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EDITORIAL

Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy: Where are we?

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Abstract

Peritoneal surface malignancies are generally associated with poor prognosis. In daily clinical routine, systemic chemotherapy is still considered the only reasonable therapy despite of encouraging results of cytoreductive surgery (CRS) along with intraperitoneal hyperthermic chemotherapy (HIPEC). The Achilles heel of CRS and HIPEC is appropriate patient selection and precise surgical technique preventing patients from excessive morbidity and mortality. Given these findings, new concepts of second look surgery for high risk patients allow detection of peritoneal spread ahead of clinical symptoms or presence of peritoneal masses reducing perioperative morbidity. In addition, personalized intraperitoneal chemotherapy might further improve outcome by appreciating individual tumor biology. These days, every physician should be aware of CRS and HIPEC for treatment of peritoneal surface malignancies. Since there is now sufficient data for the superiority of CRS and HIPEC to systemic chemotherapy in selected patients, our next goal should be providing this strategy with minimal morbidity and mortality even in the presence of higher tumor load.

Key words: Peritoneal carcinomatosis; Hyperthermic intraperitoneal chemotherapy; Cytoreductive surgery

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INTRODUCTION

In Peritoneal surface malignancies (PSM), tumor location is restricted to the abdominal cavity as opposed to systemic metastatic disease. In the past, the majority of PSM patients underwent systemic chemotherapy which was associated with poor quality of life and was ineffective with respect to prolonging survival.

Thanks to the pioneer work of Professor Paul Sugarbaker, a proactive surgical approach termed cytoreductive surgery (CRS), aiming for maximal tumor resection, along with hyperthermic intraperitoneal chemotherapy (HIPEC) evolved into a highly relevant treatment option for selected patients with limited peritoneal spread of various tumor entities[1-3]. The rationale for this particular approach is the restriction of tumor dissemination to the peritoneal compartment justifying a radical surgical procedure followed by HIPEC.

Although there is now evidence for the superiority of

CRS and HIPEC to systemic chemotherapy, this strategy has not made its way into clinical routine since peritoneal spread is still considered as stage Ⅳ cancer when surgical resection is not an option any more. However, there was a similar thinking for colorectal liver metastasis for a long time. Now, surgery represents the main strategy even though its superiority has never been proven in a randomized phase Ⅲ trial.

If cytoreductive surgery is scheduled, proactive surgery achieving total or almost total (remaining nodules < 2.5 mm) cytoreduction has to be the main aim In addition, hyperthermic intraperitoneal chemotherapy is administered for eradication of microscopic residual disease. The most frequently cited paper on this topic was published by Verwaal *et al*[4,5] who first proved the benefit of this multimodal approach in a phase Ⅲ trial comparing patients with colorectal cancer undergoing CRS and HIPEC followed by systemic chemotherapy with systemic chemotherapy. Up to now, there are several reports on long term survival if radical resection was performed[4-7]. Other entities for which this treatment is accepted are pseudomyxoma peritonei and mesothelioma[8]. For selected patients with ovarian and gastric cancer this option can be offered with good results[9-12].

Since survival does not significantly differ between completeness of cytoreduction CC0 or CC1, an oncologic resection with wide resection margins seems not necessary in this content except for primary gastrointestinal cancer with peritoneal carcinomatosis PC.

INTERDISCIPLINARY CONCEPT

The implementation of many new centers for PSM could mean that more and more patients are asking for this therapeutic option. However, the perioperative setting has to be established first rather than the surgical one. An experienced radiologist is mandatory to assess preoperative tumor load and to rule out contraindications such as diffuse infiltration of the small bowel or extraperitoneal disease. The anaesthesiologists, nurses and HIPEC technicians should visit centers and participate in workshops for HIPEC before initiating the program. Lastly, the medical oncologist becomes more and more important because there are numerous different intraperitoneal as well as pre- and postoperative chemotherapy regimens. The founding of a peritoneal surface malignancy group which meets regularly has had a great impact on scientific discussion between surgeons, radiologists, anaesthesiologists. In addition, this facilitates the initiation of clinical multi-center and experimental studies.

HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY

In most cases, intraperitoneal chemotherapy is administered after cytoreductive surgery and completion of intestinal anastomoses, either immediately intraoperatively (HIPEC) or early postoperatively (EPIC). Technically, this chemotherapy can be applied to an open or closed abdomen which varies between the different centers.

The rationale of HIPEC is the synergistic cytotoxic effect of heat, ideally 42-43 degree Celcius, and the chemotherapeutic agent itself on tumor cells.

There are various concepts varying in duration of exposition, in combination with for example intraoperative intravenous therapy and in type of the administered chemotherapy.

The effect of hyperthermic intraperitoneal chemotherapy itself has never been proven in a randomized controlled trial and is still the focus of ongoing investigations. Nevertheless, there are numerous data of how HIPEC might work and most surgeons, medical oncologists and last but not least patients believe in the effect of local chemotherapy.

The rationale for applying intraoperative chemotherapy under hyperthermic conditions is improving both tissue as well as tumor oxygenation by vasodilation enhancing the cytotoxic effect of chemotherapeutic agents. So far, however, nobody has demonstrated an effect on hyperthermia on tissue oxygenation and there is no data whether this putative effect on pO_2 (oxygen) might be sustained throughout the entire HIPEC period. As learned from wound healing research, supplemental oxygen during HIPEC might further enhance cytotoxicity since it has been shown to increase tissue oxygen tension. In addition of thinking about the best timing for HIPEC, HIPEC in combination with supplemental oxygen could be a worthwhile option in the future.

Another future important issue could be testing chemotherapeutic sensitivity to improve the cytotoxic effect of HIPEC. Such particular tests already exist for ovarian cancer with respect to platinum resistance[13,14]. This further strengthens the need for personalized intraoperative chemotherapy regimens.

NEOADJUVANT CHEMOTHERAPY

A quite high percentage of patients is not eligible for cytoreductive surgery at the time of surgical exploration. Therefore, tumor downsizing by systemic chemotherapy and subsequent surgery might be an option. In liver surgery, the concept of secondary resection after chemotherapy, both intravenous as well as regionally, is accepted and response to preoperative chemotherapy can be considered as a prognostic factor[15-17]. In PSM, neoadjuvant chemotherapy might also aid in categorizing patients in responders and non-responders with responders being more likely to profit from CRS and HIPEC. One limitation is the difficulty to evaluate response to chemotherapy since computed tomography (CT) or positron emission tomography (PET)/CT often do not sufficiently show tumor spread. One ongoing phase Ⅱ trial in Germany addressing perioperative chemotherapy is the COMBATAC trial (multimodality treatment including neoadjuvant and adjuvant chemotherapy with cetuximab and CRS and HIPEC).

ORGAN PRESERVING CYTOREDUCTIVE SURGERY

Radical cytoreduction is many times associated with multivisceral resection because of diffuse organ infiltration. When performing cytoreductive surgery, the surgeon should, however, aim for preserving as many organs as possible. Moreover, the surgeon should leave as much as possible behind but without any oncological compromise. This approach seems quite unfamiliar to surgeons who do not deal with peritoneal metastases. In many cases, the small/large bowel can be preserved when addressed with patience for meticulous tumor resection since tumor nodules are mainly located on the peritoneal surface and can be removed without opening the bowel in most cases unless there is infiltrative growth.

From an oncologic point of view, a radical oncologic colon resection, except in primary colorectal cancer with peritoneal spread is not necessary in our opinion.

The surgical expertise should ideally include a broad surgical spectrum especially colorectal surgery. One technical challenge is certainly the liver hilus with the sulcus rex, sulcus arancii and segment 1 region which is very demanding to dissect with the risk of biliary or vascular damage when a certain experience in liver surgery is helpful.

SECOND LOOK SURGERY

Peritoneal carcinomatosis index (PCI), representing intraabdominal tumor load is a prognostic factor for survival. The lower the PCI, the better the prognosis maybe also due to the fact that a complete cytoreduction becomes more likely. Clinical signs of peritoneal metastases are often not specific and current imaging methods often do not detect small tumor nodules[18,19]. Given these findings, a second look protocol with a re-laparotomy within one year of colorectal surgery in high risk patients was proposed. The high risk patient for developing PSM suffers from either a perforated tumor or a local peritoneal spread at the time of primary surgery. Current data revealed quite a high percentage of PSM in those patients.

The second look protocol was firstly described by Elias *et al*[20]. Predicting the development of PSM in high risk patients is certainly a mile stone in the treatment of peritoneal metastases[20]. Although this approach is proactive, it may further prolong survival in those patients.

The administration of HIPEC even in a patient without macroscopic peritoneal disease needs further to be elucidated in randomized trials but seems to be promising so far. The "ProphyloCHIP" trial (Trial Comparing Simple Follow-up to Exploratory Laparotomy Plus "in Principle" HIPEC in Colorectal Patients) run by Prof Elias is addressing this particular point. In this randomised phase Ⅲ trial, colorectal cancer patients at risk to develop PC receive standard adjuvant chemotherapy after curative resection. After having excluded recurrent disease within 6 mo of follow-up they are randomised to either surveillance alone or explorative laparotomy and HIPEC. With this proactive approach, disease free and overall survival may be increased.

CONCLUSION

With newer imaging modalities such as PET/CT and PET/magnetic resonance tomography a better location of the tumor may be realized in future. Pro-active second look surgery, as far as there is no optimal imaging method, realizes the anticipation of diffuse peritoneal spread.

Making "unresectable patients" resectable is one challenging goal of neoadjuvant chemotherapy protocols in the future.

Using new protocols including intraperitoneal antibodies or even intraperitoneal virotherapy in patients with unresectable disease may further improve results.

Lastly and most importantly, a dedicated surgeon, an experienced anaesthesiologist and cooperating medical oncologists are mandatory to achieve excellent results and develop new concept in the treatment of peritoneal metastases.

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FIELD OF VISION

c-Met in pancreatic cancer stem cells: Therapeutic implications

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Abstract

Pancreatic cancer is the deadliest solid cancer and currently the fourth most frequent cause of cancer-related deaths. Emerging evidence suggests that cancer stem cells (CSCs) play a crucial role in the development and progression of this disease. The identification of CSC markers could lead to the development of new therapeutic targets. In this study, the authors explore the functional role of c-Met in pancreatic CSCs, by analyzing self-renewal with sphere assays and tumorigenicity capacity in NOD SCID mice. They concluded that c-Met is a novel marker for identifying pancreatic CSCs and c-Met^{high} in a higher tumorigenic cancer cell population. Inhibition of c-Met with XL184 blocks self-renewal capacity in pancreatic CSCs. In pancreatic tumors established in NOD SCID mice, c-Met inhibition slowed tumor growth and reduced the population of CSCs, along with preventing the development of metastases.

Key words: Cancer stem cells; c-Met; Gemcitabine; Self-renewal; Tumorigenicity

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INVITED COMMENTARY ON HOT ARTICLES

Pancreatic adenocarcinoma is one of the most aggressive neoplasias and the fourth leading cause of cancer death in the United States, with 5 year survival being less than 5%[1]. Part of the reason for the fatal prognoses of patients with pancreatic adenocarcinoma is the lack of response to the available therapies. In addition to achieving early diagnosis, the identification of markers for prognosis and response to therapy is necessary for the development of specific targeting agents for the management of patients with pancreatic cancer[2].

Conventional chemotherapy is directed at tumor cells that have limited tumorigenic potential, instead of targeting the cancer stem cell population.

Cancer stem cells (CSCs) conform to a subpopulation of tumoral cells that contribute mainly to drug resistance; since they can self-renew indefinitely and are charac-

terized by their relative quiescence. These properties, together with the contribution of the epithelial to mesenchymal transition, allow them to avoid conventional chemotherapy induced-cell death, and thus patients often suffer from tumor relapse[3,4].

A better understanding and characterization of these cells will be a helpful tool in the future for designing new therapies, since the CSC hypothesis predicts that only therapies that efficiently eliminate the CSC fraction of a tumor are able to induce long-term response and halt tumor progression. Novel therapeutic strategies that could target pancreatic CSCs will allow us to induce drug sensitivity, the inhibition of invasion, and the metastasis of pancreatic cancer, which will ultimately yield better treatment outcomes[5].

Although there have many studies attempting to establish markers for these CSCs, there is no complete or defined panel for the identification of these cells. Isolated CSCs have been reported to show increased tumorigenicity, chemoresistance, stationary phase, and asymmetric division to differentiate into non-stem progeny[6,7]. Based on these characteristics, pancreatic CSCs are identified by flow cytometry using cell surface markers such as CD44, CD24, ESA, CD133, ALDH1 and low proteosome activity[8-13].

In this study, new data is presented about CSC characterization and possible related therapies that should be considered for pancreatic cancer clinical trials.

c-Met is a receptor of the tyrosine kinase family that acts as a proto-oncogene and is stimulated by hepatocyte growth factor to mediate motility, invasion, and metastasis[14]. The levels of c-Met are increased in pancreatic carcinoma where c-Met signaling induces growth and invasion. Some authors have reported c-Met as a stem cell marker in pancreatic tissue[15], as well as in other tissues and organs such as the brain and gastrointestinal tract where the molecule regulates stem cell proliferation and cell renewal[16,17].

In this study, the authors conclude that c-Met is a novel marker for pancreatic CSCs and they show the functional role of this molecule in pancreatic tumors.

Using xenografts (immunodeficient mice NOD-SCID) and injecting pancreatic cancer cells from patients, they show that there is a subpopulation of cells that express high levels of c-Met (c-Met^{high}), while another subpopulation expresses low levels or does not express this maker at all (c-Met^{low}). Part of the c-Met^{high} population also expresses other previously described CSCs makers such as CD44, CD24, CD133, and ALDH1, but compared with these other markers, c-Met^{high} is better at identifying higher tumorigenic cancer cell populations (when injecting the same number of cells, c-Met^{high} cells produce tumors in 35% of the mice, while CD133+ produces tumors in 16% and CD44+ in 25%).

The population of positive cells for both the markers c-Met and CD44 (c-Met^{high}CD44+) enhances the *in vivo* tumorigenicity compared with the population with CD44+ alone. These data together show that c-Met is an important and novel marker of pancreatic CSCs.

Using sphere formation assay, it can be shown that c-Met^{high} cells have a self-renewal capacity, as they can form tumorspheres while c-Met^{low} are unable to do so.

On the other hand, treatment with the chemical inhibitor XL184 (a small molecule kinase inhibitor for c-Met) or the inhibition of c-Met with a specific shRNA impairs sphere formation, leading to apoptosis and cell cycle arrest; showing that c-Met activity is required to maintain a CSC population.

XL184 inhibits tumor growth and reduces the number of CSCs in xenograft mice with subcutaneous tumors and in orthotopic models.

When compared with Gemcitabine, the effect produced by XL184 is different. Although both XL184 alone and Gemcitabine alone treatments can inhibit tumor growth during the period that the animal is receiving treatment, some days after the end of treatment, the tumor growth continues at the same rate as the control tumors. By contrast, when the treatments are combined (XL184 in combination with Gemcitabine), tumor growth is prevented for up 32 d following cessation of treatment. This effect is due to the cells that each treatment targets. While Gemcitabine treatment results in an increase of the c-Met^{high}CD44+ population (likely because these cells are resistant to cell death with this chemotherapy) c-Met inhibition with XL184 leads to a decrease in c-Met^{high}CD44+ cells. Combination treatment prevents the increase in the CSC population observed with Gemcitabine alone and also contributes to a decrease in c-Met^{high}CD44+ population, suggesting that XL184 targets the CSC population specifically. Additionally, using an intracardiac injection model with pancreatic cancer cells, the authors demonstrated that XL184 treatment prevents metastasis development.

A study by Li *et al*[5] contributes significantly to the investigation into the stemness of pancreatic cancer cells. The study in question shows that c-Met is a new human pancreatic CSC marker. The results demonstrate that, in addition to identifying the population of cells with a self-renewal capacity in a pancreatic tumor, c-Met is a molecule necessary for maintaining tumoral cell growth and has capacity for metastasize.

Previous experiments had characterized CSCs as EpCAM+, CD44+, CD24+, CD133, low proteosome activity, ALDH+ for pancreatic CSCs, and CD133+ CXCR4+ for CSCs with higher metastatic potential. The addition of the new marker c-Met, already identified in other types of tumor, directs research towards the development of therapies targeting this tyrosine kinase receptor. In addition to its role as a biomarker of pancreatic CSCs, the authors purpose a potential therapy, showing reduced tumor growth in xenograft mice. They also demonstrated that c-Met has an important role in metastasis development, and that this process could be blocked by targeting c-Met specifically with XL184 treatment. XL184 (Cabozantinib) is a small-molecule kinase inhibitor with potent activity toward c-Met and vascular endothelial growth

factor receptor 2, as well as a number of other receptor tyrosine kinases including rearranged during transfection, kinase receptor, axl receptor tyrosine kinase, and fms like tyrosine kinase 3[18].

The authors demonstrated that XL184 targeting c-Met could be a promising therapy in combination with Gemcitabine treatment for pancreatic cancer. This is a very important achievement, since pancreatic cancer has a fatal prognosis due to its resistance to the currently available chemotherapy and radiotherapy. Although Gemcitabine is the most common chemotherapy used in pancreatic cancer patients, it has been demonstrated that it may not be particularly useful due to the properties that CSCs confer to the tumor. Some studies have demonstrated that, after Gemcitabine treatment, the subpopulation of CSC CD133+ is enriched[11] and that pancreatic cancer cell lines can undergo epithelial to mesenchymal transition after this treatment, resulting in an increased population of CD44+CD24+ESA+ cells[19].

Li *et al*[5] showed that the combination treatment of XL184 and Gemcitabine is an effective therapy for pancreatic cancer treatment. As different studies have shown, using a combination of therapies that target CSCs and the non-tumorigenic population of pancreatic cancer cells, this neoplasia can be effectively treated.

Collectively, these data and other recently published studies concerning different tumors, suggest that c-Met is a promising CSC marker for pancreatic cancer and XL184 is effective at inhibiting tumor growth, angiogenesis, and metastasis, and both should be seriously considered for clinical trials in combination with other available chemotherapy.

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Magnets, children and the bowel: A dangerous attraction?

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Abstract

Reports of magnet ingestion are increasing rapidly globally. However, multiple magnet ingestion, the subsequent potential complications and the importance of the early identification and proper management remain both under-recognized and underestimated. Published literature on such cases could possibly represent only the tip of an iceberg with press reports, web blogs and government documents highlighting further occurrence of many more such incidents. The increasing number of complications worldwide being reported secondary to magnet ingestion point not only to an acute lack of awareness about this condition among the medical profession but also among parents and carers who will be in most cases the first to pick up on magnet ingestion. There still seems to be no consensus on the management of magnet ingestion with several algorithms being proposed for management. Prevention of this condition remains a much better option than cure. Proper education and improved awareness among parents and carers and frontline medical staff is key in addressing this rapidly emerging problem. The goal of managing such cases of suspected magnet ingestion should be aimed at reducing delays between ingestion time, diagnosis time and intervention time.

Key words: Multiple magnet ingestion; Children; Bowel injury; Fistulation; Necrosis

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INVITED COMMENTARY ON HOT ARTICLES

Background

Ingestion of foreign bodies is a common clinical problem; the occurrence of which has been steadily increasing all over the world. More than three quarters of such cases occur in children[1]. Of particular concern is the diagnostic and management dilemma that is posed by the ingestion of magnetic elements. Ingestion of a confirmed single magnet by itself does not pose a problem because it behaves just as an isolated foreign body. The single magnet in most cases moves through the gut harmlessly and silently and usually gets expelled without complications[2,3]. However, the ingestion of multiple magnets or a single magnet along with another metallic piece poses a totally different challenge as these magnetic elements can get attracted to each other with forces up to 1300 G[4] and any intervening bowel wall between the attracted parts eventually undergoing pressure necrosis. Subsequent fistulization between bowel loops can remain silent until it

leaks and peritonitis intervenes.

Magnitude of the problem

The issues of foreign body ingestion have been well discussed in the literature. However, multiple magnet ingestion, the subsequent potential complications and the importance of the early identification and proper management remain both under-recognized and underestimated. Reports of multiple magnet ingestion and its complications have been steadily increasing over the past few years, with over 15 cases being reported in the literature over 7 mo in 2012 compared to 10 cases in 2010, and two cases per year about a decade ago[3,5-8]. Published literature on such cases may represent only the tip of an iceberg with press reports, web blogs and government documents highlighting further occurrence of many more such incidents[5]. The extent of the problem is highlighted as a total of 128 published cases across 18 countries assimilated in 2010 have now expanded to over 150 cases over 22 countries in 2012[6-10]. The majority of such cases have involved the ingestion of either two or three magnetic elements, although there is one reported case of nearly 100 pieces[11].

Initial reports of this condition more than a decade ago were mainly confined to infants and toddlers. Children with a variety of psychological conditions including autism, developmental delays, history of pica, schizoid characteristics, Angelman syndrome, behavioral problems, Beckwith-Wiedemann syndrome with developmental delays, 4p syndrome, congenital hydrocephalus, mental retardation, reactive attachment, and anxiety were thought to be at a higher risk for accidental ingestion[11]. Of interest was the fact that this group comprised < 15% of the total cases reported so far.

Presently, the incidence of this problem no longer remains confined to these groups. Recent reports suggest that multiple magnet ingestion seem to be occurring with increasing frequency in fully developed older age groups[5,11,12].

The origin of most of these magnetic elements has been traced to toys, either directly belonging to the child or to an elder sibling[9,11-13]. A possible cause for the increase of such cases is the easy availability of cheap toys that contain magnetic elements[14]. New-generation magnets are made of combinations containing iron, along with other rare earth elements including boron and neodymium, and such magnets tend to be nearly 10 times stronger than standard iron magnets. This has enabled the miniaturization of magnets for inclusion in various small toys[3,15]. In many of these toys, the magnetic elements are poorly embedded in plastic moulds from which they can easily become detached[16].

Diagnosis

The key to diagnosis is to obtain a reliable history of magnet ingestion. A credible history of ingestion is crucial in the early recognition and correct management of this condition. The lack of a documented history of ingestion in nearly half of the reported cases even among the older age groups is of concern[5]. Younger children or those with developmental delays may be hindered by their inability to communicate effectively to their parents due to their limited linguistic or developmental abilities. Older children may hesitate to inform parents due to a sense of guilt or embarrassment or a fear of the consequences[9,17]. This may have a direct bearing on the time interval between ingestion and intervention. This may be shorter if there is evidence of ingestion or longer when only the occurrence of bowel complications may highlight an underlying magnetic pathology. Time intervals between ingestion and intervention have varied from a few hours to a few months[5].

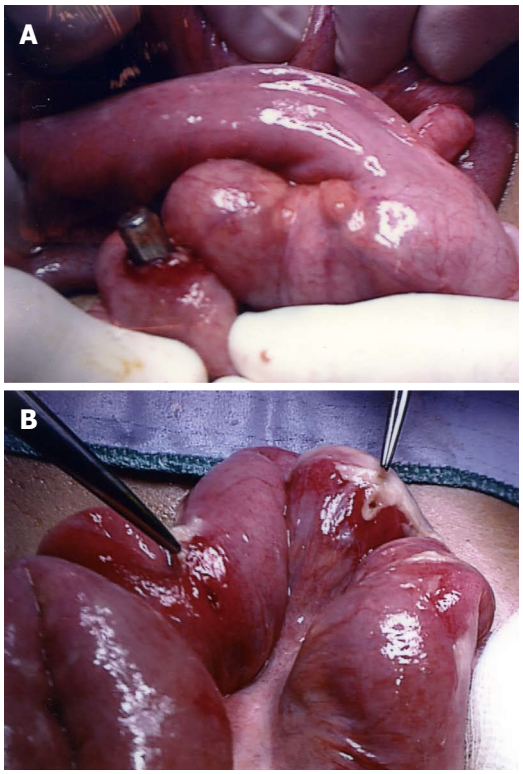

Figure 1 Symptoms may vary from totally asymptomatic or an unrelated pain to a mild flu-like illness with nonspecific symptoms of nausea, vomiting, cramps, or abdominal pain, to features of bowel obstruction or localized peritonitis. A: Magnetic cause for an acute appendicitis in an 8-year-old child; B: Laparotomy for peritonitis showed small bowel fistulation caused by two magnets.

Symptoms may vary from totally asymptomatic or an unrelated pain to a mild flu-like illness with nonspecific symptoms of nausea, vomiting, cramps, or abdominal pain to features of bowel obstruction or localized peritonitis[3,5,12,18] (Figure 1A, B).

Plain abdominal radiographs almost always pick up these objects and is a simple and quick screening test if a history of ingestion is obtained[19-21]. Plain radiography is a sensitive tool to screen and identify such cases but is poor in differentiating whether the ingested magnet is truly only single or is actually composed of multiple densely adherent magnetic elements. Although radiographs can be taken at different angles and planes, the

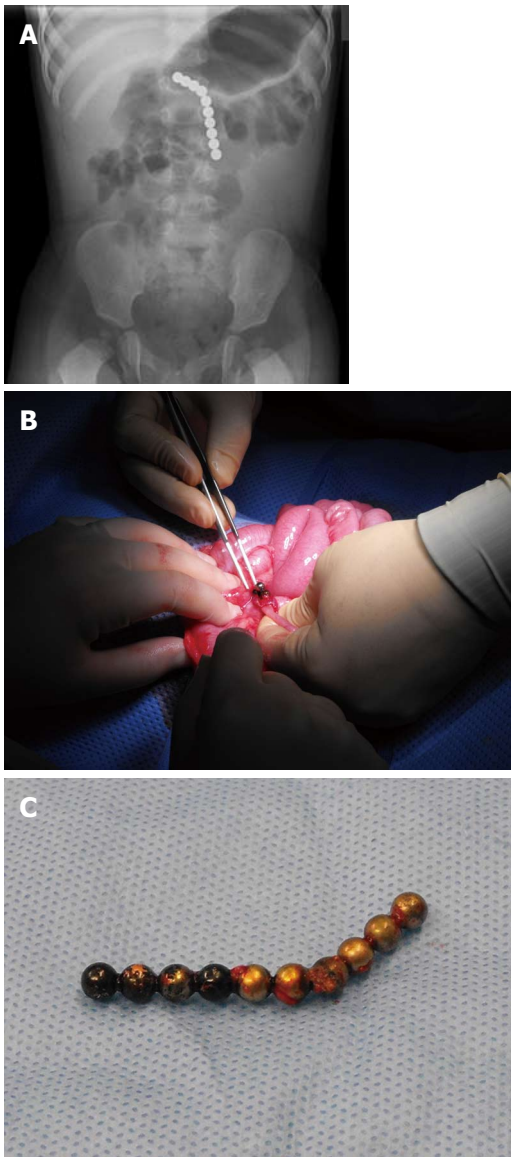

Figure 2 If multiple magnet ingestion is suspected, the entire gastrointestinal tract remains at risk of perforation even if the child is asymptomatic. A: Asymptomatic 18-mo-old child 96 h after ingestion of magnet; B: Silent gastro-jejeno-colic fistula at laparotomy; C: Ten strongly attracted magnetic balls lying across the fistula.

differences in radiographic appearances between a single magnet and multiple magnets adhered to each other may be subtle and impossible to differentiate[2]. Subtle separations or gaps between otherwise individual metallic pieces may point to the presence of multiple magnetic elements or the presence of intervening bowel between the magnets. However, this is by no means diagnostic and the absence of any gaps within the imaged magnet does not exclude more than one magnet nor the absence of bowel wall involvement[2]. In addition, the failure of the ingested magnetic element to progress through the bowel on subsequent follow-up radiographs should raise the suspicion of multiple magnetic elements with entrapped bowel, although this is not diagnostic because the multiple magnets can move *en bloc*[2,17,22,23]. Documenting the size and shape of the swallowed object on radiography and confirming the presence of only a single magnet may be challenging because such ingested magnets tend to be miniature and they usually originate from children's toys[3,12].

Computed tomography and ultrasound can be performed but may not contribute greatly because they generally lack the sensitivity to determine the multiplicity of or the presence of trapped bowel between the magnetic objects. Magnetic resonance imaging (MRI) scans should not be performed due to the magnetic nature of the ingested foreign body and bowel perforation secondary to inadvertent MRI has been reported[8].

Management

Management of ingested foreign bodies still relies to a great extent on "masterly inactivity", whereby the ingested foreign body traverses the gut and is expelled without any complications.

There still seems to be no consensus on the management of magnet ingestion, with several algorithms being proposed[2,3,5,21]. A common underlying theme is that in cases of multiple magnet ingestion, conservative management may have no appreciable role. Surgical exploration and removal remains the preferred management irrespective of the size or shape of the magnet[19].

A diagnostic and management dilemma arises if there is a doubt as to whether one or more magnets were ingested. A proper history is important to help identify between single or multiple magnet ingestion, but reliable documentation of ingestion may not always be present[5]. Clear differentiation is not always possible between the two because multiple magnets may tend to be densely adherent to each other and can mimic a single object on imaging.

Conservatively discharging the child back to the community without reliable evidence of single magnet ingestion may have the potential to cause unnecessary morbidity[3,5]. Undiagnosed multiple magnets can tend to remain asymptomatic for several weeks or months until potentially disastrous complications intervene[5].

Close observation of such cases even if they are asymptomatic may be prudent given the lack of any investigation which can effectively rule out multiple adherent magnets. If single magnet ingestion is suspected, normal progression through the bowel can be monitored closely with expulsion of the magnet through a bowel movement[21].

If multiple magnet ingestion is suspected, the entire gastrointestinal tract remains at risk of perforation even if the child is asymptomatic (Figure 2A-C). All such cases should be reviewed urgently by the surgeon with a view to magnet removal. If pediatric surgery expertise is not available, urgent transfer to an appropriate specialist center is important (Figure 3).

The aim of management in cases of suspected multiple magnet ingestion should be to shorten the delays between ingestion time, diagnosis time and intervention

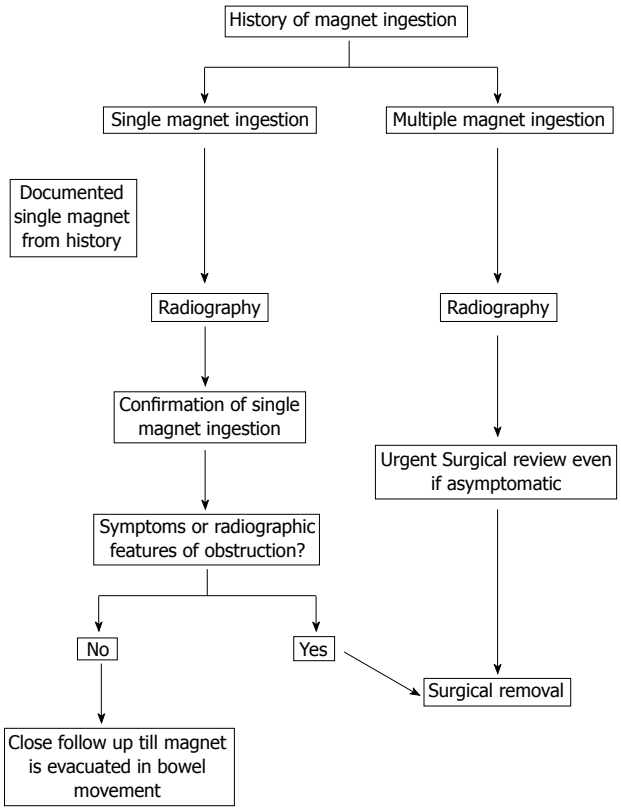

Figure 3 A view for magnet removal.

time.

Removal of the magnets is seminal and delaying the removal or waiting for evidence of bowel obstruction to develop may lead to unnecessary morbidity and even mortality[3,5]. All such reported cases with the exceptions of a few sporadic cases[11,18] have been managed with the surgical removal of the magnetic parts. Bowel injury following multiple magnet ingestion in a few conservatively managed cases may have been avoided possibly due to a near-simultaneous ingestion of the magnetic elements, which may have then behaved as a single large magnet. This however, would not be sufficient reason to recommend conservative management in such cases.

Removal of the ingested magnets can be retrieved endoscopically if they are in the esophagus, stomach or proximal duodenum[5]. Once the magnets move further into the small bowel, surgical removal either through an exploratory or a laparoscopic assisted laparotomy is required to localize and remove the magnets.

Field of vision

The field of vision regarding this condition is highly myopic. Prevention remains a much better option than cure for this rapidly increasing problem.

The increasing number of complications worldwide being reported secondary to magnet ingestion point not only to an acute lack of awareness about this condition among the medical profession but also among parents and carers who will be, in most cases, the first to pick up on magnet ingestion. Parents need to be alerted to the potential risk of silent bowel perforation and fistulation from accidental ingestion of magnets. The importance of increasing awareness regarding the potential complications of magnet ingestion is crucial. This could prompt parents and carers to identify earlier cases of suspected magnet ingestion and rapidly seek appropriate medical attention, and considerably reduce the delay between ingestion and diagnosis.

There also exists a lack of awareness among the medical profession about the potential of multiple magnet ingestion to do great harm. Improving awareness among frontline medical staff can help to reduce the time delay between ingestion and diagnosis, as well as between diagnosis and intervention.

There is also a need for tighter control and regulation of toys with magnetic components. Since 2006, there have been numerous alerts and recalls from Canadian and United States consumer product safety commissions issued in relation to children and the sale of toys with small ingestible magnetic parts[2,24]. The occurrence of such cases from over 21 countries worldwide highlights that this is no longer confined to a localized geographical region or population. Toy manufacturers all over the world can incorporate easily visible warnings regarding the presence of small magnetic parts in the toys on the labels. Highlighting age restriction on toys may not by itself cover much ground without improved awareness because younger children can accidentally ingest magnetic elements from toys that may have been appropriately bought for elder siblings in the family.

Parents, carers and medical staff globally remain under-informed and largely unaware regarding this rapidly increasing potential public health problem. The goal of managing such cases of suspected magnet ingestion should be to reduce the delays between ingestion time, diagnosis time and intervention time.

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TOPIC HIGHLIGHT

Cesare Ruffolo, MD, PhD, *Series Editor*

Laparoscopic distal pancreatectomy: Up-to-date and literature review

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Abstract

Pancreatic surgery represents one of the most challenging areas in digestive surgery. In recent years, an increasing number of laparoscopic pancreatic procedures have been performed and laparoscopic distal pancreatectomy (LDP) has gained world-wide acceptance because it does not require anastomosis or other reconstruction. To date, English literature reports more than 300 papers focusing on LDP, but only 6% included more than 30 patients. Literature review confirms that LDP is a feasible and safe procedure in patients with benign or low grade malignancies. Decreased blood loss and morbidity, early recovery and shorter hospital stay may be the main advantages. Several concerns still exist for laparoscopic pancreatic adenocarcinoma excision. The individual surgeon determines the technical conduction of LDP, with or without spleen preservation; currently robotic pancreatic surgery has gained diffusion. Additional researches are necessary to determine the best technique to improve the procedure results.

Key words: Pancreas resection; Laparoscopic distal pancreatectomy; Left pancreatectomy; Open pancreatectomy; Pancreatic fistula; Splenectomy; Spleen-preserving technique

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PANCREATIC SURGERY AND LAPAROSCOPY

Pancreatic surgery represents one of the most challenging areas in digestive surgery, and it has been historically associated with up to 50% morbidity and 5% mortality[1,2]. It is usually performed by open approaches, but following the increased experience in laparoscopic surgery of other districts and the availability of new technological devices, an increasing number of laparoscopic pancreatic procedures has been performed[3,4].

Laparoscopy has initially been used only for staging

pancreatic cancer. Cuschieri reported the first description of laparoscopic pancreatic resection in 1994[5]; few years later, Gagner published his initial experience with laparoscopic distal pancreatectomy (LDP) with spleen preservation including eight cases[6]. Actually, LDP has gained world-wide acceptance because the procedure does not require anastomosis or other reconstruction[7,8].

A comparison between open surgery and LDP confirms advantages commonly ascribed to minimal-access surgery such as reduced postoperative pain, faster recovery, fewer wound related and general morbidity[9-19]. Although the laparoscopic approach to distal pancreatectomy has become a feasible option over the last few years, it still faces two problems: firstly, sparing the spleen with or without ligation of the splenic vessels, and secondly, controlling the leak from the pancreatic remnant and pancreatic fistula[20]. However, some controversy about its indications and safety concerning long-term oncologic outcome, still exist[21-23].

Literature concerning LDP is relatively poor: Case reports, small case series and few multicentric larger studies have been published[21,24-26]. The aim of this paper is to review the most recent literature, in order to offer an up-to-date concerning the indications, the results and some technical controversial issues concerning LDP.

LAPAROSCOPIC DISTAL PANCREATECTOMY: LITERATURE REVIEW

A web search, focusing on humans, was performed by PubMed database, including papers published in the English language up to 20 November 2011, using the key words "laparoscopic distal pancreatectomy" or "left-side pancreatectomy". A total of 388 papers were found. The bibliographic research was further expanded considering the related references cited by the above-mentioned papers.

In order to avoid the confounding effect of case reports and small series, a more refined research, including series of at least 30 cases, was performed. Results published only in the abstract form were excluded; in case of multiple publications from the same authors or institutions, only the latest and largest series were considered, in order to avoid the duplication of cases.

Twenty-two papers, including 2016 operated patients were found when literature search was reviewed. The median number of cases included were 70 (range: 30-359). The results are summarized in the Table 1. Eighteen papers included retrospective series; patients were prospectively included only in four studies. Eight papers derived from multicentre group studies.

The review of the literature confirms that LDP may be considered a feasible and safe technique[21,27,28]. It represents more than 70% of the laparoscopic pancreatic resections actually performed[29]. However, the major part of the studies on LDP is represented by case series with a relatively small number of patients[23,30]; only 6% of papers includes more than 20 cases. Most of the studies have a retrospective design; subsequently, it is still difficult to trace any conclusion from the results of these experiences because of the insufficient level of evidence.

Several comparative studies have shown that the average operative time, blood loss, morbidity, mortality and length of hospital stay after laparoscopic access might favourably comparable with those after open surgery[9,11-19,29].

In particular Mehta *et al*[17] describes a tendency toward a shorter duration of surgery in laparoscopic resection compared to open, although without a significance level ($P = 0.071$).

By contrast, with these optimistic prospects, Baker has published a single-institution comparison between laparoscopic and open distal pancreatectomy, focusing on post-discharge readmission. The laparoscopic approach has been associated with a shorter hospital stay, but a higher rate of late readmission requiring interventional procedures[31]. Furthermore, LDP cannot be considered a routine laparoscopic procedure, since it requires an advanced technical laparoscopic skill.

To date, several aspects are still controversial, mainly related to the indications, the results of the procedure and some technical details.

INDICATIONS TO LAPAROSCOPIC DISTAL PANCREATECTOMY

The indications for LDP vary, depending on the study, although most operations of LDP have been performed because of benign lesions, neuroendocrine tumors or low-grade malignancies (in particular cystic tumors)[29,32]. However some cases of pancreatic adenocarcinoma have been reported[33]; the results of laparoscopic resection for left pancreatic adenocarcinoma are limited, and its safety for long-term oncologic outcome is strongly debated. This approach for the treatment of pancreatic carcinoma still requires prospective validation[34] (Table 2).

LDP has also been performed in patients with chronic pancreatitis[35]; laparoscopic necrosectomy for acute necrotizing pancreatitis has been also described[36]. Steering wheel injury typically involves pancreatic parenchyma in front of the vertebra; LDP preferably with spleen preservation, has been indicated for patients with pancreatic trauma[37-39].

Persistent hyperinsulinemic hypoglycemia of infancy is a rare disease due to focal islet cell adenomatosis that may cause severe neurogenic damage. LDP or enucleation of the focal lesion has been performed in pediatric patients, while an open near-total pancreatectomy has been indicated in an infant with sustained hypoglycemia[40,41].

Surgery is the only curative modality currently available for resectable pancreatic neuroendocrine tumors[26,42]. Spleen-preserving LDP is feasible and can be achieved in

Table 1 Laparoscopic distal pancreatectomy: Results of the literature review

Ref.	Year	Study type	*n*	Pathology
Butturini *et al*[98]	2011	Retrospective	43	SCN (14), MCN (9), SPT (4), NET (9), other (7)
Mekeel *et al*[99]	2011	Retrospective	34	SCN (11), NET (8), IPMN (6), MCN (4), other (5)
Nakamura *et al*[100]	2011	Retrospective	47	NET (9), MCN (10), IPMN (6), SPT (5), P (6), other (11)
Mehta *et al*[17]	2011	Prospective	30	NET (12), IPMN (4), ADK (7), other (7)
Song *et al*[101]	2011	Retrospective	359	SCN (51), MCN (72), SPT (52), NET (36), IPMN (76), ductal ADK (24), P (25), cyst (13), others (10)
Zerbi *et al*[102]	2011	Prospective	95	NET
Røsok *et al*[103]	2010	Retrospective	117	NET (53), carcinoma (28), metastases (5), cystic tumours (48), other (37)
Vijan *et al*[12]	2010	Retrospective	100	Cystic benign neoplasm (49), solid benign neoplasm (27), malignant neoplasm (20), other (4)
Jayaraman *et al*[104]	2010	Retrospective	107	NA
DiNorcia *et al*[16]	2010	Retrospective	95	NA
Ziegler *et al*[105]	2010	Retrospective	126	NA
Giulianotti *et al*[80]	2010	Retrospective	46	ADK (6), NET (carcinoma) (5), benign neoplasm (28), other (6)
Finan *et al*[13]	2009	Retrospective	50	ADK (6), MCN (9), SCA (9), IPMN (4), NET (9), other (13)
Weber *et al*[106]	2009	Retrospective	219	MCN (45), SCN (29), IPMN (23), cyst (14), SPT (5), NET (45), ADK (16), metastases (9), P (14), other (19)
Yoon *et al*[107]	2009	Retrospective	35	SPT (7), IPMN (4), MCN (4), NET (4), other (16)
Kooby *et al*[27]	2008	Retrospective	159	Cystic neoplasm (85), solid neoplasm (60), P (14), benign neoplasm (102), ADK (16)
Taylor *et al*[108]	2008	Retrospective	46	SCN (10), ductal ADK (9), MCN (6), other (19)
Laxa *et al*[109]	2008	Retrospective	32	SCN (10), NET (6), mucinous neoplasm (4), IPMN (4), other (8)
Sa Cunha *et al*[7]	2008	Prospective	37	NA
Melotti *et al*[110]	2007	Prospective	58	MCN (19), SCN (13), NET (9), SPT (5), ductal ADK (5), other (7)
Fernández-Cruz *et al*[28]	2007	Retrospective	82	Inflammatory tumor (8), cystic neoplasm (29), IPMN (10), NET (22), ductal ADK (13)
Mabrut *et al*[21]	2005	Retrospective	99	NA

n: Number of patients; SCN: Serous cystic neoplasm; MCN: Mucinous cystic neoplasm; ADK: Adenocarcinoma; SPT: Solid pseudopapillary tumor; IPMN: Intraductal papillary mucinous neoplasm; NA: Not available; NET: Neuroendocrine tumor; P: Pancreatitis. Number of case for each pathology are report between parenthesis.

Table 2 Indications to laparoscopic distal pancreatectomy

Benign disease	Borderline disease	Malignant disease
Acute/chronic pancreatitis	Neuroendocrine tumor	Invasive carcinoma
Trauma	Mucinous cystic neoplasm	Metastatic renal cell carcinoma
Persistent hyperinsulinemic hypoglycemia of infancy	Intraductal papillary mucinous neoplasm	
Serous cystic neoplasms		
Transplantation in the living donor		

most cases; it is indicated for insulinomas of the pancreatic body and tail. Intraoperative laparoscopic ultrasonography is essential to localize the tumor and to evaluate the gland for additional tumors. If the insulinoma is benign, solitary, and not close to the main duct, a laparoscopic enucleation of the neoplasm may be indicated[25,43,44].

Laparoscopic resections have been also performed in some patients with gastrinoma, VIPoma, glucagonoma[45], pancreatic polypeptidoma[46], or other islet neoplasms including so-called non-functioning tumors[6]. However, some of these neuroendocrine neoplasms are often malignant and conversion to open surgery is necessary in cases of gastrinomas and VIPomas complicated by lymph node metastasis[6,47,48].

In case of potentially malignant neuroendocrine neoplasms in the pancreatic body/tail, LDP might be indicated, but laparoscopic en bloc splenectomy with resection of the spleen vessels and regional lymph-nodes dissection are recommended[4]. In case of multiple endocrine neoplasia-1 (MEN-1) patients, multiple neoplasms are common (especially gastrinomas); in these cases tumors are also located at gastroduodenal sites; the intraoperative localization of the tumors by laparoscopic approach, is not always possible even by the laparoscopic ultrasound; subsequently it is not usually indicated[48]. Furthermore, since a prolonged postoperative follow-up (at least 10 years) is required in case of potentially malignant neuroendocrine tumors, consistent data concerning the cure and recurrences rate, are still not available.

Pancreatic mucinous cystic neoplasm (MCN) represents a further possible indication to LDP. It groups a spectrum of lesions ranging from benign mucinous cystadenoma to mucinous cystadenocarcinoma. MCN is characterized by a distinct ovarian type stroma; patients with these lesions are usually relatively young women, with the peak around in the fifth decade of life. These neoplasms are more common in the body/tail of pancreas and a complete resection of the lesion is indicated. In patients with MCN several authors[49,50] have reported successful treatments by LDP.

The pancreatic serous cystic neoplasms are usually benign cystic neoplasms. When serous cystic neoplasms are symptomatic or when a differential diagnosis from potentially malignant cystic neoplasm is not possible,

a resection (possibly LDP) is indicated[49-52]. In a large comparative study between open and LDP, cystic lesions represented 59% of the laparoscopically resected tumors and 46% of the tumor excised by a laparotomic approach[19].

Intraductal papillary mucinous neoplasms (IPMN), non-invasive mucin-producing, predominantly papillary, or rarely flat epithelial neoplasms arising from the main pancreatic duct (MD-IPMN) or its secondary branches (BD-IPMN), are grossly visible[53]. They involve the head of the pancreas more commonly than the body/tail and they affect older patients with the peak age in the seventh decade. For IPMN in the body/tail of the pancreas, LDP has been performed, but one should be aware that some IPMN is associated with invasive carcinoma, as it is for MCN[53,54].

LDP has also been carried out in patients with pancreatic invasive carcinoma[23], although the questions about the oncological consequences of laparoscopic pancreatic surgery remain strongly controversial[21].

The positive margin resection rate in pancreatectomy for ductal adenocarcinoma is difficult to understand because there is no defined standard for histologic margin assessment. A recent review of the large randomized trials highlight that positive margin rates ranged from 0% to 83%[55].

A prospective observational study comparing open *vs* LDP has shown that the number of lymph nodes removed during the laparoscopic procedure was significantly inferior in comparison to the open approach[10]. Another recent analysis from a multicentre group, has compared the results of laparoscopic and open distal pancreatectomy applied to pancreatic ductal carcinoma. Cancer outcomes in short-term (lymph nodes harvest and margin status) and long-term (survival) were found to be similar in both groups[32,33]. Finally, LDP have been sporadically described also for metastatic renal cell carcinoma[56] and for pancreas transplantation in the living donors[57,58].

SURGICAL TECHNIQUES

The individual surgeon determines the technical conduction of LDP; it is usually performed in a supine or in a right lateral position[59]. However, several technical variants may be used, and some controversies still exist. The main controversial aspects in LDP are related to the preservation of the spleen, by the number and location of orifices needed for approaching the pancreas, the extent of the resection and the technique used for the parenchymal transection.

Traditionally, distal pancreatectomy has been performed with splenectomy. However the spleen plays an important role in the immune system and spleen-preserving distal pancreatectomy is preferable, in patients with benign diseases or non-invasive neoplasms[8,21,51,60].

The rate of splenic conservation of LDP is reported to be between 32% and 84%[14,17,42]. Some comparative studies have assessed the outcomes preoperative intent of splenic conservation in distal pancreatectomies performed by laparoscopic and open approaches, with a higher success rate of preservation in the first group[14,17]. This is surely due to the better vision afforded by the magnification, used in laparoscopy.

Preservation of the spleen with distal pancreatectomy can be undertaken either with preservation or with sectioning of the splenic vessels by maintaining the blood flow to the spleen *via* short gastric vessels (technique of Warshaw[61]). The latter method is associated with a shorter operation time, less blood loss, and a shorter hospitalization. The subsequent appearance of gastric varices is a consequence of loss of the splenic vein but no bleeding from these collaterals during long-term follow up, has been described. However, a splenic infarction after the laparoscopic procedure of Warshaw[62] has been documented in several case reports[63].

A technical difficulty during the preservation of splenic vessels is the division of numerous shorts tributaries from the splenic vein spreading toward the pancreatic body/tail, that requires special caution[64]. The appropriate usage of modern technologies (electro thermal bipolar vessel sealer, ultrasonic coagulating shears) can achieve secure haemostasis of tributaries from splenic vessels.

The hand-assisted laparoscopic surgical techniques have been utilized in LDP, in order to facilitate the splenic vessel preservation, because incidental bleeding can be immediately stopped by finger compression, and in large cystic tumors for a safe mobilization of the tumor and adjacent tissue. Hand ports for the insertion of operator's left hand are placed through an upper midline incision, right subcostal incision, or right lower-quadrant transverse incision according to the preference of surgeons[65-68].

Single incision laparoscopic surgery has gained attention for its minimal invasiveness and aesthetic results. This approach has been commonly described for cholecystectomy and appendectomy[69-71]; recently it has also been reported for LDP[72]. It may be effective as conventional laparoscopic pancreatectomy, when performed by expert hands although it is still a challenging procedure[58]. Further studies are necessary to determine the advantages of this procedure in comparison with standard laparoscopy.

Even though laparoscopic surgery of the pancreas remains a very challenging technique, the classically available instruments have some relevant limits. Today, following the increasing use of the robotic surgery in other fields of general surgery, some robot-assisted pancreatic resections have been reported[73-75]. Robotic surgery, can bridge the gap between minimally invasive surgery and complex pancreatic surgery, thus extending the indications for minimally invasive pancreatic surgery.

Robot-assisted surgery increases the degrees of freedom of forceps manipulation and yields three-dimensional images[22,73,76-79]. It is a procedure with some technical

and oncological advantages over other minimally invasive techniques for distal pancreatic tumors, due to the stability of the operative field, the 3D, magnified vision, and the articulated robotic arms. Moreover, the robotic articulated arms permit a superior handling of vascular structures and articulated instruments minimizing manipulation of the pancreatic gland. This technique minimizes the risk of pancreatic capsule rupture as well as tumor cell dissemination, respecting oncological surgical standards and it could provide an increased chance for spleen preservation. Giulianotti *et al*[80] has highlighted that robot-assisted laparoscopic pancreatic surgery achieves complication and mortality rates comparable to open surgery approaches, but offers the advantages of minimally invasive surgery. However, robotic surgery has high costs especially concerning the installation and the operation time, which is longer than open surgery; at the same time, it also needs an adequate learning curve.

The extent of a resection in LDP is another controversial topic. It varies depending on the pathology. For example, when a non-invasive MCN is located in the tail of the pancreas, the gland can be divided to the right of the cystic lesion with a minimal margin and only the tail of the pancreas removed. For chronic pancreatitis, it is typically divided at the pancreatic neck anterior to the superior mesenteric vein[4,35]. Recently, pancreatic surgeons have performed parenchyma-sparing resections more frequently in order to decrease the rate of postoperative pancreatic insufficiency. Oncological radicality is essential and extended resections may be necessary in the setting of IPMNs, which encompass a spectrum of lesions from adenoma to invasive carcinoma.

Intraoperative examination of the transection margin is of paramount importance in the management of MD-IPMNs[81-83]. The International Association of Pancreatology guidelines for the management of IPMNs suggest that when adenoma or low-grade pancreatic intraepithelial neoplasia is found intraoperatively in a resection margin, no further resection is needed. In case of borderline neoplasms, high-grade dysplasia or invasive carcinoma, an extension of the surgical resection to a negative margin, requires total pancreatectomy[53].

MORBIDITY

The most frequent complications after distal pancreatectomy are the fistula formation and collection[21,84,85]; they are usually related to pancreatic parenchymal transection techniques, that is another controversial topic.

In 2005 the International Study Group on Pancreatic Fistula Definition consensus paper defined a postoperative pancreatic fistula as the existence of any fluid output after postoperative day three with amylase content greater than three times the upper normal serum value[86].

Mabrut reviewed a total of 897 patients who underwent open distal pancreatectomy and reported the incidence of pancreatic fistula to be 3.5%-26% (average 13%)[21]. The incidence of pancreatic fistula with laparoscopy in studies that involved at least ten patients ranged from 0% to 27%[85].

Various risk factors for fistula formation have been reported after distal pancreatectomy. It is likely to occur in a pancreas with a soft texture[21,84,85,87], and when a selective identification and ligation of the main pancreatic duct has not been performed[87-89].

Some authors have suggested that the selective ligation may be more difficult during laparoscopy and may contribute to increased fistula rates[21]. Nevertheless, comparative studies showed that the laparoscopic approach results in a similar rate of fistula formation than the open approach[14,15,17]. A meta-analysis of studies comparing minimally invasive (laparoscopic or laparoscopically assisted) to the open approach, showed a lower rate of pancreatic fistula formation for minimally invasive approach[11]. However these results need cautious interpretation, because they depend on the study by Kooby, that relied on a definition different from that of the other studies[27].

The debate regarding the technique of stump closure after distal pancreatectomy continues. All approaches, including fibrin glue, sealants, patches, stapler closure, electrocautery and suture have been tested in numerous studies[90,91].

The distal pancreatectomy trial included 352 patients that were randomly assigned to stapler or hand-sewn closure of the pancreatic remnant: both groups showed equal fistula rates of 30% and 36%[92].

The main duct ligation and parenchymal transection during LDP is most commonly performed using endoscopic linear staplers. The surgeon may oversew the staple line to ensure pancreatic ductal closure and haemostasis. In such cases, ultrasonic coagulating shears can be used, usually followed by a monofilament suture to secure the closure of the main pancreatic duct[21,63,93,94].

In a recent study, Sartori *et al*[95]describes a new technique of pancreatic transection by the electrothermal bipolar vessels sealer, which seals vessels and other tubular structures by reforming parietal collagen and elastin, particularly suitable for laparoscopic left pancreatectomy; but a possible suitability in terms of fistula reduction is still under investigation.

When using the hand assisted laparoscopic technique, the pancreas can be divided through the hand port wound as for conventional open surgery.

To prevent post-operative fistula, octreotide and its analogues, have also been used since 1990. However, despite twenty years of clinical use and performance in numerous studies, a recent Cochrane meta-analysis concluded that evidence is still lacking to give clear recommendations[96].

Intraabdominal drains are commonly used in most centres after pancreatic resections. There is no evidence that persisting drainage of postoperative wound fluid has a positive effect in avoiding fistulae; on the contrary, a recent study sustains that drains kept in situ for more than three days enhance fistula development[97].

The management of postoperative fistula remains a therapeutic challenge. Depending on patient's clinical conditions, it ranges from persisting drainage without any further measures, up to revision surgery. However in literature, after LDP, a conservative management of pancreatic fistula is usually described[85,87].

In conclusion, LDP is a feasible and safe procedure in patient with benign or low grade malignancies. Decreased blood loss and morbidity, early recovery and shorter hospital stay may be the main advantages. The introduction in particular of robotic surgery, can bridge the gap between minimally invasive surgery and complex pancreatic surgery. On the other hand, additional researches are necessary to determine the best technique for minimizing pancreatic fistula formation and to improve the results of procedure.

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REVIEW

Theoretical basis of a beneficial role for vitamin D in viral hepatitis

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Abstract

Abnormal bone metabolism and dysfunction of the calcium-parathyroid hormone-vitamin D axis have been reported in patients with viral hepatitis. Some studies suggested a relationship between vitamin D and viral hepatitis. Genetic studies have provided an opportunity to identify the proteins that link vitamin D to the pathology of viral hepatitis (i.e., the major histocompatibility complex class Ⅱ molecules, the vitamin D receptor, cytochrome P_{450}, the renin-angiotensin system, apolipoprotein E, liver X receptor, toll-like receptor, and the proteins regulated by the Sp1 promoter gene). Vitamin D also exerts its effects on viral hepatitis *via* non-genomic factors, i.e., matrix metalloproteinase, endothelial vascular growth factor, prostaglandins, cyclooxygenase-2, and oxidative stress. In conclusion, vitamin D could have a beneficial role in viral hepatitis. Calcitriol is best used for viral hepatitis because it is the active form of the vitamin D_3 metabolite.

Key words: Calcitriol; Hepatitis; Hepatitis B virus; Hepatitis C virus; Vitamin D

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INTRODUCTION

Abnormal bone metabolism and dysfunction of the calcium-parathyroid hormone (PTH)-vitamin D axis have been reported in patients with viral hepatitis. In these patients, bone mineral density (BMD) was reduced in the lumbar spine and femoral neck[1-4]. The prevalence and severity of bone loss increases based on the severity of the liver disease[2]. Biochemical markers of bone resorption, such as urinary telopeptide (NTX) and pyridinoline, bone-specific alkaline phosphatase, and serum levels of PTH, were increased in patients with chronic viral hepatitis[1,4-9]. Serum insulin-like growth factor-1 (IGF-1) and 25-hydroxyvitamin D_3 (25OHD) were lower in patients with viral hepatitis[1,8-10]. However, other studies demonstrated contradictory results with respect to bone metabolism in patients with chronic viral hepatitis. Osteosclerosis was reported in patients with hepatitis C virus (HCV) and was associated with normal levels of IGF-1. It is also associated with an increased levels of osteoproterin (OPG) and the ligand for receptor activator of nuclear factor-κB (RANK)[11,12]. Serum levels of PTH were lower in patients with HCV compared to controls[6,13]. These findings suggested that there might be a relationship between vitamin D and viral hepatitis. In this paper, we review the role of vitamin D in patients with viral hepatitis.

