL dNTP, 4 μg total RNA, 2 μL 1 μmol/μL stem-loop RT specific primer and RNase-free ddH<sub>2</sub>O. Four internal controls including U6, let7a, hsa-mir-191 and hsa-mir-103 were reverse transcribed in parallel. The reaction condition was as follows: incubated at 16 °C for 30 min, 42 °C for 30 min, and 70 °C for 15 min finally. The synthesized cDNA was diluted up to 40 μL and preserved at -20 °C until use. The qRT-PCR reaction was performed on Applied Biosystems 7500 detection system by a 20 μL reaction system including 10 μL SYBR green real-time PCR Master Mix-plus (Toyobo, Japan), 2 μL Plus solution (Toyobo, Japan), each 2 μL specific Forward Primer and Reverse Primer, 1 μL RT product of total RNA and 3 μL DEPC water. All reactions were triplicate. The reaction was performed at 95 °C for 2 min, then followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were generated for each real-time RT-PCR to verify the specificity of each

PCR reaction.

### Data analysis

The Ct value (threshold cycle) is defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Delta Ct (ΔCt) represent the expression difference between the target miRNA and the normalizer: ΔCt = C<sub>t</sub>mir - C<sub>t</sub>normalizer. Then delta delta Ct (ΔΔCt) was calculated using the equation: ΔΔCt = ΔCt<sub>cancer tissue</sub> - ΔCt<sub>normal tissue</sub>. The normalized miRNA in a sample is 2<sup>-ΔΔCt</sup>. For the matched normal tissue control sample ΔΔCt equal to zero and 2<sup>-ΔΔCt</sup> equals to one. The expression levels of normalized miRNAs were characterized by their median and range (25th-75th percentile) because they did not fit the Gaussian distribution. Paired sample *t* test was used to evaluate the difference of miRNA expression between GC tissue and paired normal tissue and *P* < 0.05 was considered to have significant difference. Non-parameter tests were used to evaluate the differences of the miRNA expression between different groups: the Wilcoxon test for 2 paired groups (the tumor group and paired normal group) and the Mann-Whitney *U* test for the 2 independent groups. The partial correlation analysis was used to evaluate the relationship between some miRNA expression and the status of LN metastasis eliminating age, gender, tumor location, tumor size, invasion depth and cell differentiation. *P* < 0.05 was considered to be statistically significant. Receiver-operating-characteristic (ROC) curves was used to evaluate the sensitivity and specificity in predicting the LN metastasis based on the miRNA expression. The area under the ROC curve (AUC) and 95 percent confidence intervals were calculated. An AUC with a confidence interval that did not include the 0.5 value was considered that the miRNA had some ability to distinguish between the two groups. All calculations were performed with the software SPSS16.0.

## RESULTS

### Expression of 60 candidate miRNAs in gastric cancer specimens

The expressions of 60 candidate miRNAs were detected in 30 gastric cancer specimens by SYBR-green-based stem-loop real-time RT-PCR. As shown in Table 2, the expressions of the miRNAs in cancer tissues were very different from those of corresponding normal tissues. The relative expression of 38 miRNAs expressions (2<sup>-ΔΔCt</sup>) in gastric cancer tissues were significantly different from those in paired normal tissues which set at 1.000 (*P* < 0.01), suggesting that those 38 miRNAs might be involved in the process of gastric tumorigenesis. Among them, 31 miRNAs were found to be up-expressed in cancer tissues and 1 miRNAs were down-expressed ≥ 1.5 fold *vs* paired normal gastric tissue. Also, 5 miRNAs (hsa-mir-221, hsa-mir-15b, hsa-mir-181b, hsa-mir-199a-3p and hsa-mir-155) were in the highest expression levels, whereas hsa-mir-30b expression was the lowest.

Among the 30 candidate gastric cancer cases, 15 cases

**Table 2** The relative expression of 60 candidate microRNAs ( $2^{-\Delta\Delta Ct}$ ) in 30 gastric cancer specimens

MiRNA	Median	Percentile		<sup>1</sup> P value
		25%	75%	
Hsa-mir-106b	1.129	0.783	1.982	0.889
Hsa-mir-143	1.231	1.095	1.378	0.779
Hsa-mir-125a-5p	1.255	0.963	1.392	0.889
Hsa-mir-145	1.084	0.931	1.280	1.000
Hsa-mir-25	1.727	1.152	2.103	0.208
Hsa-mir-133b	1.127	0.938	1.314	0.779
Hsa-mir-195	0.989	0.754	1.534	0.889
Hsa-mir-374	1.005	0.800	1.599	0.779
Hsa-mir-451	0.594	0.442	1.636	1.000
Hsa-mir-1	1.228	0.950	2.135	0.327
Hsa-mir-141	0.973	0.663	1.125	0.208
Hsa-mir-200a	0.954	0.757	1.038	0.123
Hsa-mir-29c	0.867	0.664	1.042	0.123
Hsa-mir-29b	1.217	0.964	1.500	0.674
Hsa-mir-30b	0.600	0.542	0.782	0.017
Hsa-mir-26a	1.062	0.787	1.211	0.779
Hsa-mir-26b	1.257	0.838	1.369	0.779
Hsa-mir-144	1.548	1.251	2.118	0.123
Hsa-mir-103	2.103	1.259	2.339	0.050
Hsa-mir-h450b	1.500	1.061	2.601	0.208
Hsa-mir-191	1.770	1.007	2.216	0.161
Hsa-mir-200b	1.314	0.951	1.556	0.401
Hsa-mir-200c	1.151	1.101	1.545	0.161
Hsa-mir-203	1.790	1.464	2.498	0.069
Hsa-mir-429	1.944	1.250	2.028	0.050
Hsa-mir106a	1.279	1.139	1.917	0.069
Hsa-mir-15a	1.782	1.209	1.961	0.093
Hsa-mir-16a	1.145	1.112	1.338	0.017
Hsa-mir-17	1.603	1.396	1.921	0.017
Hsa-mir-155	2.663	1.873	3.557	0.012
Hsa-mir-18a	2.149	2.040	4.214	0.012
Hsa-mir-181b	2.535	2.195	2.635	0.012
Hsa-mir-421	2.141	1.518	2.498	0.012
Hsa-mir-92a	1.848	1.524	2.092	0.012
Hsa-mir-20a	1.596	1.037	2.071	0.036
Hsa-mir-125a-3p	1.729	1.191	2.997	0.036
Hsa-mir-199a-5p	1.645	1.157	2.624	0.069
Hsa-mir-93	2.583	1.250	3.139	0.025
Hsa-mir-222	1.373	1.309	2.488	0.025
Hsa-mir-15b	3.162	1.655	3.821	0.017
Hsa-mir-199a-3p	2.346	1.872	2.779	0.012
Hsa-mir-212	1.543	1.230	2.702	0.050
Hsa-mir-221	2.961	2.440	3.609	0.025
Hsa-mir-147	2.162	1.928	6.019	0.012
Hsa-mir-205	2.008	1.727	15.946	0.017
Hsa-mir-30d	1.614	1.346	2.17	0.017
Hsa-mir-363	1.732	1.612	1.928	0.012
Hsa-mir-23b	1.744	1.339	2.116	0.017
Hsa-mir-214	1.768	1.69	1.902	0.012
Hsa-mir-497	1.520	1.204	2.328	0.017
Hsa-mir-let7c	1.759	1.276	2.300	0.012
Hsa-mir-99a	1.286	1.220	2.734	0.012
Hsa-mir-193b	1.616	1.387	2.554	0.012
Hsa-mir-31	1.749	1.355	3.234	0.012
Hsa-mir-let7b	1.287	1.155	2.071	0.017
Hsa-mir-487b	1.598	1.424	2.855	0.017
Hsa-mir-h450a	1.435	1.116	1.609	0.123
Hsa-mir-18b	1.691	1.413	3.164	0.069
Hsa-mir-19b	1.411	1.248	1.726	0.093
Hsa-mir-19a	1.687	1.209	1.823	0.093
Hsa-mir-let7e	1.195	1.061	1.459	0.123

The relative expression of miRNA ( $2^{-\Delta\Delta Ct}$ ) in tumor samples, with that in nontumor control samples set at 1.000. <sup>1</sup>P: The Mann-Whitney *U* test for the different expression of microRNA in different group.