GENETIC FACTORS RELATED TO VITAMIN D IN VIRAL HEPATITIS

The major histocompatibility complex (MHC) class Ⅱ molecules play an important role in immune functioning

and are essential to the body's defense against infection. The human MHC class Ⅱ is encoded by three different isotypes, *HLA-DR*, *HLA-DQ*, and *HLA-DP*. Studies have suggested that several genes in the MHC region promote susceptibility to viral hepatitis. Human leukocyte antigen (HLA) genes, which are located in the MHC region, have been implicated in viral hepatitis susceptibility. *HLA-DRB1*12* is significantly more common in children with autoimmune hepatitis with positive hepatitis A IgM than in children with negative hepatitis A IgM[14]. In addition, the *HLA-DPA1* and *HLA-DPB1* genes are known to be associated with hepatitis B virus (HBV) infection in Han Chinese, Japanese, and Thai populations[15-18]. However, *HLA-DPA1* was not associated with the development of cirrhosis or hepatocellular carcinoma (HCC) in Han Chinese populations[19]. Genetic variants in the *HLA-DPA1* region may also affect treatment-induced hepatitis B e antigen (HBeAg) sero-conversion[20]. In the normal human liver, mRNA expression of *HLA-DPA1* and *HLA-DPB1* are important for control of HBV[21]. *HLA-DRB1*1101* correlates with less severe hepatitis in Taiwanese male carriers of HBV[22]. *HLA-DRB1*1302* was reported to be associated with protection against persistent HBV infection in Gambian populations[23]. In South Indian populations, a significantly higher frequency of *HLA-DRB1*0701* was observed in patients with chronic viral illness compared with individuals who spontaneously recover (SR), but *HLA-DRB1*0301* was noted to be of higher frequency in the SR group than the chronic HBV group[24]. In patients from Eastern Turkey, *DQ2* and *DQ8* have been noted to be markedly higher in patients with chronic HBV than those with SR[25]. The presence of *DQw1* may protect against chronic active HBV infection[26]. In addition, patients with chronic HBV infection and the *DQB1*0303* and *DRB1*08* haplotypes may be less responsive to interferon alpha (IFNα) treatment[27]. Moreover, *DRB1*11*, *DRB1*0301*, and *DRB1*04* were found to confer a significant protective advantage against HCV infection[28-31]. These alleles might be responsible for the selection of viral epitopes for presentation to $CD4^+$ T cells, leading to a more efficient immune response against the virus. In a meta-analysis study, both *DQB1*0301* and *DRB1*1101* were protective alleles and presented HCV epitopes more effectively to $CD4^+$ T lymphocytes than other epitopes, Indeed, subjects with these two alleles were at a lower risk of developing chronic HCV infection[32]. On the other hand, calcitriol is known to stimulate phagocytosis but suppresses MHC class Ⅱ antigen expression in human mononuclear phagocytes[33,34]. In peripheral blood leukocytes, the expression of *HLA-DR* decreased after calcitriol administration in renal transplant recipients[35]. Calcitriol also decreases interferon-gamma-induced HLA-DR antigen expression on normal and transformed human keratinocytes and cultured epithelial tumor cell lines[35,36]. Both DR and DQ protein levels on the surface of a myeloma cell line were decreased after calcitriol treatment[37]. Moreover, calcitriol inhibits the expression of all three subtypes of MHC class Ⅱ antigens (*HLA-DR*, *HL-ADP*, and *HLA-DQ*) as well as the accessory activity of monocytes, both in a dose- and time-dependent manner[38]. These findings suggest that calcitriol may have an impact on viral hepatitis by suppressing the expression of MHC class Ⅱ antigens.

Genetic studies provide an opportunity to link molecular variations with epidemiological data. DNA sequence variations, such as polymorphisms, exert both modest and subtle biological effects. Vitamin D exerts immunomodulatory and anti-proliferative effects through the vitamin D receptor (VDR) in numerous diseases. VDR gene polymorphisms are reported to be associated with distinct clinical phenotypes in Taiwanese hepatitis B virus (HBV) carriers[39]. There is an association between *Taq1* and *Fok1* polymorphisms of VDR and HBV outcomes in Chinese patients[40]. The *tt* genotype of VDR polymorphism is linked to persistent HBV infection in African patients[41]. Polymorphisms in the *TT* allele of exon 9 of VDR are associated with occult HBV infection in Iranian patients[42]. Significant differences in the frequency of the allelic distribution of the *Apa1* of VDR are reported to occur more frequently in patients with HBV complicated by severe liver disease as well as those with higher viral loads[43]. These observations suggest that alterations in VDR function may play a role in viral hepatitis.

The cytochrome P_{450} (*CYP*) system is responsible for the oxidation, peroxidation, and/or reduction of vitamins and for the metabolism of steroids, xenobiotics, and various drugs. The *CYP27B1-1260* promoter polymorphism has been reported to be associated with vitamin D deficiency and an increased risk of fracture in the elderly[44]. Reduced 25OHD levels associated with the *CYP27B1-1260* promoter polymorphism results in reduced 1,25OHD levels and are associated with failure to achieve sustained virologic response (SVR) in patients with hepatitis C virus (HCV) genotypes 1, 2, and 3[45]. In Huh7.5 hepatoma cells, HCV infection increased calcitriol production by inhibiting *CYP24A1* induction, the enzyme responsible for the first step in calcitriol catabolism[46]. *CYP24A1* methylation tended to correlate with better prognosis in HCV-related HCC[47].

The primary function of the renin-angiotensin system (RAS) is to maintain fluid homeostasis and regulate blood pressure. Angiotensin converting enzyme (ACE) is a key enzyme in the RAS and converts angiotensin (AT) I to the potent vasoconstrictor AT Ⅱ[48]. Hepatic stellate cells (HSCs) are recognized as the main collagen-producing cells in injured hepatic tissue. Angiotensin Ⅱ (AT Ⅱ) mediates key biological actions involved in hepatic tissue repair, including myofibroblast proliferation, infiltration of inflammatory cells, and collagen synthesis. Activated HSCs secrete AT Ⅱ[49]. ACE2 expression is significantly increased in the context of liver injury, in both humans and rats[50]. In addition, AT Ⅱ levels are much higher in patients with HBV when compared to controls. These levels were directly related to the severity of the illness and decrease markedly with captopril, which is an ACE inhibitor[51]. A statistically significant correlation has been

noted between polymorphisms in the promoter region of the *AT* gene and the development of progressive hepatic fibrosis in patients with chronic HCV[52]. In recurrent hepatitis C infection, male liver recipients who were carriers of the *D* allele of *ACE* appeared to gain more weight after liver transplantation; in female recipients, however, carriers of the *D* allele appear to experience more severe allograft fibrosis[53]. Losartan, an AT1 receptor blocker, attenuates liver fibrosis in experimental models and in patients with chronic hepatitis C and significantly decreases the expression of several profibrogenic and NADPH oxidase (NOX) genes[54]. The administration of AT-blocking agents reduced the development of graft fibrosis in hepatitis C recurrence after liver transplantation[55]. However, there is also an interaction between vitamin D and the RAS. The combination of ACE inhibitors with the ACE *DD* genotype has been shown to decrease the level of calcitriol[56]. In Turkish populations of hypertensive patients, the presence of the *ACE D* allele is associated with a higher risk of left ventricular mass index and ambulatory blood pressure measurement, which is negatively correlated with serum 25OHD levels[57]. In addition, genetic disruptions of the VDR gene result in overstimulation of the RAS, resulting in increased renin and AT Ⅱ productions and subsequently leading to elevated blood pressure and cardiac hypertrophy. Treatment with captopril reduced cardiac hypertrophy in VDR knockout mice[58], suggesting that calcitriol could function as an hormonal suppressor of renin biosynthesis. Moreover, calcitriol suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin core promoter[59] and decreases ACE activity in bovine endothelial cells[60].

Apolipoprotein E (ApoE) is critical to systemic and local lipid transport and is a major genetic factor in viral hepatitis. The hepatitis virus is associated with serum lipoproteins, including ApoE and ApoB, and may enter cells *via* the low-density lipoprotein receptor (LDL-R). In *in vitro* models, the co-culture of hepatocytes with liver sinusoidal endothelial cells (LSEC) significantly increases the ability of hepatocytes to uptake low-density lipoprotein (LDL) and also results in a high level of HCV-like particle uptake[61]. The cell surface expression of LDL-R has been reported to correlate well with LDL-cholesterol and HCV-viral load[62]. ApoE antibody can block both HCV entry an the knockdown of the LDL-R reduced HCV infection of cells[63]. Human ApoE is required for the infectivity and assembly of HCV[64,65]. The *ApoE ε4* allele protects against severe liver disease caused by HCV[66], while *ApoE ε3* is associated with persistent HCV infection[67]. In addition, patients with chronic hepatitis C who do not carrying an *ApoE* ε3 allele, as well as carriers of a single *ApoE* ε3 allele with a serum cholesterol concentration over 190 mg/dL, were more likely to have a favorable outcome[68]. Moreover, lipoprotein abnormalities found in the early phases of acute hepatitis; low levels of serum cholesterol and ApoA associated with the severity of liver cell injury in chronic liver disease[69]. The nonstructural protein 5A (NS5A) of the HCV has been shown to interact with $ApoA_1$[70]. A decreased level of $ApoA_1$ was found in the LDL fractions of HCV-infected patients; the specific siRNA-mediated down-regulation of $ApoA_1$ led to a reduction in both HCV RNA and viral particle levels in culture[71]. On the other hand, the *ApoE4* allele is reported to be associated with decreased bone mass in postmenopausal Japanese women[72]. The common *ApoE* polymorphism has a complex effect on bone metabolism in peri-menopausal Danish women: those with *ApoE2* have lower bone mineral losses in the femoral neck and hip region than other women, whereas those with *ApoE4* gain more bone mineral than other women[73]. Calcitriol has been shown to induce macrophages to exhibit specific saturable receptors for LDL and acetyl-LDL; the LDL receptor of 1,25OHD-induced macrophages has been found to exhibit specificity for ApoB and E-containing lipoproteins[74]. In ApoE knockout mice, an animal model of dyslipidemia, high oxidative stress, and pronounced atherosclerosis after unilateral nephrectomy, animals developed less plaque growth and calcification with vitamin D analog treatment (paricalcitol) compared to healthy controls[75,76]. *ApoE ε4*, however, is associated with higher serum 25OHD levels[77]. Moreover, hypovitaminosis D is associated with reductions in serum $ApoA_1$[78] and a highly significant positive correlation was found between serum concentrations of 25OHD and *$ApoA_1$*[79]. In addition, calcitriol was reported to suppress *$ApoA_1$* gene expression at the transcriptional level in hepatocytes[80].

Lipids have been shown to play important roles in the viral life cycle and pathogenesis of infection. HBV infection of primary hepatocyte cultures is dependent on the presence of cholesterol in the viral envelope. The extraction of cholesterol from HBV purified from the plasma of HBV-infected patients leads to a strongly reduced level of infection, whereas infectivity is only regained by adding cholesterol back[81]. A number of lipid metabolic pathways were disrupted by HCV infection, resulting in an increase in cholesterol and sphingolipid levels[82]. Higher serum triglycerides, total cholesterol and LDL levels were correlated with higher HCV RNA levels[83]. Ceestatin, a novel small molecule inhibitor of hepatitis C virus replication, inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in a dose-dependent manner[84]. Polyunsaturated liposomes are reported to be antiviral against hepatitis B and C viruses by decreasing cholesterol levels in infected cells[85]. Moreover, HCV and HBV X protein increases the hepatic lipogenesis is mediated predominantly by the liver X receptor (LXR)[86-88]. LDL receptor-related protein 5 (LRP5) is essential for normal cholesterol and glucose metabolism. Mice lacking LRP5 develop both increased plasma cholesterol levels when fed a high-fat diet markedly impaired glucose tolerance when fed a normal diet[89]. HCV core protein activates Wnt/β-catenin signaling molecules, such as LRP5/6 co-receptors[90], whereas calcitriol regulates the expression of LRP5 *via* DNA sequences elements located downstream of the transcription start site[91]. Notably,

high serum 25OHD concentrations are associated with a favorable serum lipid profile, e.g., total cholesterol and high-density cholesterol (HDL-C)[92]. Low levels of active vitamin D (calcitriol) are also associated with low HDL-C levels[93]. Moreover, calcitriol has been shown to suppress foam cell formation by reducing acetylated LDL (AcLDL) and oxidized LDL (oxLDL) cholesterol uptake by macrophages[94]. In addition, calcitriol also inhibits the activity of HMG-CoA reductase, an enzyme required for cholesterol biosynthesis[95]. In male VDR knockout mice, serum total cholesterol and LXRβ levels were significantly higher than those in wild type mice[96]. The crosstalk between LXRα and VDR signaling in the regulation of bile acid metabolism suggests a possible contribution of the VDR to the modulation of bile acid and cholesterol homeostasis[97].

Toll-like receptors (TLRs) are a group of glycoproteins that functions as surface trans-membrane receptors and are involved in innate immune responses to exogenous pathogenic microorganisms. Substantial evidence supports an important role for TLRs in the pathogenesis and outcomes of viral hepatitis. There is a correlation between hypo-responsiveness to TLR ligands and liver dysfunction in HCV infection[98]. The disruption of TLR-3, TLR-7, and TLR-9 signaling was reported in viral hepatitis[99-101]. *In vivo*, TLR signaling also inhibits HBV replication[102]. TLR-2 polymorphisms that impair the recognition of HCV core and nonstructure 3 proteins may be associated with allograft failure and mortality after liver transplantation for chronic HCV[103,104]. These polymorphisms affect HCV viral loads and increase the risk of HCC in patients infected with HCV genotype 1[105]. The TLR-3 polymorphism may predispose Asian Indian populations to HCV infection[106] and protect Han Chinese populations from HBV recurrence after liver transplantation[107]. TLR-7 polymorphisms are protective against from development of inflammation and fibrosis in male patients with chronic HCV infection and are predictive of the response to IFN treatment[108-110]. TLR-2 and TLR-4 polymorphisms are not associated with liver cirrhosis in HCV infected Korean patients[111]. RNA levels of TLRs 2, 4, 6, 7, 8, 9 and 10 were up-regulated in both the monocytes and T cells of HCV-infected patients when compared to controls[112,113]. In obese rats, vitamin D deficiency increases the expression of hepatic mRNA levels of TLR-2, TLR-4, and TLR-9[114]. However, calcitriol is also known to suppress the expression of the TLR-2 and TLR-4 protein and mRNA in human monocytes; it also triggers hypo-responsiveness to pathogen-associated molecular patterns[115]. Calcitriol has also been shown to down-regulate intracellular TLR-2, TLR-4 and TLR-9 expression in human monocytes[116]. TLR activation results in the expression of VDR and 1α-vitamin D hydroxylase in human monocytes[117]. Calcitriol can cause vitamin D-induced expression of cathelicidin in bronchial epithelial cells[118] and may enhance the production of cathelicidin LL-37[119]. The addition of a VDR antagonist has been shown to inhibit the induction of cathelicidin mRNA by more than 80%; consequently, the protein expression of this antimicrobial agent was reduced by approximately 70%[118].

The HBV major surface antigen promoter contains four functional transcription factor Sp1 binding sites, which likely contribute to the level of expression from this promoter during viral infection[120-122]. HCV-core protein functions as a positive regulator of IGF-Ⅱ transcription *via* the protein kinase C (PKC) pathway, and Sp1 and Egr1 are direct targets of the transcriptional regulation of the IGF-Ⅱ, which plays an important role in HCV pathogenesis during the formation of HCC[122,123]. Steatosis is an important clinical manifestation of HCV infection. Sp1 is involved in sterol regulatory element-binding protein-1c (SREBP-1c) activation, which activates the transcription of lipogenic genes by HCV-3a NSSA[124]. Moreover, Sp1 might participate in triggering HCV core protein up-regulation of the extracellular matrix metalloproteinase (MMP) inducer expression and progression of metastasis[125]. On the other hand, binding sites for the transcription factor Sp1 have been implicated in the hormone-dependent transcription of several genes. In cultured human fibroblasts, the level of CYP24 (25-OHD 24-hydroxylase) mRNA plays a key role in the metabolism of 1,25OHD and increases responsiveness to calcitriol by 20 000-fold. Two vitamin D-responsive elements (VDREs) located upstream of the *CYP24* gene are primarily responsible increased mRNA levels, and Sp1 has been noted to act synergistically with these VDREs in this induction[126]. The mVDR promoter is controlled by Sp1 sites[127] and functions as the transactivation component of the VDR/Sp1 complex to trigger gene expression[128]. Moreover, the genes encoding Sp1, VDR, the locus for the vitamin D-dependent rickets type Ⅰ, and hepatitis B virus-positive hepatocellular carcinomas from Thai patients were mapped to human chromosome 12q[129,130].

THE NON-GENETIC ROLE OF VITAMIN D IN HEPATITIS

A high prevalence of vitamin D deficiency was reported in HCV patients[10,131]. Low serum 25OHD levels are also found in patients with human immuno-deficient virus (HIV) and HCV and are correlated with severe liver fibrosis[132,133]. Preparations containing vitamin D_3 were shown to be effective in reducing the severity of the syndrome associated with osteo-arthropathy, including a decrease in BMD in Ukrainians with chronic hepatitis B and C[134]. The combination of vitamin A (25 000 IU) and vitamin D_2 (2500 IU) enhances the re-vaccination reaction against HBV in Chinese children[135]. *In vitro*, vitamin D_2 is reported to inhibit HCV RNA replication and its combination with β-carotene and linoleic acid also causes an additive and/or synergistic effect with respect to HCV RNA replication[136]. VDR mRNA and protein were found in the rat liver throughout the animal's life span[137]. In another study, however, human and mouse hepato-

cytes were found to have very low nuclear VDR (nVDR) mRNA and protein levels, whereas the sinusoidal endothelial, Kupffer, and stellate cells of the normal rat liver as well as a mouse biliary cell line clearly expressed the nVDR gene transcript[138]. Vitamin D_3 dramatically inhibits HCV production in Huh7.5 hepatoma cells and in combination with INF-α, also synergistically suppresses HCV production in human hepatocytes[47]. Serum vitamin D levels are complimentary to the IL-28B polymorphism in enhancing the accurate prediction of the SVR in patients undergoing treatment for chronic HCV[139]. Low vitamin D is linked to severe liver fibrosis and low SVR in response to IFN-based therapy in genotype 1 chronic HCV patients[10]. Vitamin D supplementation also improves SVR in chronic HCV-naïve patients[140] and in response to antiviral treatment for recurrent HCV infection in liver transplant patients[141]. These findings suggest that vitamin D may play a role in the treatment of HCV. Chronic infection with viral hepatitis is a major risk factor worldwide for the development of HCC. Vitamin D analogs have been reported to reduce tumor volume in patients with inoperable HCC[142] and to increase apoptosis of hepatocarcinoma cells by 21.4%[143]. In another pilot study, an intra-arterial injection of calcitriol in lipiodol into the hepatic artery was given to eight refractory HCC patients and led to the stabilization of α-fetoprotein levels[144].

MMPs are proteolytic enzymes that are responsible for extracellular matrix remodeling and the regulation of leukocyte migration through the extracellular matrix, which is important step for inflammatory processes and infectious diseases. MMPs are produced by many cell types including lymphocytes, granulocytes, astrocytes and activated macrophages. During the course of chronic HCV infection, hepatic mRNA expression of MMPs has been shown to either increase steadily with disease progression (MMP-1, MMP-2, MMP-7, and MMP-14) or increase transiently (MMP-9, MMP-11, and MMP-13), depending on the type of MMP[145]. Serum and tissue MMP-9 expression were reported to decrease in chronic HCV patients treated with pegylated INF-α2b and ribavirin[146]. The ratio of MMP-9 to MMP-2 is useful in distinguishing between patients with early stage and advanced HCC[147]. Serum TIMP-1 levels decreased significantly during and after treatment in sustained responders[148]. MMP-3 polymorphisms are associated with persistent HBV infection and advanced liver cirrhosis in Korean populations[149,150]. MMP-1, MMP-3, and MMP-9 polymorphisms are associated with the progression of HCV-related chronic liver disease in Japanese populations and may be a risk factor for poor prognosis in HCC patients[151,152]. However, VDR knock-out mice demonstrated an increased influx of inflammatory cells, phospho-acetylation of NF-κB associated with increased pro-inflammatory cells, and up-regulation of MMP-2, MMP-9, and MMP-12 in the lung[153]. The VDR *TaqI* polymorphism is associated with a decreased production of TIMP-1, which is a natural inhibitor of MMP-9[154]. Calcitriol modulates tissue MMP expression under experimental conditions[155], down-regulates MMP-9 levels in keratinocytes, and may attenuate the deleterious effects caused by the excessive TNF-α-induced proteolytic activity associated with cutaneous inflammation[156]. Calcitriol inhibits both basal and the staphylococcus-stimulated production of MMP-9 in human blood monocytes and alveolar macrophages[157]. Moreover, a vitamin D analog was also reported to reduce the expression of MMP-2, MMP-9, vascular endothelial growth factor (VEGF) and PTH-related peptide in Lewis lung carcinoma cells[158]. Furthermore, calcitriol significantly attenuated *Mycobacterium tuberculosis* (*M. tuberculosis*)-induced increases in the expression of MMP-7 and MMP-10, while suppressing the secretion of MMP-7 by *M. tuberculosis*-infected PBMCs. MMP-9 gene expression, secretion and activity were significantly inhibited, irrespective of infection status[159]. Calcitriol also suppressed the production of MMPs (MMP-7 and MMP-9) and enhanced the level of TIMP-1 in tuberculosis patients[160]. In human articular chondrocytes, calcitriol significantly suppresses the increased production of MMP-9 that is induced by phorbol myristate acetate (PMA)[161]. These studies suggest that calcitriol may play an important role in the pathological process of viral hepatitis by down-regulating the levels of MMPs and regulating the levels of TIMPs.

Angiogenesis is a complex process involving the coordinated steps of endothelial cell activation, proliferation, migration, tube formation and capillary sprouting, which require the participation of intracellular signaling pathways. VEGF is a key mediator of angiogenesis. Vascular changes associated with angiogenesis usually occur in cancer; however, they have also been reported to occur in inflammatory disease processes. HCV C protein can activate the expression of VEGF in hepatoma cell lines ($HepG_2$) and might contribute to viral carcinogenesis[162]. Co-expression of the HBV X gene and the HCV core gene also increase the expression of VEGF in $HepG_2$ cells and act synergistically in carcinogenesis[163]. The expression levels of TNFα mRNA and VEGF mRNA showed a positive correlation with the progression of viral hepatitis to cirrhosis, i.e., the higher levels of TNFα and VEGF mRNA, the higher the prevalence of HCC[164]. HBV X protein is known to up-regulate the expression of VEGF, thereby promoting angiogenesis in HCC *via* NFκB signaling pathway[165]. Serum VEGF concentration is a predictor of invasion and metastasis in HCC[166] and positively correlates with the recurrence rate of HCC after curative resection[167]. In contrast, calcitriol was reported to inhibit angiogenesis *in vitro* and *in vivo*[168]. Calcitriol significantly inhibits VEGF-induced endothelial cell spouting and elongation in a dose-dependent manner and decreases the production of VEGF[169]. Calcitriol is a potent inhibitor of retinal neovascularization in a mouse model of oxygen-induced ischemic retinopathy[170]. Vitamin D and its analog also reduce the expression of VEGF in various cancer cell lines[158,171]. Moreover, *DBP-maf* was reported to inhibit angiogenesis and tumor

growth in mice[172] and inhibits the VEGF signaling by decreasing VEGF-mediated phosphorylation of VEGF-2 and ERK1/2, a downstream target of the VEGF signaling cascade[173]. These findings suggested that vitamin D modulates angiogenesis in viral hepatitis and may impact the mechanism of progression to HCC in patients with viral hepatitis.

Prostaglandins (PGs) play a role in inflammatory processes. Cyclooxygenase (COX) participates in the conversion of arachidonic acid to PGs. HBV X protein was reported to up-regulate levels of COX-2, 5-lipoxygenase and phosphorylated extracellular signal-regulated protein kinase ½ (p-ERK1/2) and releases arachidonic acid metabolites in liver cells[174]. In liver samples from patients with chronic HCV infection, there is a significant correlation between the dominant intensity of COX-2 and the presence of histological steatosis and an inverse correlation was observed between COX-2 and viral load[175]. COX-2 up-regulates VEGF expression and tumor angiogenesis in HBV-associated HCC *via* PG production; selective COX-2 inhibitors may block HCC-associated angiogenesis and an increase in platelet counts when used with pegylated TFNα2a[176,177]. Indomethacin also cleared HBV DNA in chronic healthy carriers, and 5 patients with positive HBeAg became negative after 4 mo[178]. On the other hand, calcitriol has been reported to regulate the expression of several key genes involved in the PG pathway, resulting in a decrease in PG synthesis[179]. Calcitriol and its analogs have been shown to selectively inhibit the activity of COX-2[180]. These findings suggested that vitamin D plays a role in modulating the inflammatory process in viral hepatitis.

Reactive oxygen species (ROS) are produced by activated phagocytes as a part of their microbicidal activities. Intracellular hydrogen peroxide (H_2O_2) levels are significantly higher in patients with chronic HCV infection than in asymptomatic carriers and positively correlates with alanine amino-transferase (ALT) levels[181]. ROS can also modulate the intracellular level of HBV X protein. The direct addition of H_2O_2 to cells significantly increased the level of HBV X protein in HBV X protein ChangX-34 cells, while antioxidants completely abolished the increase in HBV X protein[182]. There is a significant decrease glutathione (GSH) levels in the patients with HBV-infected[183]. Superoxide dismutase (SOD) was present in peripheral blood mononuclear cells (PBMC) but was absent in the liver of patients with chronic HCV infection[184]. Levels of lipid peroxidation products are increased in serum, leukocyte, and liver specimens in HCV patients[185]. Similarly, calcitriol has been reported to exert a receptor-mediated effect on the secretion of H_2O_2 by human monocytes[186]. Human monocytes in culture gradually lose their capacity to produce superoxide when stimulated. The addition of calcitriol, lipopolysaccharide or lipoteichoic acid restored the ability of stimulated monocytes to produce superoxide and increased their oxidative capacity when compared with unstimulated monocytes[187]. Calcitriol can also protect nonmalignant prostate cells from oxidative stress-induced cell death by eliminating ROS-induced cellular injuries[188]. Vitamin D metabolites and vitamin D analogs were reported to induce lipoxygenase mRNA expression, lipoxygenase activity and ROS in a human bone cell line[189]. Vitamin D can also reduce the extent of lipid peroxidation and induce SOD activity in the hepatic anti-oxidant system of rats[190]. These findings suggested that vitamin D modulates oxidative stress in viral hepatitis.

Nitric oxide (NO) is a reactive nitrogen species (RNS) that is critical in the redox biology of hepatocytes and is formed by nitric oxide synthase (NOS). In the liver, iNOS was found to be important in the development and propagation of inflammation. Viral hepatitis is associated with an increased iNOS expression[191,192]. HCV infection can also stimulate the production of iNOS through the activation of the iNOS gene by the viral core protein and the NS3 protein[191]. In patients with HCC, the combined negative expression of iNOS and COX-2 on histology has a significant impact on patient survival[193]. Oxidative DNA damage has been reported to increase chromosomal aberrations associated with cell transformation, and oxidative stress has also been suggested in the development of HCV-associated HCC. Oxidative DNA damage was observed in circulating leukocytes and occurs as an early event in chronic HCV infection[194]. NO often damage mitochondria, leading to the induction of double-stranded DNA breaks and the accumulation of oxidative DNA damage[195]. The viral core and NS3 proteins were shown to be responsible for inhibition of DNA repair, which is mediated by NO and ROS[196]. On the other hand, the activation of macrophage 1α-hydroxylase results in an increase in 1,25 OHD, which inhibits iNOS expression and reduces the NO produced by LPS-stimulated macrophages[197]. This calcitriol production by macrophages could provide protection against the oxidative injuries caused by the NO burst. Calcitriol is known to inhibit LPS-induced immune activation in human endothelial cells[198]. Calcitriol enhances intracellular GSH pools and significantly reduces the nitrite production induced by LPS[199]. In human macrophage-like cells, calcitriol induces iNOS and suppresses the growth of *M. tuberculosis*[200]. Moreover, calcitriol protects against doxorubicin-induced chromosomal aberrations in rat bone marrow cells[201]. Calcitriol also acts synergistically with vanadium in inhibiting diethylnitrosamine-induced chromosomal aberrations and DNA-strand breaks in the rat liver[202]. In regenerating liver cells, calcitriol regulates the synthesis of DNA polymerase-alpha, generates functional ribonucleotide reductase subunits, and induces DNA replication[203,204]. In addition, calcitriol appears to be effective in suppressing liver-specific early chromosomal damage as well as DNA damage during the process of rat hepatocarcinogenesis[205].

CONCLUSION

The relationship between vitamin D and viral hepatitis

has been discussed. Vitamin D may have a beneficial role in viral hepatitis. Genetic studies have provided the opportunity to determine what proteins link vitamin D to the pathology of viral hepatitis. Vitamin D also exerts its effect on viral hepatitis *via* non-genomic mechanisms. As a result, it is imperative that vitamin D levels in patients with viral hepatitis be followed. Many studies use the relationship between serum PTH and 25OHD to define the normal range of serum 25OHD. According to the report on Dietary Reference Intakes for vitamin D and calcium by the Institute of Medicine (IOM), persons are at risk of deficiency at serum 25OHD levels less than 30 nmol/L. Recently, Saliba *et al*[206] suggested that a 25OHD threshold of 50 nmol/L is sufficient for PTH suppression and prevention of secondary hyperparathyroidism in persons with normal renal function. Calcitriol is best used for viral hepatitis, because of its active form of vitamin D_3 metabolite and inhibits inflammatory cytokine expression. Adjusting dose for calcitriol depends on serum calcium and PTH levels. However, monitoring of serum 25OHD after calcitriol intake is not necessary because calcitriol inhibits the production of serum 25OHD in the liver[207,208]. Further investigation with calcitriol in viral hepatitis is needed.

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ORIGINAL ARTICLE

Anti-inflammatory effects of Lacto-Wolfberry in a mouse model of experimental colitis

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Abstract

AIM: To investigate the anti-inflammatory properties of Lacto-Wolfberry (LWB), both *in vitro* and using a mouse model of experimental colitis.

METHODS: The effects of LWB on lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) and interleukin (IL)-6 secretion were assessed in a murine macrophage cell line. *in vitro* assessment also included characterizing the effects of LWB on the activation of NF-E2 related 2 pathway and inhibition of tumor necrosis factor-α (TNF-α)-induced nuclear factor-κB (NF-κB) activation, utilizing reporter cell lines. Following the in vitro assessment, the anti-inflammatory efficacy of an oral intervention with LWB was tested *in vivo* using a preclinical model of intestinal inflammation. Multiple outcomes including body weight, intestinal histology, colonic cytokine levels and anti-oxidative measures were investigated.

RESULTS: LWB reduced the LPS-mediated induction of ROS production [+LPS *vs* 1% LWB + LPS, 1590 ± 188.5 relative luminescence units (RLU) *vs* 389 ± 5.9 RLU, $P < 0.001$]. LWB was more effective than wolfberry alone in reducing LPS-induced IL-6 secretion *in vitro* (wolfberry *vs* 0.5% LWB, 15% ± 7.8% *vs* 64% ± 5%, $P < 0.001$). In addition, LWB increased reporter gene expression *via* the anti-oxidant response element activation (wolfberry *vs* LWB, 73% ± 6.9% *vs* 148% ± 28.3%, $P < 0.001$) and inhibited the TNF-α-induced activation of the NF-κB pathway (milk *vs* LWB, 10% ± 6.7% *vs* 35% ± 3.3%, $P < 0.05$). Furthermore, oral supplementation with LWB resulted in a reduction of macroscopic (-LWB *vs* +LWB, 5.39 ± 0.61 *vs* 3.66 ± 0.59, $P = 0.0445$) and histological scores (-LWB *vs* +LWB, 5.44 ± 0.32 *vs* 3.66 ± 0.59, $P = 0.0087$) in colitic mice. These effects were associated with a significant decrease in levels of inflammatory cytokines such as IL-1β (-LWB *vs* +LWB, 570 ± 245 μg/L *vs* 89 ± 38 μg/L, $P = 0.0106$), keratinocyte-derived chemokine/growth regulated protein-α (-LWB *vs* +LWB, 184 ± 49 μg/L *vs* 75 ± 20 μg/L, $P = 0.0244$), IL-6 (-LWB *vs* +LWB, 318 ± 99 μg/L *vs* 117 ± 18 μg/L, $P = 0.0315$) and other pro-inflammatory proteins such as cyclo-oxygenase-2 (-LWB *vs* +LWB, 0.95 ± 0.12 AU *vs* 0.36 ± 0.11 AU, $P = 0.0036$) and phosphorylated signal transducer and activator of transcription-3 (-LWB *vs* +LWB, 0.51 ± 0.15 AU *vs* 0.1 ± 0.04 AU, $P = 0.057$). Moreover, antioxidant biomarkers, including expression of gene encoding for the glutathione peroxidase, in the colon and the plasma anti-oxidant capacity were significantly increased by supplementation with LWB (-LWB *vs* +LWB, 1.2 ± 0.21 mmol/L *vs* 2.1 ± 0.19 mmol/L, $P = 0.0095$).

CONCLUSION: These results demonstrate the anti-inflammatory properties of LWB and suggest that the underlying mechanism is at least in part due to NF-κB inhibition and improved anti-oxidative capacity.

Key words: Lacto-Wolfberry; Colitis; Nutrition; Inflammation; Wolfberry; Inflammatory bowel disease; Crohn's disease

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INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), represents a group of chronic disorders characterized by inflammation of the gastrointestinal tract, typically with a relapsing and remitting clinical course. They affect between 0.5%-1% of the Western world's population[1]. The primary focus of IBD therapy is to induce remission of acute inflammatory flare ups and to maintain the state of remission.

The innate immune system plays a central role in the acute inflammatory process. As part of the innate immune response, neutrophils are one of the early responders to local injury. Both, the circulating levels and activation of neutrophils, are increased in IBD patients with active disease[2,3]. Activated neutrophils and monocytes release a plethora of mediators including reactive oxygen species (ROS), eicosanoids and proinflammatory cytokines. In fact, the therapeutic benefit of depleting granulocytes in CD patients has been demonstrated[4]. Apart from neutrophils, monocytes and mucosal macrophages play an important role in the development of IBD as shown by an increase of the number of recruited monocytes and activated macrophages in the inflamed gut of patients with IBD[5]. Indeed during active inflammation, neutrophils recruit and activate monocytes which themselves secrete pro-inflammatory mediators such as tumor necrosis factor (TNF-α), interleukin (IL)-1β and IL-6[6].

A vast body of literature supports the role of nutritional therapy in IBD, particularly in CD (reviewed in[7,8]). While enteral nutrition is not as effective as steroid therapy in induction of remission in CD, the benefit to the patient is well established[9]. Thus, identification and characterization of novel anti-inflammatory foods may aid in improving the currently available nutritional formulations.

A variety of functional nutrients, such as glycosides[10], alkaloids[11] and black tea extracts[12], have been shown to exert their beneficial effects through inhibition of Nuclear factor-κB (NF-κB) activation. NF-κB is one of the most important regulators of pro-inflammatory cytokine expression and reducing its activity may have beneficial effects under acute inflammatory conditions[13]. Besides NF-κB, phytochemicals are also known to activate NF-E2 related 2 (Nrf2) pathway through the anti-oxidant response element causing an increase in the anti-oxidative enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase[14].

Wolfberry, the fruit of *Lycium barbarum*-also called as Goji (Gouqi or Gou QZ in Romanized Chinese), is a sweet red berry, which has been traditionally used in Chinese medicine. It is also one of the richest sources of zeaxanthin, an antioxidant that has been postulated to improve visual acuity[15]. Apart from antioxidant activity, wolfberry juice has also been demonstrated to have immunomodulatory effects[16]. However, a large part of the supporting evidence is derived either from *in vitro* experiments or animal studies wherein, wolfberry extracts were delivered parenterally. We believe that this might be due to reduced bioavailability of active ingredients when given enterally. Therefore, to improve bioavailability of its anti-oxidant components, wolfberry was processed with skimmed milk and freeze-dried to generate Lacto-Wolfberry (LWB), a water-dispersible powder[17]. This novel preparation, which contains approximately 50% wolfberry and 25% skimmed milk, has been clinically demonstrated to improve the bioavailability of zeaxanthin[17]. Subsequently, the immune-enhancing properties of LWB in both, young-adult and aged mice, have been characterized[18]. Recent studies have demonstrated that dietary supplementation with LWB enhances immune response to flu vaccine[19] and plasma oxidative capacity in elderly[20]. The aim of this study was to characterize the anti-inflammatory and anti-oxidative properties of LWB. We first demonstrate that LWB inhibits lipopolysaccharide (LPS)-induced ROS and IL-6 production in a murine macrophage cell line. Next, using reporter cell lines we show that LWB activates Nrf2 pathway, while inhibiting the NF-κB pathway. Finally, using a mice model of colitis, we demonstrate that LWB reduces the severity of colitis by mediating a reduction in pro-inflammatory cytokines, namely IL-6, IL1β and keratinocyte-derived chemokine/growth regulated protein-α (KC/GRO-α).

MATERIALS AND METHODS

Inflammatory response in LPS-challenged RAW cells

The murine macrophage cell line RAW 264.7 (ATCC, United States) was maintained in Dulbecco's modified Eagle medium (DMEM, Amimed, Bioconcept, Switzerland) supplemented with 10% heat-inactivated fetal-calf serum (FCS, Amimed) at 37 ℃ in a 50 mL/L CO_2/air incubator. Intracellular ROS was measured using a ROS-sensitive fluorescent dye, 2',7'-dichlorofluorescin diacetate (DCFH2-DA, Sigma, United States). Cells (10^5

cells/well in 96 well plates) were incubated overnight with LPS, from *Escherichia coli* serotype 055:B5 (Sigma, United States) at 0.5 mg/L, either in the absence or presence of LWB (0.1% or 1% final concentration). Control cells in the absence of LPS were also included. Cells were then treated with 10 μmol DCFH2-DA for 30 min at 37 ℃ and washed twice with phosphate-buffered saline (PBS). Fluorescence was measured at 485 nm excitation and 538 nm emission by a Fluoroskan enzyme linked immunosorbent assay plate reader (Labsystems Oy, Finland) at the indicated time points. For experiments measuring IL-6, RAW 264.7 cells were seeded in 96 well plates at 10^4 cells/well. After 3 d (approximately 80% of confluence), cells were stimulated with LPS at 0.5 mg/L and incubated in the presence of either LWB (1% final concentration), wolfberry (0.5%) or skimmed milk (0.25%) for 24 h at 37 ℃. Cell culture supernatants were then harvested and IL-6 secretion was quantified using commercial enzyme linked immunosorbant assay (ELISA) kit according to manufacturer's protocol (Murine IL-6 Eli-pair, Diaclone, France). Cell viability was determined by CellTiter-Glow Luminescent assay (Promega, United States) according to manufacturer's instructions. It should be noted that the same lots of wolfberry and skimmed milk that were used in the preparation of LWB were used for all experiments described.

NF-κB inhibition assay

The human colonic adenocarcinoma cell line, HT-29 (ATCC, United States), was stably transfected with the plasmid pNF-κB-SEAP-NPT. The plasmid was a kind gift from Prof. Kim (Natural Products Research Institute, Seoul). It contains a secreted alkaline phosphatase (SEAP) encoding sequence downstream of four tandem copies of NF-κB binding sites. Stably transfected cells (HT-29 clone 34) were maintained in high glucose (4.5 g/L) DMEM containing 1% L-glutamine, 10% heat-inactivated FCS, 1% penicillin/streptomycin, 500 mg/L G418 (Invitrogen, Switzerland) and 100 mg/L Normocin (Invivogen, France) at 37 ℃ in a 50 mL/L CO_2/air incubator. For the NF-κB inhibition assay, HT-29 clone 34 cells were seeded at 10^4 cells/well in 96-flat bottom well plates. After 3-4 d of culture (approximately 80% confluence), cells were washed with PBS and stimulated with recombinant TNF-α (10 μg/L, RD systems, England) in the absence or the presence of either LWB (1% final concentration), wolfberry (0.5%) or skimmed milk (0.25%) for 24 h at 37 ℃. SEAP release was assessed in the supernatants using the Phospha-Light™ System (Applied Biosystems, United States) according to manufacturer's protocol.

Nrf2 activation assay

AREc32 (CXR biosciences, United Kingdom) is a reporter cell line that stably expresses the anti-oxidant response element (ARE)-driven luciferase gene[21]. These cells were cultured at 12 000 cells/well in 96 well plates (Nunc) at 37 ℃ and 50 mL/L CO_2/air incubator in DMEM supplemented with 10% FCS. After 1 d, cells were washed with PBS and treated, in the absence of serum, with either, LWB (1% final concentration), wolfberry (0.5%) or skimmed milk (0.25%) for 24 h at 37 ℃. Luciferase activity was measured using the Luciferase Assay™ (Promega, United States) following manufacturer's instructions.

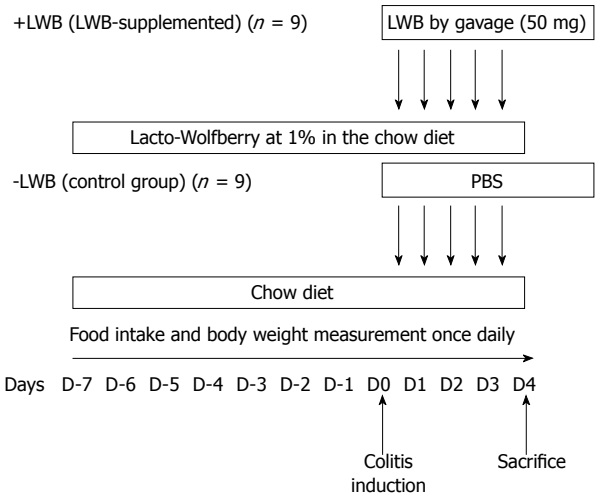

Figure 1 Animal study design. Body weight and food intake was monitored throughout the duration of the experiment (i.e., D-7 to D4). Colitis was induced on D0. For the Lacto-wolfberry (LWB) fed group, diet was supplemented with 1% LWB from D-7 until D4 and 50 mg of LWB was also gavaged from D0 to D4. Control animals were fed regular diet for the same period and gavaged with equal volume of control solution from D0 to D4. PBS: Phosphate buffered solution.

Experimental colitis model

Male mice (C57BL/6J), aged 7 wk, were obtained from Charles River Laboratories Inc. (France) and housed five per cage in a temperature-controlled room with free access to food and water. The overall study design is provided in Figure 1. Mice (n = 9 per group) were randomly assigned to either the control (LWB, chow fed) or the LWB-supplemented group (+LWB, 1% in the diet) 7 d prior to colitis induction (D-7), which was induced on D0, as previously described[22]. Chemically induced colitis was performed as described earlier[23]. Briefly, colitis was induced with an intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) at 125 mg/kg dissolved in 50% ethanol solution. In order to compensate for reduced food intake after colitis induction, mice were also supplemented by daily gavage with 50 mg of LWB from D0 to D4. Control mice were gavaged with an equal volume of PBS. Food intake and body weight measurements were taken once daily from D-7 to D4. Animals were sacrificed on D4. All experimental protocols were conducted in accordance with Swiss law and Nestlé policy on ethics and animal welfare.

Macroscopic and histological assessment

The distal colon tissue was washed with PBS and macroscopic scoring was performed using the system of Wallace *et al*[24]. Samples of the inflamed tissues (1 cm above the anal canal) were collected for histological analysis. The tissues were fixed in 4% paraformaldehyde at 4 ℃

for 24 h. Sections were prepared, stained with hematoxylin and eosin, and graded according to Ameho *et al*[25].

mRNA expression analysis

Colon tissue homogenization, RNA extraction and reverse transcription were performed as described earlier[22]. Custom-made Low Density Array (LDA) cards were purchased from Applied Biosystems (United States) and used according to manufacturer's instructions. Briefly, mixes (100 μL), containing 100 ng of cDNA, 2X TaqMan Mix and nuclease-free water, were prepared and loaded onto the LDA card. The LDA cards were then processed using an automated fluorometer ABI Prism 7900HT. Gene expression was calculated using the relative quantification method with SDS 2.2.2 software.

Electrophoresis and Western blotting analysis

Colon tissue homogenization, protein extraction, electrophoresis and Western blotting analysis were performed as described earlier[22]. Briefly, after tissue homogenization, protein was quantified using the RC DC Protein Assay (Bio-Rad, United States). Proteins were loaded and separated on a 4%-12% bis-tris gel (Invitrogen). The blot was probed with antibodies against murine cyclooxygenase-2 (COX-2) (Cayman, United States), signal transducer and activator of transcription-3 (STAT-3) and phosphorylated STAT-3 (pSTAT-3, Cell Signalling Technology, United States) and β-Actin (Sigma, United States). Relative quantitation of bands was determined using Scion Image Densitometry System (Scion Corp., United States), with normalization to β-Actin.

Myeloperoxidase assay

Protein levels for myeloperoxidase (MPO) were measured in colon protein extracts by ELISA following the manufacturer's instructions (Hycult biotechnology, The Netherlands).

Cytokine analysis

IL-1β, IL-6, IL-10, IL-12p70, KC/GRO-α, interferon-γ (IFN-γ) and TNF-α were measured in the colon protein extracts using multiplex assay kits (Meso Scale Discovery, United States) according to manufacturer's protocol. Cytokine concentrations were determined with Discovery Workbench 3.0 software, using curve 4-PL as suggested by the manufacturer.

Plasma anti-oxidant capacity

Total anti-oxidant capacity of plasma was performed using an assay, which measures inhibition of 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS®) to ABTS®+ by metmyoglobin as Trolox equivalents (Cayman, United States), according to manufacturer's protocol.

Statistical analysis

Data were analyzed by means ± SE either the Mann-Whitney test or where appropriate, a two-way analysis of variance with a Bonferroni post test. *P* values of less than 5% were considered as significant.

RESULTS

Effects of LWB in vitro

Anti-oxidant effects: ROS production from LPS-stimulated RAW 264.7 cells was evaluated in the presence and absence of LWB (Figure 2A). As expected, LPS increased ROS production, in a time dependent manner, as compared to the untreated controls (-LPS). Interestingly, LWB was able to reduce LPS-induced ROS production. The inhibitory effect of LWB was significant from the 2 h time point at both 0.1% and 1% final concentration. At the 6 h time point, LWB reduced the amount of LPS-induced ROS production by about 25% and 75%, at the concentrations of 0.1% and 1%, respectively ($P < 0.001$ for both concentrations). Next, the effects of LWB on Nrf2 activation, using a stable ARE-driven reporter gene expressing cell line, *AREc32*[21], were evaluated (Figure 2B). The data show that LWB at 1% final concentration increased Nrf2 activity by approximately 200%, whereas its individual components, i.e., wolfberry (0.5%) and milk (0.25%), induced only a mild or almost no increase in Nrf2 activity, respectively.

Anti-inflammatory effects: Finally, the effects of LWB (1%), wolfberry (0.5%) and milk (0.25%) on LPS-induced IL-6 production (Figure 2C) and TNF-α-induced NF-κB activity (Figure 2D) were assessed. As shown, LWB inhibited LPS-induced IL-6 production in RAW 264.7 cells by approximately 80% and TNF-α-induced NF-κB activity by approximately 35%. These were significantly different from the values obtained for milk, approximately 20% and 10%, respectively ($P < 0.001$ and $P < 0.05$, respectively). However, no significant differences were observed from wolfberry, which inhibited IL-6 production by approximately 65% and NF-κB activation by 10%.

Anti-inflammatory effects of LWB in vivo

The anti-inflammatory effects of LWB were characterized in chemically-induced colitis model as described in materials and methods section.

LWB attenuates colitis-induced body weight loss: Body weight and food intake of each mouse was monitored daily. Following colitis induction at D0, the percentage mean change in body weight of control mice was -6.4, -9.5, -8.9 and -7.6 at D1, D2, D3 and D4, respectively (Figure 3, LWB). The mice fed with LWB had a percentage mean change in body weight of -4.7, -4.6, -1.9 and -0.5 at D1, D2, D3 and D4, respectively (Figure 3, +LWB). Thus, while both mice have a reduction in body weight at D1, the reduction in body weight loss of the LWB fed mice were significantly lower from D2-D4 ($P < 0.05$ at D2 and $P < 0.01$ at D3 and D4). The total food intake between the two groups did not change (data not shown).

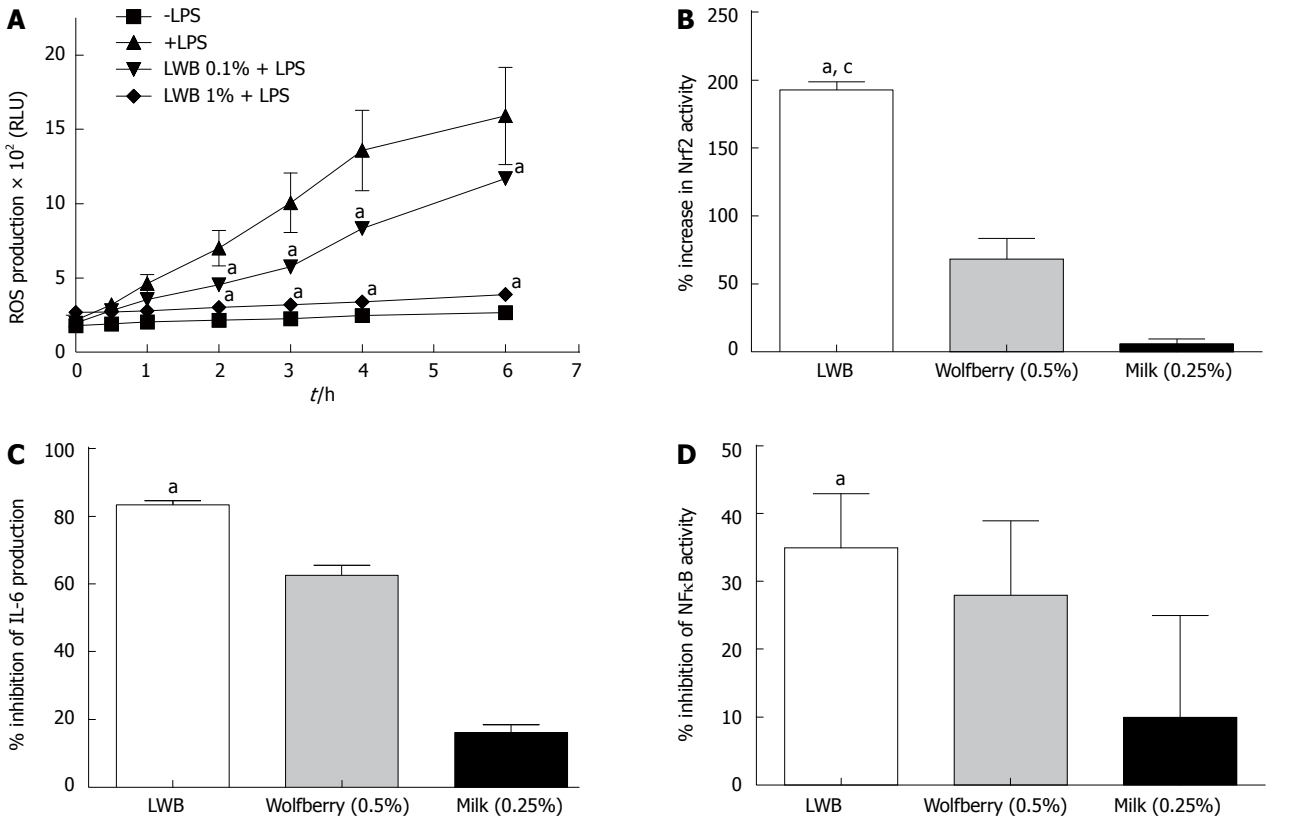

Figure 2 Anti-inflammatory and anti-oxidative effects of Lacto-Wolfberry *in vitro*. A: The effect of Lacto-Wolfberry (LWB) on lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) production in RAW 264.7 cells was measured as described in the methods section. Results are expressed in relative luminescence units (RLU). $^{a}P < 0.05$ at the given time points and dose as compared to LPS-stimulated ROS production (+LPS); B: Effects of given concentrations of LWB, milk and wolfberry on NF-E2 related 2 activity in AREc32 cells. Results are expressed as percentage of increase compared to control conditions. $^{a}P < 0.05$ *vs* milk; $^{c}P < 0.05$ *vs* wolfberry; C: Effects of given concentrations of LWB, milk and wolfberry on LPS-induced interleukin-6 production in RAW 264.7 cells. Results are expressed as percentage of inhibition compared to induction after LPS stimulation. $^{a}P < 0.05$ *vs* milk; D: Effects of given concentrations of LWB, milk and wolfberry on tumor necrosis factor-α-induced nuclear factor- κB activation in a reporter cell line. Results are expressed as percentage of inhibition compared to induction after TNF-α stimulation. All results are depicted as mean ± SE. $^{a}P < 0.05$ *vs* milk.

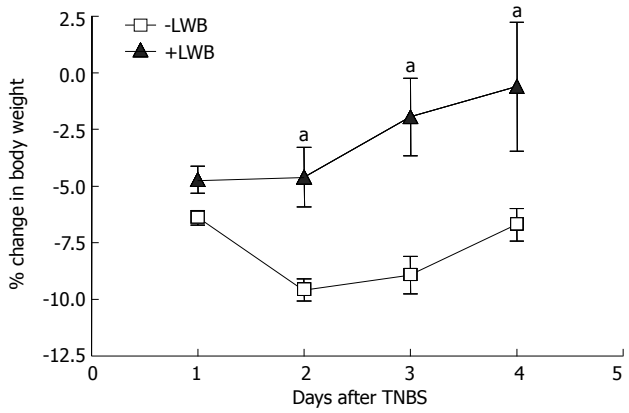

Figure 3 Lacto-Wolfberry attenuates colitis-induced body weight loss. Percentage change in body weight was calculated from a ratio of body weight measured at each day after colitis induction and body weight at D0. Results are depicted as mean ± SE. $^{a}P < 0.05$ *vs* -Lacto-Wolfberry (LWB) at the respective time points. TNBS: 2,4,6-trinitrobenzene sulfonic acid.

LWB reduces colonic inflammation: Supplementation with LWB significantly reduced the colonic inflammation as judged by macroscopic (Figure 4A) and histological (Figure 4B, C and D) evaluation of intestinal inflammation. Macroscopic lesions were assessed as delineated by Wallace *et al*[24]. Colons of control mice (-LWB) presented significantly higher scores compared to mice fed with LWB, 5.39 ± 0.61 and 3.66 ± 0.47, respectively (Figure 4A, $P < 0.05$). In agreement with the macroscopic assessment, histological evaluation showed lower inflammatory infiltrates and better mucosal integrity in mice fed with LWB (+LWB, Figure 4D) as compared to the control (-LWB, Figure 4C). This difference was reflected in the histological score, 5.44 ± 0.32 and 3.66 ± 0.59, for the control and LWB-fed mice, respectively, which was significantly different (Figure 4B, $P < 0.01$). The 70% reduction of MPO content in the LWB treated group (+LWB) as compared to control mice (-LWB) provides further support to reduced neutrophil infiltration in the LWB group (Figure 4E, $P < 0.05$).

LWB reduces pro-inflammatory effector proteins: To delve deeper into the effect of LWB, levels of various effector proteins were measured in the colon tissue. As shown in Table 1, levels of IL-1β, IL-6 and KC/GRO-α were significantly reduced in colitic mice fed with LWB (+LWB) compared to the control (-LWB). Moreover IL-10, IFN-γ, TNF-α and IL-12p70 levels were also re-

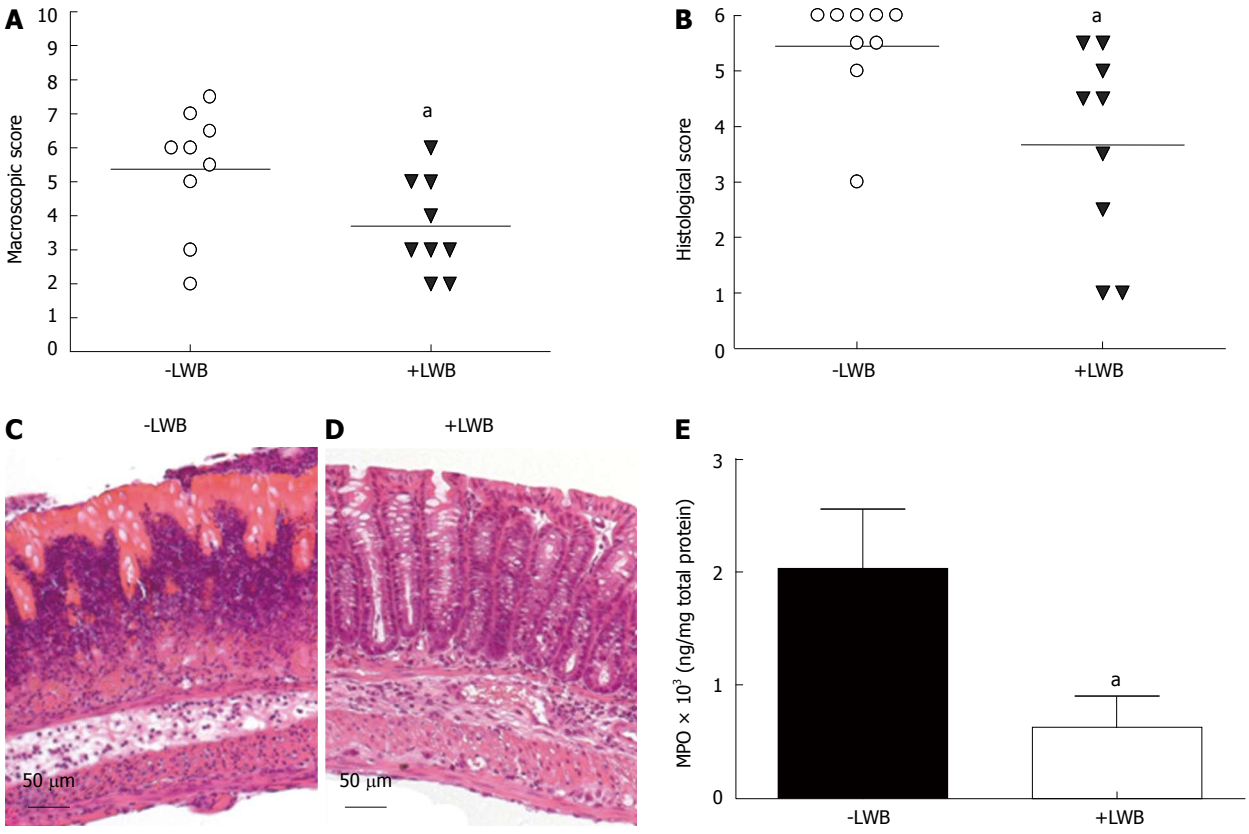

Figure 4 Lacto-Wolfberry reduces colonic inflammation. A: Macroscopic evaluation of the colon was performed according to Wallace criteria. Results are expressed in individual values and the mean value is represented by the horizontal black line; B: Histological evaluation and scoring of the colon was performed according to the Ameho criteria. The individual scores of the mice in the two groups are provided, with the mean value being represented by the horizontal line; C: Representative section of score 6 from the control fed mice [-Lacto-Wolfberry (LWB)]; D: Representative section of score 1 from LWB fed mice (+LWB); E: Colonic myeloperoxidase (MPO) levels were measured by enzyme linked immunosorbant assay. The values are represented as mean ± SE. $^{a}P < 0.05$ *vs* -LWB.

Table 1 Cytokine levels in colon samples (mean ± SE)

Cytokines (μg/L)	-LWB	+LWB	Reduction (%)	*P* value
TNF-α	80 ± 24	31 ± 4	61	0.0770
IL-1β	570 ± 245	89 ± 38	84	0.0106
IL-6	318 ± 99	117 ± 18	63	0.0315
KC/GRO-α	184 ± 49	75 ± 20	59	0.0244
IL-12p70	415 ± 129	89 ± 38	79	0.1135
IFN-γ	18 ± 6	7 ± 1	61	0.0770
IL-10	428 ± 141	165 ± 27	61	0.0625

LWB: Lacto-Wolfberry; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IL-6: Interleukin-6; KC/GRO-α: Keratinocyte-derived chemokine/growth regulated protein-α; IL-12p70: Interleukin-12p70; IFN-γ: Interferon-γ; IL-10: Interleukin-10.

duced by more than 50%, however these were not statistically significant. COX-2 and pSTAT3 levels were assessed semi- quantitatively by Western blotting analysis and densitometry. As shown in Figure 5A, colon of mice fed with LWB had approximately 65% reduction of COX-2 levels ($P < 0.01$). Mice fed with LWB also demonstrated an 80% reduction in pSTAT3 expression (Figure 5B) in the colon. However, this difference didn't reach statistical significance ($P = 0.057$).