**Table 3** Five miRNAs expression in different lymph node metastasis groups

MiRNA	LN Negative (n = 15)	LN Positive (n = 15)	<sup>1</sup> P value
miR-125a-3p	1.87 (1.27, 2.32)	0.81 (0.49, 1.15)	0.02
miR-133b	1.31 (1.08, 1.59)	0.74 (0.41, 0.97)	0.04
miR-143	1.38 (1.23, 1.57)	0.57 (0.16, 1.03)	0.04
miR-195	1.53 (1.18, 2.52)	0.46 (0.31, 0.64)	0.02
miR-212	2.70 (2.14, 3.16)	1.06 (0.93, 1.18)	0.02

<sup>1</sup>P: The Mann-Whitney *U* test for the different expression of microRNA in different groups. LN: Lymph node.

**Table 4** Partial correlation analysis of 5 miRNAs expressions and lymph node metastasis

MicroRNA	Correlation coefficient	P value
miR-125a-3p	-0.451	0.091
miR-133b	-0.014	0.961
miR-143	-0.25	0.369
miR-195	-0.57	0.026
miR-212	-0.616	0.014

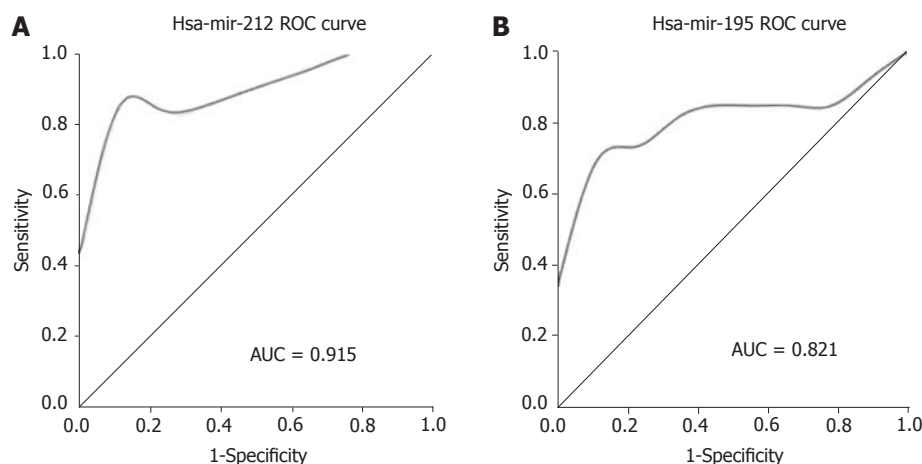
associated with LN metastasis, whereas 15 did not. Using the Mann-Whitney *U* test, we found that 5 miRNAs (hsa-mir-125a-3p, hsa-mir-133b, hsa-mir-143, hsa-mir-195 and hsa-mir-212) in gastric cancer patients with LN metastasis were quite different from those in patients without LN metastasis (Table 3). hsa-mir-125a-3p and hsa-mir-212 in patients with LN metastasis were lower than those in patients without LN metastasis while hsa-mir-143 and hsa-mir-195 were higher than those in patients without LN metastasis. But the expressions of the miRNAs did not correlate with age, gender, tumor location, tumor size and depth of invasion (data not shown).

**Correlation between the miRNAs expressions and lymph node metastasis of gastric cancer**

As the expressions of hsa-mir-125a-3p and hsa-mir-212 in patients with LN metastasis were lower than those in patients without LN metastasis while hsa-mir-143 and hsa-mir-195 were higher than those in patients without LN metastasis, partial correlation analysis for eliminating age, gender, tumor location, tumor size, invasion depth and cell differentiation showed that hsa-mir-212 and hsa-mir-195 were correlated with LN metastasis in spite of the status of cell differentiation (Table 4).

**Predicting value based on miRNAs expression in lymph node metastasis**

Five candidate miRNAs which correlated with metastasis to LN were selected and re-examined in another 22 gastric cancer biopsies to evaluate the predicting value in metastasis to LN (Figure 1). ROC analysis indicated hsa-mir-212 yielded an AUC of 0.915 (95% CI: 0.790-1.039). At the cutoff value of 1.439, hsa-mir-212 had 84.6% sensitivity and 100% specificity in discriminating gastric cancer biopsies with metastasis to LN. Whereas, hsa-mir-195 yielded AUC of 0.821 (95% CI: 0.634-1.007) with 69.2%



**Figure 1** Receiver operating characteristics curve analysis using hsa-mir-212 and hsa-mir-195 for discriminating gastric cancer biopsies with or without metastasis to lymph node. A: Hsa-mir-212 yielded an AUC (the areas under the receiver operating characteristics curve) of 0.915 (95% CI: 0.790-1.039) with 84.6% sensitivity and 100% specificity in discriminating gastric cancer biopsies with metastasis to lymph node (LN); B: Hsa-mir-195 yielded AUC of 0.821 (95% CI: 0.634-1.007) with 69.2% sensitivity and 100% specificity of in discriminating gastric cancer biopsies with metastasis to LN.

sensitivity and 100% specificity of in discriminating gastric cancer biopsies with metastasis to LN.

## DISCUSSION

Recently, many researches have demonstrated that miRNAs played an important role as either an oncogene or tumor suppressor gene in the initiation and progression. Though a few studies on miRNAs expression of gastric cancer have been carried out, and aberrant expressions in gastric cancer have been identified. However, few reports had screened the miRNAs expression specifically associated with LN metastasis, which was a prognostic factors to gastric cancer patients. In this study, firstly the expressions of 60 candidate miRNAs were detected in 30 gastric cancer specimens by SYBR-green-based stem-loop real-time RT-PCR, and 38 of 60 miRNAs expressions in gastric cancer tissues were found to be significantly different from those in paired normal tissues ( $P < 0.01$ ). Among them, 31 miRNAs were found to be up-expressed in cancer tissues and 1 miRNA were down-expressed  $\geq 1.5$  folds *vs* paired normal gastric tissue, suggesting that abnormal expression of those miRNAs may play a role in the development of gastric tumorigenesis.

The abnormal expressions of some miRNAs had been reported in several cancers, for example, hsa-mir-155 was over-expressed in pancreatic tumor<sup>[27]</sup>, thyroid tumor<sup>[28]</sup>, cervical cancer<sup>[29]</sup> and the up-regulated expression of hsa-mir-155 was related to a poor prognosis of patients with pancreatic cancer<sup>[19]</sup>. hsa-mir-15b was up-regulated in pancreatic cancer<sup>[30]</sup>, colorectal cancer<sup>[31]</sup> and cervical cancer<sup>[29]</sup>. The highly expressed hsa-mir-221, one member of mir-221/222 cluster, was up-regulated in glioblastoma<sup>[32]</sup>, bladder cancer<sup>[33]</sup>. Three reports demonstrated that mir-199a was down-regulated in bladder tumor<sup>[34]</sup>, ovarian cancer<sup>[35]</sup> while up-regulated in hepatoblastoma<sup>[36]</sup>. hsa-miR-143 and hsa-miR-145 were down-regulated in colon cancer<sup>[37]</sup> and nasopharyngeal cancer<sup>[38]</sup> and gastric cancer<sup>[17]</sup>. hsa-mir-143 and hsa-mir-145 have been identified to have a suppressive effect on cell growth and the reduction in the level of mir-143 and mir-145 positively contributed to the proliferation in gastric cancer cell<sup>[17]</sup>. hsa-mir-212 was downregulated and repressed growth by targeting

methy-CpG-binding protein MeCP2 in gastric cancer cell line<sup>[39]</sup>. Therefore, the abnormal expression of these miRNAs may be correlated to the development cancers.

Recently, miR-373 and miR-520c have been reported to be as metastasis-promoting miRNAs that promote tumor invasion and metastasis, whereas miR-335, miR-206, and miR-126 have been as suppressors of breast cancer metastasis<sup>[40,41]</sup>. The down-regulation of has-mir-195 has been observed in primary peritoneal carcinoma<sup>[42]</sup> and bladder tumor<sup>[34]</sup>. Introduction of miR-195 dramatically suppressed the ability of hepatocellular carcinoma and colorectal carcinoma cells to form colonies *in vitro* and to develop tumors in nude mice<sup>[43]</sup>. However, our data showed that hsa-mir-195 and hsa-mir-212 were down-regulated in gastric cancer with LN metastasis in spite of the status of tumor cell differentiation.

Li *et al*<sup>[44]</sup> recently reported that seven microRNAs (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p, miR-126) as a risk signature was an independent predictor of overall survival and relapse-free survival. Here we reported that the down-regulation of hsa-mir-212 and hsa-mir-195 were not only associated with lymph metastasis for the first time but also had better predicting value in LN metastasis.

Taken together, abnormal expression of miRNAs may be correlated with the development and progression of gastric cancer, the down-regulation of hsa-mir-212 and hsa-mir-195 were correlated with LN metastasis and could distinguish patients with LN metastasis from patients without LN metastasis. Further works and large samples of gastric cancer are needed to validate the diagnostic criteria of miRNA for gastric cancer with LN metastasis and identify target mRNAs from candidate.

## COMMENTS

### Background

Gastric cancer is one common solid tumor world widely. Almost one million people die from it every year. Although patients received radical gastrectomy with systemic lymph node (LN) dissection the 5-year survival is still far satisfactory. Gastric cancer with metastasis to LN is one important prognostic factor. As surgeons could get little information of metastasis to LN before the operation some patients who should receive radical gastrectomy received operation style of endoscopic mucosal resection or laparoscopy-assisted gastrectomy. So patients



could benefit from the evaluation of metastasis to LN before surgical operation.

### Research frontiers

Accumulating evidence indicate that gastric cancer metastasized to LN is the results of various genetic and epigenetic alternations of oncogens, tumor suppressor genes, DNA repair genes, cell cycle regulators and cell adhesion molecules. Hundreds of genes have been reported involved in the process of metastasis and this made the study of metastasis a more complex problem. Recently researches have focused their study on the aberrantly expressed microRNA in gastric cancer. However, there haven't been a report about the predictive value in evaluating the status of gastric cancer metastasized to LN basing on the expression level of microRNA.

### Innovations and breakthroughs

Recent reports have demonstrated many microRNAs were upregulated or down-regulated in gastric cancer. This is the first study to report that hsa-mir-212 and hsa-mir-195 had better sensitivity and specificity in distinguishing gastric cancer biopsies with metastasis to LN from gastric cancer biopsies without metastasis to LN.

### Applications

By understanding the predictive value of microRNA in evaluating the status of gastric cancer metastasized to LN, this study approached the problem to identify a diagnostic criteria for gastric cancer biopsies with metastasis to LN employing miRNA as biomarkers and help surgeons to evaluate the status of metastasis to LN before surgical operation and choose reasonable operation style for patients.

### Terminology

microRNAs are an abundant class of endogenous non-coding RNAs that regulate the stability and expression efficiency of target mRNAs at the post-transcription level. Almost 50% microRNAs located at or near to the fragile site of tumor-associated genes. MicroRNAs which were near to the gene promoting metastasis of gastric cancer should be aberrantly expressed. This signature make it feasible that microRNA could distinguish gastric cancer biopsies with metastasis to LN from gastric cancer biopsies without metastasis to LN.

### Peer review

This is a very interesting study in which the investigators approached the problem to identify a diagnostic criteria for gastric cancer biopsies with LN metastasis employing miRNA as biomarkers.

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## Two clinically relevant pressures of carbon dioxide pneumoperitoneum cause hepatic injury in a rabbit model

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### Abstract

**AIM:** To observe the hepatic injury induced by carbon dioxide pneumoperitoneum (CDP) in rabbits, compare the effects of low- and high-pressure pneumoperitoneum, and to determine the degree of hepatic injury induced by these two clinically relevant CDP pressures.

**METHODS:** Thirty healthy male New Zealand rabbits weighing 3.0 to 3.5 kg were randomly divided into three groups ( $n = 10$  for each group) and subjected to the following to CDP pressures: no gas control, 10 mmHg, or 15 mmHg. Histological changes in liver tissues were observed with hematoxylin and eosin staining and transmission electron microscopy. Liver function was evaluated using an automatic biochemical analyzer. Adenine nucleotide translocator (ANT) activity in liver tissue was detected with the atractyloside-inhibitor stop technique. Bax and Bcl-2 expression levels were detected by

western blotting.

**RESULTS:** Liver functions in the 10 mmHg and 15 mmHg experimental groups were significantly disturbed compared with the control group. After CDP, the levels of alanine transaminase and aspartate transaminase were  $77.3 \pm 14.5$  IU/L and  $60.1 \pm 11.4$  IU/L, respectively, in the 10 mmHg experimental group and  $165.1 \pm 19.4$  IU/L and  $103.8 \pm 12.3$  IU/L, respectively, in the 15 mmHg experimental group, which were all higher than those of the control group ( $P < 0.05$ ). There was no difference in pre-albumin concentration between the 10 mmHg experimental group and the control group, but the pre-albumin level of the 15 mmHg experimental group was significantly lower than that of the control group ( $P < 0.05$ ). No significant differences were observed in the levels of total bilirubin or albumin among the three groups. After 30 and 60 min of CDP, pH was reduced ( $P < 0.05$ ) and  $\text{PaCO}_2$  was elevated ( $P < 0.05$ ) in the 10 mmHg group compared with controls, and these changes were more pronounced in the 15 mmHg group. Hematoxylin and eosin staining showed no significant change in liver morphology, except for mild hyperemia in the two experimental groups. Transmission electron microscopy showed mild mitochondrial swelling in hepatocytes of the 10 mmHg group, and this was more pronounced in the 15 mmHg group. No significant difference in ANT levels was found between the control and 10 mmHg groups. However, ANT concentration was significantly lower in the 15 mmHg group compared with the control group. The expression of hepatic Bax was significantly increased in the two experimental groups compared with the controls, but there were no differences in Bcl-2 levels among the three groups. Twelve hours after CDP induction, the expression of hepatic Bax was more significant in the 15 mmHg group than in the 10 mmHg group.

**CONCLUSION:** A CDP pressure of 15 mmHg caused more substantial hepatic injury, such as increased levels of acidosis, mitochondrial damage, and apoptosis;



therefore, 10 mmHg CDP is preferable for laparoscopic operations.

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**Key words:** Carbon dioxide pneumoperitoneum; Hepatic injury; Rabbit; Mitochondria; Apoptosis

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## INTRODUCTION

Developments in technology and medicine and improvements in anesthetic and surgical techniques have led to the extensive use of laparoscopic procedures for different patient groups, including high-risk patients<sup>[1-4]</sup>. The advantages of laparoscopic surgery compared with open surgery include a shorter hospital stay, early return to work, and decreased cost. Other advantages of laparoscopic surgery include less perioperative blood loss, a reduction in, or absence of, postoperative pain, and better cosmetic healing<sup>[5]</sup>.

The creation and maintenance of a pneumoperitoneum to create space for dissection is one of the basic requirements for laparoscopic procedures, and carbon dioxide is the most commonly used gas for inducing a pneumoperitoneum. However, insufflation of carbon dioxide into the abdominal cavity for a short time may significantly and adversely impact respiration, circulation, and the acid-base balance in patients because of carbon dioxide absorption and persistently high intra-abdominal pressure<sup>[6-7]</sup>. Increased intra-abdominal pressure has some potential side effects, such as impairments of liver, kidney, and heart functions. The severity of such impairments is directly related to the degree of intra-abdominal pressure. Uncomplicated laparoscopic cholecystectomy can be performed reasonably safely with a low-pressure pneumoperitoneum. However, if available space is needed for extended resections or complicated reconstructive operations, such as laparoscopic colorectal surgery, a high-pressure pneumoperitoneum is induced. To maintain sufficient intra-abdominal space for surgical procedures, 15 mmHg carbon dioxide pneumoperitoneum (CDP) is routinely used instead of 10 mmHg to 12 mmHg. An increasing number of cases presenting hepatic injury after laparoscopic surgery have been reported, but the number of studies assessing this complication under clinical levels of intra-abdominal pressure is very limited<sup>[8-14]</sup>. Therefore,

in this study we investigated hepatic injury in response to these two clinically relevant levels of intra-abdominal pressure to investigate the safety of CDP.