LWB improves anti-oxidative capacity: Finally, changes in mRNA expression of Nrf2 target genes, such as CAT, SOD2 and glutathione peroxidase (GPx1) were examined in the colon tissue. GPx1 mRNA expression was higher by more than 20% in mice fed with LWB (+LWB) compared to control (-LWB) (Figure 6A, $P < 0.02$). No difference of gene expression was detected for CAT and SOD2 (data not shown). Finally, the measures of anti-oxidant capacity in the plasma demonstrated that mice supplemented with LWB (+LWB) had more than 70% increase in anti-oxidative capacity compared to control mice (-LWB) (Figure 6B, $P < 0.01$).

DISCUSSION

The aim of the study was to investigate the anti-inflammatory properties of LWB *in vitro* and in an animal model of intestinal inflammation. Initial experiments showed that LWB reduces LPS-induced ROS generation. The anti-oxidant effects of wolfberry are well characterized[16]. Moreover, LWB has also been proposed to have ROS scavenging activity[26]. Thus, this finding was not surprising. However, phytochemicals have also been shown to activate the Nrf2 pathway[14]. Nrf2 is a redox-sensitive transcription factor, which regulates the expression of ARE-driven anti-oxidant enzymes[14]. Thus, the effect of LWB on Nrf2 activation was tested. Interestingly, LWB

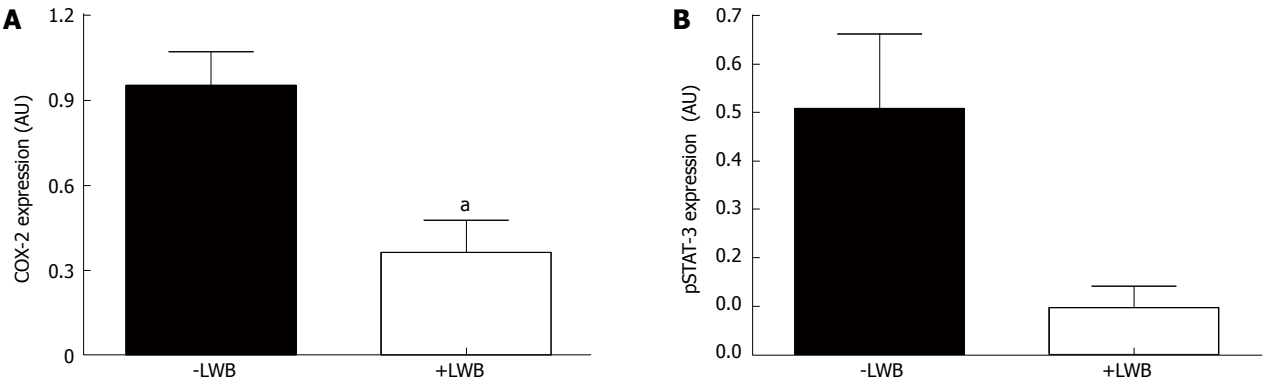

Figure 5 Effect of Lacto-Wolfberry on cyclooxygenase-2 and phosphorylated- signal transducer and activator of transcription-3 levels. A: Cyclooxygenase-2 (COX-2) levels were assessed semi-quantitatively by Western blotting and densitometry. COX-2 levels were normalized to β-actin. Results are expressed in mean ± SE. [a]*P* < 0.05 *vs* -Lacto-Wolfberry (LWB); B: Phosphorylated-signal transducer and activator of transcription-3 (pSTAT3) levels were assessed by Western blotting and densitometry. The results are expressed as a ratio of pSTAT3 to STAT3 (mean ± SE). *P* = 0.057 *vs* -LWB group.

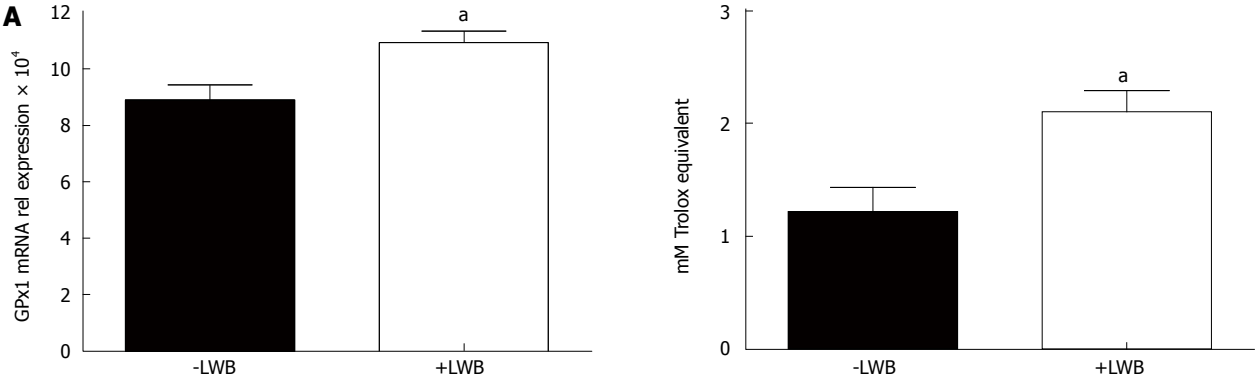

Figure 6 Anti-oxidant effect of Lacto-Wolfberry in murine colitis. A: mRNA expression of glutathione peroxidase 1 (GPx1) was measured as described in methods section. Results are expressed as mean ± SE; B: Anti-oxidant ability in plasma was measured in mmol/L Trolox equivalent. Results are expressed in mean ± SE. [a]*P* < 0.05 *vs* -Lacto-Wolfberry (LWB).

had a two fold increase in Nrf2 activation, however this effect was not fully replicated by its major components tested separately, i.e., either, wolfberry or milk. Hence, this could suggest a synergistic effect between the two major components of LWB resulting from the LWB manufacturing process. Finally, LWB demonstrated wolfberry-equivalent inhibition of LPS-induced IL-6 secretion and TNF-α-induced NF-κB activation. The beneficial effects observed *in vitro* prompted a further examination of LWB in an animal model of colitis.

The pro-inflammatory roles of ROS production, NF-κB activation and cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, have been firmly established in IBD pathology[13,27,28]. Thus, the effects of LWB were tested in a murine model of colitis. Firstly, LWB supplementation attenuated colitis-induced body weight loss. Secondly, both colonic parameters, macroscopic and microscopic, confirmed a reduction in the severity of colitis after LWB intervention. In further support, supplementation with LWB reduced neutrophil infiltration in the colon tissue. In this experimental model, the secretion of Th1 cytokines, namely, IL-1β, IL-6, TNF-α and IFN-γ, play an important role in the propagation of colitis[29]. As NFκB activation controls expression of most of these genes[13] and considering the *in vitro* inhibitory effects of LWB on NF-κB activation, the cytokine levels were measured to gain mechanistic insight. Supplementation with LWB resulted in reduced levels of majority of these cytokines, while significantly reducing the levels of IL-1β and IL-6. Further, LWB supplementation reduced not just the levels of IL-6, but also the downstream signaling *via* STAT-3 activation. Interestingly however, there was only non significant trend observed in the reduction of TNF-α. It is possible that this could be either due to technical variability or due to different kinetics of this cytokine. Moreover, the significant reduction in KC/GRO-α, an established chemokine involved in neutrophil chemotaxis[30], can explain the reduction in neutrophil infiltration. Thus, modulation of cytokine levels, perhaps *via* a decrease in NF-κB activation, is responsible for the anti-inflammatory effects of LWB in this study. The increase of Nrf2 activation *in vitro* as well as an increase in the plasma anti-oxidative capacity and upregulation of GPx1 after LWB supplementation in colitic mice also suggests a possibility of an anti-oxidant mechanism underlying the anti-inflammatory effects of LWB. However, the lack of upregulation of other Nrf2 target genes, such as, CAT and SOD2 means that the anti-oxidative properties explains only part of

the overall effects observed.

Wolfberry is believed to contain at least three different biologically active components: (1) Lycium Barbarum polysaccharides (LBP); (2) zeaxanthin dipalmitate; and (3) 2-O-β-D-glucopyranosyl-L-ascorbic acid (a Vitamin C analogue)[16]. The anti-oxidative properties of all three active ingredients of wolfberry are well documented[16]. LWB is prepared by a milk based extraction process of wolfberry, which is believed to increase bioavailability of its active ingredients, as demonstrated for zeaxanthin[17]. In addition, the *in vitro* assays suggest that the anti-inflammatory activity of LWB is attributable to wolfberry rather than its milk component. However, to the best of our knowledge, wolfberry has not been shown to reduce cytokine levels under inflammatory conditions, as observed in our present study. In fact, wolfberry has been shown to up-regulate cytokine expression[31] and both LWB and wolfberry have a demonstrated immune-enhancing effect[18,19,32-34]. On the other hand, the anti-inflammatory properties of milk components are well established[35,36]. Overall, it seems that depending on the physiological environment LWB may provide support to recover homeostasis and/or immune competence. In the present study, we can speculate that a synergistic effect between the anti-oxidative ingredients of wolfberry and the anti-inflammatory components in milk can also be a potential mechanism of the benefits observed in our model. In that respect, further studies are required to identify the active anti-inflammatory ingredients in LWB. Nutritional therapies are an effective and safe form of intervention to induce remission in active state of CD[7,8]. Despite this profile of effectiveness and safety, they have not gained widespread usage, particularly in the area of adult IBD. One of the reasons for this could be that while they are effective, a review of the clinical trials comparing the efficacy of nutritional therapies to steroids concluded that, they are not as effective as steroids in induction of remission[9]. Thus, clearly more needs to be done in this area. Our findings suggest that addition of LWB to enteral diet formulations might help improve disease outcomes in IBD patients. However, it should be noted that further work addressing efficacy in different colitis models and in-depth confirmation of mechanism of action is necessary before clinically relevant research can be undertaken.

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COMMENTS

Background

Inflammatory bowel disease (IBD) consists of a group of disorders, such as Crohn's disease (CD) and ulcerative colitis (UC). The incidence of IBD is increasing throughout the world. Both, CD and UC are characterized by relapsing-remitting disease progression. Currently, there is no known cure for IBD and the various available therapies are only palliative. Herein, the authors have identified the anti-inflammatory properties of a nutritional ingredient in a preclinical model of colitis.

Research frontiers

Due to the chronic nature of IBD and the adverse effects of existing therapies, nutritional ingredients with anti-inflammatory properties may benefit the patient in the long term. With this in mind, the characterization of anti-inflammatory properties of novel or traditional food ingredients is an important field of research.

Innovations and breakthroughs

Previously, the authors have characterized the benefits of Lacto-Wolfberry (LWB) on the adaptive immune system. In this article, they have characterized the anti-inflammatory properties of LWB. The authors first demonstrate the anti-inflammatory and anti-oxidative properties of LWB in cellular models and subsequently show that LWB can ameliorate chemically-induced colitis.

Applications

The identification of the anti-inflammatory properties of LWB raises new possibilities of developing novel nutritional solutions for patients with IBD.

Terminology

LWB is a skimmed milk extract of the traditional Chinese ingredient, wolfberry, specifically developed to increase the bioavailability of its active ingredients.

Peer review

In the original article, the authors examined the complex anti-inflammatory effect of LWB administration in 2,4,6-trinitrobenzene sulfonic acid induced colitis and in selected cell lines. The study is well designed and the results and conclusions are clear and logical.

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ORIGINAL ARTICLE

Increased tumor necrosis factor receptor 1 expression in human colorectal adenomas

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Abstract

AIM: To determine the expression statuses of tumor necrosis factor (TNF)-α, its receptors (TNF-R) and downstream effector molecules in human colorectal adenomas.

METHODS: We measured the serum concentrations of TNF-α and its receptors in 62 colorectal adenoma patients and 34 healthy controls. The protein expression of TNF-α, TNF-R1, TNF-R2 and downstream signals of the TNF receptors, such as c-Jun N-terminal kinase (JNK), nuclear factor-κ B and caspase-3, were also investigated in human colorectal adenomas and in normal colorectal mucosal tissues by immunohistochemistry. Immunofluorescence confocal microscopy was used to investigate the consistency of expression of TNF-R1 and phospho-JNK (p-JNK).

RESULTS: The serum levels of soluble TNF-R1 (sTNF-R1) in adenoma patients were significantly higher than in the control group (3.67 ± 0.86 ng/mL *vs* 1.57 ± 0.72 ng/mL, $P < 0.001$). Receiver operating characteristic analysis revealed the high diagnostic sensitivity of TNF-R1 measurements (AUC was 0.928) for the diagnosis of adenoma, and the best cut-off level of TNF-R1 was 2.08 ng/mL, with a sensitivity of 93.4% and a specificity of 82.4%. There were no significant differences in the serum levels of TNF-α or sTNF-R2 between the two groups. Immunohistochemistry showed high levels of TNF-R1 and p-JNK expression in the epithelial cells of adenomas. Furthermore, a high incidence of co-localization of TNF-R1 and p-JNK was identified in adenoma tissue.

CONCLUSION: TNF-R1 may be a promising biomarker of colorectal adenoma, and it may also play an important role in the very early stages of colorectal carcinogenesis.

Key words: Tumor necrosis factor-α; Tumor necrosis factor receptor 1; c-Jun N-terminal kinase; Colorectal adenoma; Biomarker

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INTRODUCTION

The proinflammatory cytokine, tumor necrosis factor (TNF), plays an important role in diverse cellular events, such as the induction of other cytokines' expression, cell proliferation, differentiation, necrosis and apoptosis[1,2]. Many of the TNF-induced cellular responses are mediated by one of two specific cellular membrane receptors, tumor necrosis factor receptor 1 (TNF-R1) and TNF-R2, both of which belong to the TNF receptor superfamily[3]. In response to TNF treatment, activation of nuclear factor-κ B (NF-κB), a transcription factor, as well as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase (JNK) have been reported in most cell types and, in some cases, after apoptosis or necrosis had been induced[4,5].

Overexpression of TNF-α and its receptors often accompanies tumor development and progression, and their important role in the pathogenesis of cancer is now increasingly recognized. Several studies have reported on the status of TNF-α expression in various malignancies. For example, TNF-α mRNA and protein have been detected in both malignant and stromal cells in human ovarian cancer biopsies[6,7]. The plasma levels of TNF-α have been reported to be increased in some cancer patients, especially those with poor prognoses[8-11]. Blood TNF-α concentrations have been reported to be higher in prostate cancer patients with advanced, cachectic disease[12]. These findings suggest that TNF-α might also have an important role in carcinogenesis. We now know that many of the cells and mediators of inflammation that have been detected in human and experimental cancers and inflammatory conditions increase the risk of cancer development[13-16]. There is strong evidence to suggest that cancer-related inflammation aids in the proliferation and survival of malignant cells, stimulates angiogenesis and metastasis[17-19], subverts adaptive immunity[20-22], and modulates their responses to hormones and chemotherapy[23,24].

In colitis-related colon cancer, TNF-α has been demonstrated to promote carcinogenesis, and anti-TNF-α drugs are being used therapeutically[25,26]. Moreover, serum TNF-α is known to serve as an important pathophysiologic marker for the presence and severity of inflammatory bowel disease[27]. However, TNF-α has a short half-life (20 min); therefore, accurate measurement of the blood levels is difficult[28]. There is some debate about whether TNF-α can actually be measured using some sort of physiologically active substance[28,29]. On the other hand, TNF-R1 and TNF-R2 are released into the blood as soluble TNF-R1 (sTNF-R1) and soluble TNF-R2 (sTNF-R2) after proteolysis of their extracellular domains in response to activators, including TNF-α itself[30,31]. Their presence in the peripheral blood is reflective of an inflammatory response occurring within the body. sTNF-R1 and sTNF-R2 also have very long half-lives; thus, they are more stable than TNF-α[32,33]. These receptors may provide better serum biomarkers than TNF-α. Spoettl *et al*[34] found that serum sTNF-R1 levels were significantly increased in ulcerative colitis (UC) patients compared with that of healthy controls. However, there have been few reports of the association between TNF-R1 and TNF-R2 and sporadically occurring colorectal neoplasms[35]. Thus, the association of these receptors with the risk of colorectal adenoma has not yet been fully clarified. The aim of this study was to investigate the serum levels of TNF-α, sTNF-R1 and sTNF-R2 in adenoma patients and also to investigate the expressions of downstream molecules in the TNF-α signaling pathway, including TNF-R1, TNF-R2, JNK, NF-κB and caspase-3 in adenoma tissues in comparison to those in normal colorectal mucosa.

MATERIALS AND METHODS

Patients

The study population consisted of 62 consecutive patients with colorectal adenoma who underwent colonoscopy from January 2008 to January 2009 at the Division of Gastroenterology, Yokohama City University School of Medicine. Patients with concomitant diseases, including infectious diseases, inflammatory bowel diseases, autoimmune conditions, allergy or asthma, that were likely to elevate serum TNF-α levels were excluded from the study. The patients ranged in age from 54 to 86 years (mean ± SD: 67.7 ± 8.2 years) and consisted of 39 males and 23 females. The control group consisted of 34 healthy people, matched for age. The control group consisted of people undergoing colonoscopy after positive fecal occult blood test but not found to have adenomas. The study was carried out in accordance with the Declaration of Helsinki (revised 1989) and with the approval of the Ethics Committee of Yokohama City University School of Medicine. Informed consent was obtained from each patient participating in this study and for the use of the obtained data for research purposes.

Serum samples

Before colonoscopy, blood samples were obtained from all participants after an overnight fast to determine the serum concentrations of TNF-α, sTNF-R1 and sTNF-R2. The blood samples were centrifuged at 800 g for 5 min. Serum was separated as soon as possible from the clot of red blood cells by centrifugation to avoid TNF-α production by the blood cells, which would have led to falsely increased values[36]. Then, the serum specimens were stored at -80 ℃ until the biochemical analyses.

Biochemical determinations

Serum concentrations of human TNF-α, sTNF-R1 and sTNF-R2 were determined by enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems Inc., Minneapolis, MN, United States). The minimum limits of detection of the assay were as follows: TNF-α, 1.6 pg/mL; sTNF-R1, 0.77 pg/mL; and sTNF-R2, 0.6 pg/mL. The tests were carried out as described in the product manuals.

Immunohistochemical analysis

Immunohistochemical analysis was performed in the adenomatous polyps retrieved from the study patients (n = 62). Adenomatous polyps removed during colonoscopy, together with normal colorectal mucosal biopsies from the same patients when possible, were examined. Four-micrometer sections were prepared from the formalin-fixed, paraffin-embedded tissues and mounted on slides coated with polylysine. We examined only sporadically occurring polyps by excluding specimens from patients with familial adenomatous polyposis (FAP) or hereditary non-polyposis coli (HNPCC). The protein expression levels were determined in all 62 normal and colorectal adenomatous tissue sections by immunohistochemistry. Sections were deparaffinized in xylene and rehydrated. The sections were then heated in a 750 W microwave three times for 7 min. Inhibition of endogenous peroxidase activity was performed by treating sections with 3% hydrogen peroxide for 10 min. After washing three times with Tris-Buffered Saline (TBS), the blocking of non-specific interactions was accomplished by incubating the samples with blocking serum for 30 min at room temperature. Sections were then probed with the primary antibodies (Table 1) and subsequently incubated with the Histofine simple stain max PO kit for 30 min (Nichirei Laboratories, Tokyo, Japan) in accordance with the manufacturer's instructions. The signals were visualized by treatment with diaminobenzidine (peroxidase substrate kit, Vector Laboratories, Burlingame, CA, United States), and the sections were counterstained with hematoxylin. Six random microscopic fields per sample of approximately 250 cells were counted at a magnification of 400 × under a bright-field microscope. The results were expressed as the percentage of positive cells with separating epithelial cells and stromal cells.

Immunofluorescence confocal microscopy

For immunofluorescence studies, the sections were stained with anti-TNF-R1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States) at a dilution of 1:50, anti-TNF-R2 rabbit polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:50, and anti-p-JNK mouse monoclonal antibody at Thr 183 and Tyr 185 (Santa Cruz Biotechnology) at a dilution 1:50. After thorough washes in TBS, sections were incubated with their respective Alexa594-conjugated anti-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA, United States) or Alexa488-conjugated anti-mouse IgG secondary antibodies (Invitrogen) for 1 h, washed, and mounted.

The confocal imaging was carried out on a FV1000-D (Olympus, Tokyo, Japan) confocal laser scanning microscope. Excitation and detection of the samples were carried out in sequential modes to avoid overlapping of signals. Sections were scanned simultaneously at both wavelengths (488/594 nm) with appropriate laser intensity, confocal aperture, and gain. The Black-level setting was kept constant for all samples.

Table 1 Summary of the specific antibodies used and immunohistochemistry

Protein	Antibody (catalog number)	Dilution	Incubation
TNF-α	Rabbit polyclonal (Abcam, ab6671)	1:100	Overnight
TNF-R1	Rabbit polyclonal (Abcam, ab19139)	1:2000	1 h
TNF-R2	Rabbit polyclonal (Abcam, ab15563)	1:50	30 min
p-JNK	Rabbit polyclonal (CST, #9251)	1:100	Overnight
NF-κB	Rabbit polyclonal (CST, #3034)	1:50	Overnight
Caspase-3	Rabbit polyclonal (CST, #9661)	1:200	Overnight

Abcam (Cambridge, United Kingdom). CST: Cell signaling technology (Beverly, MA, United States).

Table 2 Comparison of the clinical data and measured biochemical parameters in the serum of patients with adenomas and control subjects (mean ± SD)

Parameters	Control (n = 34)	Adenoma (n = 62)	P value
Age (yr)	67.6 ± 15.9	67.7 ± 8.2	NS
Sex (F/M)	14/20	23/39	NS
BMI	23.5 ± 5.0	23.9 ± 3.4	NS
TNF-α (pg/mL)	5.56 ± 1.3	4.83 ± 1.9	NS
sTNF-R1 (ng/mL)	1.57 ± 0.72	3.67 ± 0.86	< 0.001
sTNF-R2 (ng/mL)	2.72 ± 1.15	3.09 ± 0.91	NS

P < 0.05 was denoted significance. NS: Non-significant; TNF-α: Tumor necrosis factor-α; sTNF-R1/2: Soluble tumor necrosis factor receptor 1/2; F/M: Female/male; BMI: Body mass index.

Statistical analysis

Data were expressed as the mean ± SD unless otherwise indicated. The significances of the differences in clinical characteristics between patients with adenomas and controls were evaluated by the χ^2-test for categorical variables and Welch's test for continuous variables. One-way analysis of variance was performed to compare the prevalence of each variable between groups. To assess the diagnostic sensitivity and specificity of the sTNF-R1 and sTNF-R2 measurements, receiver operating characteristic (ROC) curve analysis was performed. P < 0.05 was considered to indicate statistical significance.

RESULTS

Biochemical determinations

Table 2 shows a comparison of the clinical data and laboratory parameters between the 62 patients with adenoma and the 34 normal controls. No differences in

Table 3 Association between the measured biochemical parameters and the number of colorectal adenomas per person

	Control (n = 34)	≤ 3 (n = 34)	≥ 4 (n = 28)	P value
TNF-α (pg/mL)	5.63 ± 1.3	4.31 ± 1.9	4.95 ± 2.0	NS
sTNF-R1 (ng/mL)	1.57 ± 0.72	3.37 ± 0.93	4.05 ± 0.55	< 0.001
sTNF-R2 (ng/mL)	2.72 ± 1.15	3.13 ± 0.88	3.04 ± 0.95	NS

Values are expressed as the mean ± SD; $P < 0.05$ denoted significance. NS: Non-significant; TNF-α: Tumor necrosis factor-α; sTNF-R1/2: Soluble tumor necrosis factor receptor 1/2.

the age, sex or body mass index were found between the two studied groups. The mean serum levels of sTNF-R1 were significantly higher in the adenoma patients compared to the control group patients. On the other hand, there were no significant differences in the serum levels of TNF-α or sTNF-R2 between the two groups. Linear contrast analysis was conducted to evaluate the correlation between each variable and the number of adenomas per person (Table 3). The serum levels of sTNF-R1 were positively correlated with the number of adenomas in the colorectum ($P < 0.001$). As assessed by nonparametric analysis of the ROC curves, sTNF-R1 measurements showed a high diagnostic sensitivity at a value of 0.928 (Figure 1). The best cut-off level for TNF-R1 as 2.08 ng/mL, which showed a sensitivity of 93.4% and a specificity of 82.4%.

Immunohistochemical analysis

Immunohistochemistry showed varying expression intensities of TNF-α, TNF-R1, TNF-R2, p-JNK, NF-κB and caspase-3 in different areas of the samples. The expression of TNF-α in the stromal area of adenomas was significantly higher than that in the stromal areas of the normal mucosa ($P < 0.05$); however, the expression of TNF-α in the epithelial cells of adenomas was not as high as that in the epithelial cells of the normal mucosa (Figure 2). The expression of TNF-R1 in the epithelial cells of the adenomas was significantly higher than that in the epithelial cells of the normal mucosa ($P < 0.05$); however, in the stromal areas, the expression of TNF-R1 was equivalent between the adenomas and the normal mucosa. No significant difference in the expression of TNF-R2 was noted in either the epithelial cells or the stromal areas between the adenomas and the normal mucosa. In relation to the expressions of the downstream molecules in the TNF-α signaling pathway, the expression of p-JNK in both the epithelial cells and stromal areas of the adenoma were significantly higher than those in the normal mucosa ($P < 0.05$) (Figure 3). However, there were no significant differences in the expressions of NF-κB or caspase-3 in either the epithelial cells or stromal areas between the adenomas and the normal mucosa.

To investigate the consistency of TNF-R1 and p-JNK expression, we performed immunofluorescence staining of the adenoma tissues, because the results of immunohistochemistry revealed high levels of TNF-R1 and p-JNK expression in the epithelial cells of the adenomas. The results revealed expression of TNF-R1 and p-JNK co-localized predominantly in the epithelial cells of the adenomas. Merged images of TNF-R1 and p-JNK immunofluorescence staining demonstrated this co-localization (Figure 4). On the other hand, the expression of TNF-R2 was localized predominantly to the stromal area of the adenomas, and merged images of TNF-R2 and p-JNK immunofluorescence staining showed a lack of co-localization.

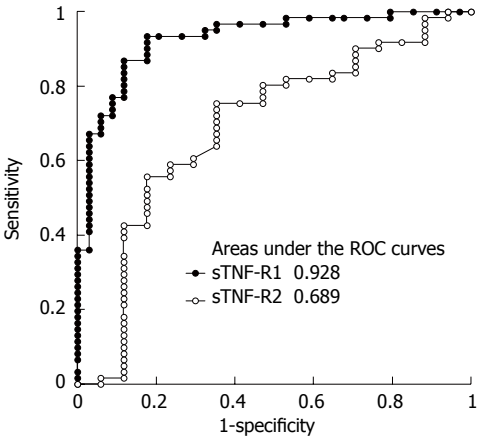

Figure 1 Receiver operating characteristic curves for soluble tumor necrosis factor receptor-1 and 2 in patients with adenomas. ROC: Receiver operating characteristic.

DISCUSSION

This is the first study to show marked changes in the expression levels of TNF-R1 in colorectal adenoma tissues. The serum sTNF-R1 levels were also significantly higher in colorectal adenoma patients than in the control subjects. To evaluate the possible usefulness of TNF-R1 as a biomarker for colorectal adenoma, we evaluated the correlation between the number of adenomas in the colorectum and the serum sTNF-R1 level. The results showed that the serum sTNF-R1 level positively correlated with the number of colorectal adenomas. A ROC analysis revealed the high diagnostic sensitivity and specificity of sTNF-R1 measurements for diagnosis of colorectal adenoma. These results may suggest that TNF-R1 is a promising biomarker for colorectal adenomas. In addition, our results agreed with the Kaminska *et al*[37] study, which showed that sTNF-R1 had the highest diagnostic sensitivity in colorectal cancer patients. Other previous reports have shown a high diagnostic and prognostic accuracy of serum sTNF-R1 levels for various diseases. Determination of the TNF-R1 levels in several body fluids, including the serum, provides valuable insight into a variety of pathological conditions. For example, in cervical adenocarcinoma patients, serum sTNF-R1 is reported to be a useful marker, especially in the early stages of disease[38]. In patients with breast cancer, serum sTNF-R1 is considered to be an independent and clinically useful

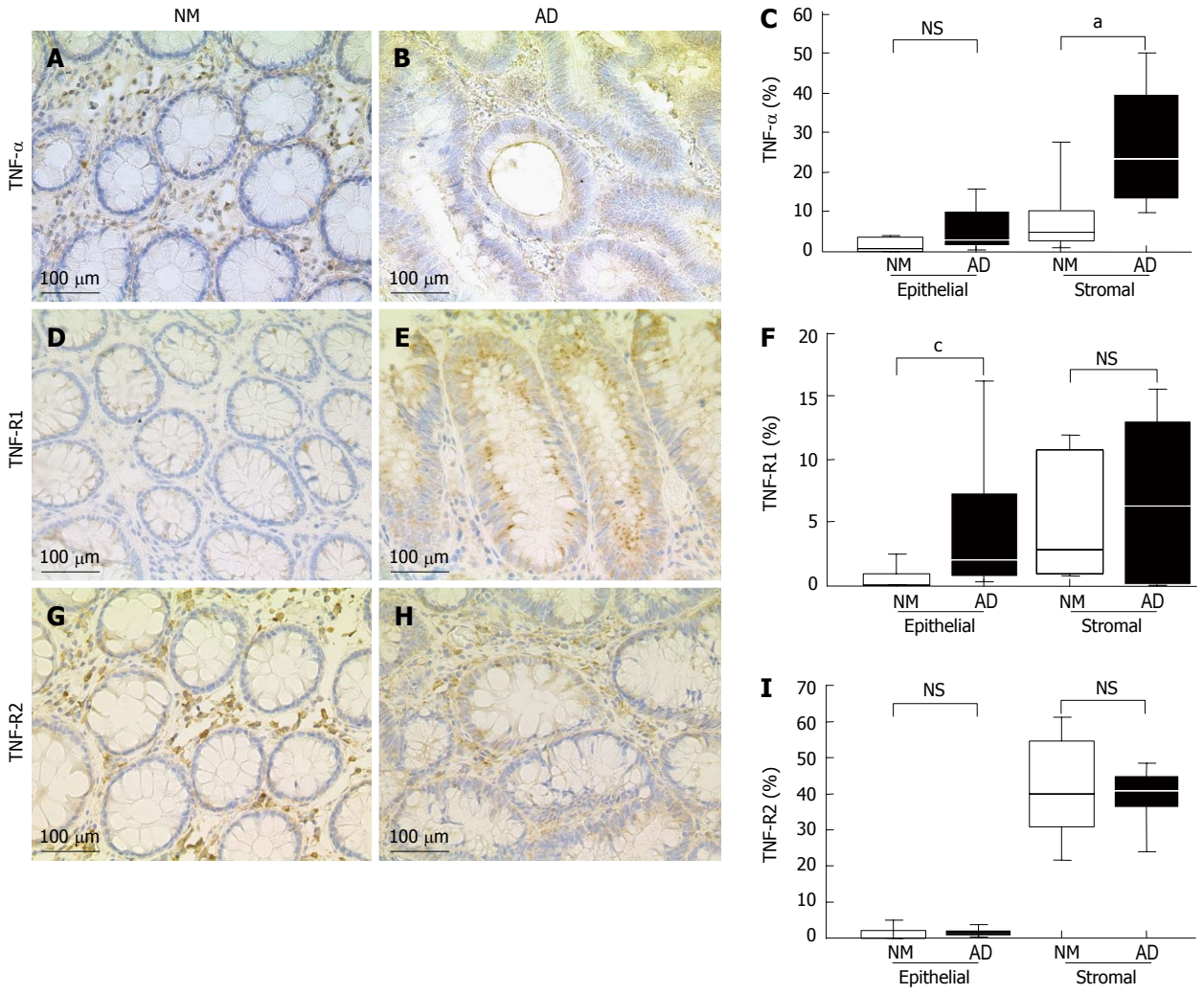

Figure 2 Immunohistochemical analyses in the normal colorectal mucosa and adenoma tissues. A: Tumor necrosis factor (TNF)-α expression in the normal colorectal mucosa; B: TNF-α expression in the adenoma tissues; C: The percentage of TNF-α-positive cells; D: Tumor necrosis factor-receptor 1 (TNF-R1) expression in the normal colorectal mucosa; E: TNF-R1 expression in the adenoma tissues; F: The percentage of TNF-R1-positive cells; G: TNF-R2 expression in the normal colorectal mucosa; H: TNF-R2 expression in the adenoma tissues; I: The percentage of TNF-R2-positive cells. Box plots display median values and interquartile ranges (C, F, I). The non-outlier range is also shown. $^{a}P < 0.05$ between NM and AD in stromal of TNF-α; $^{c}P < 0.05$ between NM and AD in epithelial of TNF-R1. NS: Non-significant; NM: Normal mucosa; AD: Adenoma.

indicator of a poor prognosis[39].

TNFR-1 is an important member of the death receptor family, which is capable of inducing apoptotic cell death[40]. In addition to its involvement in apoptotic signaling, TNF-R1 has been widely studied because it is a dual-role receptor. In addition to inducing apoptosis, it also has the ability to transduce cell survival signals. When TNF-R1 transduces cell survival signals, TNFR-associated factor 2 (TRAF-2) is recruited to the complex, which inhibits apoptosis *via* the cytoplasmic inhibitor of apoptosis protein (cIAP). The binding of TRAF-2 initiates a sequence of phosphorylation steps resulting in the activation of cFos/cJun transcription factors *via* MAPK and JNK[41]. The cFos/cJun transcription factors induce transcription of antiapoptotic, proliferative, immunomodulatory, and inflammatory genes.

TNF-α is a major mediator of cancer-related inflammation[13,42], and most of the pro-tumor actions of TNF-α appear to be mediated by TNF-R1. Mouse experiments have revealed that the development of primary cancers and metastases is attenuated in mice deficient in TNF-R1. For example, TNF-R1-/- mice are resistant to DMBA-TPA carcinogenesis, as are TNF-α-/- mice[43]. Experimental formation of lung and liver metastases was attenuated in TNF-R1-/- mice compared with that in their normal counterparts[44,45]. In wild-type mice with bone marrow cells repopulated using cells from TNF-R1-/- mice, the likelihood of colitis and colon cancer development was reduced[46] suggesting that TNF-α in the tumor microenvironment enhanced tumor development through its actions on TNF-R1-positive myeloid cells. In various pathologic states, it has been reported that the production and release of TNF-R1 may mediate host responses and determine the course and outcome of the disease by binding with TNF-α and competing with cell surface receptors.

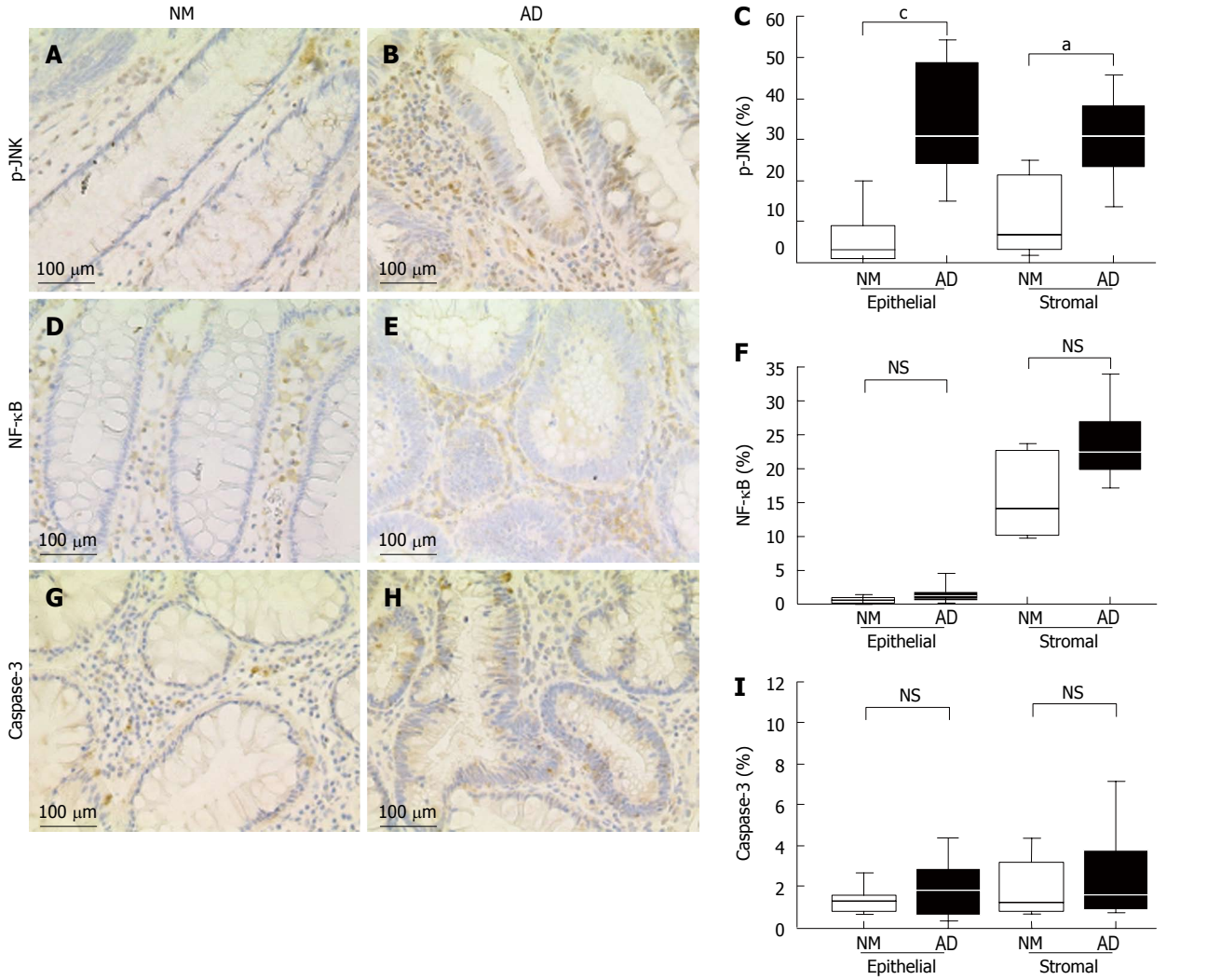

Figure 3 Immunohistochemical analyses in the normal colorectal mucosa and adenoma tissues. A: Phospho-c-Jun N-terminal kinase (p-JNK) expression in the normal colorectal mucosa; B: p-JNK expression in the adenoma tissues; C: The percentage of p-JNK positive cells; D: Nuclear factor-κ B (NF-κB) expression in the normal colorectal mucosa; E: NF-κB expression in the adenoma tissues; F: The percentage of NF-κB-positive cells; G: Caspase-3 expression in the normal colorectal mucosa; H: Caspase-3 expression in the adenoma tissues; I: The percentage of caspase-3-positive cells. Box plots display median values and interquartile ranges (C, F, I). The non-outlier range is also shown. $^{a}P < 0.05$ between NM and AD in stromal of p-JNK; $^{c}P < 0.05$ between NM and AD in epithelial of p-JNK. NS: Non-significant; NM: Normal mucosa; AD: Adenoma.

In addition to the systematic analysis of serum sTNF -R1, we also investigated, by immunohistochemical analysis, the local expressions of downstream molecules in the TNF-α signaling pathway in normal colorectal mucosa and adenoma tissues. The results revealed high levels of TNF-R1 and p-JNK expression in the epithelial cells of adenomas. Moreover, we identified co-localization of TNF-R1 and p-JNK in adenomas by immunofluorescence confocal microscopy. In the past, expression of p-JNK in adenomas has only been described by Hardwick *et al*[47]. These authors found that the expression of p-JNK was observed mainly in stromal T-lymphocytes, and epithelial cells were not stained. The discrepancy in our results was presumably due to differences in the immunohistochemical staining protocol used. The Hardwick study used phosphate- buffered saline (PBS) as the wash buffer, while we used TBS. Additionally, different antibodies were used in the two studies, which may have resulted in the absence of phospho-JNK staining in the epithelial cells of their study.

Our results suggest that the TNF-R1/p-JNK pathway is upregulated in adenomas and that this pathway may play an important role in adenoma formation, which represents the very early stages of colorectal carcinogenesis because this pathway is not upregulated in the normal colorectal mucosa. Expression of JNK in colon cancer has already received some attention[48,49]; studies have found increased activity of JNK in both rat models of colon cancer and human colorectal tumors. In addition, Zhang *et al*[50] indicated that the TNF-R1/JNK signaling cascade can functionally promote tumorigenesis of human epithelial cancers, such as squamous cell carcinomas. Moreover, a recent study conducted by our group showed that JNK/c-Jun may play an important role in promoting colorectal carcinogenesis and epithelial cell proliferation under high-fat dietary conditions[51].

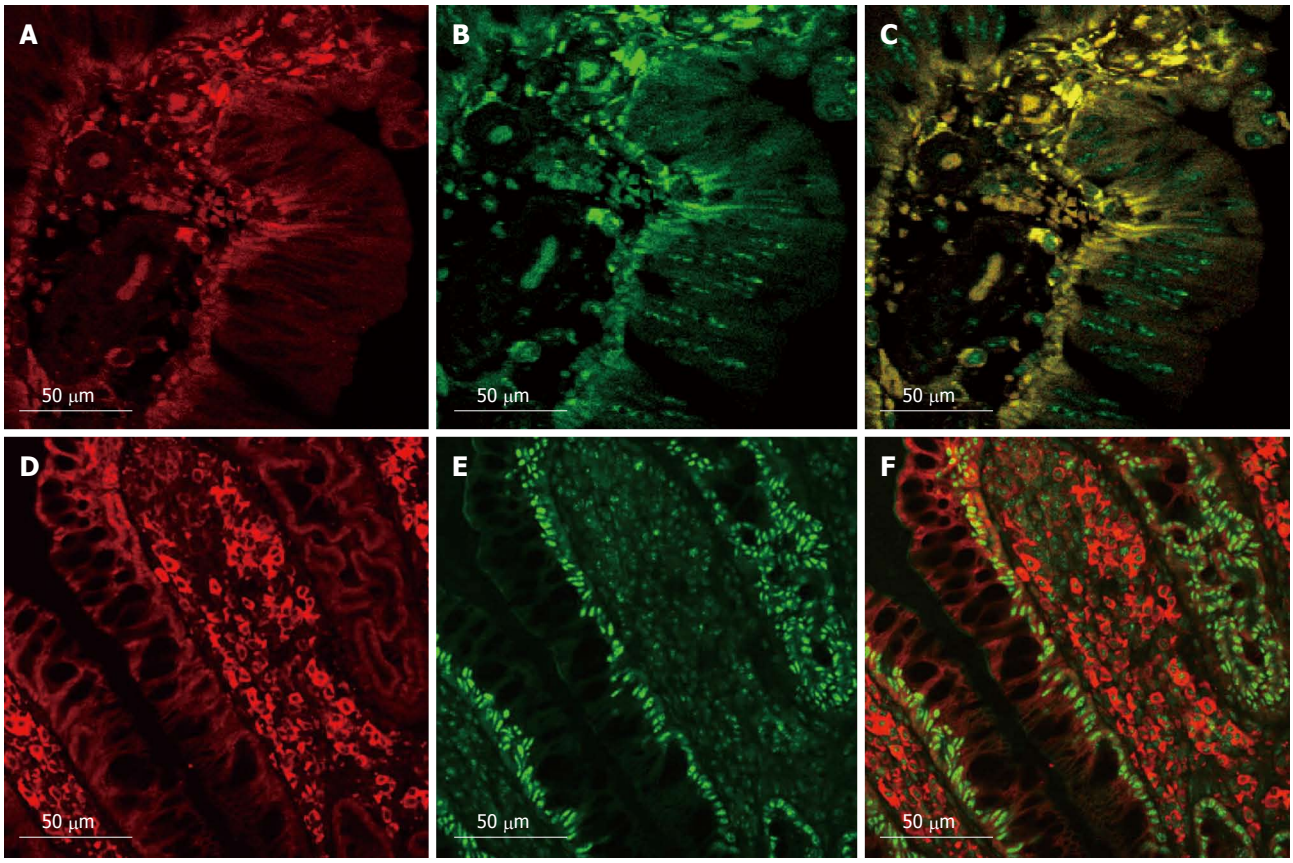

Figure 4 Confocal laser microscopic analyses of colorectal adenoma sections. A: Tumor necrosis factor receptor 1 (TNF-R1) expression; B: Phospho-c-Jun N-terminal kinase (p-JNK) expression; C: Merged image of A and B. Co-localized expression of TNF-R1 and p-JNK was noted predominantly in the epithelial cells of adenomas. Merged images showing co-localization; D: TNF-R2 expression; E: p-JNK expression; F: Merged image of D and E. Expression of TNF-R2 was localized predominantly to the stromal area of the adenoma. Merged images showed a lack of co-localization.

Our study had several important novelties. We showed for the first time that the serum levels of sTNF-R1 may have high diagnostic sensitivity and specificity as a biomarker for colorectal adenoma. Second, TNF-R1 was relatively undetectable in the normal colorectal mucosa, whereas adenomas showed high expression levels of TNF-R1. In addition, co-expression of p-JNK with TNF-R1 was observed in adenomas, although a previous study reported that the epithelial cells of adenoma showed a lack of p-JNK staining[47]. These results suggest that the TNF-R1/JNK pathway may play an important role in the development/progression of colorectal adenoma.

The present study also had some limitations. First, our research target was colorectal adenoma and not colorectal cancer. Thus, further studies targeting colorectal cancer are needed. Secondly, the control population was small. Third, different cell death receptors are able to activate JNK. Therefore, the increased p-JNK expression in adenoma tissue may be related to TNF-R1 or other effectors. The expression of cell death receptors may also be altered in inflammatory cells, which may certainly be involved in cancer progression. Fourth, it was considered that TNF-R1 may directly stimulate JNK activation, but this would also be difficult to prove.

In conclusion, this is the first study to report elevated serum levels of sTNF-R1 in patients with adenomas compared to a control group. We also found, by immunohistochemical analysis, enhanced expression of TNF-R1 in colorectal adenomas. These results suggest that TNF-R1 may not only be a promising biomarker of colorectal adenoma but also plays an important role in the very early stages of colorectal carcinogenesis. More studies are needed to elucidate the exact functions of TNF-R1 in colorectal adenomas.

ACKNOWLEDGMENTS

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COMMENTS

Background

The proinflammatory cytokine, tumor necrosis factor (TNF)-α, is a major mediator of cancer-related inflammation. However, little has been reported on the relationship between TNF-α and sporadically occurring colorectal neoplasms. Studies on human colorectal adenoma were performed to determine the expression statuses of TNF-α and its receptors.

Research frontiers

Overexpression of TNF-α and its receptors often accompanies tumor development and progression, and their important role in the pathogenesis of cancer is

now increasingly recognized.

Innovations and breakthroughs

The authors show, for the first time, that the serum levels of tumor necrosis factor-receptor 1 (TNF-R1) may have high diagnostic sensitivity and specificity as a biomarker of colorectal adenoma. TNF-R1 was hardly detected in the normal colorectal mucosa, whereas adenomas showed high expression levels of TNF-R1. In addition, co-expression of phospho-c-Jun N-terminal kinase (p-JNK) with TNF-R1 was observed in adenomas. These results suggest that the TNF-R1/JNK pathway may play an important role in the development/progression of colorectal adenoma.

Applications

TNF-R1 may be a promising biomarker of colorectal adenoma, and further studies may show that TNF-R1 expression can be used to screen for adenomas in patients as an alternative or in addition to hemoccult screening or colonoscopy screening.

Terminology

TNF-R1 belongs to the TNF receptor superfamily. In response to TNF treatment, activation of the transcription factor nuclear factor-κ B and mitogen-activated protein kinase, as well as Extracellular Signal-regulated Kinase, p38, and JNK, has been reported in most types of cells and, in some cases, apoptosis or necrosis was also induced.

Peer review

This is an interesting study investigating the importance of TNF-R1/JNK co-expression in colorectal adenoma. The major finding of the study was that serum levels of TNF-R1 were higher in patients with colorectal adenomas, while immunohistochemistry showed high expression of both TNF-R1 and p-JNK in the adenomatous tissues.

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ORIGINAL ARTICLE

Axl glycosylation mediates tumor cell proliferation, invasion and lymphatic metastasis in murine hepatocellular carcinoma

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Abstract

AIM: To investigate the effects of Axl deglycosylation on tumor lymphatic metastases in mouse hepatocellular carcinoma cell lines.

METHODS: Western blotting was used to analyze the expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell line Hca-F treated with tunicamycin and PNGase F 3-(4,5)-dimethylthiazol(-zyl)-3,5-diphenyltetrazolium bromide (MTT) assay, extracellular matrix (ECM) invasion assay (*in vitro*) and tumor metastasis assay (*in vivo*) were utilized to evaluate the effect of Axl deglycosylation on the Hca-F cell proliferation, invasion and lymphatic metastasis.

RESULTS: Tunicamycin and PNGase F treatment markedly inhibited Axl glycoprotein synthesis and expression, proliferation, invasion, and lymphatic metastasis both *in vitro* and *in vivo*. In the MTT assay, proliferation was apparent in untreated Hca-F cells compared with treated Hca-F cells. In the ECM invasion assay (*in vitro*), treated cells passed through the ECMatrix gel in significantly smaller numbers than untreated cells (tunicamycin 5 μg/mL: 68 ± 8 *vs* 80 ± 9, $P = 0.0222$; 10 μg/mL: 50 ± 6 *vs* 80 ± 9, $P = 0.0003$; 20 μg/mL: 41 ± 4 *vs* 80 ± 9, $P = 0.0001$); (PNGase F 8 h: 66 ± 7 *vs* 82 ± 8, $P = 0.0098$; 16 h: 49 ± 4 *vs* 82 ± 8, $P = 0.0001$; 24 h: 34 ± 3 *vs* 82 ± 8, $P = 0.0001$). In the tumor metastasis assay (*in vivo*), average lymph node weights of the untreated Hca-F group compared with treated Hca-F groups (tunicamycin 5 μg/mL: 0.84 ± 0.21 g *vs* 0.72 ± 0.19 g, $P = 0.3237$; 10 μg/mL: 0.84 ± 0.21 g *vs* 0.54 ± 0.11 g, $P = 0.0113$; 20 μg/mL: 0.84 ± 0.21 g *vs* 0.42 ± 0.06 g, $P = 0.0008$); (PNGase F 8 h: 0.79 ± 0.15 g *vs* 0.63 ± 0.13 g, $P = 0.0766$; 16 h: 0.79 ± 0.15 g *vs* 0.49 ± 0.10 g, $P = 0.0022$; 24 h: 0.79 ± 0.15 g *vs* 0.39 ± 0.05 g, $P = 0.0001$). Also, average lymph node volumes of the untreated Hca-F group compared with treated Hca-F groups (tunicamycin 5 μg/mL: 815 ± 61 mm^3 *vs* 680 ± 59 mm^3, $P = 0.0613$; 10 μg/mL: 815 ± 61 mm^3 *vs* 580 ± 29 mm^3, $P = 0.0001$; 20 μg/mL: 815 ± 61 mm^3 *vs* 395 ± 12 mm^3, $P = 0.0001$); (PNGase F 8 h: 670 ± 56 mm^3 *vs* 581 ± 48 mm^3, $P = 0.0532$; 16 h: 670 ± 56 mm^3 *vs* 412 ± 22 mm^3, $P = 0.0001$; 24 h: 670 ± 56 mm^3 *vs* 323 ± 11 mm^3, $P = 0.0001$).

CONCLUSION: Alteration of Axl glycosylation can attenuate neoplastic lymphatic metastasis. Axl N-glycans may be a universal target for chemotherapy.

Key words: Axl; Glycosylation; Hepatocellular carcinoma; Lymphatic metastasis

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INTRODUCTION

The receptor tyrosine kinases (RTKs) constitute a large family of transmembrane proteins that relay signals from extracellular growth factors into the cell[1,2]. The Tyro-Axl-Mer (TAM) subfamily shares the vitamin K-dependent ligand Gas6 (growth arrest specific 6). TAM receptors contain a combination of two immunoglobin-like domains and dual fibronectin type Ⅲ repeats in the extracellular region, and a cytoplasmic kinase domain[3,4]. The TAM receptors regulate a diverse range of cellular responses including cell survival, proliferation, autophagy, migration, angiogenesis, platelet aggregation, and natural killer cell differentiation[4].

The Axl receptor (also called UFO, Tyro7, and Ark) is a RTK originally identified as a transforming gene in chronic myeloid leukemia[5,6]. Axl is expressed in various organs, including the brain, suggesting its involvement in mesenchymal and neural development[7,8]. Axl has been shown to have transforming potential when overexpressed during development. Axl overexpression is clearly associated with invasiveness and metastasis in several cancer cell types, including myeloid leukemia[6,9], esophageal[10], metastatic lung[11], metastatic colon[12], renal cell[13], prostate[14], breast[15], gastric[16], and thyroid[17] cancers. Axl also affects multiple pathways in angiogenesis[11]. Thus, Axl may play an important role in tumor progression, although its mechanism remains unknown.

Protein glycosylation is one of the major types of posttranslational modifications that has profound biological implications[18,19]. Specific changes in the glycosylation pattern of cell surface glycoproteins have been shown to correlate with metastatic efficiency in tumor cells[20]. In particular, protein N-glycosylation is one of the most prominent biochemical alterations in tumorigenesis and metastatic spread[21,22]. A cell surface transmembrane glycoprotein, little is known about the mechanism of Axl deglycosylation.

The mouse hepatocellular carcinoma cell line Hca-F is highly aggressive, with a metastasis rate over 80%. Hca-P, on the other hand, has a lymphatic metastasis rate of less than 30%. Both cell lines are derived from 615-mice ascites-type hepatocellular carcinoma cells. Hca-F and Hca-P cells metastasize only to lymph nodes, and not extrahepatic organs. However, the relationship between Axl glycosylation and lymphatic metastasis of mouse hepatocellular carcinoma cells remains unclear.

Our aim was to investigate whether Axl glycosylation regulates lymphatic metastasis. We demonstrated a possible correlation, based upon regulation of Axl glycosylation in mouse hepatocellular carcinoma cells.

MATERIALS AND METHODS

Cell culture and animals

Mouse hepatocellular carcinoma cell lines Hca-F and Hca-P, grown and stored in our institution (Department of Pathology, Dalian Medical University) were cultured in 90% Roswell Park Memorial Institute (RPMI)-1640 (Gibco) and supplemented with antibiotics (1 × penicillin/streptomycin 100 U/mL, Gibco) and 10% fetal bovine serum (FBS) (Gibco). Cells were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2. 615-mice (8 wk old males) were obtained from the Experimental Animal Center of Dalian Medical University.

Whole protein extract

10^7 cells were centrifuged at room temperature at 1000 × *g* for 10 min. Cells were rinsed twice with phosphate buffered saline (PBS) at 1000 × *g* for 5 min, and lysed with a protease inhibitor cocktail (whole protein extraction kit KGP2100, KeyGEN). Cells were suspended on a swing bed at 4 ℃ for 15 min, and centrifuged at 4 ℃ at 14 000 × *g* for 15 min. Protein concentration of the whole cells was measured with a bicinchoninic acid protein assay kit (KGPBCA, KeyGEN).

Western blotting analysis

Western blotting analysis was performed to evaluate Axl (with or without tunicamycin or PNGase F treatment) protein levels. Extracted proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Pall Corporation). After blocking for 2 h with 5% skimmed milk in PBS containing 0.1% Tween 20 (PBST), membranes were incubated with rabbit anti-mouse Axl polyclonal antibody (Santa Cruz Biotech Inc., 1/200 diluted) overnight in 5% powdered skim milk buffer, washed thrice with PBS with 0.1% Tween 20, and then incubated with secondary antibody anti-rabbit-HRP (Santa Cruz Biotech Inc., 1/3000 diluted). Glyceraldehyde-3-phosphate dehydrogenase antibody (Santa Cruz Biotech Inc., 1/200 diluted) was used as controls. All blot analysis was performed with a ECL Western blotting kit (Amersham Biosciences, United Kingdom).

Tunicamycin treatment

To inhibit N-linked glycosylation of newly synthesized proteins, Hca-F cells were washed once with PBS and cultivated for 12 h in fresh culture media (90% RPMI 1640 supplemented with antibiotics) with or without tunicamycin (Sigma Aldrich, St. Louis, MO) in a dose-dependent manner (0 μg/mL, 5 μg/mL, 10 μg/mL, or 20 μg/mL). Cells were washed with PBS and subjected to Western blotting analysis, 3-(4,5)-dimethylthiazol(-zyl)-3,5-diphenyltetrazolium bromide (MTT), migration *in*

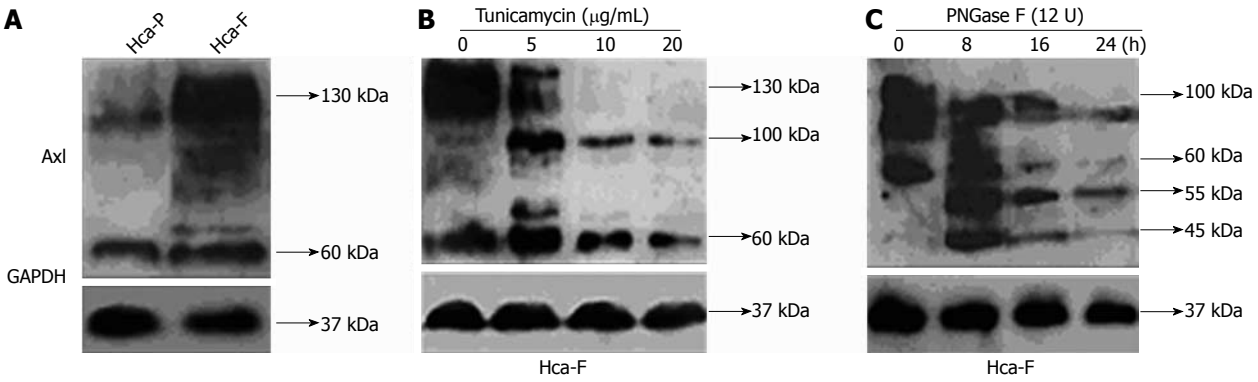

Figure 1 Expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell lines. A: Axl glycoprotein levels by Western blotting analysis in Hca-P and Hca-F cell lines. Relative signal intensities of Axl protein were compared with GAPDH by LabWorks (TM ver4.6, UVP; BioImaging Systems), ($^{a}P < 0.05$ *vs* untreated Hca-F cells); B: Hca-F cells were treated with 0 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL tunicamycin for 12 h. Total protein extracts were loaded for each sample; C: Hca-F cell protein was deglycosylated with 12 units of PNGase F in lysis buffer. Probes were incubated at 37 ℃ in a time-dependent manner (0 h, 8 h, 16 h, 24 h). Protein was separated on a gel for Western blotting Analysis. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane, and were detected by rabbit anti-mouse Axl polyclonal antibody. GAPDH blotting was used as the control. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

vitro, and tumor metastasis *in vivo* assays.

PNGase F treatment

To remove N-glycans, protein fractions (100 μg) from Hca-F cells were deglycosylated with 25 units of PNGase F (Elizabethkingia meningoseptica; Sigma Aldrich, St. Louis, MO) in lysis buffer. Probes were incubated for 8 h, 16 h and 24 h at 37 ℃. The reaction was terminated with Laemmlie's sample buffer and proteins were separated on a gel as described earlier.