## MATERIALS AND METHODS

### Animals

Thirty healthy male New Zealand rabbits weighing 3.0 to 3.5 kg were randomly divided into three groups ( $n = 10$  in each group) and submitted to different CDP pressures: a control group (no gas), 10 mmHg group (carbon dioxide pressure was 10 mmHg), and 15 mmHg group (carbon dioxide pressure was 15 mmHg). Rabbits were given no water or food for 8 h prior to the experiments. They received 1 mg/kg midazolam and 20 mg/kg ketamine before surgery. A tracheal incision was made, and a 4.5 F canal was inserted for mechanical ventilation. The tidal volume was maintained at 10 mL/kg at a frequency of 30 times per minute. Anesthesia was sustained with injections of ketamine (5 µg/kg per minute) and vecuronium bromide (0.1 mg/kg per minute). All chemical treatments were halted 10 min before CDP induction. The rabbits in the experimental groups underwent CDP for 1 h. Mechanical ventilation was stopped once the rabbits recovered from CDP. All operations were approved by the animal care guidelines of the General Hospital of Chengdu Military Command.

### Reagents

<sup>3</sup>H-ADP and atractyloside were obtained from Sigma Corporation (United States). Rabbit anti-Bax polyclonal IgG and anti-Bcl-2 polyclonal IgG were purchased from Santa Cruz Biotechnology (United States). The goat anti-rabbit horseradish peroxidase-conjugated antibody was purchased from Zhongshan Golden Bridge Biotechnology Co. (China).

### Liver function assay

Blood samples were collected from ear-edge veins 12 h after the commencement of CDP and allowed to clot, and sera were isolated by centrifugation at 1000 r/min for 10 min and stored at -20°C until the assay. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and albumin were determined by routine laboratory methods using a Hitachi Automatic Analyzer (Hitachi, Inc., Japan).

### Blood gas analysis

Femoral artery blood samples were collected at 0 min, 30 min, 60 min, and 12 h after the CDP operation and analyzed using a blood gas analyzer (AVL 995). When the samples were collected at 0 min, 30 min, and 60 min, the rabbits underwent mechanical ventilation.

### Histological examinations of liver tissue

Liver biopsies were collected 12 h after the beginning of CDP, and the specimens were fixed in 10% formalde-

**Table 1** Changes in liver function (mean  $\pm$  SD)

Group	TB	ALT	AST	A	Pre-A
Control	1.5 $\pm$ 0.3	52.4 $\pm$ 9.6	41.0 $\pm$ 9.1	32.6 $\pm$ 2.1	154.5 $\pm$ 17.7
10 mmHg	1.6 $\pm$ 0.3	77.3 $\pm$ 14.5 <sup>a</sup>	60.1 $\pm$ 11.4 <sup>a</sup>	31.7 $\pm$ 2.0	146.9 $\pm$ 15.7
15 mmHg	1.7 $\pm$ 0.5	165.1 $\pm$ 19.4 <sup>a</sup>	103.8 $\pm$ 12.3 <sup>a</sup>	30.5 $\pm$ 1.5	118.0 $\pm$ 14.9 <sup>a</sup>

<sup>a</sup> $P < 0.05$  vs control group. TB: Total bilirubin; ALT: Alanine transaminase; AST: Aspartate transaminase; A: Albumin; Pre-A: Pre-albumin.

hyde for 12 h to 24 h, embedded in paraffin, sliced into 5- $\mu$ m-thick sections, and stained with hematoxylin and eosin. Histological changes in the liver tissues were observed using a micrographic system (Olympus).

### Transmission electron microscopy

Liver tissues were fixed using 3% glutaraldehyde, post-fixed in 1% osmium tetroxide in 0.1 mol/L cacodylate buffer, dehydrated with acetone, and embedded in EPON 812. After location by semi-thin sectioning, the samples were sectioned to a thickness of 50-80 nm and poststained with 2% aqueous uranyl acetate. All samples were examined and photographed by transmission electron microscopy (PHILIPS TECNAI 10, Netherlands) at an accelerating voltage of 100 kV.

### Atractyloside-inhibitor block technique

Mitochondria in the liver tissues were isolated by centrifugation. The activity of adenine nucleotide translocator (ANT) in the liver tissue was detected using the atractyloside-inhibitor stop technique. Mitochondrial function was initiated by adding <sup>3</sup>H-ADP and terminated after 12 s by adding adriamycin. The radioactivity in each group was measured, and ANT activity was expressed as 10<sup>-9</sup> mol/min per g protein.

### Western blotting assay

Liver tissue samples (100 mg) were homogenized in a liquid nitrogen-cooled grinding bowl and lysed in cold RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with Halt™ Protease Inhibitor Cocktail. Whole cell lysates were obtained by subsequent centrifugation at 15 000 *g* for 10 min at 4°C. Protein concentrations were determined using a Bradford Protein Assay Kit with bovine serum albumin as a standard. Protein extracts (40  $\mu$ g) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Protran nitrocellulose membrane. This membrane was incubated with rabbit anti-Bax polyclonal antibody or rabbit anti-Bcl-2 polyclonal antibody at 4°C overnight after being blocked with a 10% bovine serum albumin solution. The membrane was washed with TBST buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, and 0.1% Tween-20), incubated with a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody for 2 h at room temperature, and finally detected with a DAB Kit. Beta-actin was used as an internal control for data

analysis.

### Statistical analysis

All experimental data are expressed as the means  $\pm$  SD and were analyzed by a *t*-test using SPSS 10.0 statistical software. Probability values of  $< 0.05$  were considered to be statistically significant.

## RESULTS

### CDP operation disturbs liver function

Liver function in both CDP groups was disturbed compared with the control group (Table 1). After the CDP operation, the ALT and AST levels were 77.3  $\pm$  14.5 and 60.1  $\pm$  11.4 IU/L, respectively, in the 10 mmHg group and 165.1  $\pm$  19.4 and 103.8  $\pm$  12.3 IU/L, respectively, in the 15 mmHg group; each of these were higher than the control group ( $P < 0.05$ ). Compared with the control group, there was no significant difference in the serum concentration of pre-albumin in the 10 mmHg group; however, it was significantly lower in the 15 mmHg group compared to the control group ( $P < 0.05$ ). No significant difference was observed in the levels of total bilirubin or albumin among the three groups.

### Blood gas analysis

Blood pH in the 10 mmHg group was significantly decreased compared with the control group at 30 min and 60 min after CDP induction (Figure 1A), and PaCO<sub>2</sub> was significantly increased (Figure 1B). Blood pH and PaCO<sub>2</sub> levels were much higher in the 15 mmHg group than in the controls at these time points (Figure 1A and B). However, there were no significant differences in PaO<sub>2</sub>, SpO<sub>2</sub>, or pH levels for the experimental groups at 12 h post-CDP induction compared with the control group (Figures 1C and D).

### Histological changes in liver tissue

Hematoxylin and eosin staining and transmission electron microscopic images were analyzed for each group. The morphological changes of liver tissues in the 10 mmHg and 15 mmHg groups were similar and included mild hyperemia and mitochondrial swelling (Figures 2B, C, E and F). The hyperemia was more severe in the 15 mmHg group than in the 10 mmHg group, and the mitochondrial swelling was more apparent in the 15 mmHg group. In addition, the rough endoplasmic reticulum was slightly expanded in cells of the 15 mmHg group (Figure 2F).

### ANT Activity in liver mitochondria

In the control group, ANT activity was 10.83  $\pm$  1.11 (10<sup>-9</sup> mol ADP/min per g protein), while the activity of ANT was 9.03  $\pm$  0.89 in the 10 mmHg group; there was no significant difference between these two groups. In the 15 mmHg group, ANT activity was only 6.64  $\pm$  0.77, which was significantly lower than the control group ( $P < 0.05$ ), indicating that the energy metabolism of liver mitochondria was damaged by CDP (Table 2).