For deglycosylation of membrane proteins, intact Hca-F cells were incubated with 25 units of PNGase F for 24 h, washed, and treated as described for the MTT, migration *in vitro*, and tumor metastasis *in vivo* assays.

3-(4,5)-dimethylthiazol(-zyl)-3,5-diphenyltetrazolium bromide assay

10^6 cells in 200 μL RPMI 1640 were seeded in duplicate into 96-well culture plates, and 100 μL MTT (5 mg/mL, Sigma) was added at 24 h, 48 h, 72 h, 96 h, and 120 h, respectively. After 4 h incubation at 37 ℃ in 5% CO_2, 100 μL/well DMSO (final concentration 25%, Gibco) was pipetted to solubilize the formazan product for 30 min at room temperature. Absorbency (490 angstroms) was measured using a microplate reader (Bio-Rad).

In vitro extracellular matrix invasion assays

Cell invasion *in vitro* was demonstrated using 24-well transwell units (Corning, NY, United States) with a 8 μm pore size polycarbonate filter coated with ECMatrix gel (Chemicon) to form a continuous thin layer[23]. Cells (3×10^5) were harvested in serum-free medium containing 0.1% BSA and added to the upper chamber. The lower chamber contained 500 μL RPMI 1640. Cells were incubated for 24 h at 37 ℃, 5% CO_2 incubator. At the end of incubation, cells on the upper surface of the filter were completely removed with a cotton swab. The filters were fixed in methanol and stained with Wright-Giemsa. Cells invading the matrigel that reached the lower surface of the filter were counted with light microscopy at a magnification of 400 ×. Samples were acquired in triplicate and data expressed as the average cell number in 5 fields.

In vivo tumor metastasis assay

Forty eight 615-mice were provided with sterilized food and water and equally divided into eight groups. 10^7 Hca-F cells (with or without tunicamycin or PNGase F treatment) were subcutaneously inoculated into the footpads. After 3 wk, mice were sacrificed and their axillary lymph nodes were isolated, weighed, and photographed.

Statistical analysis

Each assay was performed at least three times. Data were presented as the mean ± SD. Statistical differences between test groups was assessed by one-way analysis of variance and Scheffe's test for post hoc analysis. A *P*-value of less than 0.05 was considered statistically significant. SPSS version 13.0 software was used for statistical analysis.

RESULTS

Expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell lines

Axl glycoprotein relative expression was determined by Western blotting analysis using whole-cell extracts (Figure 1A). Axl expression varied among cell lines, with higher and lower levels in Hca-F and Hca-P cells, respectively (Figure 1A, $P < 0.05$).

Tunicamycin, an inhibitor of endogenous N-linked glycosylation of newly synthesized proteins, was used to inhibit Axl glycosylation of Hca-F cells. Treatment in a dose dependent manner (0 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL) for 12 h showed N-linked glycosylation to be highly sensitive to tunicamycin inhibition (Figure 1B).

Axl appears as broad bands, with molecular weights ranging from 60 kDa to 140 kDa. With tunicamycin treatment, 130 kDa Axl band density decreased, 60 kDa

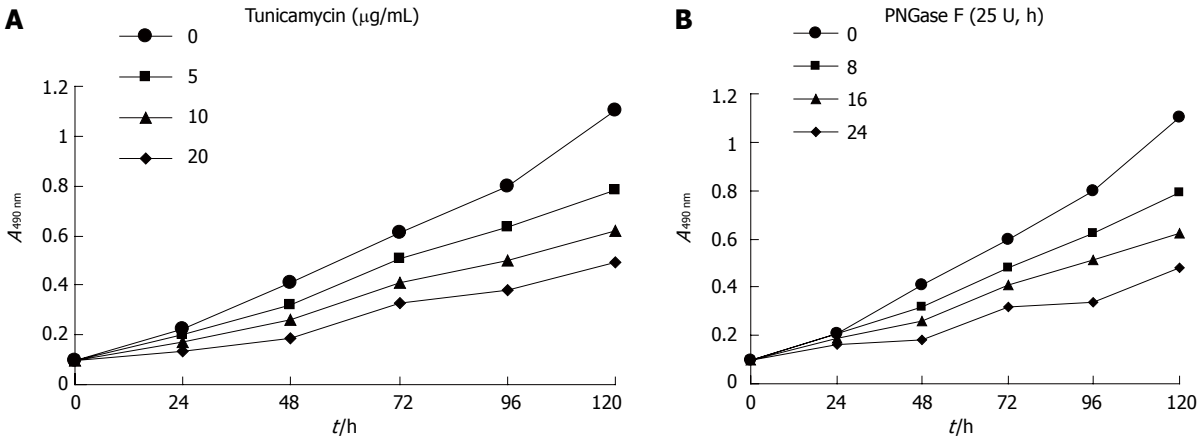

Figure 2 Axl deglycosylation effects on cell proliferation *in vitro*. Hca-F cells were exposed to tunicamycin or PNGase F and harvested at 24 h, 48 h, 72 h, 96 h, and 120 h. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Decreased proliferative ability was detected in cells treated with tunicamycin (A) or PNGase F (B), compared with untreated Hca-F cells. Data was obtained in triplicate.

band density increased, and a 100 kDa band appeared. As the dose of tunicamycin increased, the 130 kDa Axl band completely disappeared.

Whole protein aliquots extracted from Hca-F cells were exposed to exogenous PNGase F for deglycosylation (Figure 1C). 55 kDa and 45 kDa Axl bands appeared with PNGase F treatment. However, 60-140 kDa Axl band density significantly decreased. These results suggest that the N-glycosylation process for Hca-F cells responded to tunicamycin and PNGase F treatment.

Axl deglycosylation reduces cell proliferation in vitro

Hca-F cells treated with tunicamycin or PNGase F were measured for proliferative activity. Proliferation was apparent in untreated Hca-F cells compared with treated Hca-F cells (Figure 2A, B). Thus, Axl deglycosylation inhibited Hca-F cell proliferation *in vitro*.

Axl deglycosylation alters invasiveness of Hca-F cells in vitro

To examine whether Axl deglycosylation affects invasiveness of Hca-F cells, we performed *in vitro* ECMatrix gel analysis. We found that untreated and tunicamycin (Figure 3A) or PNGase F (Figure 3B) treated Hca-F cells or PNGase F had differing abilities to pass through an ECMatrix coated filter; therefore, the numbers of invading cells were unequal. Treated cells with tunicamycin passed through the ECMatrix gel in significantly smaller numbers than untreated cells (5 μg/mL: 68 ± 8 *vs* 80 ± 9, $P = 0.0222$; 10 μg/mL: 50 ± 6 *vs* 80 ± 9, $P = 0.0003$; 20 μg/mL: 41 ± 4 *vs* 80 ± 9, $P = 0.0001$). Similar results were shown with PNGase F treated Hca-F cells at 8 h, 16 h, and 24 h compared with untreated cells (66 ± 7 *vs* 82 ± 8, $P = 0.0098$; 49 ± 4 *vs* 82 ± 8, $P = 0.0001$; 34 ± 3 *vs* 82 ± 8, $P = 0.0001$). These results indicated that Axl deglycosylation reduced the invasiveness of Hca-F cells *in vitro*.

Axl deglycosylation inhibits the metastatic ability of Hca-F cells to peripheral lymph nodes in vivo

To further evaluate whether Axl deglycosylation was essential for tumor lymphatic metastasis *in vivo*, we tested the effect of Axl deglycosylation on the metastatic ability of Hca-F cells in mice peripheral lymph nodes. Treated and untreated Hca-F cells were injected in the footpads of 615-mice. After 3 wk' inoculation, a significant reduction in positive lymph nodes in the deglycosylation groups was observed, compared with untreated controls (Figure 4). Average lymph node weights of the untreated Hca-F group compared with dose-adjusted tunicamycin treated Hca-F groups (5 μg/mL: 0.84 ± 0.21 g *vs* 0.72 ± 0.19 g, $P = 0.3237$; 10 μg/mL: 0.84 ± 0.21 g *vs* 0.54 ± 0.11 g, $P = 0.0113$; 20 μg/mL: 0.84 ± 0.21 g *vs* 0.42 ± 0.06 g, $P = 0.0008$) (Figure 4A left). The average lymph node volumes of these groups were 815 ± 61 mm^3 *vs* 680 ± 59 mm^3, $P = 0.0613$; 815 ± 61 mm^3 *vs* 580 ± 29 mm^3, $P = 0.0001$; 815 ± 61 mm^3 *vs* 395 ± 12 mm^3, $P = 0.0001$ (Figure 4A right).

The average lymph node weights in the untreated Hca-F compared with PNGase F treated groups were: 8 h: 0.79 ± 0.15 g *vs* 0.63 ± 0.13 g, $P = 0.0766$; 16 h: 0.79 ± 0.15 g *vs* 0.49 ± 0.10 g, $P = 0.0022$; 24 h: 0.79 ± 0.15 g *vs* 0.39 ± 0.05 g, $P = 0.0001$ (Figure 4B left). The average lymph node volumes of these groups were: 670 ± 56 mm^3 *vs* 581 ± 48 mm^3, $P = 0.0532$; 670 ± 56 mm^3 *vs* 412 ± 22 mm^3, $P = 0.0001$; 670 ± 56 mm^3 *vs* 323 ± 11 mm^3, $P = 0.0001$ (Figure 4B right). These results demonstrate Axl deglycosylation may reduce Hca-F cells to peripheral lymph nodes *in vivo*.

DISCUSSION

Axl has garnered attention because of its high expression in many tumor cells, and its key role in neoplastic invasion and metastasis. In this study, we demonstrated Axl protein expression varied based on antineoplastic treatment of mouse hepatocellular carcinoma cell lines Hca-F and Hca-P. We found Axl protein expression to be higher in Hca-F cells, which have high lymphatic metastasis potential compared with Hca-P cells, which have low lymphatic metastasis potential. This confirms previ-

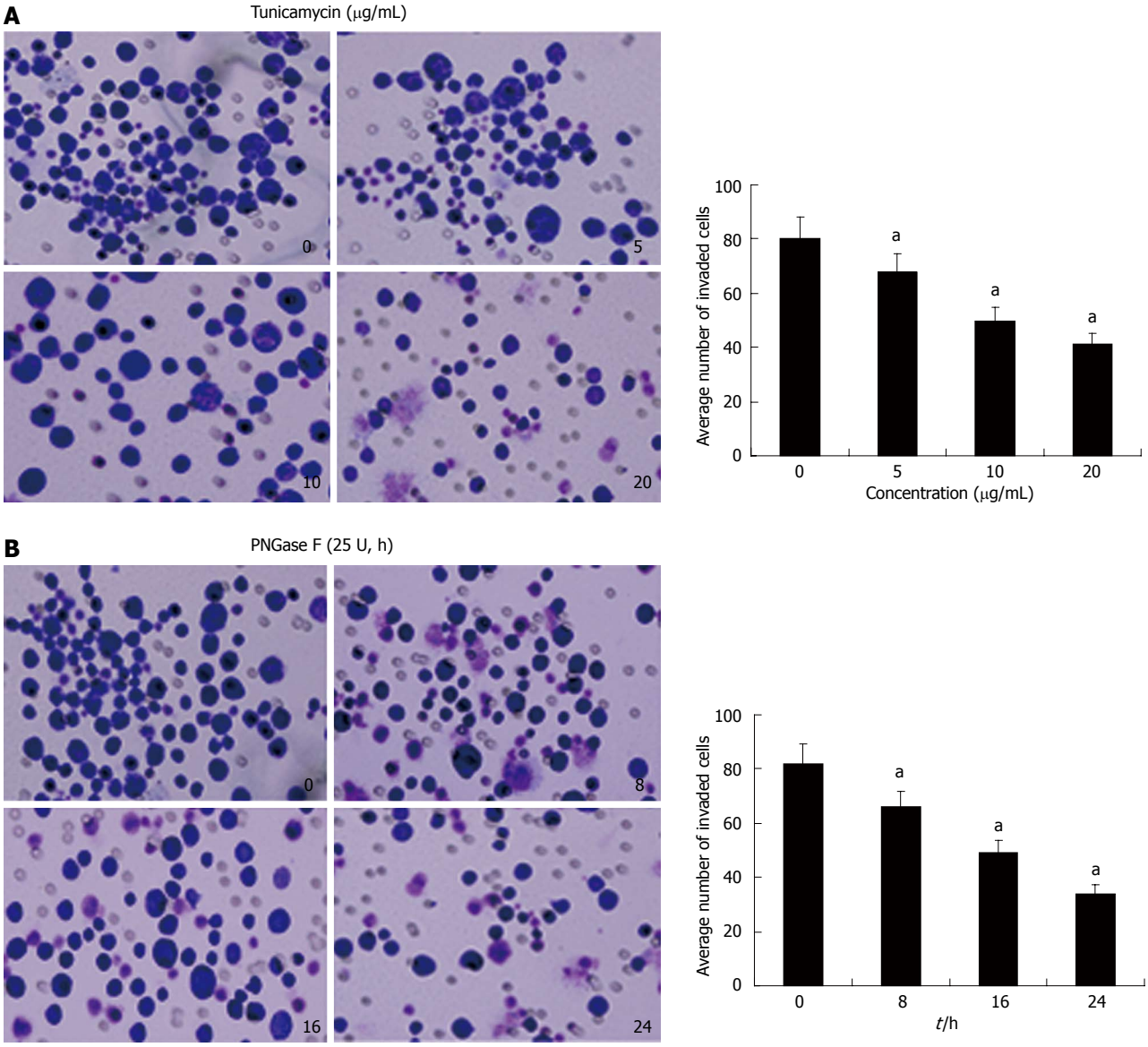

Figure 3 Axl deglycosylation alters the invasive ability of Hca-F cells *in vitro*. *In vitro* ECMatrix gel analysis. Wright-Giemsa staining results of the lower surface filter showed that the cells passed through the filter and attached to the lower side (400 ×). The average number of cells invading the filter was counted. Cells treated with tunicamycin (A) or PNGase F (B) were significantly less invasive ($^aP < 0.05$ *vs* untreated Hca-F cells) than untreated Hca-F cells. Data was obtained in triplicate.

ously reported findings of Axl overexpression in highly invasive lung adenocarcinoma cell lines, compared with their less invasive counterparts[11]. This suggests that high Axl expression may be associated with tumor lymphatic metastasis, and that Axl may be associated with tumor metastatic potential.

In our study, we achieved Hca-F deglycosylation with two methods. First, we inhibited N-glycan biosynthesis with tunicamycin; secondly, we extracted protein in the presence of PNGase F enzyme, which digests N-glycans. Both treatments resulted in significant effects on cell surface N-glycans by Western blotting assays.

Among post-translational modification reactions involving proteins, glycosylation is the most common; nearly 50% of all proteins are glycosylated[24]. Alterations of glycan structures are frequently observed in various cancer cells[25]; and this appears to be one association in cancer invasion and metastasis. We found Axl deglycosylation to be a possible factor in tumor progression, including cell proliferation, invasion, and lymphatic metastasis. In this study, we detected a significant inhibition of proliferation and invasion in Axl deglycosylated Hca-F cells *in vitro*, by both MTT and extracellular matrix assays. These results confirmed prior reports that cell proliferation requires growth factors signalling through cell surface glycoprotein receptors, which may be inactive when underglycosylated[26]. Although our findings support the role of Axl deglycosylation in reducing cell proliferation and invasion *in vitro*, its mechanism had not been elucidated. Further experiments showed that Axl deglycosylation led to a significant reduction in metastatic lymph node burden *in vivo*. These results were consistent previous reports of changes in N-linked oligosaccharide branching associated with malignancy and metastasis[27].

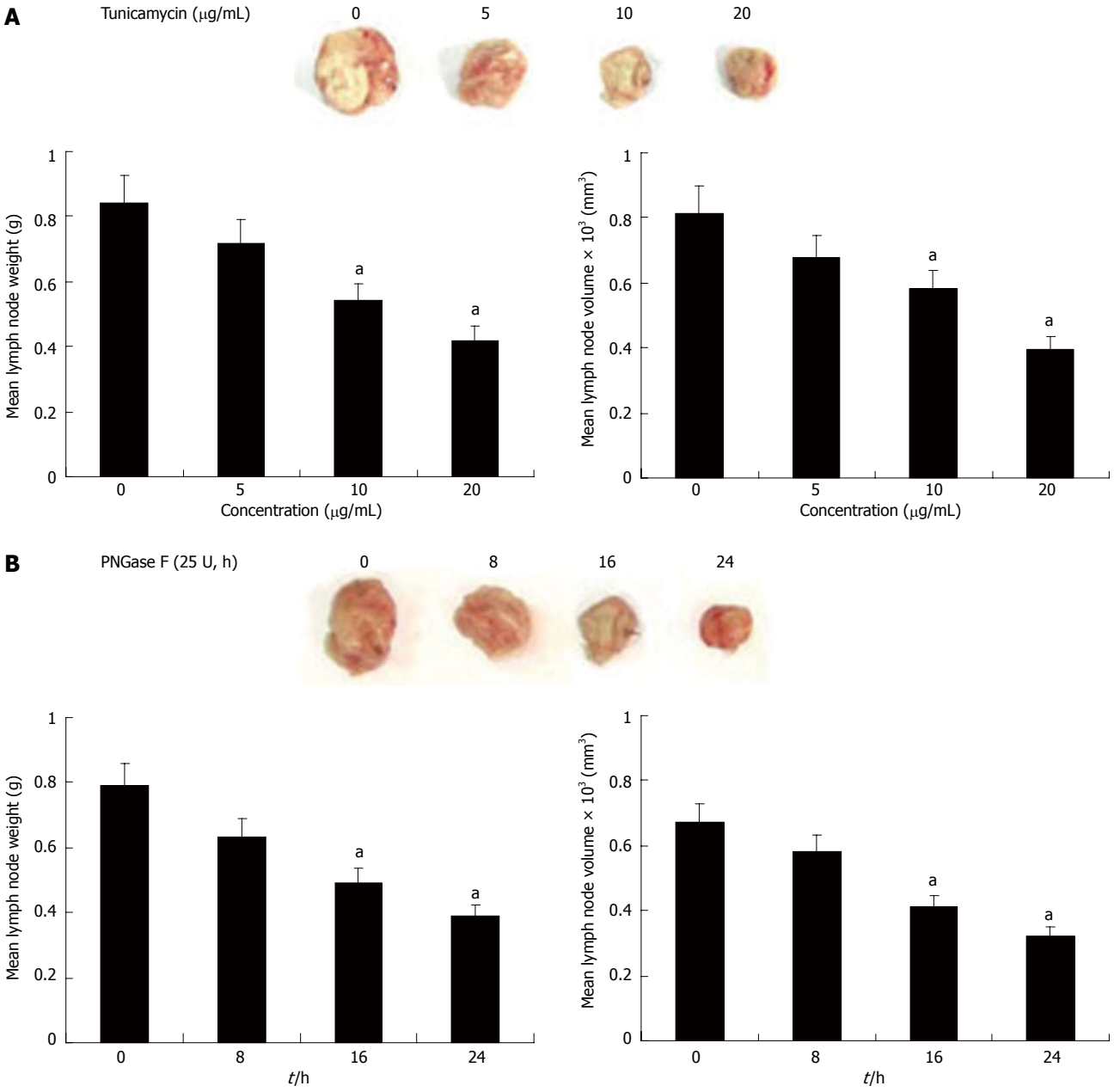

Figure 4 Axl deglycosylation inhibits the ability of Hca-F cells to metastasize to peripheral lymph nodes *in vivo*. Untreated and treated Hca-F cells were injected into the footpads of 615-mice. After 3 wk' inoculation, the mice were sacrificed and axillary lymph nodes isolated, weighed, measured, and photographed. A significant reduction in mean tumor weight ($n = 6$) of Axl tumor deglycosylation was observed, as compared with untreated Hca-F cells ($^aP < 0.05$ *vs* untreated Hca-F cells).

Some authors reported that the addition of exogenous Gas6 mediated the migration and invasion of Hca-F cells both *in vitro* and *in vivo* through the Axl pathway[28]. RNAi-mediated knockdown of Axl expression decreased the ability of YAP-expressing MIHA cells and of the primary HCC cell line to proliferate and invade[29]. In our study, we were unable to elucidate the mechanism by which Axl deglycosylation inhibits lymphatic metastasis in murine Hca-F cells. However, in many glycoproteins, N-linked oligosaccharides contribute to the folding, stability, and biological function of adhesion molecules and growth factor receptors on cell surfaces[30-32]. An increasing body of evidence indicates that glycoprotein glycans are involved in the regulation of cellular functions, including cell-cell communication and signal transduction[33,34]. The products of N-acetylglucosaminyltransferase (GnT)-IV, GnT-V and 1,6-fucosyltransferase (1,6-FucT) are all increased in hepatocellular carcinoma[35]. The presence of 1,6-GlcNAc structures in N-glycans and the expression of GnT-V, which catalyzes the addition of the 1,6-branching, were shown to promote metastasis[36-39]. At the very least, these reports demonstrate the relationship between metastasis and N-glycans to be extremely complicated. This area requires additional research.

In conclusion, we have found a role of Axl glycosylation in mediating tumor cell proliferation and invasion, and have provided the first evidence that Axl deglycosylation is required for lymphatic metastasis in murine

hepatocellular carcinoma cell lines. These results may at least partially explain the role of Axl glycosylation in the promotion of lymphatic metastasis. This study may provide new insights into regulatory mechanisms of mouse hepatocellular carcinoma with lymphatic metastasis.

COMMENTS

Background

Axl has been shown to have transforming capability when overexpressed. Prior studies have revealed Axl overexpression to be clearly associated with cancer invasiveness and metastasis. Axl also has multiple effects in angiogenesis. While Axl may play an important role in tumor progression, its mechanisms of action have not been understood.

Research frontiers

The authors investigated the potential effect of Axl deglycosylation the regulation of tumor lymphatic metastasis in mouse hepatocellular carcinoma cell lines. The authors evaluated the expression profile of Axl glycoprotein in the mouse hepatocellular carcinoma cell line Hca-F, which was treated with tunicamycin and PNGase F. Furthermore, the authors analyzed the effect of Axl glycosylation by tunicamycin and PNGase F treatment in Hca-F cells with regards to proliferation, invasion, and lymphatic metastasis both *in vitro* and *in vivo*.

Innovations and breakthroughs

Protein N-glycosylation is increasingly being recognized as one of the most prominent biochemical alterations in tumorigenesis and metastatic spread. However, as a cell surface transmembrane glycoprotein, little is known about Axl deglycosylation and its mechanism of action. Axl glycosylation was attenuated by tunicamycin and PNGase F to determine the effect on Hca-F cell proliferation, invasion, and lymphatic metastasis.

Applications

The authors have found the role of Axl glycosylation in mediating tumor cells proliferation, invasion and provided the first evidence that deglycosylation of Axl is required for metastasis of hepatocellular carcinoma cells to lymph nodes. This study may provide new insights into regulatory mechanisms of mouse hepatocellular carcinoma with lymphatic metastasis.

Terminology

The Axl receptor (also named UFO, Tyro7, and Ark) is a receptor tyrosine kinase (RTK) originally identified as a transforming gene in chronic myeloid leukemia. The RTKs constitute a large family of transmembrane proteins that relay signals from extracellular growth factors into the cell.

Peer review

In this study, the effect of Axl deglycosylation on lymphatic metastasis was investigated in mouse hepatocellular carcinoma cell lines. Differing Axl expression levels were found in mouse Hca-F and Hca-P cell lines, which are characterized by high and low metastatic potential, respectively. A decrease in Axl glycosylation by tunicamycin or PNGase F treatment resulted in a reduced proliferation, invasion, and lymphatic metastasis, both *in vitro* and *in vivo*. This work is potentially relevant in understanding hepatocellular carcinoma.

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ORIGINAL ARTICLE

Protection of ghrelin postconditioning on hypoxia/reoxygenation in gastric epithelial cells

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Abstract

AIM: To investigate the protective effect and mechanisms of ghrelin postconditioning against hypoxia/reoxygenation (H/R)-induced injury in human gastric epithelial cells.

METHODS: The model of H/R injury was established in gastric epithelial cell line (GES-1) human gastric epithelial cells. Cells were divided into seven groups: normal control group (N); H/R postconditioning group; DMSO postconditioning group (DM); ghrelin postconditioning group (GH); D-Lys3-GHRP-6 + ghrelin postconditioning group (D + GH); capsazepine + ghrelin postconditioning group (C + GH); and LY294002 + ghrelin postconditioning group (L + GH). 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect GES-1 cell viability. Hoechst 33258 fluorochrome staining and flow cytometry were conducted to determine apoptosis of GES-1 cells. Spectrophotometry was performed to determine release of lactate dehydrogenate (LDH). Protein expression of Bcl-2, Bax, Akt, and glycogen synthase kinase (GSK)-3β was determined by western blotting. Expression of vanilloid receptor subtype 1 (VR1), Akt and GSK-3β was observed by immunocytochemistry.

RESULTS: Compared with the H/R group, cell viability of the GH group was significantly increased in a dose-dependent manner (55.9% ± 10.0% *vs* 69.6% ± 9.6%, 71.9% ± 17.4%, and 76.3% ± 13.3%). Compared with the H/R group, the percentage of apoptotic cells in the GH group significantly decreased (12.38% ± 1.51% *vs* 6.88% ± 0.87%). Compared with the GH group, the percentage of apoptotic cells in the D + GH group, C + GH group and L + GH groups significantly increased (11.70% ± 0.88%, 11.93% ± 0.96%, 10.20% ± 1.05% *vs* 6.88% ± 0.87%). There were no significant differences in the percentage of apoptotic cells between the H/R and DM groups (12.38% ± 1.51% *vs*13.00% ± 1.13%). There was a significant decrease in LDH release following ghrelin postconditioning compared with the H/R group (561.58 ± 64.01 U/L *vs* 1062.45 ± 105.29 U/L). There was a significant increase in LDH release in the D + GH, C + GH and L + GH groups compared with the GH group (816.89 ± 94.87 U/L, 870.95 ± 64.06 U/L, 838.62 ± 118.45 U/L *vs* 561.58 ± 64.01 U/L). There were no significant differences in LDH release between the H/R and DM groups (1062.45 ± 105.29 U/L *vs* 1017.65 ± 68.90 U/L). Compared with the H/R group, expression of Bcl-2 and Akt increased in the GH group, whereas expression of Bax and GSK-3β decreased. Compared with the GH group, expression of Bcl-2 decreased and Bax increased in the D + GH, C + GH and L + GH groups, and Akt decreased and GSK-3β increased in the L + GH group. The H/R group also upregulated expression of VR1 and GSK-3β and downregulated Akt. The number of VR1-positive and Akt-positive cells in the GH group significantly increased, whereas the number of GSK-3β-positive cells significantly decreased. These effects of ghrelin were

reversed by capsazepine and LY294002.

CONCLUSION: Ghrelin postconditioning protected against H/R-induced injury in human gastric epithelial cells, which indicated that this protection might be associated with GHS-R, VR1 and the PI3K/Akt signaling pathway.

Key words: Human gastric epithelial cells; Ghrelin; Pharmacological postconditioning; Hypoxia/reoxygenation; Apoptosis

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INTRODUCTION

Gastric ischemia-reperfusion (GI/R) injury is a common clinical pathophysiological process. In case of clinical multiple organ dysfunction syndrome, gastrointestinal mucosal ischemia due to the redistribution of blood is the earliest to occur, and is more obvious than other organ ischemia-reperfusion (I/R) injury. Thus, the stomach is considered the earliest organ to be involved[1,2]. In recent years, ischemic postconditioning (Ipost) has been discovered to be an important endogenous protective mechanism and hypoxia postconditioning (HPost) can be elicited after hypoxia and reoxygenation in cell culture[3-5]. It has been demonstrated that IPost can effectively reduce myocardial injury[6]. Pharmacological postconditioning is the extension of Ipost, in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the first few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury[7].

Ghrelin is a 28-amino-acid peptide, which was initially identified from rat stomach[8]. In humans, it acts as the endogenous ligand for the growth hormone secretagogue receptor (GHSR)[9]. GHSR, a seven transmembrane-domain G-protein coupled receptor was cloned and identified as the receptor for a family of synthetic ligands. GHSR has been observed in the human gastrointestinal tract[10]. Ghrelin is predominantly produced in the stomach by endocrine cells of the fundic mucosa, formerly known as X/A-like cells[9,11,12]. Several studies have suggested that the many physiological actions are associated with ghrelin, including neuroendocrine, cardiovascular and gastrointestinal functions[13,14]. Ghrelin plays an important role in mucosal defense, such as resistance against a variety of ulcerogenic stimuli, including ethanol, stress and I/R[15-17].

Vanilloid receptor subtype 1 (VR1) is a nonselective cation channel, primarily expressed in central and peripheral terminals of nonmyelinated primary afferent neurons[18]. VR1 may be activated by physical and chemical mediators that contain noxious thermal stimulation, noxious protons, and vanilloid compounds such as capsaicin[19,20]. Our study aimed to establish whether the effect of ghrelin was mediated by VR1, and whether ghrelin served as a new activator of VR1.

The phosphoinositide 3-kinase (PI3K/Akt) signaling pathway has been implicated in the control of major cellular responses including cell proliferation, survival, development, differentiation, cell cycle, and apoptosis[21,22]. The PI3K/Akt signaling pathway is a type of significant antiapoptotic factor that plays an important protective role by reducing apoptosis-associated protein kinase caspase family, Bcl-2 and other apoptotic factors[23].

The primary aim of this study was to investigate whether ghrelin postconditioning had a protective effect on hypoxia/reoxygenation (H/R) injury in human gastric epithelial cells, and whether GHSR, VR1 and the PI3K/Akt signaling pathway are involved in this protection.

MATERIALS AND METHODS

Materials

The materials and reagent were as follows: human gastric epithelial cell line (GES-1) from Beijing Cancer Hospital; ghrelin, D-Lys3-GHRP-6, capsazepine and LY294002 (Sigma-Aldrich, United States) were dissolved in 100% DMSO and stored at -20 ℃; Dulbecco's Modified Eagle's Medium) (DMEM; Gibco Service Co., United States); fetal bovine serum (FBS) from Tianjin Hao Yang Biological Manufacture Co. Ltd. (China); 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Amresco (United States); Hoechst 33258 from Beyotime Institute of Biotechnology (China); Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Assay kit from Beijing Biosea Biotechnology Co. Ltd. (China); lactate dehydrogenase (LDH) assay kit from Nanjing Jiancheng Bioengineering Institute (China).

Cell culture

Cells were cultured in phenol-red-free DMEM containing 10% FBS at a density of 10^5 cells/mL, and placed in a humidified incubator with 95% air and 5% CO_2 at 37 ℃ until cells reached 70%-80% confluence.

Experimental protocol

The complete medium was replaced with DMEM containing 1% FBS 1 d before the experiment for cell synchronization. The cells were randomized into five groups and treated as follows. Cells in the normal control group (N) were kept in normoxic culture for 6 h. In the H/R group, DMEM was replaced with Krebs-Ringer bicarbonate buffer prior to hypoxia induction, and the cells

were transferred into a hypoxic incubator in a humidified atmosphere equilibrated with 94% N_2 + 1% O_2 + 5% CO_2 for 2 h (hypoxia). Afterwards, the Krebs-Ringer bicarbonate buffer was replaced with DMEM for reperfusion simulation, followed by 4 h normoxic culture (for reoxygenation). In the DMSO vehicle postconditioning group (DM), the Krebs-Ringer bicarbonate buffer was replaced with DMEM containing 0.05% DMSO prior to reoxygenation, with no modification of other procedures in the H/R group. In the ghrelin postconditioning group (GH), the Krebs-Ringer bicarbonate buffer was replaced with DMEM containing 10^{-9}, 10^{-8} and 10^{-7} mol/L ghrelin before reoxygenation, with no modification of other procedures in the H/R group. In the D-Lys3-GHRP-6 + ghrelin postconditioning group (D + GH), capsazepine + ghrelin postconditioning group (C + GH) and LY294002 + ghrelin postconditioning group (L + GH), the DMEM was replaced with Krebs-Ringer bicarbonate buffer containing D-Lys3-GHRP-6, capsazepine and LY294002, respectively, followed by 2 h hypoxia. Thereafter, the buffer was replaced with DMEM containing 10^{-7} mol/L ghrelin before reoxygenation, with no modification of other procedures in the H/R group.

MTT assay

The cells in logarithmic growth phase were cultured in a 96-well plate at a density of 10^4 cells/well. Following the experiment in each group, 20 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. Then, 200 μL DMSO was added to each well and the plate was vortexed for 10 min at 37 ℃. A 96-well microplate reader (Thermo, United States) was used to determine $A_{490\ nm}$. Viability (%) was determined as (experimental group $A_{490\ nm}$/normal control group $A_{490\ nm}$) × 100%. Each assay was repeated at least three times.

Hoechst 33258 staining assay

Cells were cultured in six-well plates at a density of 104 cells/well and incubated for 48 h. Following each experiment, cells were fixed with paraformaldehyde for 20 min and washed with 0.01 mol/L phosphate buffered solution (PBS) twice for 3 min, followed by Hoechst 33258 (0.5 mL) staining at 37 ℃ in darkness for 15 min, and rewashed with 0.01 mol/L PBS for 2 min to remove excess background stain. Apoptotic cells were observed by fluorescence microscopy, with excitation wavelength at 350 nm and emission wavelength at 460 nm. The photographs were preserved.

Flow cytometric analysis

Cells were plated in 100 mL culture flasks and incubated for 48 h. Following each experiment, cells were collected, washed twice with ice-cold PBS, followed by cell density determination and dilution in 1 × Annexin-binding buffer to 10^6 cells/mL. Cells was suspended in 200 μL binding buffer and 5 μL Annexin V-FITC for 15 min in darkness. Finally, 300 μL binding buffer and 5 μL propidium iodide (PI) were added to each sample. Apoptosis percentage was analyzed by flow cytometry (Becton Dickinson, United States).

LDH assay

The cells were cultured in 96-well plates at a density of 10^4 cells/well. At the end of each experiment, 20 μL supernatant was drawn from each well and transferred into test tubes, followed by determination of LDH release using the LDH assay kit.

Western blotting

Cells were plated in 100 mL cell culture flasks and incubated for 48 h. At the end of each experiment, the cells were washed thrice in ice-cold PBS prior to lysis with lysis buffer containing 150 mL/L protease inhibitor, followed by cell collection and centrifugation at 4 ℃ for 15 min. The resulting protein-containing supernatant was collected for protein quantification or storage at -80 ℃. The protein contents were determined by bicinchoninic acid assay. Protein samples were adjusted to equal concentration and volume by lysis buffer and then mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The protein samples were heated at 100 ℃ for 5 min, and then 100 μg protein was loaded into each well for 12.5% SDS-PAGE, and the targeted protein was transferred onto a nitrocellulose membrane. Immunoblotting was performed with the following antibodies: mouse anti-Bcl-2 (Zhongshan Golden Bridge Biotechnology Co. Ltd., China), mouse anti-Bax (Zhongshan Golden Bridge Biotechnology), mouse anti-β-actin (Zhongshan Golden Bridge Biotechnology), rabbit anti-VR1 (Zhongshan Golden Bridge Biotechnology), rabbit anti-Akt (Zhongshan Golden Bridge Biotechnology), and rabbit anti-glycogen synthase kinase (GSK)-3β (Wuhan Boshide Biotechnology Co. Ltd., China). The secondary antibodies were alkaline phosphatase goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology) and alkaline phosphatase horse anti-mouse IgG (Zhongshan Golden Bridge Biotechnology). Protein expression was quantified by Image J software.

Immunocytochemistry assay

Exponentially growing cells were cultured in a 24-well plate. Immunohistochemistry detection reagent and DAB kit (Zhongshan Golden Bridge Biotechnology) were used to examine the expression of VR1, Akt and GSK-3β in human gastric epithelial cells. Following each experiment, cells were washed thrice in PBS and fixed with 4% paraformaldehyde at 4 ℃ for 30 min. Cells were incubated with 0.3% Triton X-100 for 20 min and 3% H_2O_2 for 10 min. Cells were blocked with 10% normal goat serum for 1 h at room temperature. Primary antibodies were added and incubated at 4 ℃ overnight. With cells washed thrice in cold PBS, the secondary antibody was added and incubated at room temperature for 3 h. Finally, cells were washed thrice in 0.01 mol/L PBS, followed by incubation with DAB complexes for 20 min. Cells were observed for

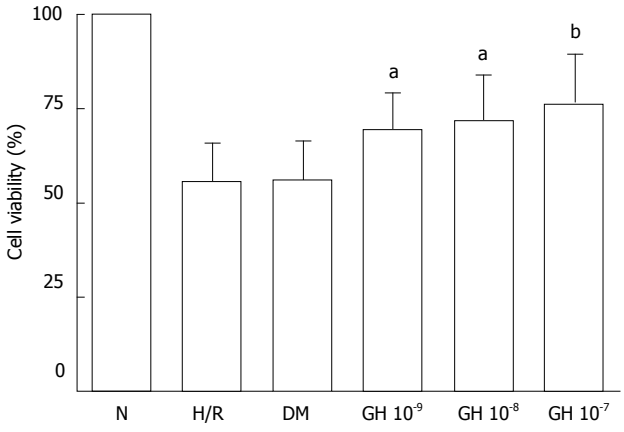

Figure 1 Effects of different doses of ghrelin on cell viability in human gastric epithelial cells induced by H/R. Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4 h reoxygenation (H/R), alcohol vehicle postconditioning (DM) and ghrelin postconditioning at three doses (10^{-9} mol/L, 10^{-8} mol/L and 10^{-7} mol/L). Cell viability was detected by 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. mean ± SD. $n = 6$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* H/R.

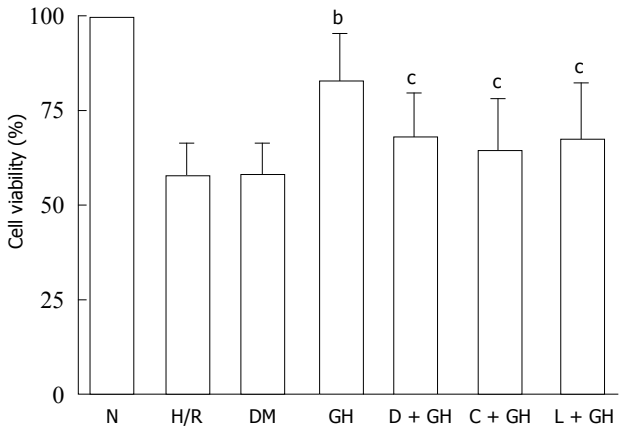

Figure 2 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on cell viability in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4h reoxygenation (H/R), DMSO vehicle postconditioning (DM), ghrelin postconditioning (10^{-7} mol/L) (GH), D-Lys3-GHRP-6 + ghrelin postconditioning (D + GH), capsazepine + ghrelin postconditioning (C + GH) and LY294002 + ghrelin postconditioning (L + GH). Cell viability was detected by 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. mean ± SD. $n = 6$. $^{b}P < 0.01$ *vs* H/R; $^{c}P < 0.05$ *vs* GH.

photography under a phase contrast microscope.

Statistical analysis

All data were presented as mean ± SD. One-way analysis of variance was performed to determine differences among groups and LSD test was performed to determine differences between groups. All statistical analyses were performed with the SPSS version 13.0 (SPSS, Chicago, IL, United States). The difference was considered statistically significant at $P < 0.05$.

RESULTS

Effects of different doses of ghrelin on cell viability in human gastric epithelial cells induced by H/R

The MTT assay indicated that the GES-1 cells were treated with ghrelin postconditioning at 10^{-9} mol/L, 10^{-8} mol/L and 10^{-7} mol/L. The viability of the GH group was 69.6% ± 9.6%, 71.9% ± 17.4%, and 76.3% ± 13.3%, respectively, in a dose-dependent manner. Compared with the H/R group (55.9% ± 10.0%), the viability significantly increased ($P < 0.05$), suggesting that 10^{-7} mol/L ghrelin was the optimal protective dose, which was used in the subsequent experiments. There were no significant differences between the H/R and DM groups (55.9% ± 10.0% *vs* 56.1% ± 10.5%, $P > 0.05$, Figure 1).

Effects of ghrelin postconditioning on viability of human gastric epithelial cells induced by H/R

To investigate whether GHS-R, VR1 and the PI3K/Akt signaling pathway were related to this effect, their inhibitors D-Lys3-GHRP-6, capsazepine and LY294002 were administered prior to ghrelin postconditioning. The GH group had significantly increased cell viability ($P < 0.01$ *vs* H/R group), whereas the D + GH, C + GH and L + GH groups had significantly decreased cell viability ($P < 0.05$ *vs* GH group, Figure 2), which indicated that D-Lys3-GHRP, capsazepine and LY294002 could reverse the protective effect of ghrelin postconditioning on GES-1 cell viability induced by H/R.

Effects of ghrelin postconditioning on apoptosis of human gastric epithelial cells induced by H/R

Hoechst 33258 staining showed that apoptotic cells were apparent in normal gastric epithelial cells. Compared with the H/R group, there were fewer apoptotic cells in the GH group, whereas many apoptotic cells were observed in the D + GH, C + GH and L + GH groups compared with the GH group (Figure 3).

Flow cytometric analysis showed that the percentage of apoptotic cells in the GH group significantly decreased (12.38% ± 1.51% *vs* 6.88% ± 0.87%, $P < 0.01$) compared with the H/R group. Compared with the GH group, the D + GH, C + GH and L + GH groups had a significantly increased percentage of apoptotic cells (11.70% ± 0.88%, 11.93% ± 0.96%, 10.20% ± 1.05% *vs* 6.88% ± 0.87%, $P < 0.05$). There were no significant differences in the percentage of apoptotic cells between the H/R and DM groups (12.38% ± 1.51% *vs* 13.0% ± 1.13%, $P > 0.05$, Figure 4).

Effects of ghrelin postconditioning on LDH release in human gastric epithelial cells induced by H/R

There was a significant decrease in LDH release in the GH group compared with the H/R group (561.58 ± 64.01 U/L *vs* 1062.45 ± 105.29 U/L, $P < 0.01$). There was a significant increase in LDH release in the D + GH, C + GH and L + GH groups compared with the GH group (816.89 ± 94.87 U/L, 870.95 ± 64.06 U/L, 838.62 ± 118.45 U/L *vs* 561.58 ± 64.01 U/L, $P < 0.01$). There were no significant differences in LDH release between the H/R and DM groups (1062.45 ± 105.29 U/L

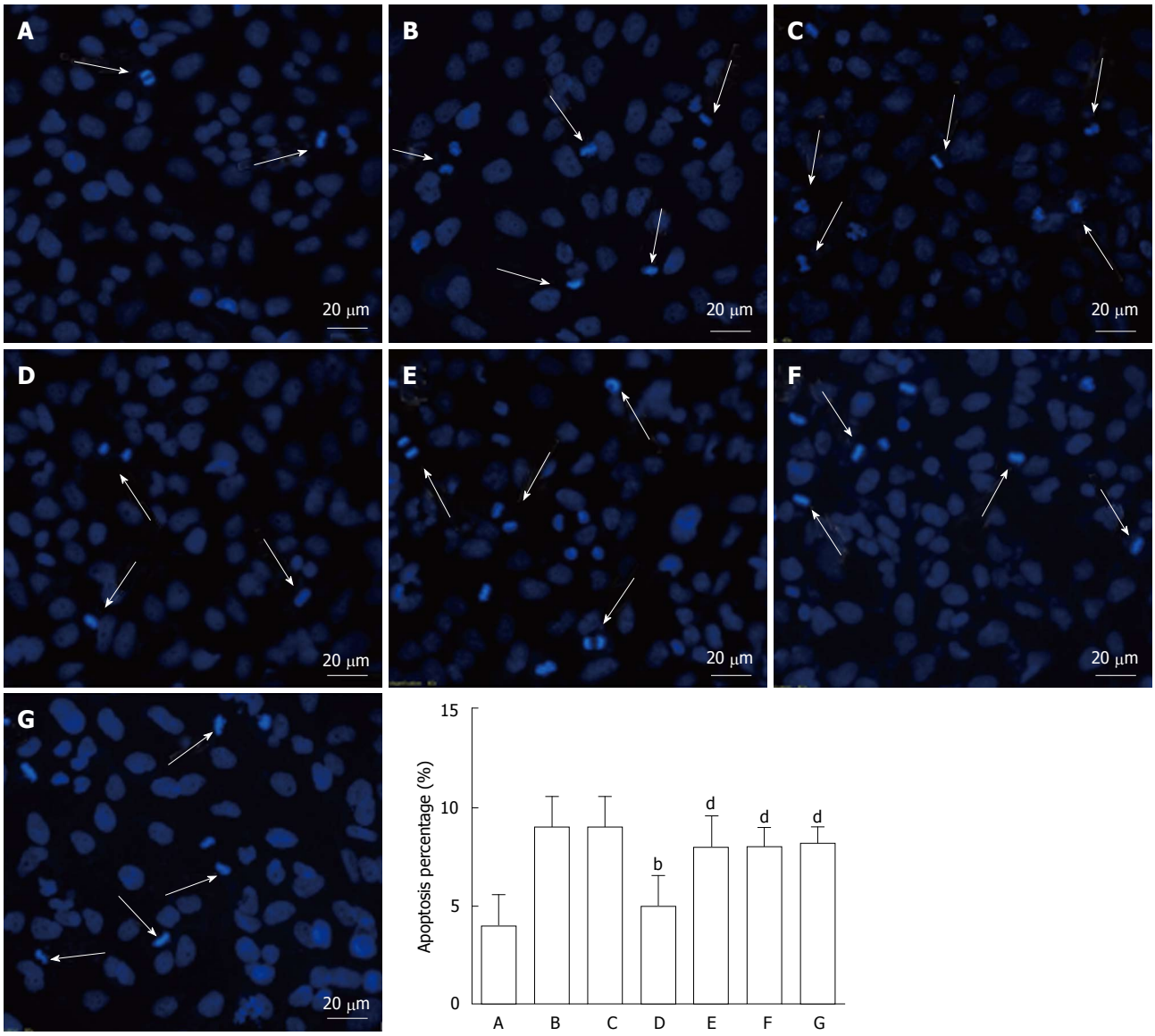

Figure 3 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on cell apoptosis in human gastric epithelial cells induced by H/R. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), D-Lys3-GHRP-6 + ghrelin postconditioning (E), capsazepine + ghrelin postconditioning (F), and LY294002 + ghrelin postconditioning (G). mean ± SD. n = 6. $^{b}P < 0.01$ *vs* B; $^{d}P < 0.01$ *vs* D. Cells were then stained by Hoechst33258. The arrows indicate apoptotic cells.

Table 1 Effects of ghrelin postconditioning on lactate dehydrogenate release

Groups	LDH release (U/L)
N group	672.73 ± 64.79
H/R group	1062.45 ± 105.29
DM group	1017.65 ± 68.90
GH group	561.58 ± 64.01[d]
D + GH group	816.89 ± 94.87[b]
C + GH group	870.95 ± 64.06[b]
L + GH group	838.62 ± 118.45[b]

$^{b}P < 0.01$ *vs* GH group; $^{d}P < 0.01$ *vs* H/R group. N: Normal control group; H/R: Hypoxia/reoxygenation; DM: DMSO postconditioning; GH: Ghrelin postconditioning (10^{-7} mol/L); D + GH: D-Lys3-GHRP-6 + ghrelin postconditioning; C + GH: Capsazepine + ghrelin postconditioning; L + GH: LY294002 + ghrelin postconditioning; LDH: Lactate dehydrogenate.

vs 1017.65 ± 68.90 U/L, $P > 0.05$, Table 1).

Effects of ghrelin postconditioning on expression of Bcl-2, Bax, VR1, Akt and GSK-3β in human gastric epithelial cells induced by H/R

Western blotting demonstrated that Bcl-2, Bax, Akt and GSK-3β were expressed in normal gastric epithelial cells. Compared with the H/R group, expression of Bcl-2 and Akt increased in the GH group, whereas there was a decrease in expression of Bax and GSK-3β ($P < 0.01$). Compared with the GH group, Bcl-2 expression decreased and Bax expression increased in the D + GH, C + GH and L + GH groups ($P < 0.01$). Expression of Akt decreased and expression of GSK-3β increased in the L + GH group ($P < 0.01$, Figure 5).

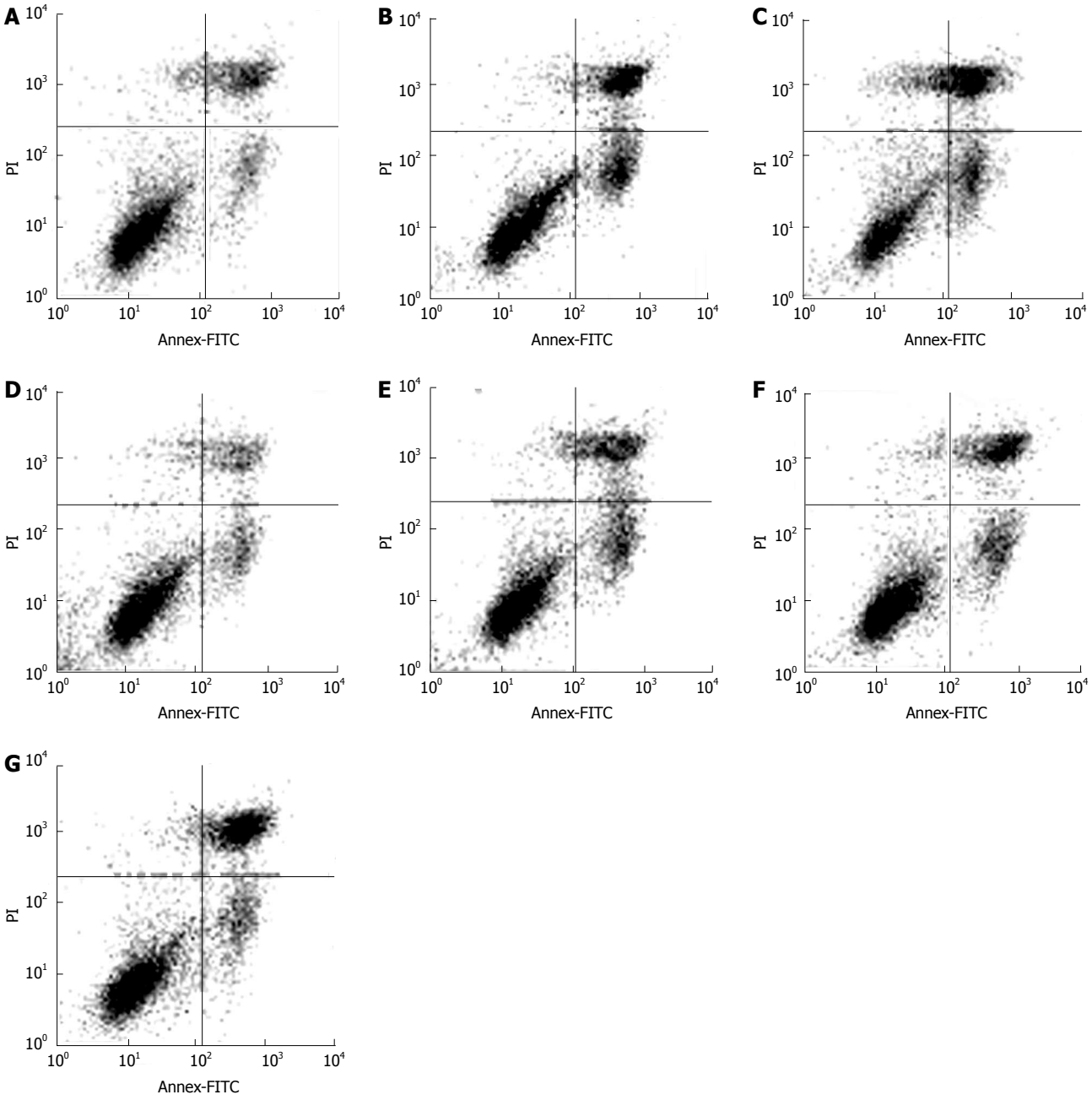

Figure 4 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on cell apoptosis in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), D-Lys3-GHRP-6 + ghrelin postconditioning (E), capsazepine + ghrelin postconditioning (F), and LY294002 + ghrelin postconditioning (G). Subsequently, the cells were stained by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Apoptosis was detected by flow cytometry.

Immunocytochemistry demonstrated expression of VR1 (Figure 6A), Akt (Figure 7A) and GSK-3β (Figure 8A) in normal human gastric epithelial cells. Two hours hypoxia followed by 4 h incubation under normoxic conditions also upregulated expression of VR1 (Figure 6B) and GSK-3β (Figure 8B) and downregulated Akt (Figure 7B). There was a significant increase in the number of VR1-positive (Figure 6D) as well as Akt-positive (Figure 7D) cells in the GH group, whereas the number of GSK-3β-positive cells significantly decreased (Figure 8D). However, these effects of ghrelin were reversed by capsazepine and LY294002 (Figures 6E, 7E and 8E).

DISCUSSION

Gastric mucosal lesions are frequently observed in clinical situations, such as stress-induced GI/R injury, a major cause of acute gastric mucosal lesions[2,24]. GI/R injury is a common clinical pathophysiological process, which is associated with such factors as excessive generation of oxygen free radicals (OFRs) in gastric mucosa, intracellular calcium overload, increased gastric acid secretion and gastric microcirculation disturbance[25]. Excessive generation of OFRs and intracellular calcium overload may lead to apoptosis[26]. In recent years, there

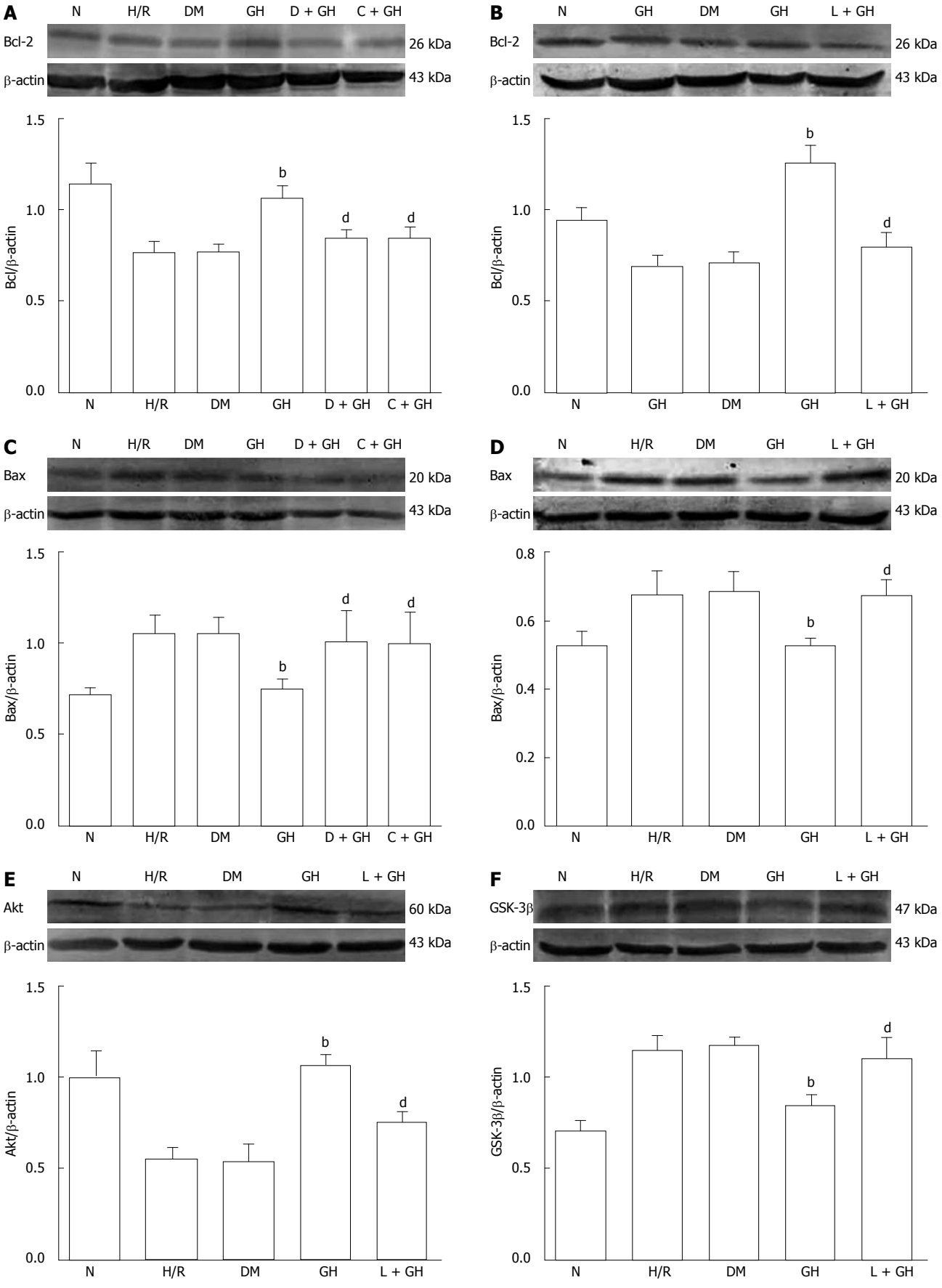

Figure 5 Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on the expression of Bcl-2, Bax, Akt and glycogen synthase kinase-3β in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4 h reoxygenation (H/R), DMSO vehicle postconditioning (DM), ghrelin postconditioning (10^{-7} mol/L) (GH) D-Lys3-GHRP-6 + ghrelin postconditioning (D + GH), capsazepine + ghrelin postconditioning (C + GH) and LY294002 + ghrelin postconditioning (L + GH). The expression of β-actin was detected as an internal standard. Densitometry results are expressed as ratio of test over normal group. A, B: Bcl-2 expression; C, D: Bax expression; E: Akt expression; F: Glycogen synthase kinase (GSK)-3β expression. mean ± SD. $n = 6$. $^{b}P < 0.01$ *vs* H/R, $^{d}P < 0.01$ *vs* GH.

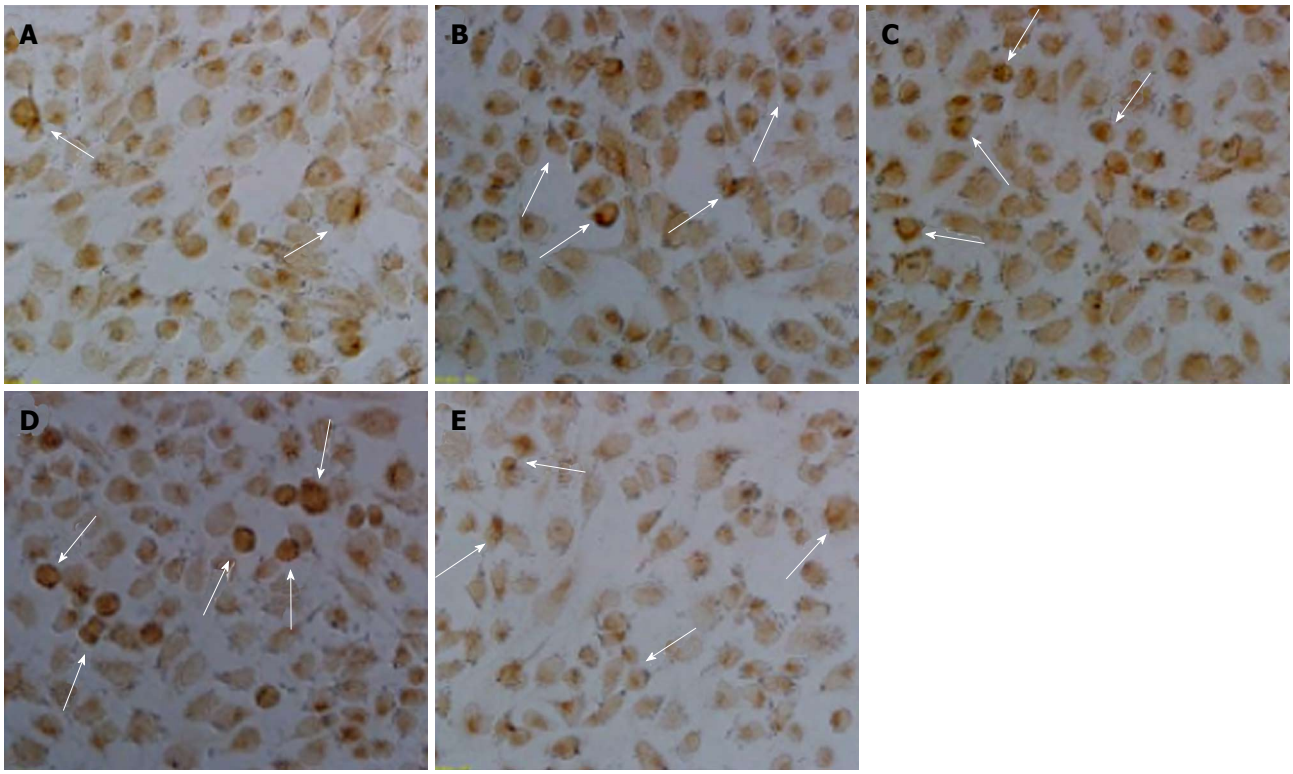

Figure 6 Effects of capsazepine in ghrelin postconditioning on the expression of vanilloid receptor subtype 1 in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and capsazepine + ghrelin postconditioning (E). The expression of vanilloid receptor subtype 1 (VR1) in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express VR1.

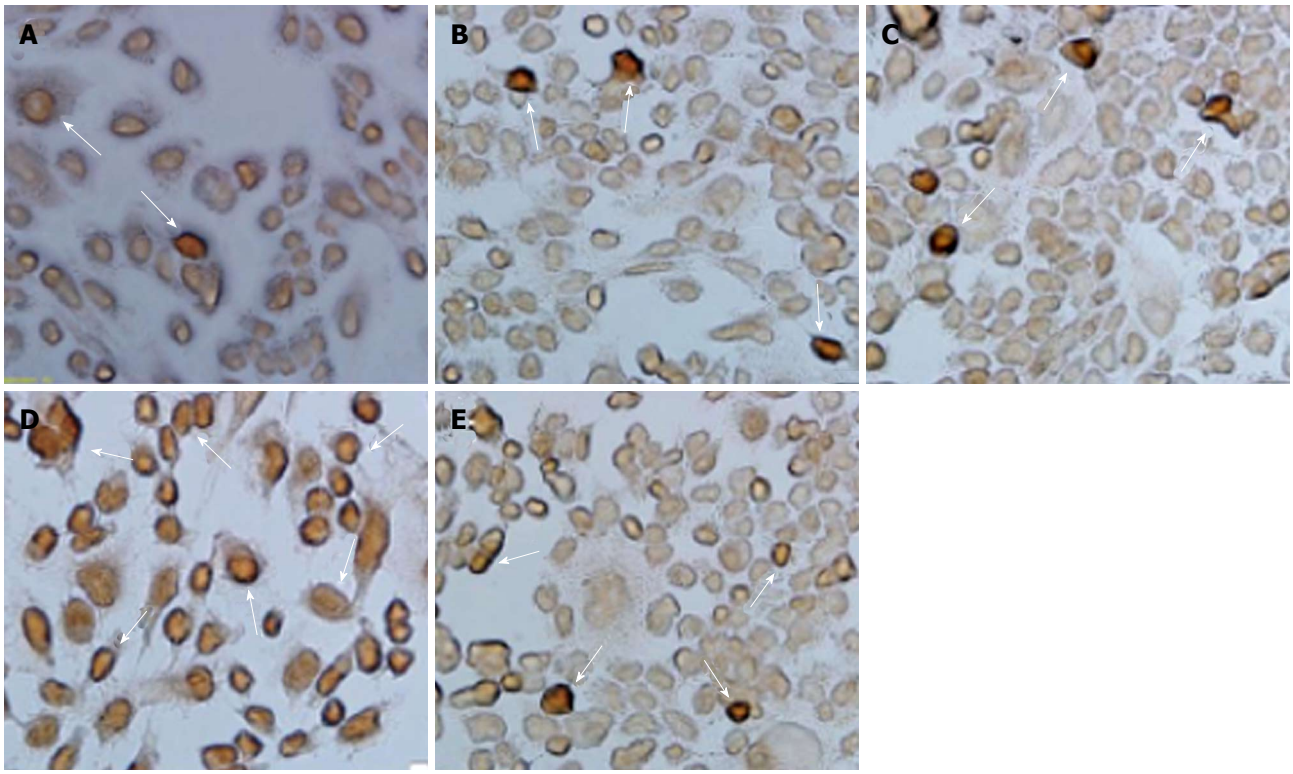

Figure 7 Effects of LY294002 in ghrelin postconditioning on the expression of Akt in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and LY294002 + ghrelin postconditioning (E). The expression of Akt in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express Akt.

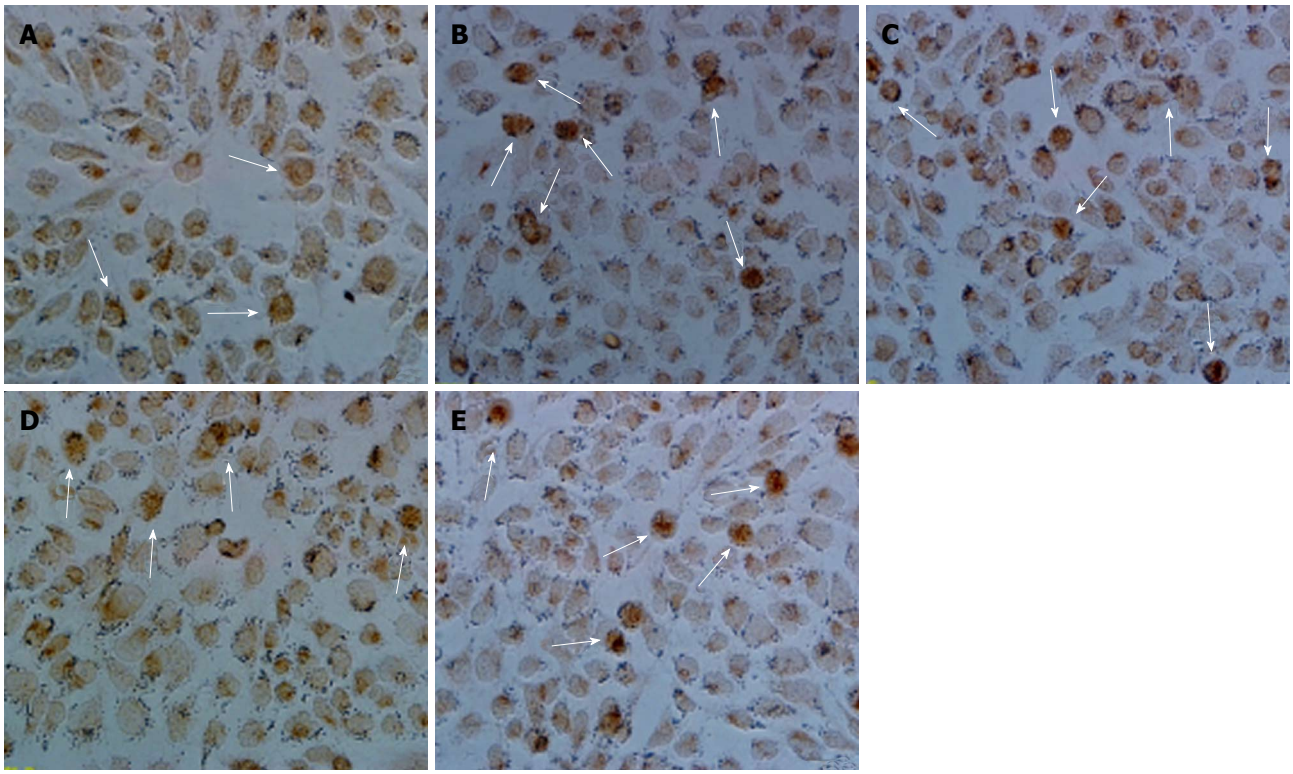

Figure 8 Effects of LY294002 in ghrelin postconditioning on the expression of glycogen synthase kinase-3β in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and LY294002 + ghrelin postconditioning (E). Expression of glycogen synthase kinase (GSK)-3β in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express GSK-3β.

has been a significant rise in the gastric mucosa cell apoptosis in gastric mucosa injury due to alcohol and nonsteroidal anti-inflammatory drugs, suggesting that there is a process of programmed cell death in acute gastric mucosal injury[27].

Apoptosis is the main mechanism of cell death, and is mediated by a cell-intrinsic suicide program, with the relative balance of pro- and antiapoptotic signaling pathways determining the fate of the cell. There are two main pathways in mammals, one of which is the mitochondrial signaling pathway, in which Bcl-2 plays an important role and Bcl-2/Bax is the key point of apoptosis[28]. In our study, the cell viability significantly decreased and apoptosis percentage increased in the model of 2 h hypoxia/4 h reoxygenation compared with normal control group. They also show that, in the same model, the expression of antiapoptotic protein (Bcl-2) decreased and the expression of proapoptotic protein (Bax) increased. These results suggest that we have successfully established a model of H/R-induced injury in a human GES-1. MTT assays, flow cytometric analysis, LDH assays and western blotting were used to test cell viability, apoptotic percentages, cell LDH release, and apoptosis-related protein expression.

Ghrelin is well known as a potent activator of growth hormone release[8,9]. Given the implication of growth hormone in the tissue regeneration and maintenance of integrity, ghrelin is supposed to contribute to the processes of healing and regeneration. A number of studies have confirmed the effect of ghrelin in gastroprotection. Brzozowski *et al*[17] have found that ghrelin inhibits stress-induced gastric injury[15]. Ghrelin can also inhibit I/R injury. Sibilia *et al*[16] have reported that ghrelin inhibits ethanol-induced gastric ulcers. Unfortunately, all of these were studies *in vivo*. In recent years, many studies have demonstrated that ghrelin confers protection in some cell types *in vitro*, but no study has clearly elucidated the effects of ghrelin in human gastric epithelial cells[29,30]. Therefore, in our study, we demonstrated that ghrelin post-conditioning has protective effects against H/R injury in human gastric epithelial cells, and confirmed that ghrelin could increase cell viability, and decrease apoptosis and LDH release. We also found that ghrelin post-conditioning increased VR1, Akt and Bcl-2 expression, and attenuated GSK-3β and Bax expression subsequent to H/R in gastric epithelial cells *in vitro*. The results showed that ghrelin seems to be involved in the regulation of gastroprotection, which also supports our hypothesis that ghrelin could effectively attenuate H/R-induced injury via the mitochondrial antiapoptotic pathway in human gastric epithelial cells.

In our study, we also demonstrated that the protective effects of ghrelin against H/R-induced injury might be mediated by GHS-R, VR1 and activation of the PI3K/Akt pathway, in that the protective effects of ghrelin were reversed by the GHS-R antagonist D-Lys3-GHRP-6, VR1 antagonist capsazepine and PI3K/Akt antagonist LY294002 during H/R. The result showed that ghrelin in combination with D-Lys3-GHRP-6, capsazepine and

LY294002 decreased cell viability, and increased the percentage of apoptotic cells and LDH release compared with ghrelin post-conditioning. The result also showed a decrease in Bcl-2 expression and increase in Bax expression. In the C + GH group, expression of VR1 was downregulated, and in the L + GH group, expression of Akt decreased, and GSK-3β expression increased. These data suggested that GHS-R, VR1 and the PI3K/Akt signaling pathway might be involved in ghrelin-induced antiapoptotic effects. However, the precise underlying mechanism of their activation by ghrelin remains to be determined.

Ishii *et al*[31] showed that treatment with GHS-R antagonist, D-Lys3-GHRP-6 could partially reverse diabetic hyperphagia. GHS-R mRNA is mainly expressed in the arcuate nucleus of the brain, ventral median nucleus and the hippocampus. It has been demonstrated that GHS-R mRNA is also expressed in peripheral organs[32]. The multifunction of ghrelin coincides with the distribution of GHS-R in various tissues[33]. GHS-R acts as the receptor for a family of synthetic ligands known as growth hormone secretagogues. Therefore, GHS-R is involved in the regulation of physiological actions when activated by its ligands, such as ghrelin. On the grounds of its distribution in the gastrointestinal tract, GHS-R plays an important role in the regulation of gastrointestinal functions[10]. Some studies have shown that GHS-R mediates ghrelin gastroprotection against I/R-induced injury, while this protective effect is inhibited by the GHS-R antagonist D-Lys3-GHRP-6[17].

Capsazepine is a specific and competitive antagonist of the VR. VR1 is a nervous-system-specific receptor. However, recent studies have indicated that VR1 is also distributed in some non-nervous tissues, such as the liver, gastric epithelial cells, bronchi, and bladder epithelium[34-37]. These findings suggest that VR1 might be related to the regulation of a variety of physiological functions of different tissues and organs. Many studies have demonstrated that activation of VR1 by its agonists exerts a gastroprotective effect[35,38-40].

PI3K antagonists, such as LY294002, at low concentrations are considered to be selective PI3K inhibitors and valuable tools for the study of cardioprotection[41]. Many studies have discovered that the activation of the PI3K/Akt signaling pathway is involved in the antiapoptotic effect of ghrelin in many cell types[42-44]. Some studies have shown that the PI3K/Akt pathway is a significant antiapoptotic factor that has an important protective role in reducing the apoptosis-associated protein kinase caspase family, Bcl-2 and other apoptotic factors[23]. The PI3K/Akt pathway has been implicated in the control of major cellular responses including cell proliferation, survival, development, differentiation, cell cycle, and apoptosis[21,22]. GSK-3β is a multifunctional Ser/Thr kinase that plays important roles in necrosis and apoptosis of cardiomyocytes. GSK-3β activity has been associated with many cell processes, including the regulation of multiple transcription factors, the Wnt pathway, nuclear factor κB, endoplasmic reticulum stress, embryogenesis, apoptosis and cell survival, cell cycle progression, and cell migration[45,46]. GSK-3β, serving as an Akt downstream effector, plays an important role during I/R-induced apoptosis of cells in the heart and brain[47,48].

In summary, our study demonstrated that ghrelin postconditioning had a protective effect against H/R-induced injury in human gastric epithelial cells *in vitro*, and the effect might be mediated by the receptors GHS-R and VR1 as well as activation of the PI3K/Akt signaling pathway, resulting in activation of the intracellular antiapoptotic signaling pathway, hence the inhibition of apoptosis.

COMMENTS

Background

Gastric ischemia-reperfusion injury is a common clinical pathophysiological process, and the mobilization of the endogenous protection is the most effective manner against ischemia-reperfusion (I/R) injury. In recent years, ischemic postconditioning has been regarded as important endogenous protection.

Research frontiers

Ghrelin is a 28-amino-acid peptide that was initially identified in rat stomach. Several studies have shown the effect of ghrelin in gastroprotection. In recent years, many studies have demonstrated that ghrelin confers protection in some cell types *in vitro*. Unfortunately, no study has clearly shown the effect of ghrelin in human gastric epithelial cells. In this study, the authors demonstrated that ghrelin post-conditioning has protective effect against hypoxia/reoxygenation (H/R)-induced injury in human gastric epithelial cells.

Innovations and breakthroughs

The injury of visceral I/R is a common pathological occurrence. Nowadays, ischemic preconditioning and ischemic postconditioning have been confirmed to be important approaches in endogenous protection. The notion of ischemic postconditioning was first proposed in 2003. Pharmacological postconditioning is the extension of ischemic postconditioning in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the initial few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury. This study suggests that the authors have successfully established a model of H/R-induced injury in the human gastric epithelial cell line gastric epithelial cell line (GES-1), and have demonstrated that ghrelin postconditioning has protection against H/R-induced injury in human gastric epithelial cells *in vitro*.

Applications

The results of the study suggest that the ghrelin postconditioning is a potential therapeutic approach that could be applied to the prevention of gastric mucosal lesions induced by ethanol, stress and I/R.

Terminology

Ghrelin, a 28-amino-acid peptide initially identified in rat stomach, is noted for its potent activation of growth hormone release. Pharmacological postconditioning is the extension of ischemic postconditioning in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the initial few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury.

Peer review

The present study was well-organized and well-investigated. The authors demonstrated the antiapoptotic effects of ghrelin against H/R-induced apoptosis of human gastric epithelial cells.

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BRIEF ARTICLE

Carbon dioxide accumulation during analgosedated colonoscopy: Comparison of propofol and midazolam

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Abstract

AIM: To characterize the profiles of alveolar hypoventilation during colonoscopies performed under sedoanalgesia with a combination of alfentanil and either midazolam or propofol.

METHODS: Consecutive patients undergoing routine colonoscopy were randomly assigned to sedation with either propofol or midazolam in an open-labeled design using a titration scheme. All patients received 4 μg/kg per body weight alfentanil for analgesia and 3 L of supplemental oxygen. Oxygen saturation (SpO_2) was measured by pulse oximetry (POX), and capnography ($PcCO_2$) was continuously measured using a combined dedicated sensor at the ear lobe. Instances of apnea resulting in measures such as stimulation of the patient, a chin lift, a mask maneuver, or withholding of sedation were recorded. $PcCO_2$ values (as a parameter of sedation-induced hypoventilation) were compared between groups at the following distinct time points: baseline, maximal rise, termination of the procedure and 5 min after termination of the procedure. The number of patients in both study groups who regained baseline $PcCO_2$ values (± 1.5 mmHg) five minutes after the procedure was determined.

RESULTS: A total of 97 patients entered this study. The data from 14 patients were subsequently excluded for clinical procedure-related reasons or for technical problems. Therefore, 83 patients (mean age 62 ± 13 years) were successfully randomized to receive propofol (n = 42) or midazolam (n = 41) for sedation. Most of the patients were classified as American Society of Anesthesiologists (ASA) Ⅱ [16 (38%) in the midazolam group and 15 (32%) in the propofol group] and ASA Ⅲ [14 (33%) and 13 (32%) in the midazolam and propofol groups, respectively]. A mean dose of 5 (4-7) mg of Ⅳ midazolam and 131 (70-260) mg of Ⅳ propofol was used during the procedure in the corresponding study arms. The mean SpO_2 at baseline (%) was 99 ± 1 for the midazolam group and 99 ± 1 for the propofol group. No cases of hypoxemia (SpO_2 < 85%) or apnea were recorded. However, an increase in $PcCO_2$ that indicated alveolar hypoventilation occurred in both groups after administration of the first drug and was not detected with pulse oximetry alone. The mean interval between the initiation of sedation and the time when the $PcCO_2$ value increased to more than 2 mmHg was 2.8 ± 1.3 min for midazolam and 2.8 ± 1.1 min for propofol. The mean maximal rise was similar for both drugs: 8.6 ± 3.7 mmHg for midazolam and 7.4 ± 3.2 mmHg for propofol. Five minutes after the end of the procedure, the mean difference from the baseline values was significantly lower for the propofol treatment compared with midazolam (0.9 ± 3.0 mmHg *vs* 4.3 ± 3.7 mmHg, P = 0.0000169), and significantly more patients in the propofol group had regained their baseline value ± 1.5 mmHg (32 of 41 *vs* 12 of 42, P = 0.0004).

CONCLUSION: A significantly higher number of patients sedated with propofol had normalized $PcCO_2$ values five minutes after sedation when compared with patients sedated with midazolam.

Key words: Colonoscopy; Deep sedation; Propofol; Hypoventilation; Blood gas monitoring; Transcutaneous

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INTRODUCTION

Colonoscopies are usually performed under sedation with an intravenous sedative that is often combined with an analgesic drug[1,2]. A combination of the benzodiazepine midazolam with an opioid is the most commonly used regimen in Western countries[3]. During the last few years, propofol (2,6-diisopropylphenol) sedation has gained increasing attention among endoscopists as an alternative sedative in GI endoscopy[4-6]. With a fast onset of action of 30-60 s, a distribution half-life of 2-4 min, and a rapid recovery time, propofol combines the major characteristics of an ideal sedative[7].

In recent years, several randomized trials have compared midazolam *vs* propofol with regard to patient safety and satisfaction[4,8-10]. In most studies, recovery time, measured as completely regained alertness after the endoscopic procedure, was used as the main endpoint. Although the advantages of propofol regarding these pharmacokinetic properties are unquestionable, there is still an ongoing debate on the potential respiratory impairment hazards of propofol when used by non-anesthesiologists. In contrast to benzodiazepines, a reversal agent for propofol does not exist; consequently, the use of propofol requires special attention. Furthermore, a deeper level of sedation may be reached with propofol that carries the risk of unintentional deep sedation or even general anesthesia[11,12].

Standard monitoring with pulse oximetry (POX) may miss hypoventilation, which is much better reflected by an increase in arterial carbon dioxide tension[13,14]. Arterial blood gas analysis is the 'gold standard' method to measure the arterial partial pressure of carbon dioxide ($PaCO_2$). However, arterial sampling, including arterial catheterization, is invasive and expensive. Transcutaneous carbon dioxide tension ($PcCO_2$) measurement is used as a noninvasive surrogate measure of $PaCO_2$ and to estimate $PaCO_2$ or determine trend changes in the measurement. Recently, considerable progress has been made in the technical aspects of $PcCO_2$ monitoring. A single earlobe sensor can now measure $PcCO_2$ and pulse oximetry simultaneously. Transcutaneous carbon dioxide tension measurement has been shown to be a reliable monitoring technique that corresponds well with $PaCO_2$ values measured in arterial blood gas samples[15-17].

The aim of this study was to evaluate the profile of $PcCO_2$ as a marker of hypoventilation during sedation with propofol or midazolam in colonoscopies.

MATERIALS AND METHODS

This was an open-labeled, blinded, randomized prospective study. Consecutive outpatients undergoing elective colonoscopy and opting for sedation were randomly assigned to receive propofol or midazolam. The patients were assigned using randomly numbered opaque envelopes. As a standard procedure, all patients received 3 L/min supplemental oxygen and analgesia with 4 μg/kg per body weight (BW) alfentanil (Rapifen®, Janssen-Cilag, Baar, Switzerland) prior to sedation[18]. The first bolus of the sedative drug was administered exactly one minute after the alfentanil. Midazolam (Dormicum®, Hoffmann La Roche AG, Basel, Switzerland) was administered in a first dose of 2 mg; further boluses of 1 mg were administered with an interval of at least 1 min or more depending on the clinical outcome. Propofol (Disoprivan®, AstraZeneca, Zug, Switzerland) was administered in two boluses of 20 mg followed by further boluses of 10 mg after an interval of at least 20 s. The sedative drugs were administered by registered nurses under the supervision of the endoscopist based on the clinical response of the patient; the nurses followed our institutional protocol as published elsewhere (nurse-administered propofol sedation or NAPS)[18]. The nurse administering the sedation had no tasks except to monitor the patient and administer sedation. A different nurse assisted the endoscopist with the technical performance of the procedure. Monitoring consisted of the measurement of continuous oxygen saturation, electrocardiography and heart rate, as well as regular measurements of blood pressure. The primary method of monitoring was the nurse's clinical assessment of the patient, including measurement of respiratory effort by visual assessment and by palpation of the chest wall and abdominal excursion and/or palpation of exhaled breath.

A short personal history was obtained from all the patients, and their general physical condition was assessed using the American Society of Anesthesiologists (ASA) classification. Exclusion criteria were as follows: (1) a known history of intolerance to propofol (including sensitivity to eggs and soybeans); (2) an age less than 18 or more than 85 years; (3) an ASA score of Ⅳ or Ⅴ; (4) a known high grade of aortic (gradient > 80 mmHg) or carotid stenosis (> 75%); and (5) intravenous drug abuse.

All endoscopic examinations were performed according to the department's standard operating procedures with different types of Pentax video colonoscopies (Pentax, Hamburg, Germany) using regular room air to insufflate the colon. The colonoscopies were conducted by seven different expert endoscopists, including four experienced gastroenterologists and three residents in their last year of gastroenterology training, who all had performed more than 400 colonoscopies each. The pro-

cedures were performed in an x-ray suite equipped for fluoroscopy (straightening maneuvers and confirming instrument position when necessary). The decision to perform an ileal intubation or an endoscopic intervention (e.g., polypectomy) depended on the clinical situation and was decided by the endoscopist.

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We used a recently developed combined POX/$PcCO_2$ sensor (V-Sign™, Sentec AG, Therwil, Switzerland) weighing 3 g that was placed at the right earlobe with a dedicated ear clip. We used a recently developed combined POX/$PcCO_2$ sensor (V-Sign™, Sentec AG, Therwil, Switzerland) weighing 3 g that was placed at the right earlobe with a dedicated ear clip[17]. This fully digital sensor combines the elements of an electrochemical Severinghaus-type carbon dioxide tension sensor with those of conventional optical POX sensors, thus providing noninvasive and continuous estimation of $PaCO_2$ and SaO_2[19]. The sensor was warmed to a constant surface temperature of 42 ℃ to improve local arterialization and to accelerate carbon dioxide diffusion. After the sensor was positioned, the SpO_2 values were available immediately, whereas the $PcCO_2$ values required an equilibration time of approximately 4-5 min. The system is designed to be "ready-for-use" by automated recalibration every time the sensor is placed on the docking station between measurements. The system was previously shown to deliver accurate and reproducible results for $PcCO_2$ and POX[17]. The system was also shown to have an excellent correlation between oxygen saturation and carbon dioxide measurements when the combined sensor was compared with arterial blood gas analysis[17,20].

Readings from the POX/$PcCO_2$ sensor (placed at the right ear lobe) were continuously recorded and stored on a personal computer. An independent observer who was blinded to the type of sedation recorded the readings. Similarly, the procedural personnel were blinded to the $PcCO_2$ data. All collected data were visualized using statistic graphics software (Igor Pro 4.01, WaveMetrics Inc., Lake Oswego, OR). Values at defined time points were thereafter identified manually. $PcCO_2$ values (as a parameter of sedation-induced hypoventilation) were compared between the study groups at the following distinct time points: at baseline, at the time point of maximal increase, at the time point when the cecum was reached, at the end of the procedure and 5 min after the end of the procedure.

The primary endpoint was defined as the number of patients in both study groups who regained their baseline $PcCO_2$ value (± 1.5 mmHg) five minutes after the end of the procedure. Secondary end points included the mean time lag between the application of the sedative drug and an increase of the $PcCO_2$ curve of more than 2 mm Hg, safety parameters defined as apnea with the need for intervention (in case of a decrease in SaO_2 values below 85% for more than 20 s), the frequency of SaO_2 decreases below 90% and a decrease of the heart rate below 50 bpm. Apnea was defined as a lack of spontaneous respiratory effort for more than 20 s and was assessed clinically by the nurse administrating the sedation. An increase of $PcCO_2$ above the baseline was defined as hypoventilation. The target sedation level was a quiet patient in both of the groups as implemented using our NAPS protocol, which has been in practice at this center for several years. The recovery time for all of the patients was defined as the time required for completion of the procedure, i.e., a complete withdrawal of the instrument and simultaneous conclusion of all sedation. As a standard of practice, the patients were transferred to a quiet recovery room following the final 5-min recording of the$PcCO_2$ readings in the endoscopy suite.

All of the parameters were analyzed using descriptive statistics (mean, standard deviation). Categorical outcomes were analyzed using the χ^2 or F test as appropriate. Continuous parameters were analyzed using an analysis of variance, and for non-continuous parameters, the Mann-Whitney Test was used. $P < 0.05$ was defined as statistically significant. All tests were two-sided. For all statistical calculations, SPSS software (SPSS for Windows, Version 11.0, SPSS Inc., Chiago, Illinois) was used. The sample size calculation was based on the primary outcome of this study of detecting a $PcCO_2$ difference of < 1.5 mmHg from the baseline at 5 min after the end of the procedure in the propofol group. In a pilot study in a similar population, a difference in values between midazolam and propofol was observed in 4 of 26 patients (15.4%). As we expected a clinical/physiological relevant effect, the sample size was determined to detect an ad-

Table 1 Demographic data of the randomized groups

	Sedation with Midazolam (*n* = 42)	Sedation with Propofol (*n* = 41)	*P* value
Age (yr, mean ± SD)	62 ± 13	62 ± 13	NS
M : F (*n*)	19:23	20:21	NS
ASA Ⅰ	12 (29%)	13 (32%)	NS
ASA Ⅱ	16 (38%)	15 (37%)	NS
ASA Ⅲ	14 (33%)	13 (32%)	NS
Smoker	6 (15%)	6 (15%)	NS
Mean dosage of sedative in mg (range)	5 (4-7)	131 (70-260)	-

ASA: American Society of Anesthesiologists classification; NS: Not significant.

Table 2 Oxygen saturation measured by pulse oximetry at baseline and changes during endoscopy according to sedatives

	Midazolam (*n* = 42)	Propofol (*n* = 41)	*P* value
SpO_2 at baseline (%)	99 ± 1	99 ± 1	NS
Mean max decrease of SpO_2 (%)	6 ± 3	4 ± 2	NS

SpO_2: Oxygen saturation measured by pulse oximetry; NS: Not significant.

Table 3 Procedure outcomes according to the defined endpoints (*n*)

	Midazolam (*n* = 42)	Propofol (*n* = 41)	*P* value
$\Delta PcCO_2$ < ± 1.5 mmHg (from baseline and five min after end)	12	32	0.0004
SpO_2 < 85%	0	0	NS
SpO_2 < 90%	6	0	0.05
HR < 50 bpm	5	1	NS
Decrease MAP > 25%	17	17	NS

HR: Heart rate; MAP: Mean arterial pressure; SpO_2: Oxygen saturation measured by pulse oximetry; $\Delta PcCO_2$: Difference of transcutaneous carbon dioxide tension; NS: Not significant.

ditional increase of 75% in the midazolam group with a power of 80%. Thirty-eight patients in each group would be required to detect such a difference ($P = 0.05$) (nQuery Advisor, Version 5.0, Statistical Solutions, Saugus, MA, United States).

RESULTS

Among the 133 colonoscopies performed during the study period, 97 patients were successfully randomized to the study. Fourteen randomized patients had to be subsequently excluded for the following reasons: in 5 patients, the procedure was not completed because of incomplete bowel preparation; in 5 patients, a short disconnection of the sensor provided no continuous data; in two patients, a calibration fault occurred because of the prototype calibration unit used, and the endoscopist refrained from recalibration; and in two patients, the sedative drug was not correctly administered according to the protocol. Therefore, the final study population contained 83 patients. Demographic characteristics of the study groups are shown in Table 1. The mean duration of the procedures was 26 ± 13 min for the midazolam group and 27 ± 18 min for the propofol group (not significant).

Decreased ventilation activity was detected in all patients to whom a sedative was administered; on average, the $PcCO_2$ values increased by 8.0 ± 3.7 mmHg. The mean SpO_2 values at baseline and the mean maximal decrease during sedation are shown in Table 2.

When comparing midazolam and propofol, different profiles were observed for the $PcCO_2$ readings, as shown in Figure 1. The increase in $PcCO_2$ was mostly related to a short delay in the administration of incremental dosages of the sedatives. The mean time interval after the first application of the drug until the $PcCO_2$ value had increased by more than 2 mmHg was 2.8 ± 1.3 min for midazolam and 2.8 ± 1.1 min for propofol. Although there was no significant difference in the mean increase in $PcCO_2$ following administration of midazolam or propofol, the patients who received midazolam tended to remain in a prolonged state of decreased ventilation when compared with the patients receiving propofol. The difference in $PcCO_2$ values (baseline compared with the end of the procedure) was significantly higher in the patients receiving midazolam. Therefore, the patients who received propofol had a $PcCO_2$ level that was significantly closer to the baseline five minutes after the end of the procedure when compared with the patients who received midazolam (Tables 2, 3, Figure 2).

None of the study patients manifested apnea or hypoxemia below 85%. Drops in oxygen saturation below 90% tended to occur more often during sedation with midazolam than with propofol (Table 3).

DISCUSSION

Achieving higher safety standards for sedation in routine endoscopy has been a priority over the last few years. In keeping with this objective, the present study showed that patients undergoing colonoscopy under sedation developed relative hypoventilation (as reflected by retention of CO_2) that persisted for a significantly longer period in patients sedated with midazolam than in patients sedated with propofol. The results support the findings of a meta-analysis that suggested that propofol sedation during colonoscopy is associated with a lower risk of complications when compared with traditional sedative agents[21].

Although the use of oxygen saturation monitoring during sedation is routinely used by most gastroenterologists and the administration of supplemental oxygen has become a widely accepted practice, little attention has been paid to the development of alveolar hypoventilation[17]. Because of the buildup of CO_2 in the patient's alveoli and blood, hypoventilation can be effectively detected by capnography, which has recently become a focus of interest as an additional monitoring parameter during gastrointestinal procedures. This procedure has become

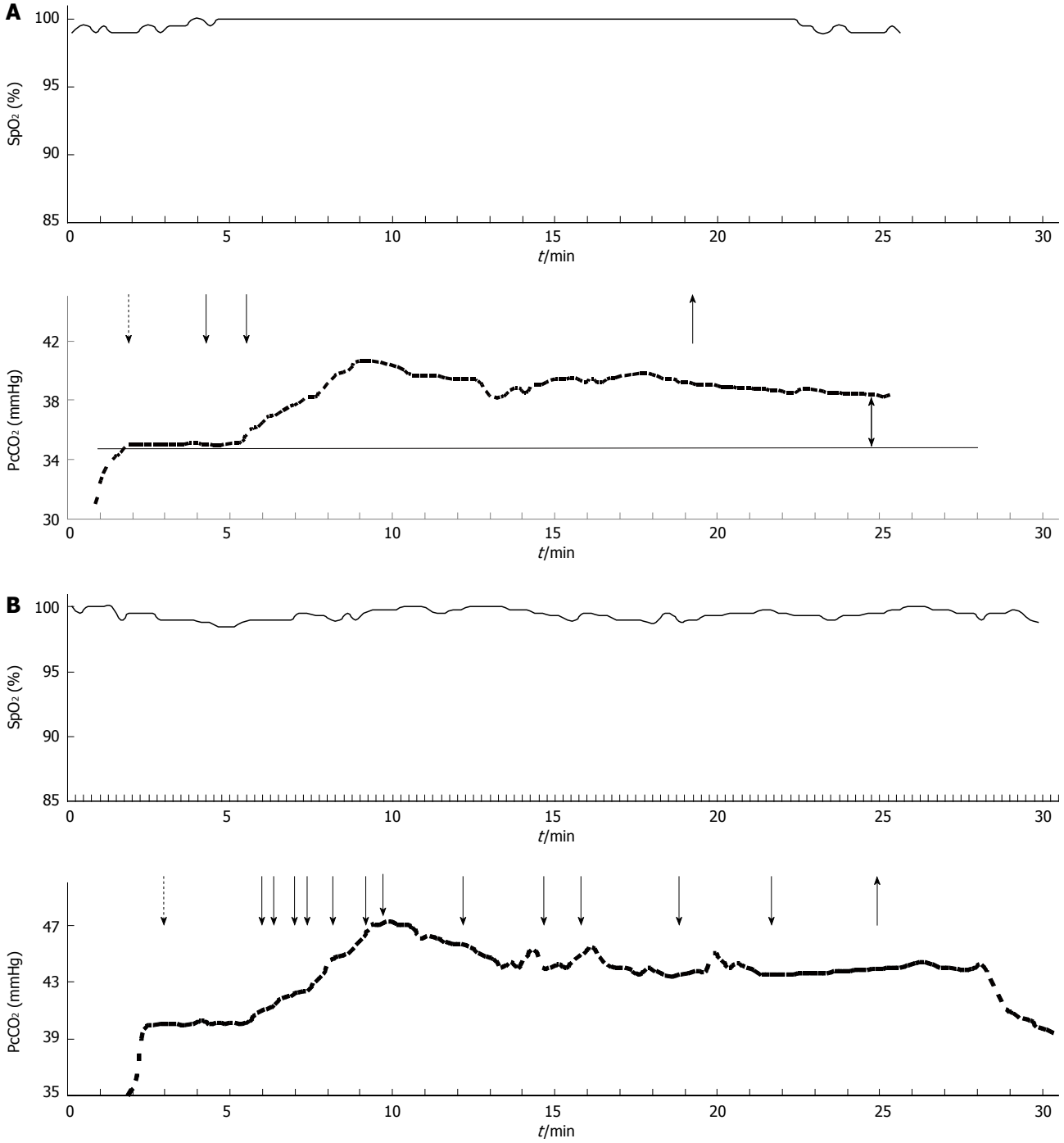

Figure 1 A typical course of oxygen saturation measured by pulse oximetry and transcutaneous carbon dioxide tension following administration of alfentanil (dashed arrow) and midazolam (A) or propofol (B) (solid arrows as indicated). The upright arrow indicates the end of the procedure. The double arrow highlights the difference after termination of the procedure.

relevant as the use of newer anesthetic drugs such as propofol has increased[15].

Several studies to date have evaluated the importance of CO_2 buildup during endoscopic procedures[14-16,22]. Freeman *et al*[15] were the first to show that profound hypoventilation may frequently occur undetected during a gastrointestinal endoscopy, especially if additional oxygen is given and the decline of oxygen saturation is thus prevented; we observed a similar effect in our study. Freeman *et al*[15] also found that the degree of hypoventilation was more closely related to the sedative drug dose than to the underlying medical illness. In 30 colonoscopies, with 90% receiving fentanyl and 37% additionally receiving midazolam, Freeman *et al*[15] recorded a mean PCO_2 increase of 6.4 ± 3.8 mmHg, whereas during ERCP with a higher rate of deeper sedation, the mean values were 14.2 ± 10.2 mmHg. Our results showed a mean increase in $PcCO_2$ of 8.6 ± 3.7 mmHg despite a continuous SpO_2 above 90%; these results are similar to the experience of Freeman *et al*[15].

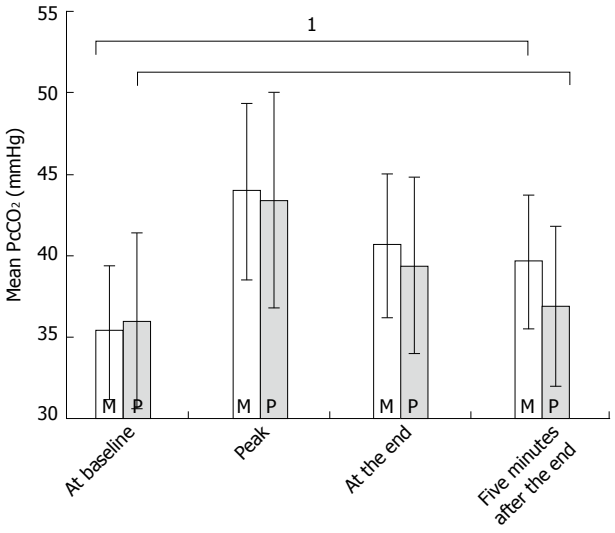

Figure 2 Mean transcutaneous carbon dioxide tension values in mmHg (± SD) at four distinct time points during colonoscopies according to sedative use (for the whole group). [1]A significant difference in the transcutaneous carbon dioxide tension values measured five minutes after the end of the procedure when compared with the baseline values. M: Midazolam; P: Propofol.

The pattern of the registered $PcCO_2$ readings differed noticeably in our study according to the pharmacological properties of the administered drugs. The pattern of the initial rise of $PcCO_2$ was similar, but the $PcCO_2$ level decreased much earlier after the administration of propofol than after the administration of midazolam. Although propofol had to be administered more often in a repeated fashion to maintain the desired sedation level, its effect ceased much faster than the effect of midazolam. This pharmacologic pattern seems to be reflected by the shape of the $PcCO_2$ curve.

We did not observe severe hypoxemia or apnea in either of the study groups; the increase in CO_2 could, however, indicate silent risk during poor sedative practice. In the study by Freeman *et al*[15]. We did not observe severe hypoxemia or apnea in either of the study groups; the increase in CO_2 could, however, indicate silent risk during poor sedative practice. In the study by Freeman *et al*[15], one case demonstrated an increase of the $PcCO_2$ curve above 80 mmHg prior to respiratory arrest. Nelson *et al*[16] showed that the monitoring of $PcCO_2$ may be useful for the endoscopist to guide sedation using midazolam and fentanyl and that this monitoring can help to prevent severe carbon dioxide retention. Thus, we believe that determining the $PcCO_2$ level can be helpful for the endoscopist when deciding whether to administer a further incremental dose of the sedative.

The combination used in this study of alfentanil, which is a potent opioid with a rapid onset of action, and midazolam is uncommon. Typically, this substance alfentanil is administered in combination with propofol for patient-controlled sedation or for short sedoanalgesia in emergency medicine[23]. The rationale to use this substance alfentanil as a single bolus at the beginning of the procedure was (1) to provide the patients with optimal analgesia during the most painful passage of the sigmoid colon; and (2) to determine whether differences in alveolar hypoventilation at the end of the procedure can be completely attributed to the pharmacologic effect of the sedatives. Furthermore, if propofol is used in outpatient procedures, alfentanil may be an ideal drug to use in combination because of its pharmacokinetic properties and analogous profile of action. Because alfentanil (similarly to all opioids) can induce or enhance alveolar hypoventilation, our protocol prescribed a single low dose of 4 μg/kg per BW of alfentanil and a strict time interval of administration one minute before the first titration dose of the sedative was given.

Alveolar hypoventilation exists when the arterial $PaCO_2$ increases above 45 mmHg, which can occur as the result of various underlying factors. Sedation directly or indirectly influences alveolar hypoventilation by a predominant central effect, thereby causing an increase in the $PaCO_2$. Therefore, recognition and adequate monitoring of this physiological change through indirect means such as transcutaneous monitoring of $PcCO_2$ could play an important role during sedation. The peak $PcCO_2$ value may not be clinically relevant; however, the time period during which reduced ventilation occurs may represent a period of increased risk for some patients. Because of the S-shape of the oxygen dissociation-curve, hypoventilation accompanied by a decrease in PaO_2 may remain unnoticed over time. Although a patient would have adequate arterial saturation with the administration of supplemental oxygen, an adverse physiologic trend that may be reflected by changes in the $PaCO_2$ may go unnoticed.

Transcutaneous CO_2 monitoring in adults has yielded conflicting results because of technological limitations, such as the time required for calibration, the need to warm the skin to 42 degrees, the effect of sweating and the influence of skin metabolism and thickness. Technical problems precluded an accurate interpretation of the data in 7 of the 97 patients and thus represent a limitation in our study.

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warm the skin to 42 degrees, the effect of sweating and the influence of skin metabolism and thickness. Technical problems precluded an accurate interpretation of the data in 7 of the 97 patients and thus represent a limitation in our study.

The main focus of this study was to evaluate the impairment of ventilation induced by midazolam or propofol during colonoscopies. Most trials comparing the use of propofol and midazolam in the endoscopy suite have focused on differences in recovery time (assessed using a discharge scoring system, for example)[24]. The present study suggests that there is also a significant difference in the duration of hypoventilation during the post-procedural period. Although alveolar hypoventilation is generally well tolerated by most patients, it may nevertheless be of clinical relevance in patients with compromised health. Iber *et al*[25] showed that in 4% of patients sedated with midazolam, a relevant decrease of oxygen saturation below 89% occurred during the 30 min after the endoscopic procedure, which is contrast to our practical experience with propofol, where the effect occurs exclusively during the time when the attention on the patient is greatest. During the endoscopic procedure, the $PcCO_2$ monitoring indicated no increased hypoventilation risk for propofol when compared with midazolam.

Insufflation of the colon with carbon dioxide (CO_2) rather than air has been shown to reduce pain and discomfort because CO_2 is rapidly absorbed by the intestinal lining. In previous studies, measurement of end tidal CO_2 ($ETCO_2$) and the mean pCO_2 demonstrated these procedures to be safe. However, no studies have used transcutaneous continuous pCO_2 monitoring, which could be valuable given the increasing use of this insufflation technique for pain relief during colonoscopies[26].

A metanalysis by Qadeer *et al*[21] showed that propofol sedation had a lower rate of cardiopulmonary complications than traditional agents used during colonoscopy procedures. This current study highlights another physiological mechanism that may be detrimental when propofol is used in larger cohorts. Therefore, assessing the $PcCO_2$ during sedation could serve as an added safety measure to detect alveolar hypoventilation.

In conclusion, hypoventilation occurs frequently during sedation for colonoscopy and is often undetected during routine pulse oximetry. A significantly higher number of patients sedated with propofol had normalized $PcCO_2$ values five minutes after sedation when compared with patients sedated with midazolam. Understanding the role of CO_2 retention will be important in increasing the further safety standards of sedation during endoscopy. More studies are required to identify and prevent hypercapnia and thus ensure the safe practice of sedation during routine gastrointestinal endoscopies.

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COMMENTS

Background

Colonoscopies are usually performed under sedation and monitored by pulse oximetry. With the increasing use of newer sedative agents such as propofol, there is an ongoing discussion about safety and monitoring requirements.

Research frontiers

The surveillance of carbon dioxide tension (e.g., measuring end tidal CO_2 by capnography) could provide more accurate information than pulse oximetry regarding ventilation impairment. Until now, little experience has been reported for transdermal CO_2 measurement systems used for this purpose.

Innovations and breakthroughs

Monitoring studies with capnography showed that hypoventilation and even short apneas that are not detected by pulse oximetry (POX) may occur during endoscopic sedation. However, an increasing body of scientific data shows that sedation during endoscopy with benzodiazepines and propofol is a safe procedure. The development of a new sensor combining transcutaneous carbon dioxide and pulse oximetry measurements provides the opportunity to explore a new monitoring method during endoscopic sedation.

Applications

The study results suggest that hypoventilation occurs often during endoscopic sedation but lasts for a shorter period if patients are sedated with propofol rather than midazolam.

Terminology

$PcCO_2$: Transcutaneous carbon dioxide tension is measured electrochemically using a Severinghaus-type sensor placed on the earlobe; POX: Pulse oximetry measures the oxygen saturation of the blood using an optical sensor.

Peer review

In this study, the authors investigated carbon dioxide accumulation after sedation with propofol or midazolam during colonoscopies. Non-invasive, continuous transcutaneous carbon dioxide tension ($PcCO_2$) monitoring was performed using a recently developed $POX/PcCO_2$ sensor that was placed at the earlobe. The results of the study show that a significantly higher number of patients sedated with propofol had normalized $PcCO_2$ values five minutes after sedation when compared with the patient group sedated with midazolam. The study is well designed and performed, and the $POX/PcCO_2$ sensor could be used in future studies rather than capnography.

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BRIEF ARTICLE

Similar fecal immunochemical test results in screening and referral colorectal cancer

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Abstract

AIM: To improve the interpretation of fecal immunochemical test (FIT) results in colorectal cancer (CRC) cases from screening and referral cohorts.

METHODS: In this comparative observational study, two prospective cohorts of CRC cases were compared. The first cohort was obtained from 10 322 average risk subjects invited for CRC screening with FIT, of which, only subjects with a positive FIT were referred for colonoscopy. The second cohort was obtained from 3637 subjects scheduled for elective colonoscopy with a positive FIT result. The same FIT and positivity threshold (OC sensor; ⩾ 50 ng/mL) was used in both cohorts. Colonoscopy was performed in all referral subjects and in FIT positive screening subjects. All CRC cases were selected from both cohorts. Outcome measurements were mean FIT results and FIT scores per tissue tumor stage (T stage).

RESULTS: One hundred and eighteen patients with CRC were included in the present study: 28 cases obtained from the screening cohort (64% male; mean age 65 years, SD 6.5) and 90 cases obtained from the referral cohort (58% male; mean age 69 years, SD 9.8). The mean FIT results found were higher in the referral cohort (829 ± 302 ng/mL *vs* 613 ± 368 ng/mL, P = 0.02). Tissue tumor stage (T stage) distribution was dif-

ferent between both populations [screening population: 13 (46%) T1, eight (29%) T2, six (21%) T3, one (4%) T4 carcinoma; referral population: 12 (13%) T1, 22 (24%) T2, 52 (58%) T3, four (4%) T4 carcinoma], and higher T stage was significantly associated with higher FIT results ($P < 0.001$). Per tumor stage, no significant difference in mean FIT results was observed (screening *vs* referral: T1 498 ± 382 ng/mL *vs* 725 ± 374 ng/mL, $P = 0.22$; T2 787 ± 303 ng/mL *vs* 794 ± 341 ng/mL, $P = 0.79$; T3 563 ± 368 ng/mL *vs* 870 ± 258 ng/mL, $P = 0.13$; T4 not available). After correction for T stage in logistic regression analysis, no significant differences in mean FIT results were observed between both types of cohorts ($P = 0.10$).

CONCLUSION: Differences in T stage distribution largely explain differences in FIT results between screening and referral cohorts. Therefore, FIT results should be reported according to T stage.

Key words: Screening population; Referral cohort; Fecal immunochemical test; Tumor stage distribution; Colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is a disease well suited for population-based screening. In industrialized countries, CRC is one of the three malignancies with the highest incidence and mortality[1,2]. CRC and adenomas can cause lower gastrointestinal bleeding, and early detection by guaiac-based fecal occult blood tests (g-FOBTs) can decrease mortality[3-5]. Recently, fecal immunochemical tests (FITs) have been found to be superior over g-FOBTs[6-10].

Studies on FITs have used different designs, e.g., in terms of populations studied. Screening and referral cohorts each have their pros and cons in this respect[6,7]. Studies in screening cohorts, consisting of individuals with average-risk for CRC, best reflect the true target population. However, in most such studies, only subjects who test positive on FIT are referred for colonoscopy, which means that sensitivity and specificity cannot be determined directly[6]. In addition, the number of cases detected, particularly cancers, is usually low. Other designs include high risk or referral cohorts. Here, an important advantage is that colonoscopy is performed in all patients allowing for calculation of direct sensitivity and specificity[7]. In addition, these studies often yield more cases, allowing for more detailed subgroup analyses[7,11].

It has been suggested that conclusions from referral studies cannot be extrapolated to the screening setting[12,13]. So far, no comparative data have been published to verify or falsify this hypothesis, and arguments both in favor of, as well as against this hypothesis exist. Due to the higher pretest likelihood and presence of symptomatic individuals included in referral cohorts, the risk of work-up bias exists, limiting extrapolation to population-based screening. On the other hand, CRC stage distribution has been shown to influence sensitivity of blood-based CRC markers[14], and is likely to have a large influence on FIT results[15]. Therefore, the aim of the present study was to compare FIT results between subjects with CRC found in either a screening or a referral cohort, and determine if differences can be explained by tumor characteristics.

MATERIALS AND METHODS

Study design

The present study aimed to compare FIT results in CRC cases derived from two methodologically different cohorts. Here, CRC cases with a FIT result ⩾ 50 ng/mL from both a screening and a referral cohort were compared for mean FIT result, age, sex and tumor stage. Both these studies were initiated in the same time period in the same country, and both used the same FIT.

Setting

Screening population: The screening cohort in this study was prospectively selected from June 2006 to February 2007 by a randomized selection from a Dutch population. Details from this study are described elsewhere[6,16]. In short, eligible individuals 50-75 years of age were invited by mail to perform either a g-FOBT or a FIT ($n = 20\,623$). Participants with a positive test were offered colonoscopy in two academic centers. Exclusion criteria were institutionalization and bowel symptoms[6]. In the current study, only participants enrolled in the FIT arm ($n = 10\,322$) were included.

Referral population: The referral cohort in this study was derived from a previous and ongoing study on FIT performance, and a more detailed description can be found elsewhere[7]. In short, from June 2006 to October 2009, all ambulatory patients (aged ⩾ 18 years) scheduled for elective colonoscopy in five Dutch hospitals (including an academic center and large teaching hospital), were selected regardless of the indication for colonoscopy. Eligible subjects were invited to participate in a prospective study on FIT performance. All participants performed a FIT before bowel preparation. Until June 2008, subjects were invited to perform both a g-FOBT and an FIT[7]. In

an ongoing study after that period, subjects were invited to perform an FIT only, but twice (on two consecutive days). From the latter cohort, the FIT performed 1 d before colonoscopy was selected for the current study for comparability with the screening population. Exclusion criteria were hospitalization, colostomy, inflammatory bowel disease or total colectomy.

Outcomes and measurements

FIT: In both cohorts, an identical semi-quantitative FIT was used: OC-sensor (Eiken Chemical Co., Tokyo, Japan). No restrictions for diet or medication in the week prior to FIT were given. Participants were educated by illustrated and written instructions to sample their feces, ensuring that no contamination with water or urine occurred. In the screening population, participants were asked not to perform the test if blood was visible.

The FIT used in this study consists of a sampling tube, filled with stabilizing buffer. Participants were instructed to scrape the probe at different parts of the stool. The amount of feces that can be inserted into the sample bottle is regulated to approximately 10 mg[17]. In the referral population, subjects performed the FIT within 72 h before colonoscopy, and returned the test and informed consent form on the day of colonoscopy. All samples were placed at -5 ℃ on arrival and analyzed according to the manufacturer's instructions within 1 wk, or were frozen at -20 ℃. The analyses were performed by two experienced technicians, blinded to the clinical data[18]. In the screening population, participants were instructed to return the test by mail as soon as possible. If the test could not be returned immediately, storage in a refrigerator was advised. Again, two specially trained technicians who were blinded to the clinical data processed all samples.

All FITs were analyzed with the OC sensor MICRO desktop analyzer (Eiken Chemical Co.). The agglutination reaction is dependent on the hemoglobin concentration in the sample. A prozone effect may occur if the concentration is too high and the excess amount of antigen limits its agglutination. Measured values can then be higher or lower than the actual concentration in the sample[19]. The risk of the prozone effect gradually increases well above 1000 ng/mL. Therefore, every measurement above 1000 ng/mL was classified as 1000 ng/mL. The quantitative nature of the test was maintained, because 1000 ng/mL is at least 10 times higher than the most usual cut-off values between 50 ng/mL and 100 ng/mL.

Colonoscopy and detected malignancies: Colonoscopies were performed under conscious sedation with midazolam and fentanyl at the discretion of the endoscopist. In both cohorts, all colonoscopies were performed or supervised by experienced gastroenterologists. Colonoscopy was considered complete if the cecum was intubated with visualization of the ileocecal valve or the appendiceal orifice, or by intubation up to CRC. Incomplete colonoscopies were excluded. In addition, subjects were excluded in case of insufficient bowel cleansing, as judged by the individual endoscopist. In the screening cohort, an incomplete colonoscopy was followed by a second colonoscopy with propofol anesthesia. If necessary, a computed tomographic colonoscopy was performed followed by a second colonoscopy. If an incomplete colonoscopy in the referral cohort was followed by a complete second colonoscopy, virtual colonoscopy or x-colon within 6 mo, the results were included in analysis.

In the screening study, histology of tissue samples obtained during colonoscopy was evaluated by one experienced pathologist. In the referral cohort, lesions were evaluated according to routine procedures. In both studies, the outcome variable CRC was classified according to tissue tumor stage (T stage) of the TNM-classification (6th edition) according to the AJCC cancer staging manual[20].

Statistical analysis

The primary aim of the study was to compare mean FIT scores in CRC cases found in the referral and screening setting, with and without correction for CRC T stage. For analyses, only individuals with CRC and a FIT result $\geqslant$ 50 ng/mL were selected, because this cut-off value was used for colonoscopy referral in the screening population.

FIT scores do not follow a Gaussian curve. On average, even after correction for the prozone effect[19], the curve is considerably skewed to the left. Logarithmic transformation of the FIT scores allowed for using the *t* test, as a normal distribution was achieved. Multivariate logistic regression analysis was used to evaluate which variables could explain the differences in FIT scores between CRC patients found in the screening and referral cohorts. In logistic regression analysis, the outcome variable was mean FIT score, and the independent variables were population of origin, T stage, age, and sex. Logistic regression analysis was performed both by forward and backward selection.

Statistical analysis was performed with SAS for Windows, version 8.02 (SAS Institute Inc., Cary, NC). Two-sided P values < 0.05 were considered statistically significant.

Ethical approval and consent

In both studies, informed consent was obtained from all participants. Approval and consent from the screening arm of this study was obtained by the Dutch Health Council (2005/03WBO, The Hague, The Netherlands, www.gezondheidsraad.nl)[6]. In all centers participating in the referral arm of this study, local Medical Ethics Review Board approval was obtained prior to the start of the study[7].

RESULTS

Participants

In the screening population, 10 322 subjects were invited to FIT sampling. Of these, 6157 completed and returned the test. Five hundred and twenty-six participants were scheduled for colonoscopy because the FIT result was $\geqslant$ 50 ng/mL (i.e., positivity rate of 8.5%). In 428 patients, colonoscopy was performed, and considered complete in

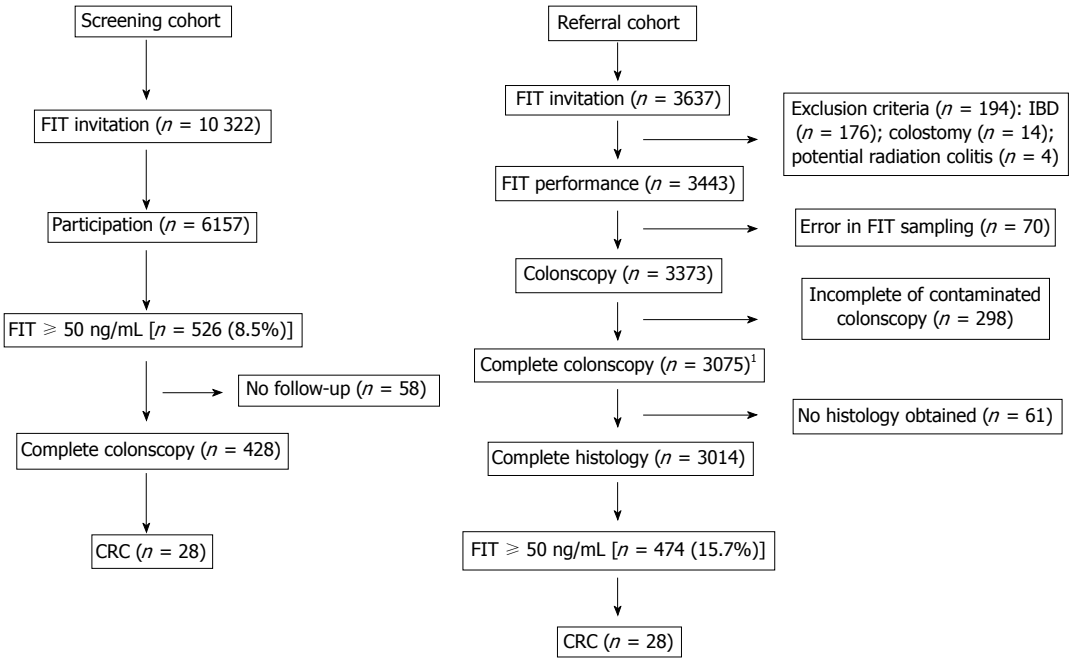

Figure 1 Study flow of the screening and referral cohort compared in the present study. [1]This includes cases in which an incomplete initial colonoscopy was followed by a second complete colonoscopy, virtual colonoscopy or x-colon. FIT: Fecal immunochemical test; CRC: Colorectal cancer; IBD: Inflammatory bowel disease.