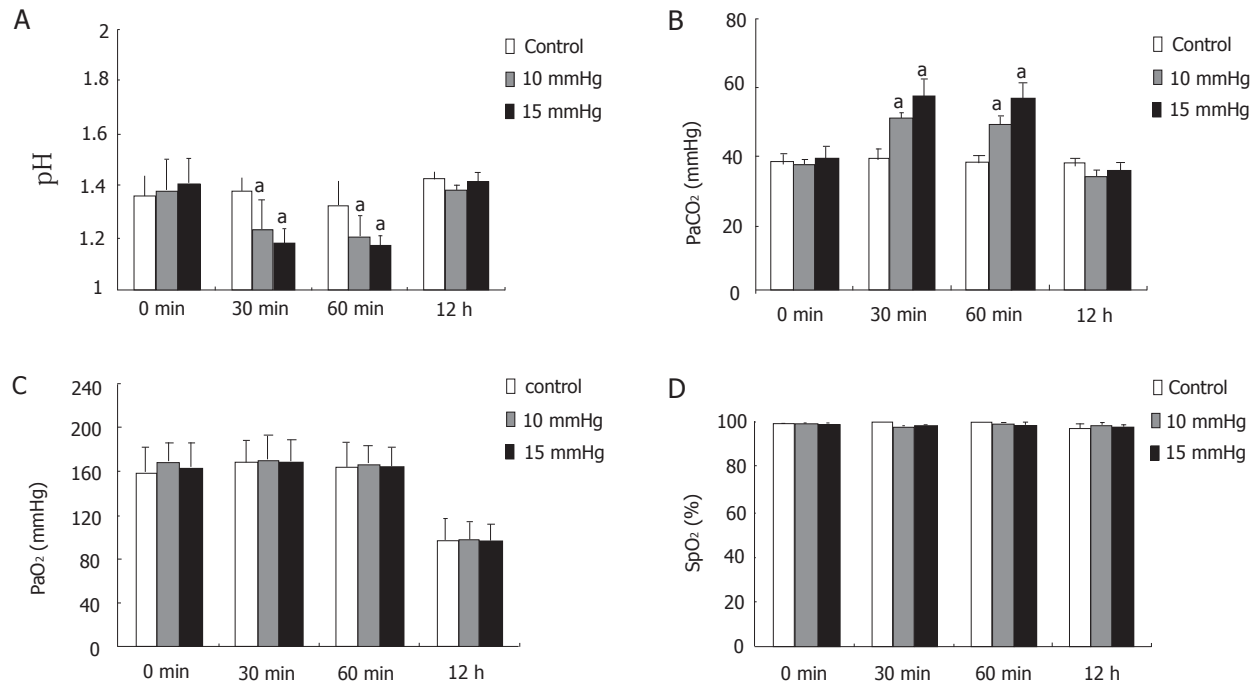


Figure 1 Arterial blood gas analysis. Data are presented as the mean  $\pm$  SE ( $n = 10$ ). <sup>a</sup> $P < 0.05$  vs control.

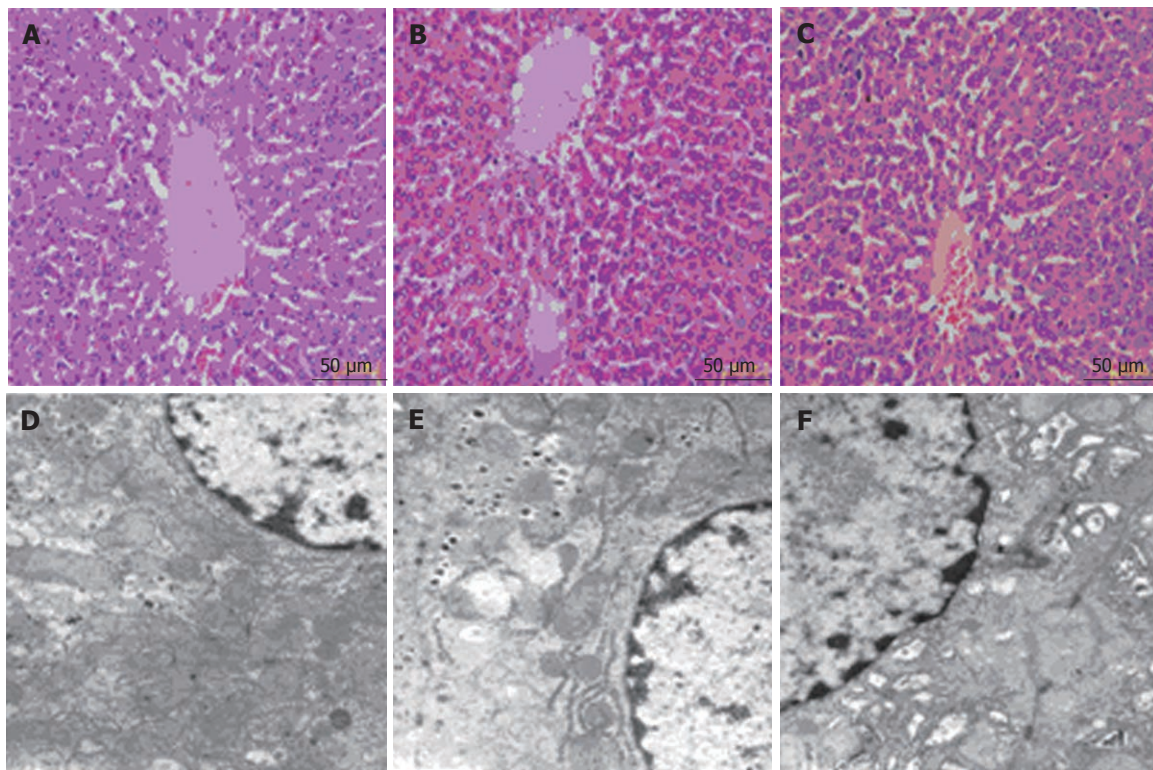


Figure 2 Histological changes in liver tissue. A-C: Hematoxylin eosin observation; D-F: Transmission electron microscopic observation. A, D: The normal structure of hepatocytes and mitochondria in the control group; B, E: Mild hyperemia in liver tissue and mitochondrial swelling were observed in the 10 mmHg group. C, F: The hyperemia was more serious: Mitochondrial swelling and expanded rough endoplasmic reticulum were observed in the 15 mmHg group.

### Expression of Bax and Bcl-2 in liver tissue

Bax and Bcl-2 protein levels were analyzed by western blot assay. The expression of Bax was significantly elevated in the 10 mmHg and 15 mmHg groups, but there was

no significant change in Bcl-2 expression among the three groups. Compared with the 10 mmHg group, hepatic Bax expression in the 15 mmHg group was more significantly increased 12 h after the initiation of CDP (Figure 3).



**Table 2** Activity of adenine nucleotide translocator in liver mitochondria (mean  $\pm$  SD)

Group	ANT (9-10 mol/min per g protein)
Control	10.83 $\pm$ 1.11
10 mmHg	9.03 $\pm$ 0.89
15 mmHg	6.64 $\pm$ 0.77 <sup>a</sup>

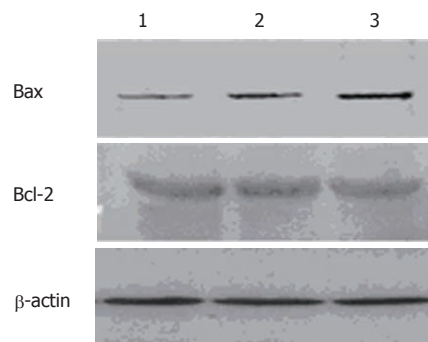
<sup>a</sup>*P* < 0.05 vs control group; ANT: Adenine nucleotide translocator.

## DISCUSSION

Laparoscopic procedures are favored by both surgeons and patients because of convenience, the minor degree of trauma, rapid healing, and a good cosmetic prognosis<sup>[5]</sup>. A pneumoperitoneum is a necessary requirement during laparoscopic operations, and carbon dioxide is the most frequently used gas to create a pneumoperitoneum. Previous studies have noted that the increased abdominal pressure caused by CDP in a short time span can impact the hemodynamics, hemoperfusion, and function of critical abdominal organs, such as the liver<sup>[15-17]</sup>. Technical advancements have led to laparoscopic surgery becoming more complicated, and a lower-pressure CDP (e.g., 10-12 mmHg) is no longer sufficient. To create a sufficient operation space and decrease complications during laparoscopic procedures, many surgeons have increased the CDP abdominal pressure to 15 mmHg. However, the safety and effects of this pressure on abdominal organs have not been fully elucidated. The liver is one of the most important abdominal organs, and it is quite sensitive to harm. Therefore, in this study we investigated the effects of two clinically relevant CDP pressures on liver function, hepatocyte morphology, and protein expression.