Table 1 Primary indications for colonoscopy

Indication group	Indication for colonoscopy	*n*
Symptomatic/suspect	Weight loss	52
	Clinical suspicion of diverticulitis	23
	Clinical suspicion of IBD	40
	Abdominal pain	310
	Anemia	174
	Hematochezia	418
	Altered bowel habits	416
	Clinical or radiological suspicion of CRC	49
	Colonoscopy for polypectomy	57
	Diarrhea	115
	Constipation	71
	Total	1725
Screening/surveillance	Average risk	69
	Familial history of CRC	387
	Lynch syndrome	42
	Polyp surveillance	491
	Post CRC surveillance	157
	Total	1146
Other	Not specified/others	143
Grand total		3014

Primary indications for colonoscopy among 3014 patients included in the referral arm of this study in a comparison of fecal immunochemical test results in screening and referral colorectal cancer (CRC) cases (exclusion criteria used in the referral arm of the study were age < 18 years, hospitalization, colostomy, inflammatory bowel disease (IBD) or total colectomy).

402 (colonoscopy completion rate 94%; Figure 1). In the 26 cases in which the cecum was not visualized, a second complete colonoscopy was performed. In total, 28 cases of CRC were detected.

In the referral population, 3637 subjects were invited for participation. Six hundred and 23 subjects were excluded because of FIT sampling violating the study protocol, incomplete or contaminated colonoscopy, or missing histology of lesions found. Therefore, 3014 individuals who had a complete colonoscopy and histology remained for analysis (completion rate colonoscopy 91%, Figure 1). The indication for colonoscopy was evaluation of symptoms in 57%, screening and surveillance in 38%, and unspecified in 5% (Table 1). The positivity rate (⩾ 50 ng/mL) was 15.7%. In total, 105 subjects with CRC were found, of whom 96 (91.4%) had a positive FIT. The sensitivity of FIT for detection of CRC at cut-off values of 50 mg/mL, 75 mg/mL and 100 mg/mL was 91.4%, 90.5% and 89.5%, respectively. The respective specificity for these cut-off values was 83.7%, 85.7% and 87.0%.

In total, 124 patients with CRC and a positive FIT result were found: 28 derived from the screening population and 96 from the referral population. From the referral population, six cases were excluded because the actual T stage could not be determined due to neoadjuvant radiotherapy or palliative treatment. The mean age of the remaining 90 CRC cases from the referral cohort was significantly higher compared with the 28 cases from the screening cohort; 69 (SD 9.8) *vs* 65 (SD 6.5) years, respectively ($P = 0.04$). As expected, the proportion of males was higher in both populations and not statistically significantly different between the referred and screened population (58% and 64%, $P = 0.54$, Table 1). Other abnormalities that might cause (minor) mucosal bleeding in the colon in addition to CRC, potentially influencing the FIT results were seen in 64% of the referral and 79% (not significant) of the screening population (Table 2).

T stage distribution and FIT results

The 28 CRC cases from the screening population had a

Table 2 Colorectal cancer patient characteristics and potential explanations for positive fecal immunochemical test results in addition to colorectal cancer

	Screening population (*n* = 28)	Referral population (*n* = 90)
Male %	64	58[1]
Age (yr, mean ± SD)	65 ± 6.5	69 ± 9.8[2]
Location of CRC (% left sided)	64	62
CRC only (%)	6 (21)	32 (36)
CRC including (%)		
Advanced adenomas	16 (57)	14 (16)
Other adenomas	5 (18)	17 (19)
Other polyps	0 (0)	14 (16)
Diverticula	1 (4)	9 (10)
Hemorrhoids	0 (0)	4 (4)

[1]χ^2 test: $P = 0.54$; [2]t test: $P = 0.04$. The percentages of other abnormalities in addition to colorectal cancer (CRC) in the referral population totaled 65% due to rounding. The total percentage of CRC cases with additional pathology was 64%.

mean FIT score of 613 ng/mL (SD 368 ng/mL), which was significantly lower ($P = 0.02$) than the mean FIT score of the 90 CRC cases from the referral population 829 ng/mL (SD 302 ng/mL, Table 3).

The CRC stage distribution was different between the populations: early stages were more frequently found in the screening population (Figure 2). Of the 28 cases from the screening population, 13 cases were classified as T1 (46%), eight as T2 (29%), six as T3 (21%) and one as T4 carcinoma (4%). In the referral population, 12 patients had stage T1 tumor (13%), 22 stage T2 (24%), 52 stage T3 (58%) and four stage T4 (4%).

After stratifying mean FIT scores by T stage, none of the tumor stages had a significant difference in FIT results (Table 2): T1, $P = 0.22$; T2, $P = 0.79$; T3, $P = 0.13$. There was only one T4 case in the screening population, therefore, T4 cases were combined with T3 cases. Again, for the combined T3 and T4 stage category, no significant difference in FIT score was seen between both populations ($P = 0.19$).

Logistic regression analysis

By univariate analysis, FIT results from the referral cohort were significantly higher compared with the screening cohort ($P < 0.01$). However, after adding T stage to the model, the difference in FIT results lost statistical significance ($P = 0.10$). The P values per T stage were 0.23 (T1), 0.79 (T2), and 0.11 (T3/T4). By multivariate analysis including the variables sex, age, T, N and M stage, only T stage ($P < 0.001$) and (marginally) age ($P = 0.05$) could significantly explain the differences between the screening and referral cohorts.

DISCUSSION

The current study compared FIT results in individuals with CRC from two different prospective study designs, i.e., a population-based screening study and a referral cohort, to study the uncertainty about the link between the results obtained from these kind of studies. Cases of CRC from the screening cohort were found to have significantly lower FIT results compared to those from the referral cohort, but after stratifying for tissue tumor stage, no difference remained. In the screening cohort, 75% (21/28) and in the referral cohort only 38% of cases had a T1 or T2 tumor (34/90). Logistic regression analysis confirmed that not the type of population, but only T stage and to a lesser extent age could explain the differences in FIT results of CRC patients between these screening and referral cohorts.

The results of this study are relevant for the evaluation of CRC screening tests and guidance of future study designs. Several studies in screening populations have been performed comparing, e.g., FIT with g-FOBT[6,9,12,21]. However, despite the large number of screened individuals in these studies, the absolute number of individuals with CRC was relatively low, hampering subgroup analysis. Furthermore, colonoscopy was missing in subjects with a negative FIT, impeding calculation of sensitivity and specificity. Indeed, for the investigation of the performance of a screening test like FIT, the ultimate prospective study design would contain full colonoscopic evaluation of all participants. However, in a screening population, this is considered unethical or unfeasible because the capacity and/or funds are lacking. In referral populations, FIT negatives do indeed all undergo colonoscopy, and in addition, in much less time and at a substantially lower cost, a much larger number of CRC patients can be included. This enables, e.g., more elaborate subgroup analysis of early stage CRC. The present study shows that tumor stage is the major contributor to the outcomes of FIT between cohorts. Possible differences in characteristics between the cohorts did not have much influence on FIT outcomes. It is indicated here that, if correction or stratification for CRC tumor stage distribution is applied, both screening as well as referral cohorts can be used to answer several important screening-related research questions. Research questions on accuracy of screening tests with sufficient power, could therefore initially be explored using referral populations. In line with Tao and colleagues, who found that sensitivity of blood-based CRC markers is dependent on tumor stage[14], results for test characteristics should be presented per tumor stage. By merging results from different sources, the strength of the evidence available will be enlarged.

Some considerations need to be discussed for proper interpretation of the present results. For evaluation of sensitivity and specificity, data from referral studies could be extrapolated, or the incidence of interval cancers could be used as false negatives. The latter requires intensive follow-up over many years before sensitivity can be estimated; time in which tumors may further evolve. Second, the number of screening cases is limited, although comparable with other screening studies[9,12,21]. This limits the power to determine any existing differences. However, from Figure 2 and Table 2 it is clear that it is unlikely that adding more screening cases could change the results substantially. Supported by the results from other

Table 3 Fecal immunochemical test results in patients with colorectal cancer derived from a screening and referral cohort according to tissue tumor stage

	Population	*n*	mean ± SD	25th %	Median	75th %	*P* value[1]
All colorectal cancer cases	Screening	28	613 ± 368	283	662	1000	0.02
	Referral	90	829 ± 302	709	1000	1000	
T1	Screening	13	498 ± 382	79	384	871	0.22
	Referral	12	725 ± 374	428	1000	1000	
T2	Screening	8	787 ± 303	559	936	1000	0.79
	Referral	22	794 ± 341	550	1000	1000	
T3	Screening	6	563 ± 368	269	454	1000	0.13
	Referral	52	870 ± 258	888	1000	1000	
T4	Screening	1	NA	NA	NA	NA	NA
	Referral	4	793 ± 415	586	1000	1000	

[1]*t* test after logarithmic transformation because of non-normality of fecal immunochemical test results (ng/mL). All results ⩾ 1000 ng/mL were classified as 1000 ng/mL. T: Tumor stage; NA: Not available. (i.e., values like mean and median having a single observation are meaningless and a *t* test cannot be performed because no error term can be estimated for a single observation).

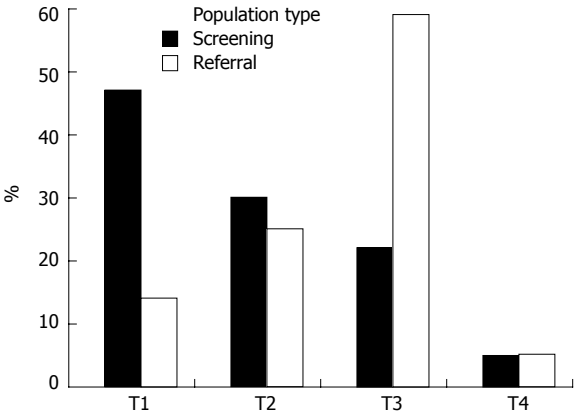

Figure 2 Colorectal cancer tumor stage distribution in screening (*n* = 28) and referral (*n* = 90) populations in studies on diagnostic performance of an fecal immunochemical test. Difference between screening and referral $P < 0.001$.

studies[13,16,17,22], CRC was considered the major contributor to the overall FIT results. Still, it cannot be excluded that other sources of bleeding (like minor polyps) could have contributed to the overall FIT result. In addition, although probably limited considering the moderate temperatures in the Netherlands, time lag between sampling and analysis could have influenced FIT results found[23]. In this study, the existing difference between both cohorts could therefore be even less, because time to analysis in the screening cohort was on average somewhat longer. Finally, in the screening cohort, no information about preoperative radiotherapy was available and therefore tumor stage could have been underestimated in a few cases. In summary, the correction for other potential bleeding sources, time lag to analysis, and radiotherapy would even decrease the differences between the referral and screening cohorts and therefore support our conclusions.

The present study aimed to compare test performance in two study designs, each with pros and cons, knowing the essential differences between the two cohorts. It can be concluded that T stage reflects the majority of the differences in overall FIT results between the two studied cohorts.

In conclusion, apparent differences in FIT results between screened and referred CRC patients can be attributed to tissue tumor stage. Based on these findings, we conclude that results from both cohorts could strengthen the evidence available. Using referral populations for studying FIT, and potentially also new CRC screening tests, can be useful to stimulate progress in CRC research. Here, sensitivity and specificity should be studied as these measures are independent of the prevalence of the disease[24], and test characteristics should be stratified by tumor stage. This will be of particular benefit in research questions that require large numbers of cases or colonoscopy confirmation in all individuals, and do not seek predictive values as outcome.

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COMMENTS

Background

Colorectal cancer (CRC) is a disease well suited for population-based screening. Subjects who test positive on fecal immunochemical tests (FITs) should be referred for colonoscopy. FIT performance has been studied in two different types of populations. In studies in screening cohorts, only subjects who test positive on FIT are referred for colonoscopy. Sensitivity and specificity cannot be calculated directly. In addition, the number of cases detected is usually low. Designs with referral cohorts do not study average but high-risk individuals. However, as colonoscopy is performed in all subjects direct sensitivity and specificity can be calculated. In addition, in referral populations, more cases are found. The aim of the present study was to compare FIT results between subjects with CRC found in a screening and a referral cohort.

Research frontiers

FITs detect occult human blood, what might be derived from adenomas or CRC. FITs are more sensitive than guaiac fecal occult blood tests, by which screening has been shown to decrease CRC-related mortality. However, exact FIT characteristics are the subject of debate.

Innovations and breakthroughs

The present study is the first to compare results obtained from both screening and referral populations to gain insight into the comparability of results derived from both study designs. It was shown that referral populations have a different tumor stage (T stage) distribution compared to screening populations (i.e., a higher percentage of high T stage cancers). This was accompanied by higher mean FIT results. After correction for T stage, mean FIT results were similar in both populations.

Applications

Apparent differences in FIT results between screened and referred CRC patients can be attributed to tissue tumor stage. Results from both cohorts could strengthen the evidence available. Using referral populations for studying FIT, and potentially also new CRC screening tests, can be useful to stimulate progress in CRC research, when test characteristics are stratified by tumor stage.

Peer review

This is an important study comparing the appropriate interpretation of FIT in screening population and in patients referred for colonoscopy regardless of the indication. According to the authors' conclusion differences in T-stage distribution largely explained differences in FIT results between screening and referral cohorts. Therefore the absolute value of the FIT results should be reported according to T-stage.

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BRIEF ARTICLE

Different risk factors influence peptic ulcer disease development in a Brazilian population

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Abstract

AIM: To investigate age, sex, histopathology and *Helicobacter pylori* (*H. pylori*) status, as risk factors for gastroduodenal disease outcome in Brazilian dyspeptic patients.

METHODS: From all 1466 consecutive dyspeptic patients submitted to upper gastroscopy at Hospital das Clinicas of Marilia, antral biopsy specimens were obtained and subjected to histopathology and *H. pylori* diagnosis. All patients presenting chronic gastritis (CG) and peptic ulcer (PU) disease localized in the stomach, gastric ulcer (GU) and/or duodenal ulcer (DU) were included in the study. Gastric biopsies (n = 668) positive for *H. pylori* by rapid urease test were investigated for vacuolating cytotoxin A (*vacA*) medium (m) region mosaicism by polymerase chain reaction. Logistic regression analysis was performed to verify the association of age, sex, histopathologic alterations, *H. pylori* diagnosis and *vacA* m region mosaicism with the incidence of DU, GU and CG in patients.

RESULTS: Of 1466 patients submitted to endoscopy, 1060 (72.3%) presented CG [male/female = 506/554; mean age (year) ± SD = 51.2 ± 17.81], 88 (6.0%) presented DU [male/female = 54/34; mean age (year) ± SD = 51.4 ± 17.14], and 75 (5.1%) presented GU [male/female = 54/21; mean age (year) ± SD = 51.3 ± 17.12] and were included in the comparative analysis. Sex and age showed no detectable effect on CG incidence (overall χ^2 = 2.1, P = 0.3423). Sex [Odds ratios (OR) = 1.8631, P = 0.0058] but not age (OR = 0.9929, P = 0.2699) was associated with DU and both parameters had a highly significant effect on GU (overall χ^2 = 30.5, P < 0.0001). The histopathological results showed a significant contribution of ageing for both atrophy (OR = 1.0297, P < 0.0001) and intestinal metaplasia (OR = 1.0520, P < 0.0001). Presence of *H. pylori* was significantly associated with decreasing age (OR = 0.9827, P < 0.0001) and with the incidence of DU (OR = 3.6077, P < 0.0001). The prevalence of m1 in DU was statistically significant (OR = 2.3563, P = 0.0018) but not in CG (OR = 2.678, P = 0.0863) and GU (OR = 1.520, P= 0.2863).

CONCLUSION: In our population, male gender was a risk factor for PU; ageing for GU, atrophy and metapla-

sia; and *H. pylori* of *vacA* m1 genotype for DU.

Key words: *Helicobacter pylori*; Gastric ulcer disease; Duodenal ulcer disease; Gastric atrophy; *Helicobacter pylori* vacuolating cytotoxin A medium region mosaicism

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a Gram-negative microaerophylic bacterium, is associated with a broad spectrum of digestive tract diseases such as chronic gastritis, gastric and duodenal ulcers, gastric cancer and lymphoproliferative disorders[1]. *H. pylori* infection prevalence and clinical outcome of the colonized patients varies according to several considerations including bacterial factors and host and environmental characteristics such as age, ethnic group, genera, geography and socioeconomic conditions[2].

The role of *H. pylori* in the interaction with the host has an impact on the pattern and severity of gastritis and its clinical outcome[3]. The physiologic mechanisms involved in these *H. pylori*-induced pathological differences are still unknown; however, one of the major bacterium virulence factors, the vacuolating cytotoxin A (*vacA*), seems to be involved. The vacA protein encoded by the polymorphic *H. pylori vacA* gene, is produced and secreted by all bacterium strains and induces the formation of intracellular vacuoles in epithelial cell lines *in vitro*[4-6]. Polymorphism of the *vacA* gene is distributed in three principal regions: the signal (s), intermediate (i), and middle (m) regions, each being divided in two main types, numbered 1 and 2[7,8], which are differently associated with several mechanisms of pathogenicity *in vitro* and *in vivo*[6,9-15]. The s1, i1, and m1 types have been shown to be independently associated with more severe forms of *H. pylori*-induced diseases[8,16].

Studies conducted in several countries have shown that type 1 and 2 alleles of *vacA* polymorphisms are both widespread in all populations examined, except in the Japanese, among whom type 2 alleles are rare[17,18]. Thus, outside Asia, *vacA*-type 1 and *cagA*-positive *H. pylori* strains are more frequently associated with severe *H. pylori*-induced peptic ulcer diseases than *vacA*-type 2 *cagA*-negative bacterium strains[7,19-22]. In Brazil, a country of continental dimensions, this association has been observed in children[23,24] but is controversial in the adult population[25-27].

Environmental and demographic data also interfere with the pathophysiology of *H. pylori*-associated gastric diseases. In the adult population of Brazil, *H. pylori* infection can range from 60%-90%[28-31]. In Marilia, a city of São Paulo State, the serological prevalence of *H. pylori* determined in blood donors was 57% and a risk factor associated with IgG and/or IgA *H. pylori* antibodies was low educational level[32]. Considering the large geographical dimensions of Brazil, with its regionally specific socioeconomic and cultural conditions reflected by the high and variable prevalence of *H. pylori*, there are few epidemiological studies on gastric diseases.

So far, all comparative Brazilian studies on gastric disease epidemiology, *H. pylori* prevalence and *vacA* gene mosaicism have generally been carried out on small populations and on *H. pylori* strains isolated in culture. Therefore, considering that gastric and duodenal ulcer diseases depend on different physiological trigger mechanisms, we comparatively investigated the role of age, gender, histopathologic antral gastric alterations and the *H. pylori* status, including the *vacA* m region mosaicism detected directly in biopsies, as risk factors for gastric ulcer (GU), duodenal ulcer (DU) and chronic gastritis (CG) in patients consecutively attending at Hospital das Clinicas of Marilia, São Paulo, Brazil, during a period of four years.

MATERIALS AND METHODS

Patients

Adult patients (n = 1466) resident in Marilia city, São Paulo State, Brazil, aged 19 to 91 years, underwent esophagogastroduodenoscopy (EGD) for upper abdominal pain or dyspeptic symptoms from January 2003 through December 2006 at the Gastroenterology Outpatient Clinic of the Hospital das Clínicas of Marília Medical School. All who presented GU, DU (by endoscopy) and/or CG (by histology), were enrolled in this study.

Endoscopy and biopsies

The EGD was accomplished by fibroendoscope (GIF-XP20, GIF-XQ20) or video-endoscope (GIF-100), both from Olympus Medical Systems, Shinjuku-ku, Tokyo, Japan. Gastric or duodenal ulcer diagnosis was defined by endoscopy and two fragments of the antrum were collected to perform rapid urease (RUT) and histopathological tests. The biopsy used for RUT was further submitted to DNA extraction. The protocol used is in agreement with the Helsinki Declaration and was approved by the Ethical Committee in Human Research from Marilia Medical School, under reference number 388/01.

Histology

One antral specimen was fixed in 40 g/L of formaldehyde and embedded in paraffin. Sections were Giemsa stained for *H. pylori* evaluation and were stained with he-

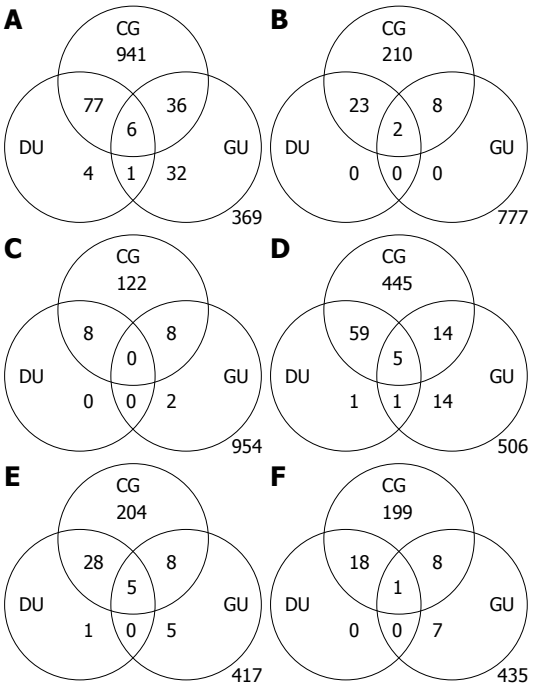

Figure 1 Incidence of chronic gastritis, duodenal ulcer and gastric ulcer disease and distribution of the investigated independent variables. A: Incidence of gastric diseases in patients who underwent endoscopy (n = 1466); B: Distribution of the histopathological parameters of atrophy (n = 1020); C: Intestinal metaplasia (n = 1094); D: Positive histological diagnosis of *Helicobacter pylori* (*H. pylori*) (n = 1045); E: *H. pylori vac*A m1 genotypes (n = 668); F: *H. pylori vacA* m2 genotypes (n = 668). CG: Chronic gastritis; DU: Duodenal ulcer; GU: Gastric ulcer; *vacA*: Vacuolating cytotoxin A. Values outside circles are negative tests.

matoxylin and eosin for assessment of histopathologic alterations according to the Sydney classification[33].

Helicobacter pylori vacA genotyping

The same biopsy used for RUT was submitted to DNA extraction with the employment of the GFx DNA extraction kit purchased from Amersham/Pharmacia Biotec, following the manufacturer's instructions. DNA was quantified in agarose gel electrophoresis using the Invitrogen low mass ladder and 50-100 μg of total DNA were used in the polymerase chain reaction (PCR) reactions with the oligonucleotides[34]: MA sense (5'CACAGCCACTTTYAATAACGA3') and MB antisense (5' CGTCAAAATAATTCCAAGGG3'), which amplify a fragment of 400 bp or 476 bp corresponding to the *vacA* m regions 1 and 2, respectively. PCR condition was 94 ℃ for 5 min followed by 40 cycles of 94 ℃ 1 min/60 ℃ 1 min/72 ℃ 1 min and one cycle at 72 ℃ 7 min, with a total volume of 25 μL containing 1 × PCR buffer, 200 μmol dNTPs, 2.0 mmol $MgCl_2$, 1 μmol of each oligonucleotide, 1.25 U Taq DNA Polimerase Platinum Brazil (Invitrogen). In all PCR reactions a negative and a positive control were used corresponding to, respectively, sterile water and *H. pylori vacA* m1 and/or m2 PCR positive gastric biopsies. The products of PCR reactions were resolved in 15 g/L agarose gels, stained with ethidium bromide and photographed under ultraviolet light.

Statistical analysis

Incidences of CG, DU, and GU in patients submitted to endoscopy were investigated using age (in years) and sex as independent factors. Only for patients who developed at least one of these diseases, the role of age, sex, CG, GU, and DU were verified as independent factors to evaluate the presence of atrophy, intestinal metaplasia, *H. pylori* and *vacA* m1 and m2 genotypes, each assessed individually. Incidences were coded as "1" and absences (of evidence) as "0" (males were also coded as "1", females as "0"). Cases without the complete records needed for each analysis were discarded. Multivariable screenings were performed by additive logistic regression models for detection of significant effects of the independent factors on each response variable. New models were made after discarding irrelevant variables and significant parameters of these last models were used to describe the relationship among factors and responses by means of logit functions. Critical P-values were considered after a Bonferroni correction based on the number of similar tests. All logistic regression analyses were performed using a free on-line device for Logistic Regression calculation provided by Pezzulo (2012)[35].

RESULTS

Among 1466 patients submitted to endoscopy, 1060 (72.3%) presented CG [male/female = 506/554; mean age (year) ± SD = 51.2 ± 17.81], 88 (6.0%) presented DU [male/female = 54/34; mean age (year) ± SD = 51.4 ± 17.14], and 75 (5.1%) presented GU [male/female = 54/21; mean age (year) ± SD = 51.3 ± 17.12], and were included in the comparative analysis. More than one of these diseases was presented by 120 (8.2%) individuals and 369 (25.2%) patients were free of them (Figure 1). Most of the other endoscopic and histopathologic alterations were related to gastroesophageal tract diseases (data not shown). Mean age and gender of the included CG, GU and GC patients are summarized.

Distribution of atrophy, intestinal metaplasia, *H. pylori* histological diagnosis and *vacA* m region mosaicism in patients presenting CG, DU and GU are summarized in Figure 1.

Atrophy and intestinal metaplasia were investigated among antral gastric biopsies of 1020 and 1094 patients, respectively, with 243 (23.8%) positive for atrophy and 140 (12.8%) for intestinal metaplasia.

Detection of *H. pylori* was performed directly from biopsy specimens by two different tests: histology and RUT. Histology is the gold standard *H. pylori* diagnostic test employed in our clinical routine which together with histopathological analysis is used to decide for *H. pylori* eradication therapy. RUT showed a very low positive predictive value for *H. pylori*-associated gastric diseases and a high discrepancy when compared to histology; consequently these data were excluded from the study (data not shown). Among 1045 patients investigated for *H. pylori* by histology, 539 (51.6%) were positive.

Among 668 biopsies of patients positive for RUT in-

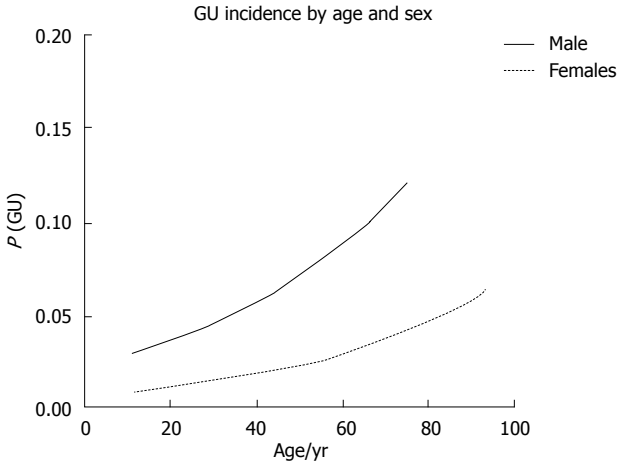

Figure 2 Logistic regression curves of age and sex as independent factors to explain gastric ulcer disease incidence. The model has a significant fit, with contributions of both coefficients (χ^2 = 30.5, P < 0.0001). Additional test for the age/sex interaction had a nonsignificant result (β_3 = -0.011, SE = 0.0161, P = 0.4844, OR = 0.989, CI- = 0.958, CI+ = 1.021). OR: Odds ratios; CI: Confidence intervals; GU: Gastric ulcer.

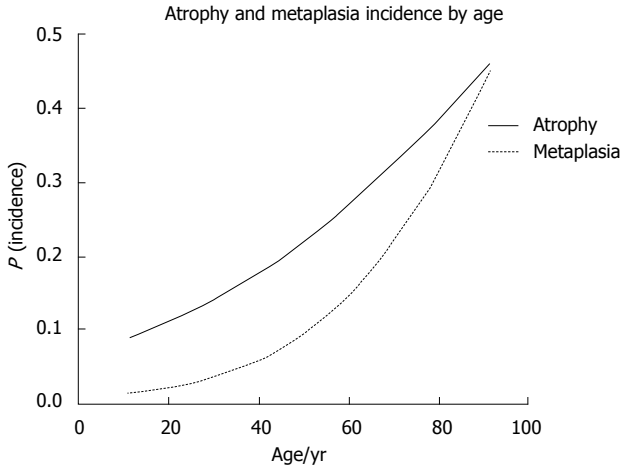

Figure 3 Logistic regression curves of age as an independent factor to explain atrophy and intestinal metaplasia incidences. All patients who underwent endoscopy and who had at least one of the three diseases were included in the analysis. Both models had highly significant fits (atrophy OR = 1.0297, P < 0.0001; intestinal metaplasia OR = 3.6077, P < 0.0001). OR: Odds ratios.

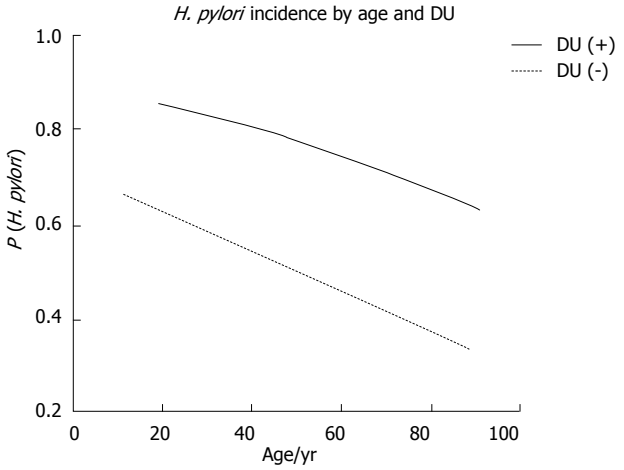

Figure 4 Logistic regression curves of age and duodenal ulcer disease as independent factors to explain *Helicobacter pylori* incidence. The model had a highly significant fit (age OR = 0.9827, P < 0.0001; DU OR = 3.6077, P < 0.0001). A posterior test for the age/DU interaction had a nonsignificant result (β_3 = 0.0085, SE = 0.0168, P = 0.6142, OR = 1.0085, CI- = 0.9758, CI+ = 1.0423). DU: Duodenal ulcer; OR: Odds ratios; CI: Confidence intervals; *H. pylori*: *Helicobacter pylori*.

vestigated for *vacA* m region mosaicism by PCR directly on biopsy specimens, 484 were positive, with 251 (51.8%) of m1 and 233 (48.2%) of m2 genotypes.

Results of the additive logistic regression models to evaluate the contribution of age and sex to the incidence of CG, DU and GU among patients who underwent gastroscopy are summarized in Table 1. Sex and age showed no detectable effect on CG incidence (overall χ^2 = 2.1, P = 0.3423). Sex (OR = 1.8631, P = 0.0058) but not age (OR = 0.9929, P = 0.2699) affected DU, and both parameters had a highly significant association with GU (overall χ^2 = 30.5, P < 0.0001), with contributions of both coefficients (age OR = 1.0233, P = 0.0017; sex OR = 3.0790, P < 0.0001). A posterior test for the age/sex interaction showed it to be nonsignificant (OR = 0.9888, P = 0.4844). The incidence of GU increased with age and further, at a given age, males had a higher probability of developing GU (Figure 2).

Additive logistic regression models were constructed to investigate the contribution of age, sex, CG, DU and GU to atrophy, intestinal metaplasia, *H. pylori* presence and *vacA* m1 and m2 mosaicism (Table 2).

The results for histopathological parameters showed a significant contribution of age for both atrophy (OR = 1.0297, P < 0.0001) and intestinal metaplasia (OR = 1.0520, P < 0.0001). Atrophy incidence increased with age (Figure 3) and a new model with only age as the independent factor also had a highly significant fit (overall χ^2 = 35.2, P < 0.0001; age OR = 1.0273, P < 0.0001) though more parsimonious (Table 2). In the case of intestinal metaplasia, it was necessary to check for the contribution of CG, which was discarded after the Bonferroni correction. A new model with only age as the independent factor also had a highly significant fit (overall χ^2 = 71.7, P < 0.0001; age OR = 1.0509, P < 0.0001) though more parsimonious (Table 2). Intestinal metaplasia increased with age, but at a lower level than atrophy for a given age (Figure 3).

Presence of *H. pylori* was significantly associated with age (OR = 0.9827, P < 0.0001) and occurrence of DU (OR = 3.6077, P < 0.0001) (Table 3). A new model with only age and DU as independent factors also had a highly significant fit (overall χ^2 = 47.8, P < 0.0001; age OR = 0.9826, P < 0 .0001; DU OR = 3.5063, P < 0.0001) but was more parsimonious (Table 3). An additional model showed that age/DU had no effect on the presence of *H. pylori* (β_3 = 0.0085, SE = 0.0168, P = 0.6142, OR = 1.0085, CI- = 0.9758, CI+ = 1.0423). *H. pylori* incidence decreased with age and DU incidence contributed to higher probabilities in developing detectable levels of the bacterium, despite individual age (Figure 4).

The five independent factors studied for the *H. pylori* *vacA* m1 and m2 genotypes resulted in nonsignificant ad-

Table 1 Additive logistic regression models to evaluate the contribution of age and sex among patients who underwent gastroscopy (n_{total} = 1466)

Factor		CG	DU	GU
Overall	n (+)	1060	88	75
	χ^2 (v = 2)	2.14	8.99	30.51
	P	0.3423	0.0112[1]	< 0.0001[1]
	β_0	0.738	-2.724	-4.836
Age	β_1	0.003	-0.007	0.023
	SE	0.0034	0.0065	0.0073
	P	0.3511	0.2699	0.0017[1]
	OR	1.003	0.993	1.023
	CI-	0.997	0.980	1.009
	CI+	1.010	1.006	1.038
Sex	β_2	0.124	0.622	1.125
	SE	0.1102	0.2256	0.2636
	P	0.2598	0.0058[1]	< 0.0001[1]
	OR	1.132	1.863	3.079
	CI-	0.912	1.197	1.837
	CI+	1.405	2.899	5.162

[1]Significant effects. n_{total}: Number of investigated cases; n (+): Number of positive cases; CG: Chronic gastritis; DU: Duodenal ulcer; GU: Gastric ulcer; OR: Odds ratios; CI: Confidence intervals. Critical P-values were adopted after a Bonferroni correction for the number of similar tests: P = 0.05/3 = 0.0167 for overall models; and P = 0.05/2 = 0.025 for each independent factor.

Table 2 Logistic regression models to evaluate the contribution of age, sex, chronic gastritis, duodenal ulcer

Factor		Atrophy	Metaplasia	*H. pylori*	m1	m2
Overall	n_{total}	1020	1094	1045	668	668
	n (+)	243	140	539	251	233
	χ^2	35.18	71.67	47.83	9.88	5.85
	v	1	1	2	1.00	1
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	0.0017[1]	0.0156
	β_0	-2.614	-4.733	0.881	-0.589	-0.038
Age	β_1	0.027	0.050	-0.018	-	-0.012
	SE	0.0047	0.0063	0.0039	-	0.0048
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	-	0.0162
	OR	1.027	1.051	0.983	-	0.989
	CI-	1.018	1.038	0.975	-	0.979
	CI+	1.037	1.064	0.990	-	0.998
DU	β_2	-	-	1.255	0.857	-
	SE	-	-	0.2702	0.2739	-
	P	-	-	< 0.0001[1]	0.0018[1]	-
	OR	-	-	3.506	2.356	-
	CI-	-	-	2.065	1.377	-
	CI+	-	-	5.954	4.031	-

[1]Significant effects after Bonferroni correction. n_{total}: Number of investigated cases; n (+): Number of positive cases; CG: Chronic gastritis; DU: Duodenal ulcer; OR: Odds ratios; CI: Confidence intervals; *H. pylori*: *Helicobacter pylori*.

ditive regression logistic models after Bonferroni correction. However, the evaluation of the contribution of the variables indicates a possible effect of DU (Table 3). A new model with only DU as the independent factor had a highly significant fit (overall χ^2 = 9.9, P = 0.0017) (Table 2). The incidence probability of m1 in DU patients was 0.5667 and in DU negative patients was 0.3569 (OR = 2.3563, P = 0.0018). In the case of m2, the evaluation of the variable contributions indicated a possible effect of age (Table 2). A new model with only age as the independent factor also had a nonsignificant fit, and was discarded by the adopted critical P-value (overall χ^2 = 5.8, P = 0.0156; age OR = 0.9885, P = 0.0162) (Table 2).

DISCUSSION

In order to verify the differential contribution of age, gender, histopathological outcome, *H. pylori* and *vacA* m region mosaicism with incidence of PU and CG, we investigated comparatively all cases of DU (88), GU (75) and CG (1060) found after analysis of 1466 patients consecutively submitted to gastroscopy in Hospital das Clinicas of Marilia, São Paulo, Brazil, over four years.

The most prevalent gastric disease found in our consecutive dyspeptic patients was CG (72.3%) followed by gastroesophageal alterations not included in this study. Peptic ulcer disease was prevalent in 11.1% of the patients, with 6.0% of GU and 5.1% of DU. Male gender had a statistically significant association with both PU incidences. Our results are in accordance with research done in the southern region of Brazil where DU and GU had a similar prevalence and were associated with male gender[36]. However, in a recent large scale PU epidemiological study carried out in a tertiary care hospital in another city of São Paulo State, Brazil, the prevalence of DU was four times higher than GU[37] and in this population there was a significant predominance of woman in the PU group[38]. These regional differences reinforce the specificity of risk factors associated with severe gastric diseases and the need to perform local investigations to improve health care strategies.

There are differences in the gastrointestinal physiological modifications that lead to gastric and duodenal ulcers, whose causes are multifactorial and also related to population characteristics and to specific gastroduodenal alterations due to association of *H. pylori* with host mucosa[39]. In our study, increasing age was a risk factor for the development of GU. The mean age of GU and DU patients in the Brazilian ulcer study performed in a hospital in São Paulo also differed significantly, being higher in GU[37]. Thus, more epidemiological studies have to be done in order to identify the risk factors associated with age and the onset of GU in the Marilia population.

The eradication of *H. pylori* infection cured both gastric and duodenal ulcers, and the cure rates are similar, suggesting that *H. pylori* is the key factor in peptic ulcer diseases independent of the ulcer site[40]. However, a number of studies have shown the participation of other risk factors such as smoking, alcohol intake, and nonsteroidal antiinflammatory drug (NSAID) use in the etiology of PU, which are principally associated with GU[39,41,42]. In our study the prevalence of *H. pylori* infection observed by histology was significantly higher in DU than in GU and CG (P < 0.0001). Also, in the Danish epidemiologic PU study, IgG against *H. pylori* was higher in DU (87.2%) when compared to GU (60%) patients. In another city of São Paulo, there was also

Table 3 Additive logistic regression models to evaluate the contribution of age, sex, chronic gastritis, duodenal ulcer and gastric ulcer disease

Factor		Atrophy	Metaplasia	*H. pylori*	m1	m2
Overall	n_{total}	1020	1094	1045	668	668
	n(+)	243	140	539	251	233
	$\chi^2(v = 5)$	64.34	78.63	50.14	13.46	10.64
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	0.0194[1]	0.0591[1]
	β_0	-24.434	-6.616	0.832	-1.448	-0.225
Age	β_1	0.029	0.051	-0.017	-0.003	-0.012
	SE	0.0047	0.0064	0.0039	0.0048	0.0048
	P	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	0.5646	0.0139[1]
	OR	1.030	1.052	0.983	0.997	0.988
	CI-	1.020	1.039	0.975	0.988	0.979
	CI+	1.039	1.065	0.990	1.007	0.998
Sex	β_2	0.064	0.101	-0.179	0.035	-0.327
	SE	0.1530	0.1906	0.1283	0.1635	0.1659
	P	0.6775	0.5969	0.1629	0.8320	0.0485
	OR	1.066	1.106	0.836	1.035	0.721
	CI-	0.790	0.761	0.650	0.752	0.521
	CI+	1.438	1.607	1.075	1.426	0.998
CG	β_3	21.676	1.807	0.135	0.985	0.378
	SE	> 100	0.8427	0.4533	0.5742	0.5710
	P	0.9978	0.0320	0.7661	0.0863	0.5082
	OR	> 100	6.090	1.144	2.678	1.459
	CI-	0.000	1.168	0.471	0.869	0.477
	CI+	> 100	31.761	2.782	8.253	4.469
DU	β_4	0.552	-0.313	1.283	0.856	-0.175
	SE	0.2658	0.3972	0.2712	0.2771	0.2931
	P	0.0377	0.4308	< 0.0001[1]	0.0020[1]	0.5496
	OR	1.737	0.731	3.608	2.353	0.839
	CI-	1.032	0.336	2.120	1.367	0.472
	CI+	2.924	1.593	6.139	4.050	1.491
GU	β_5	-0.065	0.483	-0.032	0.419	0.267
	SE	0.3825	0.4260	0.3185	0.3927	0.4038
	P	0.8660	0.2567	0.9200	0.2863	0.5083
	OR	0.938	1.621	0.969	1.520	1.306
	CI-	0.443	0.704	0.519	0.704	0.592
	CI+	1.984	3.736	1.808	3.282	2.883

[1]Significant effects. n_{total}: Number of investigated cases; n (+): Number of positive cases; CG: Chronic gastritis; DU: Duodenal ulcer; GU: Gastric ulcer; OR: Odds ratios; CI: Confidence intervals; *H. pylori*: *Helicobacter pylori*. Critical *P*-values were adopted after a Bonferroni correction for the number of similar tests: $P = 0.05/5 = 0.01$ for overall model and for each independent factor.

found to be a higher prevalence of *H. pylori* in DU (64%) than in GU (57%) patients[37]. These results suggest the participation of a major number of risk factors not associated with *H. pylori* involved in the development of GU rather than DU. Moreover, a very important and large scale follow-up study of ulcer patients performed in Europe[43] showed that GU can be a risk factor in the development of gastric cancer, while in DU this relationship was inversely observed, which suggests that GU and gastric cancer have etiologic factors in common that are not found in DU.

As a consequence of *H. pylori* gastric mucosa colonization, gastric acid secretion is altered with it being induced or impaired in response to the release of factors produced or induced by the bacterium, resulting in different topographic phenotypes of gastritis and the presence of atrophy. Gastritis associated with atrophy in the corpus is accompanied by hypochlorhydria and carries the highest risk for GU and cancer, whereas hyperchlorhydria produced by gastritis in the antrum predisposes to DU[43,44]. In our patients, antrum atrophy was more prevalent in DU than in GU, corroborating the hypothesis that DU can be a result of antral gastric atrophy. However, increasing age and not *H. pylori* presence was significantly related to the occurrence of atrophy and intestinal metaplasia. These results are in agreement with an epidemiological study performed in a rural population of Korea[45] and also in previous gastric physiological studies which have demonstrated the association of atrophic gastritis with increasing age[46,47].

In Northern Peninsular Malaysia, the prevalence of *H. pylori* increased in adult old-age groups, even when old and geriatric adults were compared[48]. In our investigated population, *H. pylori* incidence decreased significantly with age (Figure 4). These results can be indicative of a populational specific characteristic associated with the clearing of *H. pylori* during the course of a chronic infection, or can be related to the diagnosis of *H. pylori* in antrum biopsies since the expression of gastritis in the antrum, but not in the cardia or corpus, seems to decrease with age[49]. New research is necessary to answer these questions.

Polymorphisms in the m region of the *H. pylori vacA* gene affect the cell tropism of the toxin[50]; the m1 type of *vacA* shows toxicity toward a broader range of cells than the m2 type[51,52]. In Asia, where there is a high predominance of s1 alelle in the s region polymorphism of the *vacA* gene, the *vacA* m region mosaicism shows a variation within East Asia[53], with m1 strains being more prevalent in regions where there is a higher prevalence of gastric cancer, suggesting that m1 strains of *H. pylori* are more pathogenic. In Brazil, the involvement of *vacA* gene mosaicism with gastric diseases in adults is controversial[25-27]; in all these studies the size of investigated patient populations has been small and bacterium strains were isolated before genotyping which can cause a bias due to selection pressure by *in vitro* growth conditions. Our work was performed during a period of four years and the investigation of *vacA* m region mosaicism was done directly on biopsy specimens by PCR. Our results are the first in Brazil to find association of *vacA* m1 allele specific to adult DU patients when compared to GU and CG ($P < 0.0018$), indicating that in our region high prevalence of *H. pylori* and the strains harboring the *vacA* m1 genotype are more frequently associated with the development of DU.

Nowadays, PU remains the cause of significant morbidity, especially in older age groups, representing an important world health problem[54]. Its etiology in either the stomach (GU) or duodenum (DU) is multifactorial and depends on the interplay of a gastritis phenotype and of physiological gastroduodenal alterations as a result of environment, *H. pylori* genetic background and host interactions, which vary regionally. In this study we found that PU is associated with male gender when compared to CG and that there were risk factor differences associated with DU and GU. *H. pylori* and *vacA* m1 genotype

were associated with DU while older age at disease commitment was associated with GU. Thus, in spite of the few large scale Brazilian studies on epidemiological characteristics of gastric diseases with stratification of PU in GU and DU, we find high regional variation, indicating that local population investigation has to be carried out in order to improve treatment and prevention of severe gastric diseases.

COMMENTS

Background

Helicobacter pylori (*H. pylori*), a Gram-negative microaerophylic bacterium, is associated with a broad spectrum of digestive tract diseases such as chronic gastritis, gastric and duodenal ulcers, gastric cancer and lymphoproliferative disorder. *H. pylori* infection prevalence and clinical outcome of the colonized patients varies according to several considerations including bacterial factors and host and environmental characteristics such as age, ethnic group, genera, geography and socioeconomic conditions.

Research frontiers

The physiologic mechanisms involved in these *H. pylori*-induced pathological differences are still unknown; however, one of the major bacterium virulence factors, the vacuolating cytotoxin A (*vacA*), seems to be involved. The s1, i1, and m1 types have been shown to be independently associated with more severe forms of *H. pylori*-induced diseases.

Innovations and breakthroughs

Gastric biopsies positive for *H. pylori* by rapid urease test were investigated for *vacA* medium (m) region mosaicism by polymerase chain reaction. Logistic regression analysis was performed to verify the association of age, sex, histopathologic alterations, *H. pylori* diagnosis and *vacA* m region mosaicism.

Applications

These results can be indicative of a populational specific characteristic associated with the clearing of *H. pylori* during the course of a chronic infection, or can be related to the diagnosis of *H. pylori* in antrum biopsies since the expression of gastritis in the antrum, but not in the cardia or corpus, seems to decrease with age. New research is necessary to answer these questions.

Peer review

This is a good descriptive study in which authors analyze age, sex, histopathology and *H. pylori* status, as risk factors for gastroduodenal disease outcome in Brazilian dyspeptic patients. The results are interesting and suggest that male gender was a risk factor for peptic ulcer; ageing for gastric ulcer, atrophy and metaplasia; and *H. pylori* of *vacA* m1 genotype for duodenal ulcer.

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BRIEF ARTICLE

Argon plasma coagulation for superficial esophageal squamous-cell carcinoma in high-risk patients

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Abstract

AIM: To evaluate the usefulness and safety of argon plasma coagulation (APC) for superficial esophageal squamous-cell carcinoma (SESC) in high-risk patients.

METHODS: We studied 17 patients (15 men and 2 women, 21 lesions) with SESC in whom endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), and open surgery were contraindicated from March 1999 through February 2009. None of the patients could tolerate prolonged EMR/ESD or open surgery because of severe concomitant disease (e.g., liver cirrhosis, cerebral infarction, or ischemic heart disease) or scar formation after EMR/ESD and chemoradiotherapy. After conventional endoscopy, an iodine stain was sprayed on the esophageal mucosa to determine the lesion margins. The lesion was then ablated by APC. We retrospectively studied the treatment time, number of APC sessions per site, complications, presence or absence of recurrence, and time to recurrence.

RESULTS: The median duration of follow-up was 36 mo (range: 6-120 mo). All of the tumors were macroscopically classified as superficial and slightly depressed type (0-Ⅱc). The preoperative depth of invasion was clinical T1a (mucosal cancer) for 19 lesions and clinical T1b (submucosal cancer) for 2. The median treatment time was 15 min (range: 10-36 min). The median number of treatment sessions per site was 2 (range: 1-4). The median hospital stay was 14 d (range: 5-68 d). Among the 17 patients (21 lesions), 2 (9.5%) had recurrence and underwent additional APC with no subsequent evidence of recurrence. There were no treatment-related complications, such as bleeding or perforation.

CONCLUSION: APC is considered to be safe and effective for the management of SESC that cannot be resected endoscopically because of underlying disease, as well as for the control of recurrence after EMR and local recurrence after chemoradiotherapy.

Key words: Argon plasma coagulation; Superficial esophageal cancer; Squamous-cell carcinoma; High-risk patient; Endoscopic therapy

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INTRODUCTION

In Japan, 329 314 persons died of malignant neoplasms in 2006; esophageal cancer was the cause of death in 11 345 persons (3.45%). Esophageal cancer is the sixth most common type of cancer in men[1]. Squamous-cell carcinoma accounts for 90% of all esophageal carcinomas. Other histologic types, including adenocarcinoma, account for approximately 5%[2]. Approximately 90% of all esophageal cancers arise in the thoracic esophagus, and the middle thoracic esophagus is the most frequent location.

Progress in endoscopic diagnostic techniques, such as iodine staining and magnifying endoscopy with narrow band imaging, has led to the detection of increased numbers of superficial carcinomas of the esophagus[3-8]. In Japan, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) are widely used as standard procedures for the treatment of superficial esophageal squamous-cell carcinoma (SESC)[9-13]. Radiofrequency ablation (RFA) is used as an endoscopic treatment for superficial esophageal cancer in Western countries, but it is not used in Japan because this procedure is not covered by the National Health Insurance system for the treatment of superficial esophageal carcinoma[14].

Several studies have reported that argon plasma coagulation (APC) is useful for the management of Barrett's esophagus[15], Barrett's esophageal cancer[16] and early gastric cancer[17,18], but few studies have evaluated APC in patients with SESC[19,20]. We retrospectively studied the safety and usefulness of APC for SESC in high-risk patients with underlying diseases that precluded EMR and ESD and in those in whom APC was performed to control local recurrence after EMR or chemoradiotherapy.

MATERIALS AND METHODS

The study group comprised 17 patients (15 men and 2 women; 21 lesions) who underwent ablation of their tumors by APC at the Department of Gastroenterology, Kitasato University East Hospital, from March 1999 through February 2009 (Table 1). The median age of the subjects was 68 years (range: 57-80 years). At the time of diagnosis, 11 patients were asymptomatic, and SESC was diagnosed incidentally on screening examinations for other diseases (peptic ulcer in 5 patients, esophageal varices in 2, gastritis in 2, after surgery for gastric cancer in 1, and after surgery for tongue cancer in 1). The other 6 patients exhibited symptoms (epigastric pain in 3, heartburn in 2, and hoarse voice in 1), and SESC was diagnosed upon further evaluation. All of the tumors were macroscopically classified as superficial and slightly depressed type (0-Ⅱc). The tumor diameter ranged from approximately 1 cm to approximately half of the circumference of the esophagus. The preoperative depth of invasion was clinical T1a (mucosal cancer) for 19 lesions and clinical T1b (submucosal cancer) for 2. Histopathologically, all of the tumors were confirmed to be esophageal squamous-cell carcinomas. EMR and ESD could not be performed because of underlying disease in 11 patients (15 lesions). APC was used to treat local recurrence after EMR or multiple metachronous tumors in 4 patients (4 lesions) and to manage local recurrence after chemoradiotherapy in 2 patients (2 lesions). Of the 17 patients, 13 (76.5%) had the following underlying diseases: liver cirrhosis in 5 patients, heart disease in 4, cerebral infarction in 1, after right lung resection with thrombophlebitis in 1, colon cancer operation in 1, and dementia in one patient. After informed consent was obtained from patients in whom anticoagulants could not be discontinued, those in whom prolonged endoscopic therapy was precluded by respiratory failure, and a patient aged 80 years, these patients asked to undergo APC among other possible treatment options. APC was therefore performed.

Table 1 Patient and lesion characteristics

Gender (men/women)	15/2
Mean age (range), yr	68 (57-80)
Location Ce/Ut/Mt/Lt/Ae	0/0/14/7/0
Macroscopic type	0-Ⅱc 21
Depth of invasion	T1a (m) 19
	T1b (sm) 2
Previous treatment	6 (35.3%) (EMR 3, ESD 1, and CRT 2)
Concomitant disease	13 (76.5%) (liver cirrhosis 5, heart disease 4, cerebral infarction 1, other 3)

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; CRT: Chemoradiotherapy.

Before APC, endoscopy was performed to evaluate the macroscopic characteristics of the tumor. Next, 1.5% Lugol's solution (a diluted iodine-potassium solution) was sprayed on the mucosa to assess the lesion margins, and a biopsy specimen was taken and examined histopathologically. Endoscopic ultrasonography (probe, UM-DP20-25R, Olympus, Tokyo, Japan) was also performed before treatment to assess the depth of invasion. The absence of metastasis was confirmed by computed tomography. All of the tumors were macroscopically classified as superficial in type according to the Guidelines for Clinical and Pathologic Studies on Carcinoma of the Esophagus by the Japanese Society for Esophageal Disease[21]. If possible, oral anticoagulants and antiplatelet agents were discontinued before treatment. APC was performed using an Olympus videoscope, a high-frequency oscillator unit (Erbotom ICC200, 1999 to 2004 or APC 2, 2004 to 2009, Elektromedizin Co. Ltd., Tuebingen, Germany), an argon gas supply unit, and a flexible argon plasma coagulator with an argon gas flow of 2 L/min and a high-frequency output of 40 W. The treatment technique was as follows. After conventional endoscopy, the lesion was stained with iodine (Figure 1A, B), and its circumference was marked by APC (Figure 1C). Subsequently, the entire

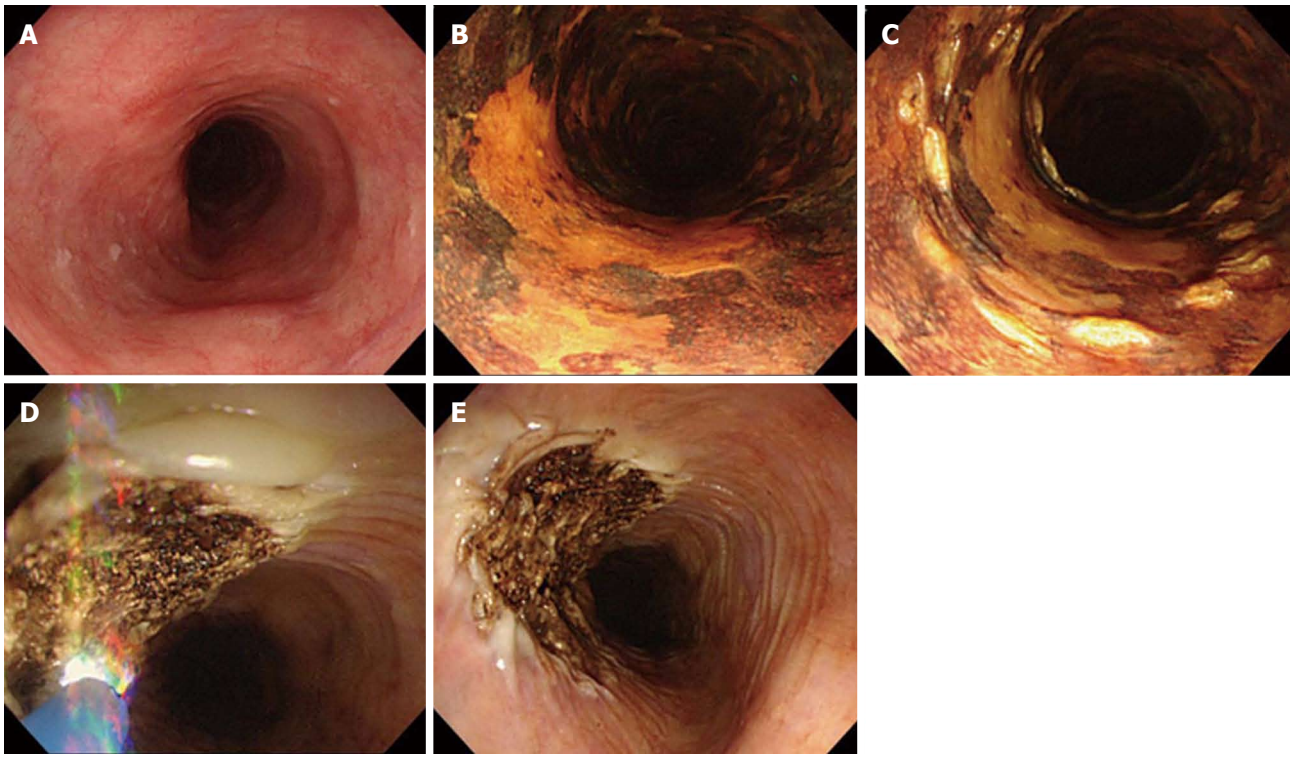

Figure 1 Treatment procedure using argon plasma coagulation. A: Conventional endoscopy; B: Chromoendoscopy with iodine staining; C: Marking by argon plasma coagulation (APC); D: Marked area was treated by APC; E: The region was completely coagulated.