Serum ALT and AST are two most commonly used markers of hepatocyte damage. In our study, a 1-h CDP operation resulted in hepatocyte injury at both 10 mmHg and 15 mmHg CDP. Increased ALT and AST levels were observed, suggesting that hepatocytes were damaged by both carbon dioxide pressures. More pronounced changes were detected in the 15 mmHg CDP group, indicating a more severe level of hepatocyte injury. Albumin and pre-albumin levels are markers of hepatocyte protein synthesis. Our data show that 10 mmHg CDP did not impact serum albumin levels, suggesting that 10 mmHg does not affect the rate of hepatocyte protein synthesis, despite its influence on ALT and AST levels. However, 15 mmHg CDP resulted in a decrease in pre-albumin, indicating that this CDP pressure could disturb hepatocyte activity as well as liver function. No significant changes in albumin levels were observed in the two CDP groups compared with the control group. The relatively long half-life of albumin might have been responsible for this phenomenon. In this experiment, we only measured albumin levels up to 12 h after the operation, which is quite short given the 14-d half-life of albumin.

The mechanisms underlying the influence of CDP



**Figure 3** Expression of Bax and Bcl-2 in rabbit liver tissues. 1: Control group; 2: 10 mmHg group; 3: 15 mmHg group.

on liver function might be related to hemodynamic changes and imbalanced acid-base levels. Many studies have shown diminished portal venous flow during increased intra-abdominal pressures, which possibly leads to decreased liver blood supply and impaired hepatic function<sup>[9,15,18-21]</sup>. Hepatic perfusion is characterized by a unique autoregulatory mechanism known as the hepatic arterial buffer response. Under physiological and pathological conditions, alterations in portal venous flow are counteracted by changes in hepatic arterial flow, thereby maintaining total hepatic blood flow and preserving a sufficient supply of oxygen to the liver<sup>[22,25,26]</sup>. However, several studies have demonstrated that during CDP, hepatic arterial blood flow does not compensate for the reduction in portal venous inflow<sup>[9]</sup>. Furthermore, there is a linear relationship between intra-abdominal pressure and portal venous pressure as well as a reciprocal correlation between increased intra-abdominal pressure and decreased portal venous flow<sup>[9,20,21,27]</sup>. In this study, CDP resulted in increased PaCO<sub>2</sub> levels and decreased blood pH, and this effect was more pronounced at 15 mmHg than at 10 mmHg. These results indicate that increased abdominal pressure leads to more severe acidosis. These changes are thought to result from the absorption of insufflated carbon dioxide or ventilation-perfusion mismatching during the procedure<sup>[28,29]</sup>. Absorption of carbon dioxide through the peritoneum may result in an accumulation of carbon dioxide and subsequent acidosis. However, increased abdominal pressure during CDP could reduce abdominal blood flow and result in local hypoxia, which is another cause of acidosis<sup>[21,30]</sup>. Some researchers have adopted this view, which has been further discussed in studies demonstrating splanchnic hypoperfusion, regardless of whether intra-abdominal pressure was increased without the use of gas<sup>[20,21]</sup> or insufflation of CO<sub>2</sub>, N<sub>2</sub>O, helium, or argon was used to induce pneumoperitoneum<sup>[17,19,31-33]</sup>. Neither PaO<sub>2</sub> nor SpO<sub>2</sub> was affected by the two CDP pressures, possibly because of the use of intermittent positive-pressure mechanical ventilation.

In addition to influencing hemodynamics and the acid-base balance, CDP operations resulted in changes in hepatocyte morphology. Neither pressure led to ap-

parent tissue damage (based on hematoxylin and eosin staining), but both pressures resulted in mild liver hyperemia, the severity of which was related to CDP pressure. CDP also impacted hepatocyte ultrastructure, including mitochondrial swelling and expanded rough endoplasmic reticulum. The activity of ANT, a marker of mitochondrial energy metabolism<sup>[34]</sup>, was reduced after 15 mmHg CDP, but not by 10 mmHg CDP. This suggests that a relatively lower pressure might not impact hepatocyte energy metabolism, despite mitochondrial swelling.

Bcl-2 and Bax are 2 important apoptotic regulatory genes. They are widely distributed in tissues and cells, and they coordinate with each other to regulate apoptosis. When Bax expression is upregulated, Bax/Bax homodimers are formed to induce apoptosis. However, increased Bcl-2 expression results in isodimers of Bcl-2 and Bax that inhibit apoptosis<sup>[35]</sup>. In this study, neither 10 mmHg nor 15 mmHg CDP resulted in increased Bcl-2 expression. However, both pressures led to elevated Bax expression, especially in the 15 mmHg group, suggesting that CDP procedures promote hepatocyte apoptosis in a pressure-dependent manner. Elevated levels of hepatocyte apoptosis might be responsible for the disturbed liver function caused by CDP.

To summarize, we investigated the presence and mechanisms underlying liver damage caused by two clinically relevant CDP pressures. Liver injury has been shown to be pressure-dependent<sup>[11]</sup>. Although a relatively high CDP pressure is required for laparoscopic procedures, such as the 15 mmHg pressure used in this study, we must bear in mind that this pressure can cause serious damage to liver function. The liver damage resulting from CDP may not cause severe complications, but the potential for such damage in patients with liver diseases is of particular importance. Some reports have shown that a shorter duration of carbon dioxide pressure during pneumoperitoneum might help to alleviate liver injury<sup>[8]</sup>. Stepwise increases in carbon dioxide insufflation might also be an ischemic preconditioning method to reduce liver injury<sup>[36]</sup>. Future studies are needed to elucidate the mechanisms underlying CDP-induced liver function damage, and the safety of CDP under different surgical conditions should be carefully evaluated<sup>[1-4,37]</sup>.

## COMMENTS

### Background

Laparoscopic surgery is widely used, and the traditional low carbon dioxide pneumoperitoneum (CDP) pressure no longer meets the requirements of complicated operations. The safety of high CDP pressure in clinical practice is the subject of much attention.

### Research frontiers

Some studies have shown that liver injury is pressure-dependant, and hepatocyte apoptosis was observed in the present study.

### Innovations and breakthroughs

The liver is a critical organ that is sensitive to many harmful factors. Liver changes during CDP operation were evaluated in this study by assaying several different markers. The data suggest that the different pressures cause hepatic injury.

### Applications

As indicated by the experimental data, although higher pressure provides more space for CDP operation, the resulting hepatic injury must be considered.

Therefore, the appropriate CDP pressure should be carefully chosen for laparoscopy.

### Terminology

CDP is the abdominal space created by insufflating carbon dioxide to provide operation space for laparoscopy.

### Peer review

This is an interesting study even if on a well studied subject.

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## A giant gas-filled abdominal mass in an elderly female: A case report

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### Abstract

We report an extremely rare case of gas-filled abdominal mass caused by an ovarian teratoma fistulating to the sigmoid colon. The patient was an 85-year-old female, who presented with severe abdominal distension. Urgent computed tomography scan showed a huge abdominal mass with air fluid level and fecal matter inside. Communication between the mass and the sigmoid colon was suspected. She underwent emergency laparotomy. The mass was resected with the involved segment of colon. Pathology confirmed squamous cell carcinoma arising from mature cystic teratoma of the ovary.

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**Key words:** Mature cystic teratoma; Fistula; Squamous cell carcinoma

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### INTRODUCTION

Abdominal distension is one of the most common presenting symptoms encountered in general surgery. Although there are numerous differential diagnoses, most of the diagnoses would become apparent after radiological workup. We report here a very interesting and extremely rare cause of abdominal distension, which posed a great diagnostic challenge.

### CASE REPORT

An 85-year-old lady was known to have had a sizable cystic lesion of undetermined nature for more than 40 years, causing abdominal distension. Though surgical excision was once offered, she refused intervention and defaulted clinic follow-up.