Table 2 Patients characteristics

No.	Age (yr)	Sex	Depth	Time (min)	Times	Hospitalization days	Interval until recurrence (mo)	Retreatment	Illness
1	66	M	m	14	1	11	-	-	After right lung resection, thrombophlebitis[1]
2	59	M	m m	22	4	35	-	-	LC (HCV), esophageal varices
3	68	M	m	26	4	68	7	APC	LC (alcoholic), IHD, remnant stomach
4	67	M	m1	30	1	12	-	-	OMI (three vessel stenosis)[1]
5	67	M	m	15	1	53	-	-	LC (HCV), HCC, esophageal varices, DM
6	79	M	m	10	1	13	-	-	Dementia
7	66	M	sm	34	2	47	-	-	LC (alcoholic), esophageal varices, DM
8	60	M	m	16	2	14	-	-	Arrhythmia, mitral regurgitation
9	74	M	m1	14	3	11	-	-	Cerebral infarction
10	66	M	m2	36	2	17	-	-	Post tongue cancer operation
11	71	M	m1 m1 m1 m1	28	2	12	-	-	Multiple SESC
12	68	F	m1	15	1	0	-	-	Post colon cancer operation
13	76	M	m1	15	1	5	-	-	lesion of near after ESD scar
14	73	M	m1	15	1	0	-	-	Recurrence after EMR
15	70	M	m1	25	2	16	-	-	IHD[1]
16	57	M	sm1	12	4	12	3	APC	LC, recurrence after CRT
17	80	F	m2	15	2	14	-	-	Senior age, IHD, recurrence after CRT

[1]Anticoagulant therapy; m: Mucosa; sm: Submucosa; m1: Epithelium; m2: Lamina propria mucosae; sm1: Superficial layer of submucosa; APC: Argon plasma coagulation; LC: Liver cirrhosis; IHD: Ischemic heart disease; OMI: Old myocardial infarction; HCC: Hepatocellular carcinoma; DM: Diabetes mellitus; SESC: Superficial esophageal squamous-cell carcinoma; ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; CRT: Chemoradiotherapy.

lesion inside the marked area was ablated until it turned black (Figure 1D, E). If residual tumor was suspected on follow-up endoscopy 1 wk after APC, the procedure was repeated, exercising care to avoid excessive ablation. Thereafter, follow-up endoscopy was performed at approximately 2 mo, 6 mo, 12 mo, 18 mo, 24 mo, and 36

Table 3 Outcomes of endoscopic argon plasma coagulation treatment (n = 21)

Median duration of follow up (mo)	36 (6-120)
Median time required for treatment (min)	15 (10-36)
Median number of treatment sessions(times)	2 (1-4)
Median duration of hospitalization (d)	14 (5-68)
Complications	
Postoperative bleeding	0
Perforation	0
Residual tumor and recurrence	2 (9.5%)

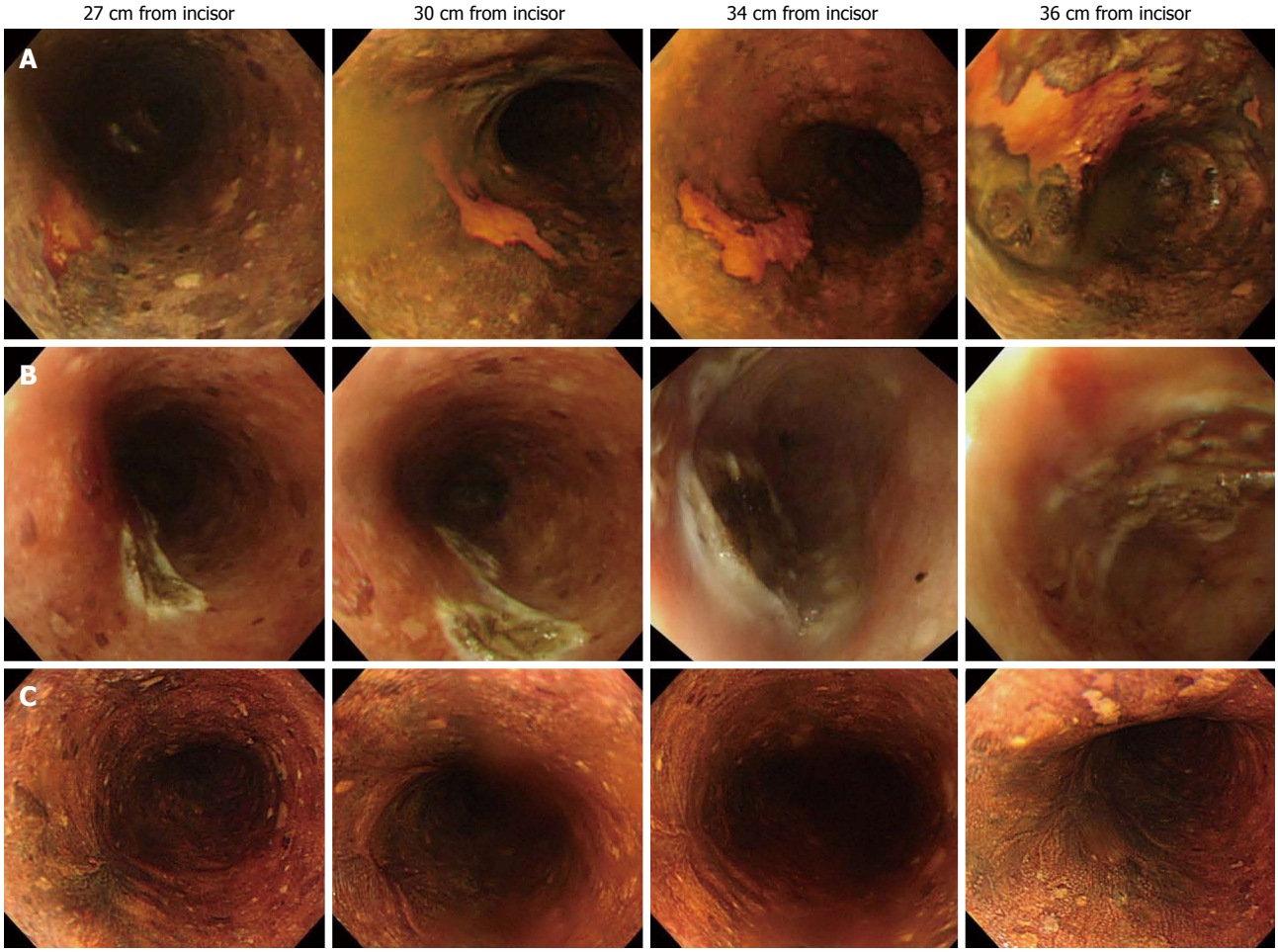

Figure 2 Case presentation: Superficial esophageal carcinoma with four lesions. A: Endoscopic findings before treatment; B: Endoscopic findings after treatment; C: Endoscopic findings 2 years after treatment.

mo. Recurrence was confirmed by endoscopic examination after the application of iodine staining and biopsy. We retrospectively studied the treatment time, the number of APC sessions per site, the presence or absence of recurrence, the time to recurrence, and complications. The median follow-up period was 36 mo (range: 6-120 mo). Informed consent was obtained from all patients in accordance with the ethical guidelines of the Declaration of Helsinki.

RESULTS

The patients' demographic characteristics are shown in Tables 1 and 2. The results of treatment and follow-up are shown in Table 3. The median time per one-treatment session was only 15 min (range: 10-36 min). The median number of treatment sessions per site was 2 (range: 1-4). There were no treatment-related complications, such as bleeding or perforation. The median duration of follow-up was 36 mo (range: 6-120 mo). Among the 21 lesions, 19 (90.0%) showed no evidence of recurrence. The 2 other lesions recurred locally 3 mo and 7 mo after APC, respectively. Both recurrences were treated by repeat APC ablation. At the time of this writing, 1 patient had died of tongue cancer and the others were alive, with no distinct evidence of metastasis or recurrence.

Case presentation

The patient was a 71-year-old man who had 4 lesions. APC was selected for treatment because prolonged ESD would have been difficult. Superficial and slightly depressed type (0-Ⅱc) lesions unstained with iodine staining were found at 4 sites, located 27 cm, 30 cm, 34 cm, and 36 cm from the incisor teeth (all of the lesions were classified as clinical T1a) (Figure 2A). APC ablation was performed at all 4 sites (Figure 2B). Follow-up endoscopy after 2 years showed no lesions *via* iodine staining and no other evidence of recurrence (Figure 2C).

DISCUSSION

APC is a non-contact technique that uses argon gas to induce tissue coagulation. In the field of gastrointestinal endoscopy, APC has been used to arrest gastrointestinal bleeding[22], ablate Barrett's esophagus[14], perform sclerotherapy of esophageal varices, and treat early gastric cancer[17,18]. At present, EMR and ESD are the endoscopic treatments of choice for superficial esophageal cancer, and studies of APC in patients with SESC are scarce[19,20].

However, EMR and ESD are associated with an increased risk of bleeding in patients with liver cirrhosis or esophageal varices and those who are receiving anticoagulant therapy[23]. Akiyama *et al*[24] used endoscopic variceal ligation for minute cancer of the esophagogastric junction accompanied by esophageal varices associated with a high risk of bleeding. ESD is thus often difficult to perform safety[25,26].

In this study, we evaluated the usefulness and safety of APC in 11 patients (15 lesions) in whom EMR was precluded by concurrent disease, 4 with recurrence after EMR, and 2 with local recurrence after chemoradiotherapy. Of the 17 patients, 13 (76.5%) had underlying diseases, such as cardiopulmonary disease and liver cirrhosis (Table 1). Two patients (9.5%) experienced tumor recurrence but received repeat APC with no subsequent recurrence. No patient experienced complications such as bleeding or perforation.

Gastrointestinal perforation caused by APC has been reported[27,28]. Grund *et al*[29] performed APC in a large series of patients and reported that the incidences of gastrointestinal perforation and intestinal emphysema were 0.25% and 0.46%, respectively. The possibility of complications should thus be borne in mind when APC ablation is performed.

Patients with recurrence after EMR or local recurrence after chemoradiotherapy have scars caused by prior treatment, increasing the risk of perforation on repeat EMR. Nomura *et al*[30] used APC to control local recurrence after chemoradiotherapy and recurrence after EMR. Both conditions could be treated safely within a short time. Two of 10 patients experienced recurrence, but APC exhibited a low risk of perforation and allowed oral intake to be resumed earlier than EMR. APC was therefore recommended for patients in whom EMR is difficult to perform. In our study, APC was completed safely and within a short time.

Photodynamic therapy is another option for the endoscopic treatment of superficial esophageal cancer but requires pretreatment and light shielding after application[31,32]. In contrast, APC is more convenient and straightforward, with a simple treatment procedure.

In conclusion, our results showed that APC effectively ablated SESC in patients with underlying disease and in those in whom EMR and ESD were not feasible. By repeating APC as required, the tumors were reliably ablated. APC is thus considered to be a safe and effective treatment that can be accomplished within a relatively short time. To confirm our results, additional prospective studies of larger numbers of patients are needed.

COMMENTS

Background

In Japan, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) are standard treatments for superficial esophageal squamous-cell carcinoma (SESC). However, the use of these procedures is contraindicated by underlying disease in certain patients.

Research frontiers

Argon plasma coagulation (APC) is useful for the management of Barrett's esophageal cancer and early gastric cancer, but few studies have evaluated APC in patients with SESC.

Innovations and breakthroughs

APC effectively ablated SESC in patients with underlying disease, as well as in those for whom EMR and ESD were not feasible.

Applications

APC is considered to be safe and effective for the management of superficial carcinoma of the esophagus that cannot be resected endoscopically because of underlying disease, as well as for the control of recurrence after EMR and local recurrence after chemoradiotherapy.

Peer review

In this study, the authors performed retrospective study that assessed the clinical usefulness and safety of APC for SESC in high risk patient. This study showed APC would be one of the good option to treat SESC especially in high risk patient, although further prospective study in large sample size is necessary to conclude it.

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BRIEF ARTICLE

Hepatitis B virus pre-S2 start codon mutations in Indonesian liver disease patients

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Abstract

AIM: To identify the prevalence of pre-S2 start codon mutations and to assess their association with liver disease progression.

METHODS: The mutations were identified by direct sequencing from 73 asymptomatic carriers, 66 chronic hepatitis (CH), 66 liver cirrhosis (LC) and 63 hepatocellular carcinoma (HCC) patients. Statistical significances were determined using Fisher's exact test, χ^2 test, and *t*-test analyses whenever appropriate. Pre-S mutation as a risk factor for advanced liver disease was estimated by unconditional logistic regression model adjusted with age, sex, and hepatitis B e antigen (HBeAg). $P < 0.05$ was considered significant.

RESULTS: Mutation of the hepatitis B virus (HBV) pre-S2 start codon was found in 59 samples from 268 subjects (22.0%), with higher prevalence in patients with cirrhosis 27/66 (40.9%) followed by HCC 18/63 (28.6%), chronic hepatitis 12/66 (18.2%) and asymptomatic carriers 2/73 (2.7%) ($P < 0.001$). Logistic regression analysis showed that pre-S2 start codon mutation was an independent factor for progressive liver disease. Other mutations, at T130, Q132, and A138, were also associated with LC and HCC, although this was not statistically significant when adjusted for age, sex, and HBeAg. The prevalence of pre-S2 start codon mutation was higher in HBV/B than in HBV/C (23.0% *vs* 19.1%), whilst the prevalence of T130, Q132, and A138 mutation was higher in HBV/C than in HBV/B. The prevalence of pre-S2 start codon mutation was higher in LC (38.9%) and HCC (40.0%) than CH (5.6%) in HBeAg(+) group, but it was similar between CH, LC and HCC in HBeAg(-) group.

CONCLUSION: Pre-S2 start codon mutation was higher in Indonesian patients compared to other Asian countries, and its prevalence was associated with advanced liver disease, particularly in HBeAg(+) patients.

Key words: Hepatitis B virus; Pre-S2 start codon; Liver disease; Hepatitis B e antigen seroconversion; Indonesia

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important infectious diseases worldwide. It is the major cause of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). About 40% of the world's population has had contact with, or are carriers of, HBV and it is estimated that more than 350 million people world wide have chronic liver infection[1]. Indonesia has a moderate to high endemnicity of HBV infection[2], perhaps due to the lack of proper health facilities, poor economical status, less public awareness, or incomplete vaccination.

HBV is a relaxed circular, partially double stranded 3.2 kb DNA virus[3]. It has four overlapping reading frames that encode Polymerase, Core, X and Envelope proteins[4]. The Envelope proteins are produced from a single open reading frame with three different translation sites, pre-S1, pre-S2, and S. It produces three forms of HBV surface proteins (HBs), which are the large (L), middle (M) and small (S) HBs[5]. The major component of the Envelope protein is S protein which consists of 226 amino acids and drives particle budding. The M protein is composed of the S protein with an additional of 55 amino acids termed pre-S2 attached to the N-terminus. The L protein is the M protein with an additional 108 or 119 genotype-dependent amino acids attached to the N-terminus[6].

The pre-S1 and pre-S2 region is the region with the most variability in the HBV genome[7]. This variability is in the form of deletions, insertions, or synonymous or non-synonymous nucleotide substitutions. The pre-S1 and pre-S2 regions encode the T- and B-cell epitopes which play roles in allowing neutralizing antibodies to bind, and consequent immune protection[8,9]. Therefore, HBV pre-S mutant variants may emerge as a result of selective immune pressure.

The high prevalence of HBV pre-S mutations in Asia, including pre-S deletion and pre-S2 start codon mutations, has been reported, and it has been demonstrated that these mutations were associated with progressive liver disease[10]. In addition, amino acid substitution from Phenylalanine to Lysine at codon 141 in pre-S2 region (F141L) is also associated with HCC in patients infected with HBV genotype C[11]. Furthermore, study of hepatitis B e antigen (HBeAg) (-) patients in China revealed that pre-S deletions alone, or in combination with mutations in precore and basal core promoter (BCP) of HBV, are also associated with advanced liver disease[12].

Studies on the prevalence of HBV genotypes, BCP and precore mutations, and their association with severity of liver disease in Indonesia have previously been reported[13-15]. We have recently published a molecular epidemiological study on the prevalence of pre-S deletion mutation in Indonesian subjects[16], but this paper did not describe the incidence of synonymous and non-synonymous mutations in the pre-S region. Therefore, we extended this study to investigate the prevalence of pre-S2 start codon mutation and its association with severe liver disease in Indonesian patients. In addition, other mutations in the pre-S2 regions were analyzed.

MATERIALS AND METHODS

Samples

Blood samples were obtained from 270 HBV carriers. Two subjects with genotype D were excluded due to its relatively low prevalence in the collective samples and also in Asian population in general. A total of 268 subjects were included in the study: 73 asymptomatic carriers (AC) and 195 liver disease patients which composed of 66 CH, 66 LC, and 63 HCC. Asymptomatic carrier samples were taken from donors with positive HBsAg from the Blood Transfusion Unit (Red Cross Makassar, South Sulawesi, Indonesia), between February and August 2007. Chronic hepatitis samples were from patients positive for HBsAg for more than six months, and who had more than twice the normal alanine aminotransferase (ALT) level. Liver cirrhosis was diagnosed by liver function tests and ultrasonography, whilst the diagnosis of HCC was either on the basis of ultrasonography as well as an elevated serum-fetoprotein (AFP) level (≥ 200 ng/mL), or by needle aspiration liver biopsy for samples in which the AFP level was low. Samples from CH, LC, and HCC patients were collected from Cipto Mangunkusumo Hospital, Gatot Soebroto Hospital, Klinik Hati, Jakarta, Siloam Hospital Lippo Karawaci, Tangerang, Mataram General Hospital, Mataram, Wahidin Sudirohusodo Hospital, Makassar, and M. Djamil Hospital, Padang, from May 2006 to February 2010. None of the samples had coinfection with hepatitis C virus. The HBsAg test for all samples was performed using a commercially available enzyme-linked immunosorbent assay, kit (Abbott Laboratories, Chicago, IL, United States). The HBeAg status of samples was tested by using rapid test (Intec, Xiamen, China) as described previously[16]. Blood samples were separated into plasma and stored at -70 ℃ until use. The study was

Table 1 Demographic data and the prevalence of pre-S mutations associated with progressive liver disease

	AC	CH	LC	HCC	All	*P* value (all)	*P* value (AC-CH *vs* LC)	*P* value (AC-CH *vs* HCC)
n (%)	73 (27.2)	66 (24.6)	66 (24.6)	63 (23.5)	268 (100.0)	-	-	-
Gender, *n* (%)								
Male	69 (94.5)	49 (74.2)	46 (69.7)	49 (77.7)	213 (79.5)	0.002	0.027	0.314
Female	4 (5.5)	17 (25.8)	20 (30.3)	14 (22.2)	55 (20.5)			
Age (yr, mean ± SD)	32.3 ± 9.1	41.9 ± 13.3	50.1 ± 10.9	48.6 ± 11.6	42.9 ± 13.3	< 0.001	< 0.001	< 0.001
Genotype, *n* (%)								
B	56 (76.7)	46 (69.7)	46 (69.7)	52 (82.5)	200 (74.6)	0.267	0.453	0.393
C	17 (23.3)	20 (30.3)	20 (30.3)	11 (17.5)	68 (25.4)			
HBeAg[1]								
HBeAg(+)	13 (22.0)	36 (56.3)	18 (30.5)	15 (28.3)	82 (34.9)	< 0.001	0.222	0.144
HBeAg(-)	46 (78.0)	28 (43.8)	41 (69.5)	38 (71.7)	153 (65.1)			
Mutation								
M120	2 (2.7)	12 (18.2)	27 (40.9)	18 (28.6)	59 (22.0)	< 0.001	< 0.001	0.002
T130	8 (11.0)	19 (28.8)	18 (27.3)	25 (39.7)	70 (26.1)	0.0018	0.311	0.054
Q132	11 (15.1)	19 (28.8)	21 (31.8)	26 (41.3)	77 (28.7)	0.008	0.111	0.041
A138	10 (13.7)	21 (31.8)	22 (33.3)	26 (41.3)	79 (29.5)	0.003	0.089	0.094

[1]Number of samples for HBeAg. *n* (all) = 235, *n* (AC:CH:LC:HCC) = 59:64:59:53. AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

approved by the Committee on Health Research Ethics of the Mochtar Riady Institute for Nanotechnology and informed consent was obtained from each subject.

Viral DNA extraction, polymerase chain reaction and sequencing

HBV DNA was extracted from 200 μL plasma using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and 80 μL of eluted DNA was stored at -70 ℃ until use. Pre-S region was amplified by nested polymerase chain reaction (PCR) using PCR Core System (Promega, Madison, WI, United States) and two sets of primers as previously described[16]. The first round was performed with primers PS1 (5'-GGGTCACCTTATTCTTGGGA-3', position 2814-2833) and PS2 (5'-CCCCGCCTGTAACACGAGCA-3', position 208-189). For the second round, primers PS3 (5'-TTGGGAACAAGATCTACAGC-3', position 2828-2847) and PS4 (5'-GTCCTGATGCGATGTTCTCC-3', position 176-157) were used. First and second round PCR were performed with the same profile for 36 cycles of 94 ℃ for 1 min, 58 ℃ for 30 s and 72 ℃ for 1 min in a thermal cycler. The PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States), directly sequenced employing an ABI 3130xl Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, United States) with the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Inc.) using primers PS3 and PS4[13-16].

Hepatitis B virus genotyping and pre-S mutations analysis

The HBV genotype was determined by phylogenetic analysis of the pre-S sequences. The sequences were compared to 74 HBV reference strains of eight HBV genotypes (A-H) from GenBank. Alignment and phylogenetic trees were constructed using molecular evolutionary genetics analysis 4 software (Center for Evolutionary Functional Genomics, Tempe, AZ, United States)[17]. Pre-S amino acid sequences were aligned and compared between each group to detect the mutations.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL, United States). Statistical significances were determined using Fisher's exact test, χ^2 test, and *t*-test analyses whenever appropriate. The odds ratio and 95% CI were estimated by unconditional logistic regression model to evaluate the association of Pre-S mutation as a risk factor for advanced liver disease adjusted with age, sex and HBeAg. $P < 0.05$ was considered to be statistically significant.

RESULTS

Demographic data of subjects

Demographic data of subjects are shown in Table 1. A total of 268 subjects comprising 73 AC, 66 CH, 66 LC and 63 HCC were included in the study. The male/female ratio was significantly different among the groups ($P = 0.002$). The mean of ages for AC, CH, LC, and HCC was 32.3 ± 9.1, 41.9 ± 13.3, 50.1 ± 10.9 and 48.6 ± 11.6 years, respectively, and it was significantly different among the group ($P < 0.001$). Ratio of HBV/B to HBV/C showed no significant difference among the groups ($P = 0.267$). Of 268 subjects, 235 subjects were available for HBeAg rapid test analysis. Of 235 subjects, 82 (34.9%) had HBeAg(+) and 153 (65.1%) had HBeAg(-) and the distribution was significantly different among the group ($P < 0.001$).

Prevalence of pre-S2 start codon mutation and its association with the risk of advanced liver disease

Pre-S2 start codon mutations were found in 22.0% (59/268) of the total samples (Table 1). The mutation

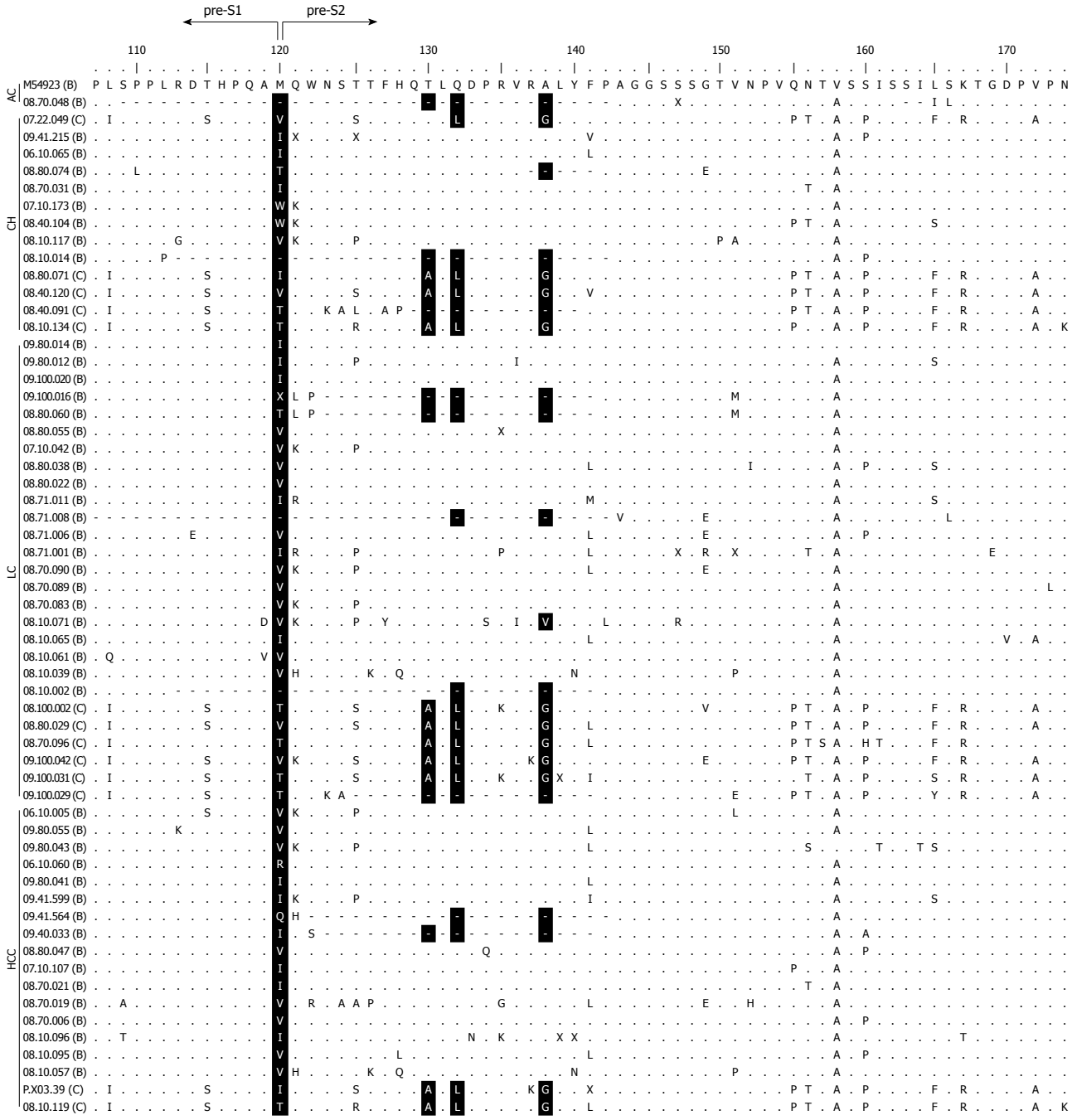

Figure 1 Amino acid alignment of 59 hepatitis B virus sequences harboring pre-S2 start codon mutation with wild type reference M54923 (genotype B). Positions of mutations are shown in black shades (M120, T130, Q132, and A138). Dots represent identical amino acids to the consensus sequence. Dashes represent deletion mutation. The names of the samples are indicated with each respective genotype in parentheses. AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver Cirrhosis; HCC: Hepatocellular carcinoma.

(M120) was either an amino acid substitution from Methionine to other amino acids or a deletion mutation (Figure 1). The prevalence of this mutation was significantly different among the groups ($P < 0.001$), and was increasingly common as disease progressed from AC (2.7%) to CH (18.2%) and LC (40.9%), but was less common in those with HCC (28.6%) than with LC but this difference was not statistically significant (Figure 2A). Multivariate regression analysis (based on 235 subjects with complete HBeAg) adjusted with age, gender and HBeAg demonstrated that M120 mutation was an independent factor in the development of progressive liver disease [OR 3.996 (1.830-8.729), $P = 0.0005$] (Table 2). These results implied that this mutation was associated with progressive liver disease and more particularly with LC than HCC.

Prevalence of other mutations in the pre-S2 region

Beside the pre-S2 start codon (M120) mutation, other mutations in the pre-S region were also analyzed. It was found that the prevalence of three amino acid changes in

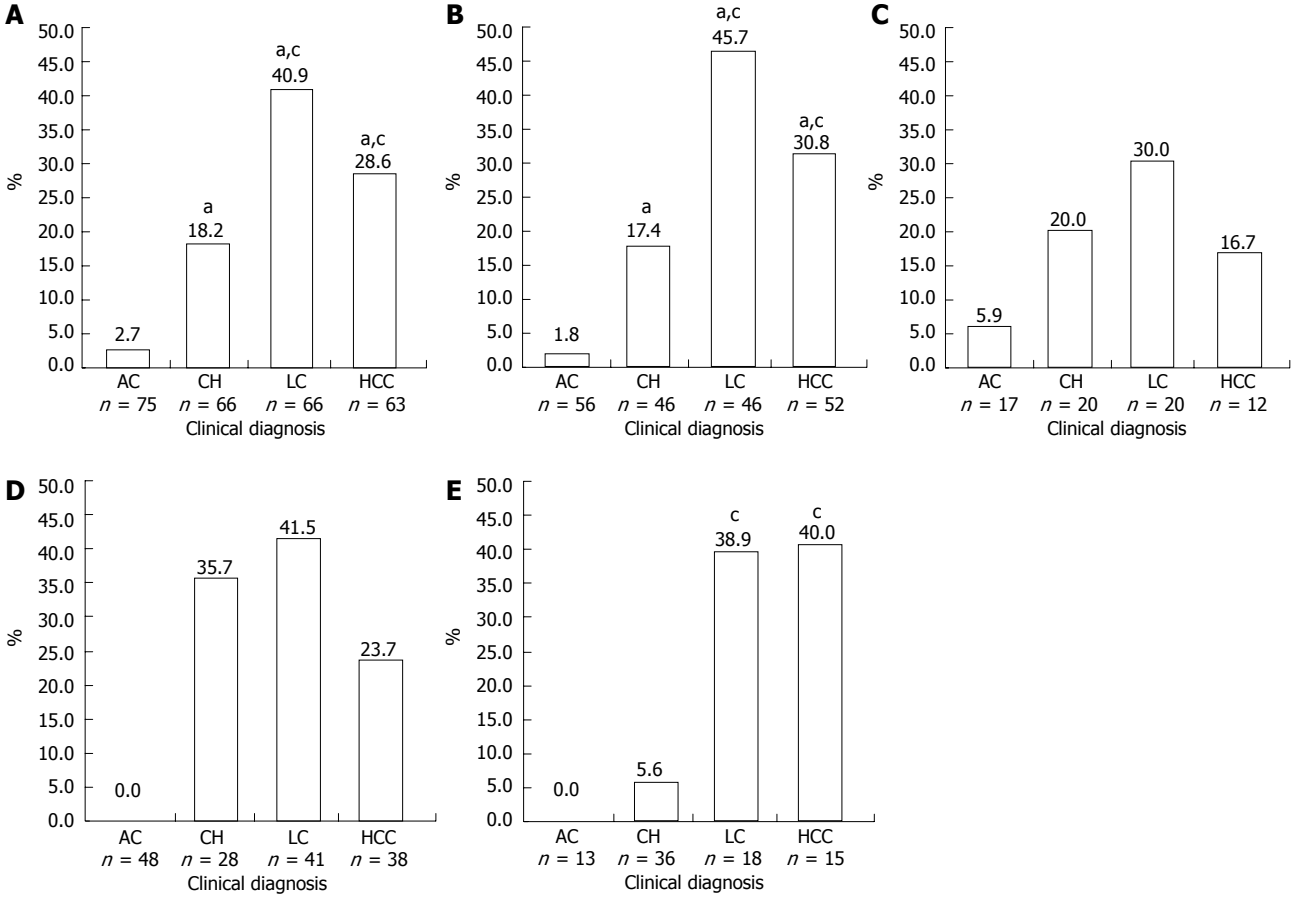

Figure 2 Prevalence of pre-S2 start codon mutation in all samples and in patients infected with hepatitis B virus/B, hepatitis B virus/C, hepatitis B e antigen (+), hepatitis B e antigen (-). A: Prevalence of pre-S2 start codon mutation in all samples; B: Prevalence of pre-S2 start codon mutation in patients infected with hepatitis B virus (HBV)/B; C: Prevalence of pre-S2 start codon mutation in patients infected with HBV/C; D: Prevalence of pre-S2 start codon mutation in patients with hepatitis B e antigen (HBeAg) (-); E: Prevalence of pre-S2 start codon mutation in patients with HBeAg(+). $^{a}P < 0.05$ *vs* AC; $^{c}P < 0.05$ *vs* CH. AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

the pre-S2 region showed significant differences among the groups (Table 1). Amino acid substitution from Threonine to other amino acids or deletion at codon 130 (T130) was detected in 26.1% of the samples, and the percentage increased in more severe liver disease ($P = 0.002$). Changes from Glutamine to other amino acids, or deletion at codon 132 (Q132), was found in 28.7% of the samples, and the prevalence was also increased with the severity of liver disease ($P = 0.008$). Similarly, amino acid substitution or deletion at Alanine 138 (A138) was found in 29.5% of samples and was highly prevalent in advanced liver disease ($P = 0.003$). Multivariate regression analysis showed that none of the mutations had significant association with progressive liver disease (Table 2).

Comparison of pre-S mutations in hepatitis B virus genotypes B and C

The samples were grouped into HBV/B and HBV/C and there were no significant differences in male/female ratios, mean age, or pattern of clinical diagnoses between the two groups (data not shown). Pre-S2 start codon mutation (M120) was more prevalent in HBV/B (23.0%) than in HBV/C (19.1%) (Table 3). In HBV/B, M120 mutation was associated with severity of liver disease ($P < 0.001$), but no association was found in HBV/C. The highest prevalence of M120 mutation in HBV/B was found in the LC group (45.7%), which was higher than that in the HCC group (30.8%), although not statistically significant (Figure 2B). Two other mutations in pre-S2 region (T130 and A138) showed significant association with the progress of liver disease in HBV/B ($P = 0.017$ and $P = 0.042$, respectively). In HBV/C, there was no association of the M120 mutation or the other mutations with progressive liver disease (Table 3, Figure 2C).

Comparison of pre-S mutations in HBeAg(-) and in HBeAg(+) patients

As shown in Table 4, the HBeAg(-) and HBeAg(+) groups differed significantly in the ratio of males to females, mean age, and pattern of clinical diagnoses. More male subjects were found in the HBeAg(-) group than in the HBeAg(+) group (83.0% *vs* 70.7%, $P = 0.035$). The mean age of subjects in the HBeAg(-) was greater than in HBeAg(+) group (44.7 ± 13.9 years *vs* 39.2 ± 11.9 years, $P = 0.003$). The prevalence of M120 mutations was higher in the HBeAg(-) group than in the HBeAg(+) group (23.5% *vs* 18.3%) (Table 5). The prevalence of this mutation was significantly different among the samples

Table 2 Pre-S mutation as risk factor for progressive liver disease adjusted with age, sex, and hepatitis B e antigen (n = 235)

	P-value	OR (95% CI)	*P*-value	OR adjusted (95% CI)
AC-CH *vs* LC-HCC				
Age, mean ± SD	1.99E-11	1.099 (1.069-1.130)		
Gender (male)	0.049	0.531 (0.281-1.002)		
HBeAg(-)	0.096	1.585 (0.921-2.730)		
Mutation				
M120	3.23E-06	4.942 (2.427-10.064)	0.0005	3.996 (1.830-8.729)
T130	0.078	1.684 (0.940-3.016)	0.531	1.246 (0.626-2.483)
Q132	0.029	1.885 (1.064-3.339)	0.352	1.379 (0.701-2.712)
A138	0.041	1.801 (1.020-3.178)	0.580	1.209 (0.617-2.370)
AC-CH *vs* LC				
Age, mean ± SD	8.86E-09	1.096 (1.063-1.131)		
Gender (male)	0.027	0.442 (0.213-0.920)		
HBeAg(-)	0.222	1.508 (0.778-2.922)		
Mutation				
M120	9.50E-07	6.343 (2.878-13.980)	4.00E-05	6.406 (2.640-15.540)
T130	0.311	1.440 (0.710-2.918)	0.672	1.198 (0.518-2.770)
Q132	0.111	1.740 (0.878-3.449)	0.364	1.457 (0.646-3.287)
A138	0.089	1.791 (0.911-3.523)	0.485	1.330 (0.597-2.964)
AC-CH *vs* HCC				
Age, mean ± SD	2.13E-07	1.084 (1.052-1.118)		
Gender (male)	0.314	0.663 (0.298-1.479)		
HBeAg(-)	0.144	1.677 (0.835-3.372)		
Mutation				
M120	0.002	3.651 (1.570-8.490)	0.052	2.538 (0.992-6.494)
T130	0.054	1.987 (0.981-4.022)	0.402	1.413 (0.629-3.174)
Q132	0.041	2.056 (1.024-4.129)	0.498	1.321 (0.590-2.953)
A138	0.094	1.811 (0.900-3.644)	0.744	1.144 (0.509-2.571)

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; CI: Confidence interval; OR: Odds ratio; HBeAg: Hepatitis B e antigen.

Table 3 Prevalence of pre-S mutations in hepatitis B virus genotype B and C associated with progressive liver disease

Mutations	Genotype B						Genotype C					
	AC n = 56	CH n = 46	LC n = 46	HCC n = 52	All n = 200	*P*-value	AC n = 17	CH n = 20	LC n = 20	HCC n = 12	All n = 68	*P*-value
M120	1 (1.8)	8 (17.4)	21 (45.7)	16 (30.8)	46 (23.0)	< 0.001	1 (5.9)	4 (20.0)	6 (30.0)	2 (16.7)	13 (19.1)	0.324
T130	6 (10.7)	11 (23.9)	10 (21.7)	19 (36.5)	46 (23.0)	0.017	2 (11.8)	8 (40.0)	8 (40.0)	6 (50.0)	24 (35.3)	0.098
Q132	9 (16.1)	11 (23.9)	12 (26.1)	20 (38.5)	52 (26.0)	0.067	2 (11.8)	8 (40.0)	9 (45.0)	6 (50.0)	25 (36.8)	0.081
A138	8 (14.3)	12 (26.1)	13 (28.3)	20 (38.5)	53 (26.5)	0.042	2 (11.8)	9 (45.0)	9 (45.0)	6 (50.0)	26 (38.2)	0.070

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

with different clinical diagnoses both in HBeAg(-) and HBeAg(+) patients. In the HBeAg(-) group, the highest prevalence of M120 mutation was found in LC (41.5%), followed by CH (35.7%) and HCC (23.7%), and no mutations were found in the AC group. However, no statistically significant differences were found between CH, LC and HCC (Figure 2D). Among HBeAg(+) patients, the highest frequency of M120 mutation was detected in those with HCC (40.0%), followed by those with LC (38.9%), and CH (5.6%), and no mutations were found in the AC group. Significant differences were found between those with CH and LC and CH and HCC, but not between those with LC and HCC (Figure 2E). On the other hand, no significant correlation of T130, Q132 and A138 mutations with severity of liver disease was found in either the HBeAg(+) or HBeAg(-) groups (Table 5).

DISCUSSION

Many studies have been reported on the association of pre-S mutations with severity of liver disease. Studies from Taiwan and China demonstrated that the pre-S deletion mutation is associated with HCC[18-20]. In addition, the pre-S2 deletion mutation was also specifically associated with increased risk of HCC in Asian children[21]. Other studies also reported that pre-S deletion and pre-S2 start codon mutations was found to be significantly associated with HCC[22,23]. A meta analysis study including 43 reports found that mutations at the promoter sites of pre-S1 and pre-S2 are significantly associated with an increased risk of HCC[24]. However, the diagnostic or predictive value of those mutations for HCC is limited because the frequencies of these mutations in the pa-

Table 4 Demographic data of samples with hepatitis B e antigen (+) and hepatitis B e antigen (-) *n* (%)

	HBeAg(+)	HBeAg(-)	*P*-value
	82 (34.9)	153 (65.1)	
Gender,			
Male	58 (70.7)	127 (83.0)	0.028
Female	24 (29.3)	26 (17.0)	
Age, mean ± SD	39.2 ± 11.9	44.7 ± 13.9	0.003
Genotype,			
B	56 (68.3)	116 (75.8)	0.215
C	26 (31.7)	37 (24.2)	
Clinical diagnosis,			
AC	13 (15.9)	46 (30.1)	< 0.001
CH	36 (43.9)	28 (18.3)	
LC	18 (22.0)	41 (26.8)	
HCC	15 (18.3)	38 (24.8)	

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

tients with HCC were low; pre-S1 and pre-S2 promoter mutations were 19.7% and 15.3%, respectively[24].

Recently, we reported on the prevalence of pre-S deletion mutation in Indonesian HBV carriers. The prevalence of pre-S deletion was 12.1% which is considered low compared to previous reports from other populations[16]. In the present study, which extends the analysis of this group of patients, pre-S2 start codon mutations were found in 22.0% of the samples. In Indonesia, it therefore appears that the pre-S2 start codon mutation is a more common type of mutation compared to the pre-S deletion mutation. The prevalence of pre-S2 start codon mutations was higher in the cirrhosis group than the HCC group, which is in accordance with the previous report[22]. Both pre-S2 deletion and start codon mutations cause alterations or loss of the M-protein, which has the T- and B-cell epitopes. These altered M-proteins can stimulate hepatocyte proliferation, and cause the formation of type II ground glass hepatocytes[25,26]. In this study, it was found that this mutation is more prevalent in the liver cirrhosis group, which was older than the other groups, indicating prolonged infection by HBV in this group. It has been suggested that pre-S2 deletion mutations, which affect the T-cell epitope and hence the ability of the virus to be neutralized, represent mutants that are selected under immune pressure, and are thus able to persist during chronic HBV infection[26]. Pre-S2 start codon mutations may have the same effect, since the mutation prevents production of M-protein. Furthermore, it has been reported that the loss of M-protein does not affect virus infectivity, but instead it may function as a spacer that supports conformational changes of L protein, or as a subdomain of the L protein in virus entry[6]. In this way it might allow mutants that lack M protein to emerge without affecting the virus life cycle.

Other mutations in the pre-S2 region were shown to be significantly associated with progressive liver disease. However regression analysis showed these amino acid changes or deletion at three codon sites, T130, Q132, and A138 were not statistically significantly associated with the increased risk of cirrhosis and HCC. The prevalence of these three mutations was considerably higher in the HCC group (38.5%-40.0%). Few studies on amino acid substitutions in the pre-S2 region and their association with progressive liver disease have been reported. An amino acid change from Glycine to Arginine at pre-S2 at codon 149 (G149R) was found in 52.9% of chronic carriers with spontaneous clearance of HBV surface antigen[27]. More recently it was found that a mutation at position 141 (F141L mutation) was associated with increased risk of HCC in patients with HBV/C infections[11]. However the mutation found in this study did not correlate with coexistence of HBsAg and anti-HBs, but may alter the B- and T-cell epitopes of the S protein, and thereby increase the risk of cirrhosis and HCC by decreasing immune recognition.

In the present study, the prevalence of pre-S2 start codon mutation was higher in HBV/B than HBV/C, although the difference was not statistically significant. In addition, a significant association of pre-S2 start codon mutation with severe liver disease was found in HBV/B, while there was no significant difference in the frequency of this mutation in HBV/C. A study in Thailand revealed a higher prevalence of pre-S2 deletions and preS2 start codon mutation in HBV/C than in HBV/B[28]. Another recent report showed a higher prevalence of pre-S2 deletions in HBV/C and this was associated with HCC in children[29]. The differences between previous studies and the present study are probably due to the difference in percentage of HBV/C samples in subjects included in the studies (the number of HBV/C infections was lower in the present study). On the other hand, in the present study, the prevalence of the three other mutations in the pre-S2 region (T130, Q132, and A138) was higher in those with HBV/C than those with HBV/B. In those with HBV/B, the incidence of T130 and A138 mutations was significantly different between the disease groups, and was increased with advanced liver disease (Table 3). However, the prevalence of these mutations was not significantly different between disease groups with HBV/C, possibly due to the low number of HBV/C samples included in this study.

The prevalence of pre-S2 start codon mutations was higher in HBeAg(-) samples than in HBeAg(+) samples (23.5% *vs* 18.3%), but interestingly in the HCC group the prevalence of this mutation was higher in HBeAg(+) samples than in HBeAg(-) samples (40.0% *vs* 23.7%) (Table 5). No mutations were found in asymptomatic carriers of either group. The prevalence of mutations in the HBeAg(+) group showed significant association between chronic hepatitis and cirrhosis as well as HCC, while in the HBeAg(-) group there were no differences found among CH, LC and HCC. Previous studies have described the roles of pre-S deletions on the progression of liver disease in HBeAg(-) patients in longitudinal study, and found that pre-S deletions were significantly associated with the development of liver cirrhosis[12]. In

Table 5 Prevalence of pre-S mutations in hepatitis B e antigen (-) and hepatitis B e antigen (+) associated with progressive liver disease

Mutation	HBeAg(-)						HBeAg(+)					
	AC *n* = 46	CH *n* = 28	LC *n* = 41	HCC *n* = 38	All *n* = 155	*P*-value	AC *n* = 13	CH *n* = 36	LC *n* = 18	HCC *n* = 15	All *n* = 82	*P*-value
M120	0 (0.0)	10 (35.7)	17 (41.5)	9 (23.7)	36 (23.5)	< 0.001	0 (0.0)	2 (5.6)	7 (38.9)	6 (40.0)	15 (18.3)	< 0.001
T130	6 (13.0)	10 (35.7)	11 (26.8)	13 (34.2)	40 (26.1)	0.082	2 (15.4)	9 (25.0)	6 (33.3)	6 (40.0)	23 (28.0)	0.474
Q132	6 (13.0)	10 (35.7)	12 (29.3)	14 (36.8)	42 (27.5)	0.057	3 (23.1)	9 (25.0)	8 (44.4)	6 (40.0)	26 (31.7)	0.389
A138	6 (13.0)	10 (35.7)	13 (31.7)	13 (34.2)	42 (27.5)	0.072	2 (15.4)	11 (30.6)	8 (44.4)	6 (40.0)	27 (32.9)	0.344

AC: Asymptomatic carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

another longitudinal study observing the natural course of HBV there was no clear relationship between HBeAg seroconversion and pre-S deletions[30]. The present study was a cross-sectional study which is limited by uncertainty whether these mutations preceded the development of advanced liver disease. However, it does suggest that pre-S2 start codon mutations can be used as a predictor for development of advanced liver disease in HBeAg(+) patients (Figure 2E), but not in HBeAg(-) patients (Figure 2D). Interestingly, the frequency of T130, Q132, and A138 mutations was similar between the two groups (Table 5), indicating that the mutations in this region are not related to HBeAg seroconversion. In summary, our study demonstrated that the pre-S2 start codon mutation has high prevalence in Indonesia. This mutation may serve as biomarker for prediction of development of advanced liver disease in HBeAg(+) patients who are infected with HBV genotype B.

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COMMENTS

Background

Hepatitis B virus (HBV) infection is a serious worldwide health problem. Indonesia is one of the countries with high endemicity of hepatitis B related disease caused by HBV. Particular mutations of the HBV genome have been associated with severe liver disease. Studies on HBV molecular epidemiology associated with the development of progressive liver disease in Indonesia are still very scarce. This study is to characterize the prevalence of pre-S2 mutation in HBV associated with advanced liver disease in Indonesia.

Research frontiers

Pre-S mutations have been reported to be associated with advanced liver disease. Pre-S deletion and pre-S2 start codon mutations were the most common form of mutations at the pre-S region of HBV. Previous study had shown the low prevalence of pre-S deletion in Indonesian patients; however the prevalence of the pre-S2 start codon mutation has not been fully investigated. In this study, the authors demonstrated that the pre-S2 start codon mutation could serve as a biomarker for liver disease progression.

Innovations and breakthroughs

Previous studies have reported the high prevalence of HBV pre-S deletions and its association with advanced liver disease. But contrary to reports from studies of other populations, previous and current studies showed that pre-S2 start codon mutations and not pre-S deletion may serve as potential biomarkers for progressive liver disease in Indonesia.

Applications

By characterizing the prevalence of the HBV pre-S2 start codon mutation among different clinical diagnoses of different HBV genotype and hepatitis B e antigen (HBeAg) presence, the pre-S2 start codon mutation may serve as a potential biomarker for prediction of development of advanced liver disease in HBeAg(+) patients who are infected with HBV genotype B.

Terminology

HBV surface (HBs) gene has three different translation sites, pre-S1, pre-S2, and S, which produce large-, middle-, and small-HBs protein, respectively. Pre-S2 start codon mutation is a substitution or deletion of Methionine at the pre-S2 translation site which abolished the M HBs protein.

Peer review

The manuscript is interesting because the authors suggest a way to predict liver disease progression according to the appearance of a mutation at pre-S2 start codon in a subset of HBV infected patients.

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BRIEF ARTICLE

Oxaliplatin-induced severe anaphylactic reactions in metastatic colorectal cancer: Case series analysis

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Abstract

AIM: To investigate oxaliplatin-induced severe anaphylactic reactions (SAR) in metastatic colorectal cancer in a retrospective case series analysis and to conduct a systemic literature review.

METHODS: During a 6-year period from 2006 to 2011 at Kaohsiung Veterans General Hospital, a total of 412 patients exposed to oxaliplatin-related chemotherapy were retrospectively reviewed. Relevant English-language studies regarding life-threatening SAR following oxaliplatin were also reviewed in MEDLINE® and PubMed® search.

RESULTS: Eight patients (1.9%, 8 of 412 cases) were identified. Seven patients were successful resuscitated without any sequelae and one patient expired. We changed the chemotherapy regimen in five patients and rechallenged oxaliplatin use in patient 3. Twenty-three relevant English-language studies with 66 patients were reported. Patients received a median of 10 cycles of oxaliplatin (range, 2 to 29). Most common symptoms were respiratory distress (60%), fever (55%), and hypotension (54%). Three fatal events were reported (4.5%). Eleven patients (16%) of the 66 cases were rechallenged by oxaliplatin.

CONCLUSION: SAR must be considered in patients receiving oxaliplatin-related chemotherapy, especially in heavily pretreated patients. Further studies on the mechanism, predictors, preventive methods and management of oxaliplatin-related SAR are recommended.

Key words: Oxaliplatin; Anaphylactic; Colorectal cancer; Metastasis

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INTRODUCTION

Colorectal cancer (CRC) accounts for 10% to 15% of all cancers and is the third leading cause of cancer deaths in Taiwan. Oxaliplatin is a third generation platinum compound frequently used in the treatment of stage Ⅲ CRC as adjuvant chemotherapy[1] and stage Ⅳ advanced CRC[2]. Similar to other platinum compounds, oxaliplatin interacts with DNA to form intra-strand/inter-strand

DNA cross-linking that can affect DNA base pairing, replication, and gene transcription and cause cell death[3]. Among the common reasons for its withdrawal are frequent peripheral neuropathy, a delayed hypersensitivity reaction, and most troublesome, anaphylaxis when patients receive accumulated doses of oxaliplatin[4]. Hypersensitivity reaction and anaphylaxis refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. These reactions may be damaging, uncomfortable, or occasionally fatal.

Multiple mechanisms of action have been proposed including the use of various neuroprotective agents in the hope of achieving adequate oxaliplatin doses with less neuropathy[5]. Much less is known about acute reactions such as anaphylaxis, and it is generally considered to be associated with immune-mediated effects[6,7]. The percentage of hypersensitivity reactions quoted in different studies ranges from 8% to 20%, but is usually around 10% to 12%[8]. Life-threatening severe anaphylactic reactions (SAR) have been reported but no systemic review of their incidence has been undertaken. Therefore, we performed a retrospective analysis of our patients who had been exposed to oxaliplatin and selected those who developed SAR requiring hospitalization with medical intervention. We also conducted a systemic literature review on this issue.

MATERIALS AND METHODS

Chart review

During a 6-year period from 2006 to 2011 at Kaohsiung Veterans General Hospital, a total of 412 patients exposed to oxaliplatin-related chemotherapy were retrospectively reviewed. Life-threatening SAR was defined as side effects including symptomatic bronchospasm, allergy-related edema/angioedema, hypotension or anaphylaxis (grade Ⅲ/Ⅳ anaphylactic reactions reference by NIH common Toxicity Criteria v3.0) requiring hospitalization and medical interventions[9]. The oxaliplatin-related chemotherapy regimen (FOLFOX) consisted of leucovorin 200 mg/m^2 as a 2-h infusion, and oxaliplatin 85 mg/m^2 given as a 2-h infusion in 500 mL of dextrose 5% *via* a Y-connector, followed by a 46-h infusion of 5-fluorouracil 2500 mg/m^2, repeated every 2 wk. Antiemetic prophylaxis with a 5HT3-receptor antagonist was administered. The use of implantable ports and disposable or electronic pumps allowed chemotherapy to be administered on an inpatient basis.

Data collection and literature review

Information collected included age, sex, allergy history, primary CRC site, tumor, nodes, metastasis classification, CRC stage, previous chemotherapy regimens, previous oxaliplatin-related chemotherapy cycles, oxaliplatin dosage, tumor response, onset time after oxaliplatin infusion, and outcome. Relevant English-language studies regarding life-threatening SAR following oxaliplatin were also reviewed. We searched the relevant studies by entering keywords "severe side effect after oxaliplatin", "life-threatening reaction after oxaliplatin" and "severe anaphylactic reaction after oxaliplatin" in MEDLINE® and PubMed® searches.

RESULTS

Patient characteristics

Eight patients (1.9%, 8 of 412 cases) were identified who developed life-threatening SAR, which occurred after infusion of oxaliplatin-related chemotherapy. The patients' characteristics were described in Table 1. There were 4 females and patients' age ranged from 36 to 72 years. Three patients had rectal cancer, 4 patients had sigmoid colon cancer, and 1 patient had descending colon cancer. Two patients had an allergy history to alcohol and flurbiprofen respectively. All patients had stage Ⅳ metastatic disease and received several lines of different chemotherapy regimens. Patients had received 5-29 cycles of oxaliplatin-related chemotherapy. Oxaliplatin dosages were 85 mg/m^2 in six patients and 90 mg/m^2 in one patient. Stable disease was achieved in three patients and progressive disease in five patients. Onset time after oxaliplatin infusion ranged from immediate to two hours. Seven patients were successfully resuscitated with oxygen support and medical interventions and fully recovered without any sequelae. However, one patient suffered from SAR and shock status 20 min after infusion of oxaliplatin. Despite cardiopulmonary resuscitation and use of inotropic agents, this patient expired 50 min later. We changed the chemotherapy regimen in five patients and rechallenged oxaliplatin use in patient 3. Because the patient 3's disease manifestations responded well to FOLFOX chemotherapy regimen, continuation was felt to be desirable. We have thus decided to attempt rechallenge of oxaliplatin by prolonging the infusion rate and using premedication with an additional 100 mg hydrocortisone plus diphenhydramine before the next treatment course. Fortunately, no anaphylactic reactions developed thereafter.

Literature review

Twenty-three relevant English-language studies, published from 1997-2011, regarding SAR following oxaliplatin-related chemotherapy were reported (Table 2). All studies were retrospective; few included the same patients. We found 59 reported cases that fitted the definition of life-threatening SAR from MEDLINE® and PubMed®[8-30]. Together with the 8 cases we presented, the median cycles of oxaliplatin given before SAR developed was 10 (range, 2-29). Most common symptoms were respiratory distress (60%), fever (55%), and hypotension (54%). Three fatal events were reported (4.5%). Eleven patients from these 66 cases were rechallenged with oxaliplatin.

DISCUSSION

According to previous studies, the estimated incidence of oxaliplatin-induced SAR was less than 2%[10,17,18,24,31]. In 2007, Lee *et al*[24] reported the incidence of SAR as 1.32%

Table 1 Clinical characteristics of patients with life-threatening severe anaphylactic reactions following oxaliplatin chemotherapy (n = 8)

Patient	Sex	Age (yr)	Allergy history	Primary CRC site	TNM classification	Stage	Previous chemotherapy regimens	Previous oxaliplatin chemotherapy cycles	Oxaliplatin dose (mg/m^2)	Tumor response	Presenting symptoms	Onset time	Outcome	Rechallenge
1	F	50	Alcohol	Rectum	T4N0M1	Ⅳ	FOLFIRI x12, FOLFOX x10	10	85	SD	Consciousness loss, dizziness, shock	30 min	Recovery	No
2	M	71	Nil	Rectum	T2N1M1	Ⅳ	FOLFOX x12, FOLFIRI + Bevacizumabx12, FOLFOX x1	13	85	PD	Consciousness loss, shock	20 min	Fatal	No
3	M	36	Nil	Sigmoid colon	T4N2M1	Ⅳ	FOLFIRI x13, FOLFOX + Bevacizumab x5, FOLFIRI + Cetuximab x5, FOLFOX x7	12	90	PD	Consciousness loss, respiratory distress, cold sweating	Immediate	Recovery	Yes
4	F	57	Nil	Sigmoid colon	T1N0M1	Ⅳ	FOLFIRI x5, FOLFOX x7, FOLFIRI + Bevacizumabx18, FOLFOX + Bevacizumab x1	8	85	PD	Respiratory distress, cold sweating	2 h	Recovery	No
5	F	68	Nil	Sigmoid colon	T3N2M1	Ⅳ	FOLFIRI x12, FOLFOX x8	8	85	SD	Angioedema, slurred speech, respiratory distress	30 m	Recovery	No
6	F	72	Nil	Descending colon	T4N1M1	Ⅳ	FOLFIRI x7, FOLFOX x3, FOLFIRI x12, FOLFOX x2	5	85	PD	Nausea, vomiting, shock,	10 min	Recovery	No
7	M	59	Flurbiprofen	Rectum	T3N2M1	Ⅳ	FOLFOX x19, FOLFIRI x8, FOLFOX x10	29	85	SD	Consciousness loss, respiratory distress, cold sweating	Immediate	Recovery	No
8	M	62	Nil	Sigmoid colon	T3N1M1	Ⅳ	FOLFIRI + Bevacizumabx6, FOLFOX x7	7	85	PD	Consciousness loss, respiratory distress, cold sweating	20 min	Recovery	No

M: Male; F: Female; CRC: Colorectal cancer; TNM: Tumor, nodes, metastasis; PD: Progressive disease; SD: Stable disease; FOLFIRI: Chemotherapy regimen including 5-fluorouracil, leucovorin, irinotecan; FOLFOX: Chemotherapy regimen including 5-fluorouracil, leucovorin, oxaliplatin.

Table 2 Studies on severe anaphylactic reactions following oxaliplatin, including data published from 1997 to 2012 in English (24 studies, *n* = 66)

Ref.	Published year/region	Patient No.	Age (yr)	Male/female	Previous oxaliplatin cycles	Oxaliplatin dose (mg/m^2)	Presenting symptoms	Onset time after oxaliplatin infusion	Outcome
de Gramont *et al*[10]	1997/France	5	NA	NA	NA	NA	NA	NA	Recovery
Tournigand *et al*[11]	1998/France	5	59-77	3/2	5-12	85-100	Reduced blood pressure, flushing, headache, tachycardia, respiratory distress	Immediate	Recovery
Larzillière *et al*[12]	1999/France	1	55	1/0	5	85	Flushing, profuse sweats, arterial hypertension, tachycardia	30 min	Recovery
Médioni *et al*[13]	1999/France	1	63	1/0	6	100	Visual disturbances, edema, tachycardia, severe hypotension, anaphylactic shock	Immediate	Recovery
Sørbye *et al*[14]	2000/Norway	1	40, 52	1/0	8	85	Severe thrombocytopenia	Immediate	Recovery
Santini *et al*[15]	2001/Italy	1	52	1/0	6	60	Chills, fever, nausea, vomiting, crampy abdominal pain, diarrhea, hypotension	15 min	Recovery
Schüll *et al*[16]	2001/Austria	1	54	1/0	5	85	Flush, generalised erythema of the trunk, nausea, hypotension	30 min	Recovery
Brandi *et al*[17]	2003/Italy	9	NA	NA	2-17	NA	Dyspnea, laryngospasm, agitation, tachycardia, precordial pain, erythema, sweating	NA	Recovery
Thomas *et al*[18]	2003/United States	1	50	0/1	9	NA	Fever, respiratory distress	2 h	Recovery
Lenz *et al*[19]	2003/Germany	2	NA	NA	NA	85	Severe abdominal, chest pain.	Immediate	Recovery
Bhargava *et al*[20]	2004/United States	1	50	0/1	12	NA	Palpitation, flushing, hypotensive, wheezing	15 min	Recovery
González-Mahave *et al*[21]	2005/Spain	2	43, 44	1/1	4, 11	NA	Respiratory collapse, fever	Immediate	Recovery
Maindrault-Goebel *et al*[22]	2005/France	3	NA	NA	NA	NA	Anaphylactic shock	NA	Recovery
Siu *et al*[8]	2006/Hong Kong	2	NA	NA	NA	100	Hypotension, oxygen desaturatio, full-blown anaphylactic reactions	NA	NA
Tze *et al*[23]	2006/China	1	60	0/1	12	NA	Anaphylactic shock	Immediate	Recovery
Lee *et al*[24]	2006/Taiwan	4	36-74	NA	6-7	85-100	Anaphylactic shock, hypertensive crisis	5-50 min	Recovery
Yanqi *et al*[25]	2007/China	1	52	1/0	6	150 mg	Anaphylactic shock	10 min	Recovery
Santodirocco *et al*[26]	2008/Italy	1	44	0/1	14	85	Acute thrombocytopenia, hemolysis, bleeding	1 h	Recovery
Shao *et al*[27]	2008/Taiwan	1	64	1/0	23	NA	Thrombocytopenia	1 h	Fatal
Chay *et al*[9]	2010/Singapore	11	36-75	4/7	NA	NA	Respiratory collapse, flushing, hypokalemia	NA	Recovery
Pietrantonio *et al*[28]	2010/Italy	1	NA	NA	NA	NA	Acute thrombocytopenia	NA	Recovery
Potenza *et al*[29]	2010/Italy	1	46	1/0	6	85	Respiratory collapse	10 h	Recovery
Teng *et al*[30]	2011/Taiwan	1	78	1/0	17	85	Pancytopenia, coagulopathy, intracranial hemorrhage	30 min	Fatal
Wang *et al*, this study	2012/Taiwan	7	36-72	3/4	5-29	85-90	Consciousness loss, chest tightness, cold sweating, nausea, vomiting, shock	immediately to 2 h	1 fatal

NA: Not applicable.