On this occasion, 7 years after default, she presented to the Accident and Emergency Department for severe abdominal pain and fever. She had experienced progressive constipation and weight loss for 6 mo prior to the presentation and had become homebound. Physical examination revealed a frail, malnourished lady with a very tense and tender abdomen. Abdominal X-ray (Figure 1) showed a well demarcated gas-filled spherical shadow. Contrast-enhanced computed tomography (CT) scan showed a huge gas-filled abdominal mass occupying the whole abdomen and pelvis, with fecal matter inside (Figure 2A). Direct communication between the mass



Figure 1 Abdominal X-ray.

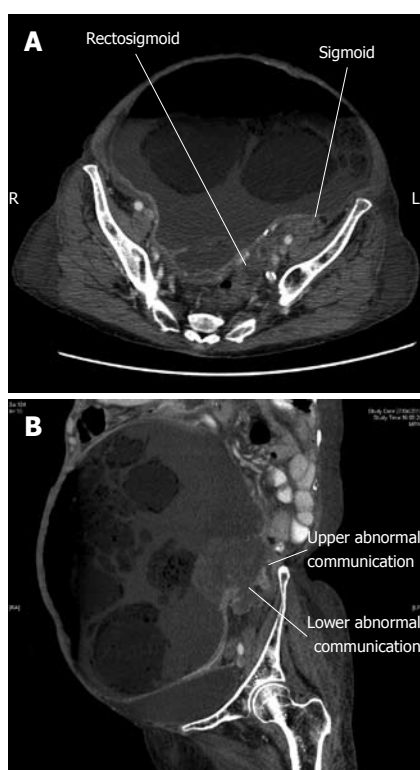


Figure 2 Computed tomography scan. A: Cross-sectional view; B: Sagittal view.

and the left-sided colon was demonstrable on sagittal-reformatted image (Figure 2B). Based on the radiological features, giant colonic diverticulum and duplication cyst of colon were suspected.

Urgent laparotomy was arranged. Intra-operatively, there was a huge cystic lesion occupying the abdomen and pelvis; the lesion was densely adhered to the anterior abdominal wall and the pelvic organs. The right ovary could not be visualized but the left ovary was grossly normal. The cyst was inseparable from the colon at the sigmoid-descending junction, where solid tumor mass was found over the cyst wall. The cyst wall was completely intact at the time of laparotomy. In order to facilitate dissection, gaseous content of the cyst was decompressed by needle aspiration. The whole lesion was

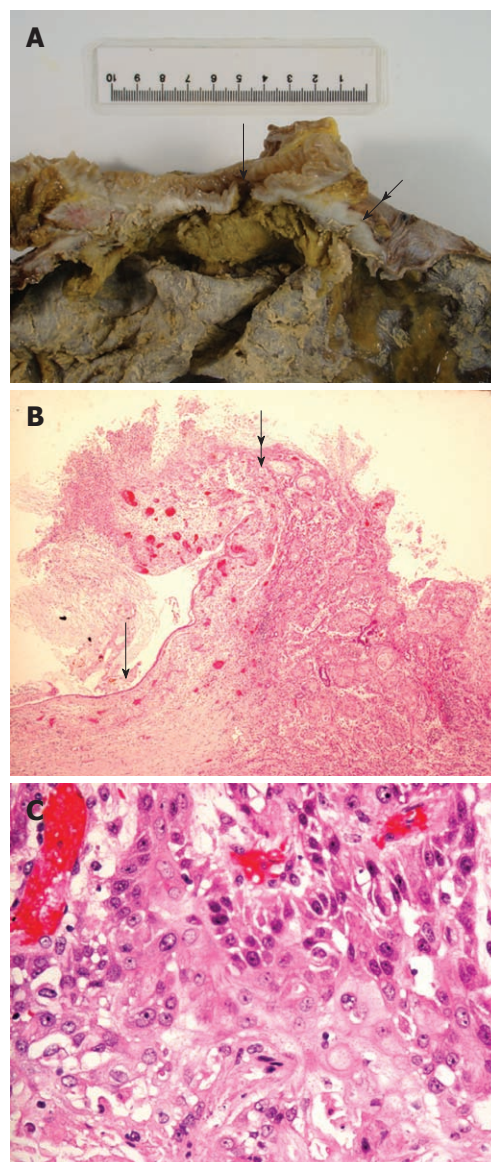


Figure 3 Pathology of the fistula and cyst wall. A: The fistula (arrow) led directly from the colon (upper part of the specimen) into the cyst wall (lower part). The tissue at the interface between the cyst wall and the colon was fleshy (double arrow); B: The benign, attenuated, ulcerative squamous cell lining of the original mature cystic teratoma (arrow) abruptly transformed to well to moderately differentiated squamous cell carcinoma (double arrow) (magnification:  $\times 100$ ); C: Higher magnification of the malignant component of the cyst wall showing typical squamous cell carcinoma ( $\times 400$ ).

completely excised *en bloc* with the involved colonic segment. End colostomy was fashioned over the left upper quadrant.

Macroscopic examination of the specimen revealed a huge complex cystic lesion containing feces, gas and necrotic material inside. It had a smooth serosal surface with focal gangrenous change. The size of the colonic fistula was 5 mm in diameter. Microscopic examination confirmed poorly differentiated squamous cell carcinoma, arising from mature cystic teratoma (MCT) of the ovary (Figure 3). Direct tumor invasion into the submucosa of the colon was evident. Most of the cyst wall was eroded and replaced by granulation tissue and fibrous tissue with

focal calcification, indicating previous episodes of cyst wall rupture, inflammation and organization.

The patient's condition gradually improved after the laparotomy. However, she was noticed to have left lower limb swelling on D5. Above knee deep vein thrombosis was confirmed by Doppler ultrasound examination and anti-coagulation therapy was initiated. Taking into consideration the advanced stage of the tumor and the poor pre-morbid state of the lady, completion hysterectomy, salpingo-oophorectomy and lymphadenectomy were not offered. She was discharged home after a 3-wk period of mobilization exercise.

Four months later, she presented to our unit again with abdominal pain. On admission, her lower abdomen was diffusely tender and distended but abdominal X-ray did not reveal any dilated bowel loops. Urgent CT scan showed features suggestive of peritoneal carcinomatosis with a 5-cm heterogenous tumor in the pelvis. She was confirmed to have urinary tract infection and treated with antibiotics according to the culture result. Despite medical treatment, her condition continued to deteriorate and she finally succumbed 1 wk after admission. Though no macroscopic peritoneal nodules were noticed during the initial operation, disease dissemination likely had occurred at the time of fistula formation, which would account for her subsequent rapid deterioration.

## DISCUSSION

The differential diagnosis of a gas-filled abdominal mass containing fecal matter includes giant colonic diverticulum<sup>[1-3]</sup>, sigmoid or cecal volvulus, and duplication of colon<sup>[4]</sup>. MCT with fistula formation to colon is an extremely remote cause. This diagnosis requires a very high index of suspicion. In hindsight, this should have been considered in our patient who had a long history of cystic lesion.

MCT is the most common type of ovarian germ cell tumor, accounting for about 10% of all ovarian neoplasms<sup>[5]</sup>. However, malignant transformation is a rare event in MCT, with an incidence of less than 1%-2%<sup>[6,7]</sup>. Any of the tissues derived from the three embryonic germ layers have the potential to undergo malignant transformation. The vast majority (> 80%) are squamous cell carcinomas<sup>[8]</sup>. In our hospital, there were 7 patients with 8 episodes of malignant transformation out of 563 patients with ovarian teratoma in the years from 1995 to 2010 (1.24%). Six of these incidents were squamous cell carcinoma, one being papillary carcinoma of thyroid tissue and one being neuroendocrine carcinoma. Pre-operative diagnosis of malignant transformation is challenging. Reported risk factors include patient's age older than 45 years, tumor size more than 10 cm and elevated tumor markers, especially the serum squamous cell carcinoma antigen<sup>[9-11]</sup>.

Fistulation is a rare complication of MCT and urinary bladder is the most commonly affected organ<sup>[12,13]</sup>. Malignant transformation is not a pre-requisite for fistulation.

Inflammation related to previous subclinical leakage of cyst content is believed to be the etiology of fistulation in some of the previous reports<sup>[12,14]</sup>. In our patient, both inflammation and direct infiltration by malignant cells might have contributed to the formation of fistulation. The presence of significant tumor bulk around the area of fistulation suggested tumor invasion being the pre-dominant factor.

Because of its rarity, management of squamous cell carcinoma arising from MCT is not well defined. In a recent review<sup>[15]</sup>, hysterectomy, bilateral salpingo-oophorectomy and lymphadenectomy were associated with better outcome; whereas omentectomy did not improve survival. Striking differences in survival existed between stage 1 disease and all other tumor stages. The 5-year survival rates reported by Chen *et al*<sup>[16]</sup> were 75.7%, 33.8%, 20.6% and 0% for stage I-IV disease, respectively.

In conclusion, squamous cell carcinoma arising from ovarian teratoma can rarely present as a surgical emergency with gross abdominal distension resembling bowel pathology such as giant colonic diverticulum and duplication cyst. Surgical resection remains the mainstay of treatment while adjuvant chemotherapy may improve survival in early stage diseases. Unfortunately, the overall prognosis is grave except for stage I disease.

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## MEETINGS

### Events Calendar 2011

January 14-15, 2011

AGA Clinical Congress of  
Gastroenterology and Hepatology:  
Best Practices in 2011 Miami, FL  
33101, United States

January 20-22, 2011

Gastrointestinal Cancers Symposium  
2011, San Francisco, CA 94143,  
United States

January 27-28, 2011

Falk Workshop, Liver and  
Immunology, Medical University,  
Franz-Josef-Strauss-Allee 11, 93053  
Regensburg, Germany

January 28-29, 2011

9. Gastro Forum München, Munich,  
Germany

February 4-5, 2011

13th Duesseldorf International  
Endoscopy Symposium,  
Duesseldorf, Germany

February 13-27, 2011

Gastroenterology: New Zealand  
CME Cruise Conference, Sydney,  
NSW, Australia

February 17-20, 2011

APASL 2011-The 21st Conference of  
the Asian Pacific Association for the  
Study of the Liver  
Bangkok, Thailand

February 22, 2011-March 04, 2011

Canadian Digestive Diseases Week  
2011, Vancouver, BC, Canada

February 24-26, 2011

Inflammatory Bowel Diseases  
2011-6th Congress of the European  
Crohn's and Colitis Organisation,  
Dublin, Ireland

February 24-26, 2011

2nd International Congress on  
Abdominal Obesity, Buenos Aires,  
Brazil

February 24-26, 2011

International Colorectal Disease  
Symposium 2011, Hong Kong, China

February 26-March 1, 2011

Canadian Digestive Diseases Week,  
Westin Bayshore, Vancouver, British  
Columbia, Canada

February 28-March 1, 2011

Childhood & Adolescent Obesity:

A whole-system strategic approach,  
Abu Dhabi, United Arab Emirates

March 3-5, 2011

42nd Annual Topics in Internal  
Medicine, Gainesville, FL 32614,  
United States

March 7-11, 2011

Infectious Diseases: Adult Issues  
in the Outpatient and Inpatient  
Settings, Sarasota, FL 34234,  
United States

March 14-17, 2011

British Society of Gastroenterology  
Annual Meeting 2011, Birmingham,  
England, United Kingdom

March 17-19, 2011

41. Kongress der Deutschen  
Gesellschaft für Endoskopie und  
Bildgebende Verfahren e.V., Munich,  
Germany

March 17-20, 2011

Mayo Clinic Gastroenterology &  
Hepatology 2011, Jacksonville, FL  
34234, United States

March 18, 2011

UC Davis Health Informatics:  
Change Management and Health  
Informatics, The Keys to Health  
Reform, Sacramento, CA 94143,  
United States

March 25-27, 2011

MedicReS IC 2011 Good Medical  
Research, Istanbul, Turkey

March 26-27, 2011

26th Annual New Treatments in  
Chronic Liver Disease, San Diego,  
CA 94143, United States

April 6-7, 2011

IBS-A Global Perspective, Pfister  
Hotel, 424 East Wisconsin Avenue,  
Milwaukee, WI 53202, United States

April 7-9, 2011

International and Interdisciplinary  
Conference Excellence in Female  
Surgery, Florence, Italy

April 15-16, 2011

Falk Symposium 177, Endoscopy  
Live Berlin 2011 Intestinal Disease  
Meeting, Stauffenbergstr. 26, 10785  
Berlin, Germany

April 18-22, 2011

Pediatric Emergency Medicine:  
Detection, Diagnosis and Developing

Treatment Plans, Sarasota, FL 34234,  
United States

April 20-23, 2011

9th International Gastric Cancer  
Congress, COEX, World Trade  
Center, Samseong-dong, Gangnam-  
gu, Seoul 135-731, South Korea

April 25-27, 2011

The Second International Conference  
of the Saudi Society of Pediatric  
Gastroenterology, Hepatology &  
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011

Neurology Updates for Primary  
Care, Sarasota, FL 34230-6947,  
United States

April 28-30, 2011

4th Central European Congress of  
Surgery, Budapest, Hungary

May 7-10, 2011

Digestive Disease Week, Chicago, IL  
60446, United States

May 12-13, 2011

2nd National Conference Clinical  
Advances in Cystic Fibrosis, London,  
England, United Kingdom

May 19-22, 2011

1st World Congress on Controversies  
in the Management of Viral Hepatitis  
(C-Hep), Palau de Congressos de  
Catalunya, Av. Diagonal, 661-671  
Barcelona 08028, Spain

May 21-24, 2011

22nd European Society of  
Gastrointestinal and Abdominal  
Radiology Annual Meeting and  
Postgraduate Course, Venice, Italy

May 25-28, 2011

4th Congress of the Gastroenterology  
Association of Bosnia and  
Herzegovina with international  
participation, Hotel Holiday Inn,  
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011

The International Digestive Disease  
Forum 2011, Hong Kong, China

June 13-16, 2011

Surgery and Disillusion XXIV  
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011

International Scientific Conference  
on Probiotics and Prebiotics-  
IPC2011, Kosice, Slovakia

June 22-25, 2011

ESMO Conference: 13th World  
Congress on Gastrointestinal Cancer,  
Barcelona, Spain

June 29-2, 2011

XI Congreso Interamericano  
de Pediatría "Monterrey 2011",  
Monterrey, Mexico

September 2-3, 2011 Falk Symposium

178, Diverticular Disease, A Fresh  
Approach to a Neglected Disease,  
Gürzenich Cologne,  
Martinstr. 29-37, 50667 Cologne,  
Germany

September 10-11, 2011

New Advances in Inflammatory  
Bowel Disease, La Jolla, CA 92093,  
United States

September 10-14, 2011

ICE 2011-International Congress of  
Endoscopy, Los Angeles Convention  
Center, 1201 South Figueroa Street  
Los Angeles, CA 90015,  
United States

September 30-October 1, 2011

Falk Symposium 179, Revisiting  
IBD Management: Dogmas to be  
Challenged, Sheraton Brussels  
Hotel, Place Rogier 3, 1210 Brussels,  
Belgium

October 19-29, 2011

Cardiology & Gastroenterology |  
Tahiti 10 night CME Cruise,  
Papeete, French Polynesia

October 22-26, 2011

19th United European  
Gastroenterology Week,  
Stockholm, Sweden

October 28-November 2, 2011

ACG Annual Scientific Meeting &  
Postgraduate Course,  
Washington, DC 20001,  
United States

November 11-12, 2011

Falk Symposium 180, IBD 2011:  
Progress and Future for Lifelong  
Management, ANA Interconti Hotel,  
1-12-33 Akasaka, Minato-ku,  
Tokyo 107-0052, Japan

December 1-4, 2011

2011 Advances in Inflammatory  
Bowel Diseases/Crohn's & Colitis  
Foundation's Clinical & Research  
Conference, Hollywood, FL 34234,  
United States





## INSTRUCTIONS TO AUTHORS

### GENERAL INFORMATION

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS.A Careaction* 2002; 1-6 [PMID: 12154804]

**Books***Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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