(4 of 303 cases) in Taiwan. In our study, the incidence is 1.9% (8 of 412 cases). Multiple suggestions to reduce the incidence of adverse reactions have been proposed, including the use of various neuroprotective agents, in the hope of achieving adequate oxaliplatin doses with less neuropathy[5]. Much less is known about acute reactions such as anaphylaxis, but this is generally considered to be associated with immune-mediated effects, as evidenced by detection of drug-dependent IgG antibodies with or without complement[6,7]. Two independent pathogenetic mechanisms have been proposed for this toxicity. Some authors described the formation of autoantibodies to erythrocytes and, more rarely, to platelets and neutrophils as a result of oxaliplatin adsorption on blood cells[32]. By contrast, other authors reported high levels of cytokines [i.e., interleukin (IL) 6, IL10 and tumor necrosis factor-α] suggesting that oxaliplatin-dependent toxicity may be triggered by a massive release of pro-inflammatory molecules[33].

In literature reviews, SAR developed after several cycles of oxaliplatin chemotherapy (median cycles before SAR is 10), suggesting a sensitization process of type I hypersensitivity due to the rapid appearance of symptoms[22,34]. Based on Chay *et al*[9], females appeared more prone to severe oxaliplatin reactions for which the reason remains unclear, and all females manifested acute hypokalemia. Recently reported ex-vivo work suggests that oxaliplatin may interfere with voltage-gated potassium channels[35] and hypothesizes that axonal membrane hyperpolarization[36,37] may account for the observed hypokalemia, with potassium ion channel activation resulting in an intracellular influx of potassium. However, in our study there were no such findings including female predominance and hypokalemia after the episode.

Theoretically, prolongation of the infusion rate with premedication including steroids and antihistamines could be a method to prevent SAR after oxaliplatin use. We adopted this strategy before rechallenging oxaliplatin in patient 3. However, in 2001 Stahl *et al*[38] reported that allergic reactions to oxaliplatin may still occur after steroid prophylaxis. In 2006, Siu *et al*[8] reported premedications with steroid and chlorpheniramine seemed ineffective in preventing SAR. In 2011, Siu *et al*[39] developed a simple rechallenge protocol for mild hypersensitivity reactions, including intravenous dexamethasone, diphenhydramine and ranitidine, as well as prolongation of the oxaliplatin infusion time with a high success rate of 70%. Why did the anaphylactic reactions disappear after rechallenge of oxaliplatin in patient 3? A possible explanation for the disappearance of symptoms may be the much lower peak plasma concentrations of the platinum compound and its metabolites in case of a protracted infusion[3], thus resulting in a minor and/or delayed, clinically negligible cytokine release reaction. In literature reviews, five of eleven rechallenged patients could tolerate oxaliplatin with no or minimal discomfort. However, there were still three patients developing SAR after receiving prolonged infusion of oxaliplatin. There were also reported cases initially having only a mild hypersensitivity reaction to oxaliplatin, who developed SAR after rechallenge with prolonged infusion schedule[22]. Therefore, it seems that prolonged infusion of oxaliplatin or using a desensitization program could only benefit a few patients who developed SAR. So, changing the chemotherapy regimen might be a better choice.

The mortality rate of oxaliplatin-related SAR was 4.5% (3 of 66 patients). In 2008, Shao *et al*[27] reported a fatal thrombocytopenia with a large intracranial hemorrhage with brain herniation after oxaliplatin chemotherapy. In 2011, Teng *et al*[30] reported another fatal pancytopenia with intracranial hemorrhage after oxaliplatin treatment. In our study, the patient who died initially presented with anaphylactic shock and loss of consciousness immediately after oxaliplatin infusion. All these three patients had been heavily pretreated with oxaliplatin and had received 23, 17 and 13 cycles of oxaliplatin treatment, respectively. To counteract the underlying immune-mediated mechanism, the use of steroids seems to be one of the most cost-effective approaches, especially when the patient's condition is life threatening[7,40]. This may also explain the fatalities in the patients reported by Shao *et al*[27], Teng *et al*[30] and our patient, who did not receive a steroid. Are there any predictors or risk factors for this rare but life-threatening event before oxaliplatin use? In 2011, Seki *et al*[41] reported a higher neutrophil count and lower monocyte count were two risk factors for grade 3/4 reactions in oxaliplatin-induced hypersensitivity reactions in Japanese patients. However, we didn't observe such a relationship in our study and the literature review.

Target therapy with monoclonal antibodies, including bevacizumab, cetuximab, and panitumumab, can also result in SAR[42]. Up till April 2012, there have been 14 698 people reported to have side effects when taking bevacizumab. Among them, 87 people (0.59%) have SAR[43]. In our study, one patient (patient 4) developed SAR after bevacizumab and oxaliplatin infusion. In our hospital, bevacizumab was started first and infused over 1-h. Oxaliplatin was infused after bevacizumab infusion. This patient developed SAR about 3 h after bevacizumab infusion and 2 h after oxaliplatin infusion. It is very difficult to differentiate the cause of SAR in this patient. But due to the time of onset of SAR, it is reasonable to suspect oxaliplatin.

Our study does have several limitations. First, being a retrospective review, it is difficult to confirm now whether those observed reactions are genuine hypersensitivity reactions or whether they developed as a result of oxaliplatin infusion only, although the temporal relationship between infusion and onset of reaction is suggestive. Therefore, it is possible that the risk may have been overestimated. We can also argue the other way round, that is, some mild reactions may have been missed resulting in underestimation.

In conclusion, SAR is rare but serious, and must be considered in patients receiving oxaliplatin-related che-

motherapy, especially in heavily pretreated patients. Physicians should be cautious when patients have repeated symptoms or signs of allergic reaction to oxaliplatin. At the moment, the mechanisms underlying oxaliplatin-related SAR remain uncertain. Prevention with prolongation of the infusion rate, steroid use and antihistamines are still in debate. Rechallenge with oxaliplatin is suggested only in carefully selected patients and should be used with caution. We recommend changing the chemotherapy regimen in patients experiencing oxaliplatin-induced SAR. Further extensive examinations with a large number of patients to determine the mechanism, the predictors, preventive methods and management strategy of oxaliplatin-induced SAR are recommended.

COMMENTS

Background

Oxaliplatin is a third generation platinum compound frequently used in the treatment of stage Ⅲ and stage Ⅳ colorectal cancer. Among the side effects of this agent, hypersensitivity reaction and anaphylaxis refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. These reactions may be damaging, uncomfortable, or occasionally fatal. The percentage of hypersensitivity reactions quoted in different studies ranges from 8% to 20%. The authors presented their experience in this retrospective study and conducted a systemic review.

Research frontiers

Much less is known about acute reactions such as anaphylaxis, but it is generally considered to be associated with immune-mediated effects, as evidenced by detection of drug-dependent IgG antibodies with or without complement. Further extensive examination with a large number of patients to determine the mechanism, the predictors, preventive methods and management strategy of oxaliplatin-induced severe anaphylactic reactions (SAR) are recommended.

Innovations and breakthroughs

Life-threatening SAR have been reported but no systemic review had been performed. Here, the authors performed a retrospective analysis of the patients who had been exposed to oxaliplatin and selected those who developed SAR requiring hospitalization with medical intervention and conducted a systemic literature review on this issue.

Applications

Physicians should be cautious when patients have repeated symptoms or signs of allergic reaction to oxaliplatin. The effectiveness of prevention with prolongation of the infusion rate, steroid use and antihistamines is still in debate. Rechallenge of oxaliplatin is suggested only in highly selected patients and should be used with caution. The authors recommend changing the chemotherapy regimen in patients experiencing oxaliplatin-induced SAR.

Peer review

This manuscript is a retrospective analysis of oxaliplatin chemotherapy-induced SAR at Kaohsiung Veterans General Hospital in Taiwan. In addition, the authors have conducted a literature review on the same issue. This side effect is rare but is a life-threatening event; the authors have made some recommendations on the use of oxaliplatin as chemotherapy. This is important information which needs to be reported.

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BRIEF ARTICLE

Myofibrillogenesis regulator-1 overexpression is associated with poor prognosis of gastric cancer patients

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Abstract

AIM: To investigate the expression of myofibrillogenesis regulator-1 (MR-1) in relation to clinicopathological parameters and postoperative survival in a group of Chinese patients with gastric cancer.

METHODS: In our previous study of human whole-genome gene expression profiling, the differentially expressed genes were detected in the gastric cancer and its adjacent noncancerous mucosa. We found that MR-1 was associated with the location and differentiation of tumors. In this study, MR-1 protein expression was determined by immunohistochemistry in specimens of primary cancer and the adjacent noncancerous tissues from gastric cancer patients. A set of real-time quantitative polymerase chain reaction assays based on the Universal ProbeLibrary-a collection of 165 presynthesized, fluorescence-labeled locked nucleic acid hydrolysis probes-was designed specifically to detect the expression of MR-1 mRNA. The correlation was analyzed between the expression of MR-1 and other tumor characteristics which may influence the prognosis of gastric cancer patients. A retrospective cohort study on the prognosis was carried out and clinical data were collected from medical records.

RESULTS: MR-1 mRNA and protein could be detected in gastric cancer tissues as well as in matched noncancerous tissues. MR-1 was up-regulated at both mRNA (5.459 ± 0.639 *vs* 1.233 ± 0.238, $P < 0.001$) and protein levels (34.2% *vs* 13.2%, $P = 0.003$) in gastric cancer tissues. Correlation analysis demonstrated that high expression of MR-1 in gastric cancer was significantly correlated with clinical stage ($P = 0.034$). Kaplan-Meier analysis showed that the postoperative survival of the MR-1 positive group tended to be poorer than that of the MR-1 negative group, and the difference was statistically significant ($P = 0.002$). Among all the patients with stage Ⅰ-Ⅳ carcinoma, the 5-year survival rates of MR-1 positive and negative groups were 50.40% and 12.70%, respectively, with respective median survival times of 64.27 mo (95%CI: 13.41-115.13) and 16.77 mo (95%CI: 8.80-24.74). Univariate and multivariate analyses were performed to compare the impact of MR-1 expression and other clinicopathological parameters on prognosis. In a univariate analysis on all 70 specimens, 6 factors were found to be significantly associated with the overall survival statistically: including MR-1 expression, depth of invasion, distant metastasis, lymph node metastasis, vascular invasion and the tumor node metastasis (TNM) stage based on the 7th edition of the International Union against Cancer TNM classification. To avoid the influence caused by univariate analysis, the expressions of MR-1 as well as other parameters were examined in multivariate Cox analysis. Clinicopathological variables that might affect the prognosis of gastric cancer patients were analyzed by Cox regression analysis, which showed that MR-1 expression

and TNM stage were independent predictors of postoperative survival. The best mathematical multivariate Cox regression model consisted of two factors: MR-1 expression and TNM stage. Our results indicated that MR-1 protein could act as an independent marker for patient overall survival [Hazard ratio (HR): 2.215, P = 0.043].

CONCLUSION: MR-1 is an important variable that can be used to evaluate the outcome, prognosis and targeted therapy of gastric cancer patients.

Key words: Myofibrillogenesis regulator-1; Gastric cancer; Real-time quantitative reverse transcriptase-polymerase chain reaction; Immunohistochemistry; Poor prognosis

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INTRODUCTION

Gastric cancer remains the fourth most common malignancy, and the second leading cause of cancer-related death worldwide. It is estimated that one million new cases of gastric cancer occur each year[1,2], and most of them come from Asia (China, Japan and South Korea) and parts of Central and South America (Costa Rica, El Salvador and Columbia)[1]. More new cases of gastric cancer are diagnosed in China than in any other countries each year[3]. Many patients are found at an advanced stage with lymph node invasion and metastasis at their initial diagnosis. Despite a curative surgery and postoperative adjuvant therapy, nearly 60% of those patients succumb to the disease[4-6]. Cancer progression and metastasis is a highly complex multi-stage process. It involves increased cell adhesion, alterations in gene expression, and changes in cell motility. During invasion and metastasis, cancer cells move within tissues, and the invasion involves multiple processes regulated by various molecules[7]. As gastric cancer is featured as a heterogeneous disease in both histology and genetics, it is hard to predict patient outcome using the classic histological classifications. Gastric carcinogenesis is thought to be a multifactorial and multistep process involving the activation of oncogenes and the inactivation of tumor suppressor genes at different stages of gastric cancer progression. However, promising molecules that have clinicopathological/prognostic significance in gastric cancer are substantially limited. It is crucial to further understand the molecular mechanisms of cancer progression and the development involved in gastric cancer and to identify more valuable prognostic markers in order to improve patient prognosis as well as to provide novel promising therapy targets[8-11].

In our recent study, we utilized human whole-genome gene expression BeadChip of Illumina Company (Human 6-V2) to compare the differentially expressed genes between the adenocarcinoma of the esophagogastric junction (AEG) group and the distal gastric cancer group, and analyzed the difference of the genes related to gastric cancer and its adjacent noncancerous mucosa. There are 1121 differentially expressed genes from the BeadChip. By further analyzing the cDNA microarray data, we found that 15 genes were differentially expressed in AEG and distal gastric cancer, 90 genes were related to the differentiation of tumors. Myofibrillogenesis regulator-1 (MR-1) was associated with the location and differentiation of tumors simultaneously. In this study, we focused on the expression of MR-1 mRNA and protein in gastric cancers.

MR-1, which is mapped to 2q35, was first cloned from a human skeletal muscle cDNA library using polymerase chain reaction (PCR) and rapid amplification of cDNA ends (Genbank™ accession no. AF417001). MR-1 is composed of three distinct exons, in which exon 3 is unique when compared with other two genes, and encodes a protein of 142 amino acids with a hydrophobic transmembrane structure from 75 to 92 amino acids[12-15]. The transcription level of MR-1 in human tissues is especially high in myocardium and skeletal muscles as revealed by Northern blot and serial analysis of gene expression[12]. Overexpression of MR-1 could promote cancer cell proliferation and migration in human hepatoma G2 (HepG2) cells[16]. MR-1 might promote cancer cell proliferation by binding to specific proteins, such as eukaryotic initiation factor 3 that is highly correlated with tumor cell growth and invasion regulation[17]. Also, overexpression of MR-1 can activate the nuclear factor κB signaling pathway, which is correlated with a wide variety of diseases, including cancer, inflammation, and autoimmune diseases[18].

Taking all the evidences listed above into account, we hypothesized that MR-1 may take part in the development and progression of gastric cancer. On the basis of these studies, we used real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR) and immunohistochemistry to examine the expression of MR-1 in gastric cancer samples and adjacent normal tissues. Our study was the first attempt to investigate the relationship between MR-1 expression and prognosis of gastric cancer patients with complete clinical and follow-up data. We analyzed MR-1 protein expression and studied the relationship between MR-1 expression and survival. We also evaluated the possible associations between MR-1 protein expression and clinicopathological characteristics.

MATERIALS AND METHODS

Patients and tissue specimens

In this retrospective study, a consecutive series of 70 paired tissue specimens were collected from the patients with gastric cancer who received subtotal or total gastrec-

tomy at the Peking University Cancer Hospital in Beijing between January 2004 and December 2005. Written informed consent was obtained before sample collection and this study was approved by the Ethics Committee of Peking University. There were 45 males and 25 females with a mean age of 56 years (range: 26-81 years). None of the patients had undergone either chemotherapy or radiotherapy before surgery and there was no other co-occurrence of diagnosed cancers. A number of clinicopathological variables such as gender, age, tumor location, histological type, tumor-node-metastasis (TNM) stage, depth of tumor invasion, lymph node metastasis, distant metastasis and vascular invasion were obtained from the histopathological records and included for survival analysis.

We classified the postoperative staging of gastric cancer according to 7th American Joint Committee on Cancer (AJCC) TNM staging classification for carcinoma of the stomach[19]. There were 6 patients with stage Ⅰ, 15 patients with stage Ⅱ, 43 patients with stage Ⅲ, and 6 patients with stage Ⅳ carcinoma. After gastrectomy, resected specimens were processed routinely for histopathological assessment; necrotic hemorrhage and connective tissues were removed and each paired bulk sample [tumor samples (T)/matched normal samples (N)] was immediately put into liquid nitrogen and stored at -80 ℃ until processed. The resected specimens of gastric cancer were also routinely subjected to macroscopic pathological assessment and fixed with 10% formalin in phosphate buffered saline (PBS) for immunohistochemistry. All tissue specimens were formalin-fixed and paraffin-embedded. Formalin fixed tissue sections were stained with haematoxylin and eosin and classified by a pathologist. These results were compared with the histopathologial records from Peking University Cancer Hospital. Final pathology was determined by consensus and reviewed if necessary. The patients were followed up from a period of 2.23 to 89.07 mo (mean, 30.78 mo). Follow-up was managed through correspondence, over the telephone or in the clinic every 3 to 6 mo for 5 years and half a year thereafter. In the clinic, history enquiry, physical examination, complete blood count, biochemical tests, imaging studies and endoscopy were routinely completed. All gastric cancer patients in our study were followed up regularly and follow-up information was complete. The primary endpoint of the follow-up was death of gastric cancer patients. Patients who did not die as a result of gastric cancer were excluded.

Isolation of total RNA

Total RNA was extracted according to the manufacturer's instructions (TRIzol, Invitrogen, United States). The integrity of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under ultraviolet (UV) light. The RNA was stored at -80 ℃ in RNase-free water until reverse transcription or fluorescence labeling.

Semiquantitative reverse transcription-polymerase chain reaction

Reverse transcription (RT) was performed in a 25 μL reaction volume with 2 μg total RNA treated with 0.5 μg of Oligo (dt), 200 U Moloney murine leukemia virus reverse transcriptase, 25 U RNase inhibitor and 2.5 mmol dNTP to synthesize the first-strand cDNA (Promega, United States), according to the manufacturer's recommendations. The reaction system was incubated at 70 ℃ for 5 min (primer annealing), 42 ℃ for 1 h (synthesis) and resulting cDNA was stored at -20 ℃. The resulting cDNA was subjected to PCR for the evaluation of the relative expression levels of β-actin (as an internal control) and MR-1. PCR was done using 1 unit HotMaster Taq DNA polymerase (Qiagen, Germany) and 1:20 of the reverse transcription reaction, with an initial hot start of 5 min at 95 ℃ followed by 30 s denaturation. Primers, annealing and extension temperatures, and number of cycles used (chosen for the exponential phase of amplification) were as follows: (1) β-actin forward primer: CATGCCATCCTGCGTCTGGAC, reverse primer: CACGGAGTACTTGCGCTCAGGAGG; 55 ℃, 45 s, 72 ℃, 45 s, 28 cycles, 72 ℃, 5-min bands of 275 bp; and (2) MR-1 forward primer: GCTTTGCAGGTGTGGTGGAG, reverse primer: AGGAACGGGTTGTAGGAGCG; 52 ℃, 40 s, 72 ℃, 45 s, 35 cycles, 72 ℃, 5-min bands of 133 bp. Two bands were detected at 133 bp and 275 bp corresponding to the molecular weight marker. PCR products were electrophoresed on 1.5% agarose gels with 0.01% ethidium bromide. Band intensities were measured under UV light using Gel Analyst software (UVP, Upland, United States).

Real-time quantitative reverse transcription-polymerase chain reaction

Real-time qPCR was performed in an ABI Prism 7500 HT (Applied Biosystems, Foster City, CA) with a Universal ProbeLibrary (UPL) probe (Roche). Primers and probe were designed by online Roche Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000). The primers for real-time qPCR were: MR-1 forward: 5'-CTTCTCAGGGGACCTGCTCT-3', reverse: 5'-TCAGCATGGTCTCTGCATTG-3'; β-actin forward: 5'-CCAACCGCGAGAAGATGA-3', reverse: 5'-CCAGAGGCGTACAGGGATAG-3'. UPL probe #76 was designed for MR-1 and UPL probe #64 for β-actin. All other reaction conditions were as described by the manufacturer. The cDNA was denatured and the Taq DNA polymerase was activated for 10 min at 95 ℃, and the cycling conditions were set as follows: 40 cycles of denaturation at 95 ℃ for 30 s, annealing at 52 ℃ for 30 s, extension at 72 ℃ for 30 s and a final step at 42 ℃ for 2 min. Standard curves were determined by running a dilution series on the housekeeping gene (β-actin) and target gene. The experiments were repeated three times independently.

Immunohistochemistry

Immunohistochemical analysis was done to study the altered protein expression in the 70 specimens of human gastric cancer tissues and non-cancerous gastric tissue controls. Four μm sections from formalin-fixed and paraffin-embedded tissues were mounted on poly-L-lysine-coated slides, baked overnight at 50 ℃ and then deparaffinized in xylene and rehydrated through alcohol to distilled wa-

ter. After hydration, endogenous peroxidase activity was blocked by incubation with 3% (v/v) hydrogen peroxidase (H_2O_2) for 20 min at room temperature. Standard antigen retrieval was then performed with heat-induced epitope retrieval (HIER) by heating the slides immersed in retrieval solution (pH 6.0) in a pressure boiler. After boiling, the slides remained in the pressure boiler for 3 min and then gradually cooled at room temperature. For the detection of MR-1, after washing with PBS three times, the sections were incubated with the polyclonal goat anti-human MR-1 antibody (HPA017068-100UL, Sigma, Germany) at 4 ℃ overnight. Then, the slides were incubated with peroxidase-labeled polymer conjugated to poly peroxidase-anti-mouse/rabbit IgG (PV-9000, Zhongshan Biotechnology Company, Beijing, China) at 37 ℃ for 30 min followed by a gentle rinse with washing buffer three times. 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining reaction was then performed and followed by Meyer hematoxylin counterstaining. The slides were then dehydrated, cleared and mounted as usual. For negative controls, the primary antibody was replaced by non-immune rabbit serum to confirm the specificity. Internal positive control was used for quality assurance. MR-1 staining was principally evaluated according to the scoring criteria. The information recorded was: subcellular location (nuclear and/or cytoplasmic), intensity of staining (negative, weak, moderate or strong) and percentage of positive immunoreactive cells. The positive group referred to the cases with > 10% cells having positive immunoreactivity. The rest was defined as negative. The slide evaluation was performed by two pathologists, and both pathologists gave almost identical reports with only minor differences. A consensus regarding controversial cases was reached after discussion.

Statistical analysis

All statistical analyses were performed using SPSS statistical analysis software, version 16.0 (SPSS, Chicago, IL, United States). A paired-samples *t* test was used to compare the MR-1 mRNA levels in the tumor tissue samples and their paired adjacent non-tumor tissue samples. Regarding MR-1 expression and the clinicopathological variables, data were cross-tabulated and a χ^2 test was performed. Cumulative survival was estimated by the Kaplan-Meier method and comparisons between groups were made with a log-rank test. Postoperative survival was measured from the date of first surgery to the date of death of gastric cancer, or the last date of information collection if no end event was documented. A multivariate analysis of Cox proportional hazards regression model (backward, stepwise) was performed to assess the influence of each variable on survival. $P < 0.05$ was considered statistically significant.

RESULTS

Myofibrillogenesis regulator-1 mRNA levels are increased in gastric cancers

We randomly selected 60 specimens from the total samples, including 30 gastric cancer tissues and 30 matched noncancerous tissues to investigate the *MR-1* mRNA expression level using semiquantitative RT-PCR. As shown in Figure 1A, *MR-1* mRNA could be detected in both gastric cancer tissues and matched noncancerous tissues. However, a significant increase in the levels of *MR-1* mRNA expression was observed in primary gastric cancer compared with matched normal tissues.

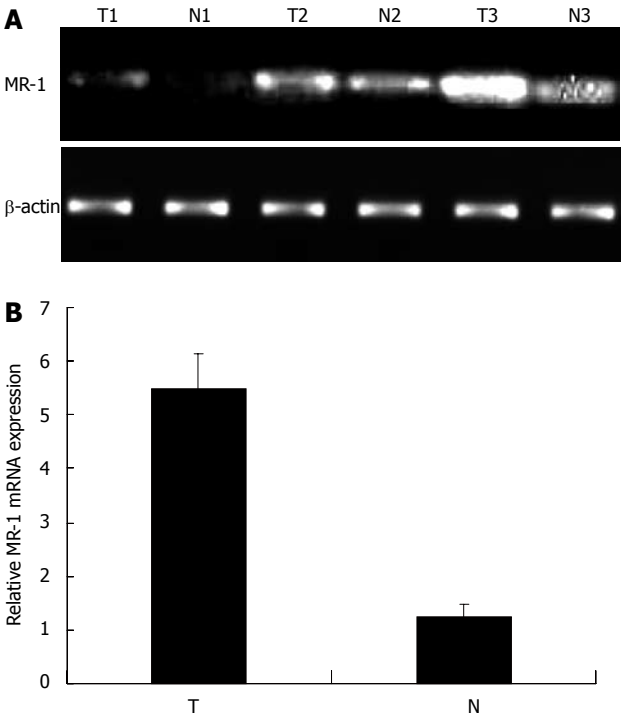

Figure 1 Expression of myofibrillogenesis regulator-1 mRNA in adjacent noncancerous mucosa and gastric cancers. A: Expression pattern of myofibrillogenesis regulator-1 (MR-1) in gastric cancer specimens by semiquantitative reverse transcription-polymerase chain reaction (PCR) (representative PCR results from 3 patients). The expression level of MR-1 mRNA showed significant difference between gastric cancer tissues and corresponding noncancerous gastric tissues; B: Results of real-time quantitative PCR assay. MR-1 mRNA expression in tumor tissue was frequently higher than that in matched normal mucosa ($P < 0.001$; Student's *t* test). β-actin was used as a control. N: Noncancerous gastric tissues; T: Gastric cancer tissues.

To validate the results of semiquantitative RT-PCR, we examined the *MR-1* mRNA expression level with real-time qPCR in the 30 paired clinical samples chosen randomly from the total cases. By real-time qPCR analysis, we found that the level of *MR-1* mRNA was increased remarkably in gastric cancer tissues. Expression of *MR-1* mRNA was measured in triplicate, and then normalized relative to the reference gene β-actin (divided by the expression level of human β-actin). The average ratios of *MR-1* mRNA to *β-actin* mRNA in gastric cancer tissues and noncancerous gastric tissues were 5.459 ± 0.639 and 1.233 ± 0.238, respectively (Figure 1B), which suggested that the expression level of *MR-1* mRNA was significantly higher in gastric cancer tissues than in the corresponding noncancerous gastric tissues ($P < 0.001$).

Myofibrillogenesis regulator-1 protein expression pattern in primary gastric cancer

MR-1 protein expression was detected in the nuclei and cytoplasm of both adjacent noncancerous mucosa and gastric cancer cells. However, the positive rate of MR-1

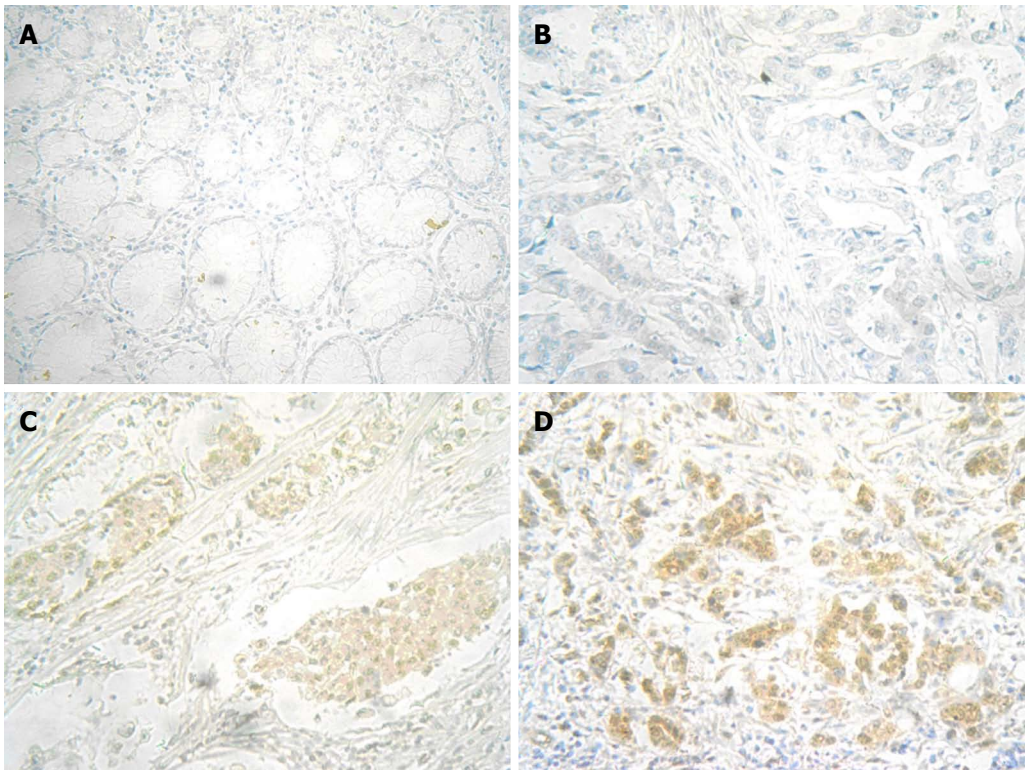

Figure 2 Myofibrillogenesis regulator-1 protein expression determined by immunohistochemical staining in adjacent noncancerous mucosa and gastric cancers. A: Negative expression in adjacent noncancerous mucosa (× 200). No myofibrillogenesis regulator-1 (MR-1) expression was detected; B: Negative expression in gastric cancers (× 200). No MR-1 expression was detected; C: Weak positive expression in gastric cancer (× 200); D: Moderate positive expression in gastric cancer (× 200). MR-1 protein was expressed in cytoplasm of the cells in (C) and (D).

expression in gastric cancer cells was much higher than that in adjacent noncancerous mucosa (34.2% *vs* 13.2%, $P = 0.003$, Figure 2).

Association of myofibrillogenesis regulator-1 protein expression with clinicopathological variables and postoperative survival in gastric cancer

We investigated the association of MR-1 protein expression with clinicopathological variables and postoperative survival. Correlation analysis demonstrated that high expression of MR-1 in gastric cancer was significantly correlated with TNM stage ($P = 0.034$). It suggested that MR-1 protein expression had no obvious association with other clinicopathological variables except TNM stage (Table 1).

Kaplan-Meier analysis showed that the postoperative survival of the MR-1 positive group tended to be poorer than that of the MR-1 negative group, and the difference was statistically significant ($P = 0.002$). For all the patients with stages I -IV carcinoma, the 5-year survival rates of MR-1 positive and negative groups were 50.40% and 12.70%, respectively, with respective median survival times of 64.27 mo (95%CI: 13.41-115.13) and 16.77 mo (95%CI: 8.80-24.74, Figure 3).

We performed univariate and multivariate analyses to compare the impact of MR-1 expression and other clinicopathological parameters on prognosis. In the univariate analysis on all 70 specimens, 6 factors were found to have statistically significant associations with overall survival: MR-1 expression, depth of invasion, distant metastasis, lymph node metastasis, vascular invasion and the TNM stage based on the 7th edition of the International Union Against Cancer TNM classification (Table 2). To avoid the influence caused by univariate analysis, the expressions of MR-1 as well as other parameters were examined in multivariate Cox analysis. Clinicopathological variables that might affect the prognosis of gastric cancer patients were analyzed by Cox regression analysis, which showed that MR-1 expression and TNM stage were independent predictors of postoperative survival (Table 2). Therefore, the best mathematical multivariate Cox regression model consisted of two factors: MR-1 expression and TNM stage. In the testing set, MR-1 was again found to be a significant independent prognostic factor for poor prognosis (hazard ratio, 2.125; 95%CI: 1.023-4.410, $P = 0.043$; Table 2) in Chinese gastric cancer patients.

DISCUSSION

Gastric cancer is reported to be the second most common cause of cancer-related death worldwide. For most cases, at the advanced stage when diagnosed, surgery is the only curative procedure for localized gastric cancer. A high recurrence rate, a low survival rate, and a poor prognosis were found in the advanced gastric cancer patients because currently available agents are not very effective. Thus, treatment of gastric tumors remains a challenge for physicians. To identify more effective approaches for cancer treatment, new targeted therapies for advanced gas-

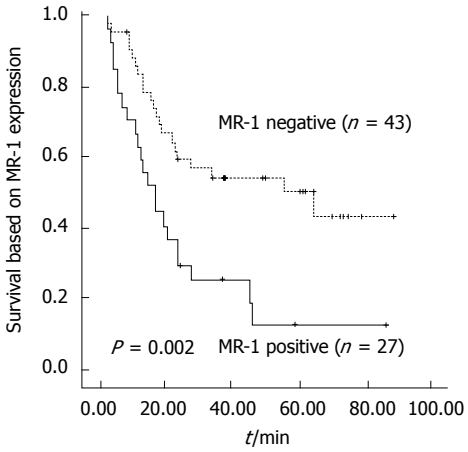

Figure 3 Postoperative survival curves in patients with stage Ⅰ-Ⅳ carcinoma. Myofibrillogenesis regulator-1 (MR-1) protein positive expression refers to cases showing weak and moderate staining.

tric cancers are needed. Right now, targeted therapy for advanced gastric carcinoma relies on target gene status evaluation[20,21]. Because of the cellular migration and metastasis, the mortality rate of gastric cancer remains high. The development of gastric cancer is a comprehensive action associated with multiple factors, such as inhibition of tumor suppressor genes, overexpression of related genes, and a failure to regulate cell proliferation. Therefore, it is urgently needed to find a sensitive biomarker for the detection of gastric cancer at the curative stage.

Li *et al*[12] identified a novel human gene, MR-1, from a human skeletal muscle cDNA library. MR-1 is located on human chromosome 2q35, having three alternatively spliced forms[22]. Based on our recent findings that MR-1 was associated with the location and differentiation of tumors simultaneously, and that MR-1 played a role in promoting the proliferation and invasion in some types of human cancers[14,18], we designed this study to comprehensively investigate the association between MR-1 and gastric cancer and the roles it may play in the pathogenesis or disease progression.

Real-time qPCR procedure combined with the Universal ProbeLibrary (UPL) technology has been successfully employed for both detection and quantification of expression levels of numerous genes, and mammalian and human viral pathogens[23-27]. UPL is a collection of 165 presynthesized, fluorescence-labeled DNA/locked nucleic acid (LNA) hybrid hydrolysis probes, which have been selected carefully to detect 8- and 9-mer motifs that are very prevalent in the transcriptomes, ensuring optimal coverage of all transcripts in a given transcriptome. On the basis of the MR-1 and β-actin sequences, UPL probe #76 and #64 with each specific PCR primer pairs were obtained from universal probe library database (Roche Diagnostics). In the present study, the prognostic relevance of MR-1 expression in gastric cancer was reported for the first time. It has been shown that MR-1 mRNA was overexpressed in gastric tumors compared with adjacent normal gastric tissues from the same individuals, in agreement with the data obtained from our microarray analysis.

In this study, immunohistochemistry was used to analyze the levels of MR-1 expression in 70 clinicopathologically characterized gastric cancer cases. The positive rate of MR-1 protein expression in gastric cancer cells was found much higher than in adjacent noncancerous mucosa. The results indicate that there are certain functions of MR-1 protein that are highly expressed in gastric cancer cells, which was confirmed by postoperative survival analysis in our study. To the best of our knowledge, there has been no report examining the role of MR-1 in gastric cancer. This is the first study showing that expression of MR-1 is increased in gastric cancer tissues compared with benign control tissues.

Table 1 Association of myofibrillogenesis regulator-1 protein expression with clinicopathological variables of gastric cancer patients

Variables	Cases	MR-1 negative *n* = 43 (%)	MR-1 positive *n* = 27 (%)	*P* value
Gender				0.855
Male	45	28 (62.22)	17 (37.78)	
Female	25	15 (60.00)	10 (40.00)	
Location				0.246
Upper	8	3 (37.50)	5 (62.50)	
Lower	62	40 (64.52)	22 (35.48)	
Age (yr)				0.400
< 55	25	17 (68.00)	8 (32.00)	
⩾ 55	45	26 (57.78)	19 (42.22)	
TNM stage				0.034
Ⅰ + Ⅱ	21	17 (80.95)	4 (19.05)	
Ⅲ + Ⅳ	49	26 (53.06)	23 (46.94)	
Depth of invasion				0.809
T1 + T2	12	7 (58.33)	5 (41.67)	
T3 + T4	58	36 (62.07)	22 (37.93)	
Lymph node metastasis				0.347
Negative	12	9 (75.00)	3 (25.00)	
Positive	58	34 (58.62)	24 (41.38)	
Distant metastasis				0.196
M0	64	41 (64.06)	23 (35.94)	
M1	6	2 (33.33)	4 (66.67)	
Vascular invasion				0.887
Negative	41	25 (60.98)	16 (39.02)	
Positive	27	16 (59.26)	11 (40.74)	
Differentiation				0.278
Well and moderately	3	1 (33.33)	2 (66.67)	
Poorly	60	36 (60.00)	24 (40.00)	
Others	5	4 (80.00)	1 (20.00)	

TNM: Tumor node metastasis; MR-1: Myofibrillogenesis regulator-1.

The clinical significance of MR-1 protein expression was studied. The result reveals that high expression of MR-1 in gastric cancer is significantly correlated with TNM stage (P = 0.034), but has no obvious association with other clinicopathological variables related to the prognosis of gastric cancer patients, such as depth of invasion and vascular invasion[28,29].

AEG has a different clinicopathological feature and poor prognosis compared with the distal gastric cancer. In this study, 5 (62.50%) of 8 cases of AEG highly expressed MR-1 protein, whereas weak expression was detected only in 22 (35.48%) of 62 cases of the distal gastric cancer. Expression was detected in two (66.67%) of three cases of well and moderately differentiated gastric cancers, whereas 24 (40.00%) of 60 cases of the poorly differentiated gastric cancer expressed MR-1 protein. These findings were in accordance with the data obtained from our microarray

Table 2 Multivariate survival analysis of the prognosis factors by Cox regression analysis

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
MR-1 expression (positive *vs* negative)	2.502 (1.365-4.586)	0.003	2.125 (1.023-4.410)	0.043
TNM stage (stages Ⅲ-Ⅳ *vs* Ⅰ-Ⅱ)	12.608 (3.845-41.345)	< 0.001	5.214 (1.155-23.526)	0.032
Depth of invasion (T3 + T4 *vs* T1 + T2)	7.094 (1.709-29.441)	0.007	3.340 (0.612-18.212)	0.164
Distant metastasis (M1 *vs* M0)	0.395 (0.166-0.943)	0.036	0.579 (0.199-1.686)	0.316
Gender (male *vs* female)	1.180 (0.635-2.192)	0.601	0.783 (0.376-1.631)	0.514
Age (yr) (< 55 *vs* ≥ 55)	0.603 (0.324-1.122)	0.110	0.717 (0.340-1.514)	0.383
Location (upper *vs* lower)	1.740 (0.726-4.168)	0.214	1.136 (0.449-2.876)	0.787
Vascular invasion (positive *vs* negative)	0.517 (0.267-1.001)	0.046	0.964 (0.456-2.038)	0.924
Lymph node metastasis (positive *vs* negative)	4.973 (1.529-16.176)	0.008	1.158 (0.249-5.382)	0.852
Differentiation (well differentiated *vs* poorly differentiated)	0.774 (0.478-1.254)	0.298	0.831 (0.485-1.426)	0.502

HR: Hazard ratio; CI: Confidence interval; TNM: Tumor node metastasis; MR-1: Myofibrillogenesis regulator-1.

analysis. However, data derived from these studies were statistically not significant, which may be because of the small sample size. Therefore, the accuracy of this first explorative result should be further tested in a larger validation study.

With respect to the results of survival analysis, in patients with stages Ⅰ-Ⅳ gastric cancer, the 5-year survival rate for those with high MR-1 expression was significantly lower than that of patients with low MR-1 expression. The results showed that MR-1 was up-regulated in gastric cancer tissues compared with normal gastric tissues and correlated significantly with prognosis. Multivariate analysis suggested that MR-1 expression and TNM stage were independent prognostic indicators for gastric cancer. The relative risk of death in patients with MR-1-positive tumors was 2.125 times higher than that in the patients with MR-1-negative tumors [hazard ratio (HR) = 2.125, 95%CI: 1.023-4.410]. Depth of invasion, distant metastasis, lymph node metastasis and vascular invasion, which were significant prognostic factors in the univariate analysis, showed no significant influence on survival in the multivariate analysis, possibly because their prognostic value was overlapped by the TNM stage. Thus, MR-1 is a potentially novel therapeutic target for the treatment of gastric cancer.

The mechanism by which MR-1 promotes tumorigenesis and cancer progression has not been well elucidated. The interaction of MR-1 with sarcomeric structural proteins involved in muscle contraction and its presence in human myocardial myofibrils indicate that MR-1 could regulate contractile proteins in the myocardium and might be associated with cardiac hypertrophy. Myosin light chain-2 (MLC-2) plays an important role in cell migration from solid cancers such as ovarian tumor, and its dephosphorylation could induce apoptosis[30]. A study showed that MLC-2 may regulate cell proliferation and migration by interacting with MR-1[31]. Knockdown of MR-1 expression in human hepatoma HepG2 cells inhibits cell migration and proliferation both *in vitro* and *in vivo*. The mechanism underlying this action is that MR-1 induces MLC-2 activation, subsequently stimulates stress fiber formation, and indirectly activates the focal adhesion kinase/protein kinase B (FAK/Akt) signaling pathway to promote cell migration and proliferation[15]. Further studies are needed to define the molecular mechanisms that govern the potential role of MR-1 expression in gastric cancer progression, clarify whether MR-1 is an early diagnostic marker for gastric cancer and to assess its full therapeutic potential.

In conclusion, our data show that a subset of patients with gastric cancer have MR-1 overexpression, which is associated with an aggressive clinical course and poor overall survival. Thus, MR-1 may be a novel biological marker and potential therapeutic target for the treatment of gastric cancer. It could also be used to monitor the effect of anti-cancer therapies. The results of our study suggest that overexpression of MR-1 in gastric cancer tissues might play an important role in the progression and metastases of the disease and that MR-1 may be a useful prognostic and survival indicator. These findings may help us explore novel therapeutic modalities and prognostic predictors for gastric cancer patients, thus improving the treatment outcomes. This is the first report to suggest a relationship between MR-1 and prognosis of patients with gastric cancer, and further prospective investigations would be worth doing in clinical settings.

COMMENTS

Background

Gastric cancer is one of the commonest malignant tumors in the alimentary tract and is characterized by delayed clinical presentation, rapid progression, and poor survival. Although this neoplasm is a serious public health problem due to its high incidence and mortality, little is known about the molecular events involved in gastric carcinogenesis.

Research frontiers

Gastric cancer, similar to other neoplasms, is a multifactorial disease that results from a combination of environmental factors and the accumulation of generalized and specific genetic and epigenetic alterations. Myofibrillogenesis regulator-1 (MR-1) protein family has been recently found associated with carcinogenesis, but not in the stomach. In this study, the authors evaluated mRNA and protein expression of MR-1 in gastric neoplasms and corresponding non-neoplastic samples.

Innovations and breakthroughs

No previous study has evaluated the gene and protein expression of MR-1 in gastric carcinogenesis. The findings from this study suggested that MR-1 may play an important role in the development of gastric cancer and it is a potential indicator that could be used to evaluate the outcome of gastric cancer treatment.

Applications

These results suggest that overexpression of MR-1 is associated with clinical stage and serves as a prognostic factor in patients with gastric cancer.

Terminology

MR-1, which is mapped to 2q35, was first cloned from a human skeletal muscle cDNA library using PCR and rapid amplification of cDNA ends (Genbank™ accession no. AF417001). It encodes a protein of 142 amino acids with a hydrophobic transmembrane structure from 75 to 92 amino acids.

Peer review

The study discovered some differential expression genes through bioinformatics and elucidated the expression level of MR-1 in gastric cancer and evaluated the link between MR-1 and the poor outcome of gastric cancer. The major finding of this study was that MR-1, which up-regulated in gastric cancer tissues comparing with matched non-tumor tissues, might be a novel prognostic marker for gastric cancer.

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BRIEF ARTICLE

Identification of deregulated miRNAs and their targets in hepatitis B virus-associated hepatocellular carcinoma

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Abstract

AIM: To identify the differentially expressed miRNAs and their targets in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC).

METHODS: Six hundred and sixty seven human miRNAs were quantitatively analyzed by Taqman low-density miRNA array (TLDA) in HBV-HCC tissues. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were used to analyze the significant function and pathway of the differentially expressed miRNAs in HBV-HCC. TargetScan software was used to predict the targets of deregulated miRNAs. Western blotting and luciferase assay were performed to verify the targets of these miRNAs.

RESULTS: Ten up-regulated miRNAs (miR-217, miR-518b, miR-517c, miR-520g, miR-519a, miR-522, miR-518e, miR-525-3p, miR-512-3p, and miR-518a-3p) and 11 down-regulated miRNAs (miR-138, miR-214, miR-214#, miR-199a-5p, miR-433, miR-511, miR-592, miR-483-3p, miR-483-5p, miR-708 and miR-1275) were identified by Taqman miRNAs array and confirmed quantitatively by reverse transcription polymerase chain reaction in HCC and adjacent non-tumor tissues. GO and KEGG pathway analysis revealed that "regulation of actin cytoskeleton" and "pathway in cancer" are most likely to play critical roles in HCC tumorigenesis. MiR-519a and ribosomal protein S6 kinase polypeptide 3 (RPS6KA3) were predicted as the most significant candidates by miRNA-mRNA network. In addition, cyclin D3 (CCND3) and clathrin heavy chain (CHC), usually up-regulated in HCC tissues, were validated as the direct target of miR-138 and miR-199a-5p, respectively.

CONCLUSION: Our data suggest an importance of miR-138 and miR-199a-5p as well as their targets CCND3 and CHC in HCC tumorigenesis, and may provide more evidence for reliability of integrative bioinformatics analysis.

Key words: Hepatocellular carcinoma; miR-138; miR-199a-5p; Cyclin D3; Clathrin heavy chain; Bioinformatics; Taqman array

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1]. The development and progression of HCC is characterized by a multi-stage process, which is believed to involve the deregulation of genes that are critical to cellular processes such as cell cycle control, cell growth, apoptosis, and cell migration and spreading. In the past decades, studies have focused on investigating the effect of genes and proteins on the development and progression of HCC[2]. Recently, an increasing number of reports have described microRNAs (miRNAs) that are implicated in HCC progression[3].

MiRNAs are endogenous non-coding RNAs (20-22 nucleotides) which regulate gene expression by catalyzing the cleavage of messenger RNA (mRNA) or repressing mRNA translation[4]. Increasing evidence showed that miRNAs play significant roles in cell development, differentiation and communication[5]. Deregulation of miRNAs has also been observed in a wide range of human diseases, including cancer[6]. In human cancer, miRNAs can function as oncogenes or tumor suppressor genes during tumor development and progression[7].

In this study, the expression of 667 miRNAs was profiled in human HCC and adjacent non-tumor tissues. A set of significantly differentially expressed miRNAs was identified in HCC tissues. Furthermore, a global analysis of miRNA-regulated signaling pathways and related genes was performed on the basis of miRNA expression profiles and bioinformatics interpretation. Cyclin D3 (CCND3) and clathrin heavy chain (CHC) which had been previously described in HCC tumorigenesis were further validated to be the direct target of miR-138 and miR-199a-5p, respectively.

MATERIALS AND METHODS

Tissue specimens

Eighteen pairs of human HCC and adjacent non-tumor tissues were obtained from surgical specimens immediately after resection from patients undergoing primary surgical treatment of HCC in the Eastern Hepatobiliary Surgery Hospital, Shanghai, China. No patient had received preoperative irradiation or chemotherapy. The samples were frozen in liquid nitrogen and stored at -80 ℃ until use. Among these samples, three pairs were used for Taqman low-density miRNA array (TLDA) analysis and all were used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Clinical and pathological information was extracted from the patients' medical charts and pathological reports (Table 1). Written consent for tissue donation (for research purposes) was obtained from the patients before tissue collection and the protocol was approved by the Institutional Review Board of Eastern Hepatobiliary Surgery Hospital and Second Military Medical University.

Taqman low-density miRNA array

Total RNA was isolated using mirVana miRNA isolation kit (Ambion, Austin, TX, United States). For miRNA cDNA synthesis, RNA was reversely transcribed using the miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, United States) in combination with the stem-loop Megaplex primer pool (Applied Biosystems). TLDA v2.0 (Applied Biosystems) was performed on the 7900HT real-time PCR system (Applied Biosystems) according to the manufacturer's protocol (667 small RNAs were profiled for each cDNA sample). PCR cycling conditions were as follows: 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. Human U6 small RNA was used as an internal control to normalize RNA input. The data were analyzed using SDS v2.3 software. The Ct value was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method and presented as the fold-expression change in tumors and their adjacent normal tissues after normalization to the endogenous control.

Table 1 Characteristics of patients (n = 18)

No.	Gender	Age (yr)	Tumor size[1] (cm)	Cirrhosis	Tumor grade[2]	TNM stage
1	M	55	1.9	No	G3	T2N0M0
2	M	40	4.1	Macronodular	G3	T2N0M0
3	M	42	4.6	No	G3	T3N0M0
4	M	42	5.6	Micronodular	G2	T3N0M0
5	M	43	5.8	Micronodular	G1	T1N0M0
6	M	61	1.9	Micronodular	G3	T2N0M0
7	M	55	12.0	Micronodular	G2	T4N0M0
8	M	40	4.0	Macronodular	G2	T3N0M0
9	M	35	2.3	Macronodular	G1	T1N0M0
10	M	57	5.9	No	G3	T1N0M0
11	M	44	9.6	No	G2	T3N0M0
12	M	45	12.2	Micronodular	G3	T2N0M0
13	M	46	4.4	Micronodular	G3	T2N0M0
14	F	46	15.8	Micronodular	G3	T2N0M0
15	M	63	6.1	No	G3	T3N0M0
16	M	61	4.6	No	G3	T2N0M0
17	F	60	9.1	No	G3	T3N0M0
18	F	50	9.3	No	G3	T2N0M0

[1]Diameter of the biggest nodule. [2]G1-2: Well-differentiated; G3: Moderately-differentiated; G4: Poorly-differentiated. M: Male; F: Female; TNM: Tumor-node-metastasis.

Quantitative real-time PCR

For miRNA expression analysis, synthesis of cDNA and qRT-PCR was carried out with TaqMan microRNA assay kits (Applied Biosystems) according to the manufacturer's protocol. Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, United States) from HCC and adjacent non-tumor tissues and used to synthesize cDNAs with gene-specific primers. Reverse transcriptase reactions contained 100 ng RNA, 50 nmol/L stem-loop RT primers, 1 × RT buffer, 0.25 mmol/L each of the dNTPs, 3.33 U/μL MultiScribe reverse transcriptase and 0.25 U/μL RNase inhibitor. The 15 μL reactions were incubated for 30 min at 16 ℃, 30 min at 42 ℃, 5 min at 85 ℃, and then kept at 4 ℃. The cDNA product was used for the following qRT-PCR analysis. The 20 μL

PCR reaction included 1.33 μL RT product, 1 × TaqMan universal PCR master mix and 1 μL primers and probe mix of the TaqMan microRNA assay kit. Reactions were incubated in a 96-well optical plate at 95 ℃ for 5 min, followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 1 min. PCR reactions were run on a StepOne Plus real-time PCR machine (Applied Biosystems) and the data were analyzed using SDS v2.3 software, the same as in TLDA.

Prediction of miRNA targets

The target genes of the deregulated miRNAs were predicted by TargetScan (http://www.targetscan.org/).

Gene oncology and Kyoto encyclopedia of genes and genomes pathway analysis based on miRNA expression profile

The miRNA targets were subjected to gene oncology (GO) analysis in order to organize genes into hierarchical categories and uncover the miR-gene regulatory network on the basis of biological process and molecular function[8]. Fisher's exact test and χ^2 test were used to classify the GO category, and the false discovery rate (FDR)[9] was calculated to correct the *P* value, the lower the FDR, the slight the error in judging the *P* value. The FDR was defined as $FDR = 1 - \frac{N_k}{T}$, where N_k refers to the number of Fisher's test *P* values less than χ^2 test *P* values. *P* values were computed for the GOs of all the differential genes. Enrichment provides a measure of the significance of the function: as the enrichment increases, the corresponding function becomes more specific, which can help find those GOs with more concrete function description in the experiment. Within the significant category, the enrichment Re was given by: $Re = (n_f/n) / (N_f/N)$ where n_f is the number of differential genes within the particular category, n is the total number of genes within the same category, N_f is the number of differential genes in the entire array, and N is the total number of genes in the array[10]. Similarly, pathway analysis was used to find out the significant pathway of the differential genes according to Kyoto encyclopedia of genes and genomes (KEGG), Biocarta and Reatome. The Fisher's exact test and χ^2 test were also used to select the significant pathway, and the threshold of significance was defined by *P* value and FDR. The enrichment Re was calculated using the same equation mentioned above[11-13]. The network of miRNA-mRNA interaction, representing the critical miRNAs and their targets, was established according to the miRNA degree.

Construction of luciferase reporter plasmids

The fragment of 3'-untranslated region (UTR) of CCND3 (1054-2061nt, Genbank accession no. NM_001136017.2) containing the two putative miR-138 binding sequences (1279-1285nt and 1346-1352nt) was amplified with the primers 5'-CCCTGGAGAGGCCCTCTGGA-3' and 5'-TTCCAAGAAGCCAAAGCCAG-3'. The partial fragment of 3'-UTR of CHC (5472-6480nt, Genbank accession no. NM_004859) containing the two putative miR-199a-5p binding sequences (5979-5986nt and 5915-5922nt) was amplified with the primers 5'-GATGAAGCGCTGATCCTGTAG-3' and 5'-TGCCTCCCTAATGCCTCAG-3'. The PCR products were cloned into firefly luciferase reporter vector pGL3 (Promega Corporation, Madison, WI, United States) respectively, termed as pGL3-CCND3-3'UTR or pGL3-CHC-3'UTR. The plasmids carrying the mutated sequence in the complementary sites for the seed region of miR-138 or miR-199a-5p, were generated based on pGL3-CCND3-3'UTR and pGL3-CHC-3' UTR plasmids by site-specific mutagenesis, termed as pGL3-CCND3-3'UTR-mut or pGL3-CHC-3'UTR-mut.

Transfection

The transfection was carried out using FuGene HD transfection reagent (Roche, Indianapolis, IN, United States) following the manufacturer's protocol. In brief, 2 × 10^4 HepG2 cells or 5 × 10^4 HEK293T cells in 24-well plate were transfected with indicated miRNA mimic (50 nmol/L, GenePharma, Shanghai, China) or plasmid DNA (100 ng) and collected 24-48 h after transfection for assay.

Dual-luciferase reporter assay

HEK293T cells were cotransfected with pGL3-CCND3-3'UTR or pGL3-CCND3-3'UTR-mut and miR-138 mimic or nonrelative control RNA duplex [non-relative control (NC) duplex, GenePharma] using FuGene HD transfection reagent. In another well, HEK293T cells were cotransfected with pGL3-CHC-3'UTR or pGL3-CHC-3'UTR-mut and miR-199a-5p mimic or nonrelative control RNA duplex using FuGene HD transfection reagent. The pRL-TK (Promega Corporation, Madison, WI, United States) was also transfected as a normalization control. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation) and recorded by multi-plate reader (Synergy 2, BioTek).

Western blotting

Protein extracts from HCC tissues and their adjacent non-tumorous tissues or HepG2 cells were prepared by a modified radioimmunoprecipitation buffer with 0.5% sodium dodecyl sulfate in the presence of proteinase inhibitor cocktail (Complete Mini, Roche). Twenty-five micrograms protein were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini-gels and transferred onto polyvinylidene fluoride membranes (Immobilon P^{SQ}, Millipore, Billerica, MA, United States). After blocking with 5% nonfat milk, the membranes were incubated with rabbit anti-CCND3 antibody (1:1000 dilution, Epitomics, Inc., Burlingame, CA, United States), rabbit anti-clathrin heavy chain (CHC) antibody (1:1000 dilution, Abcam, Cambridge, United Kingdom) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000 dilution, Epitomics, Inc.) at 4 ℃ overnight, followed by incubation with horserad-

ish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (1:10 000 dilution, KPL, Gaithersburg, MA, United States) for 1h at room temperature. Finally, signals were developed with Super Signal West Pico chemoluminescent substrate (Pierce, Rockford, Ill, United States) and visualized by the Gene Gnome HR Image Capture System (Syngene, Frederick, MD, United States).

Statistical analysis

Data were presented as mean ± SD. Comparisons were made using a two-tailed *t* test or one-way analysis of variance for experiments with more than two subgroups. $P < 0.01$ was considered statistically significant.

RESULTS

Patient characteristics

HCC and adjacent non-tumor tissues were obtained from 18 patients, whose average age was 48.5 years (ranged from 35 years to 71 years) (Table 1). All of the patients were HBsAg positive and with the diagnosis of HCC. In tumor grades, 7 (38.9%) cases were well differentiated (G1 + G2) and 11 (61.1%) cases were moderately differentiated (G3) HCC. In clinical stage, 4 (22.2%) were at invasion T1, 7 (38.9%) at invasion T2, and 6 (33.3%) at invasion T3 and 1 (5.6%) at invasion T4.

Differential expression of miRNAs in HCC

There were 86 deregulated miRNAs in total between HCC tumor and non-tumor tissues by TLDA analysis. In order to select the most significant candidates, miRNAs altered by at least 3-fold in all three pairs of the samples were selected. Under these strict criteria, 11 up-regulated miRNAs and 13 down-regulated miRNAs were identified (Figure 1A and Table 2). To validate the miRNA array data, qRT-PCR was performed in 18 pairs of HCC tissues. Four up-regulated (miR-217, miR-520g, miR-522 and miR-525-3p) (Figure 1B) and 4 down-regulated miRNAs (miR-199a-5p, miR-138, miR-483-5p and miR-511) showed consistent changes in more than 70% tumorous tissues (Figure 1C). Six up-regulated (miR-517c, miR-512-3p, miR-518a-3p, miR-519a, miR-518e and miR-518b) and 7 down-regulated miRNAs (miR-214, miR-214#, miR-592, miR-483-3p, miR-433, miR-708 and miR-1275) showed consistent changes in more than 50% tumorous tissues (data not shown). No significant difference was found between one up-regulated (miR-888) and two down-regulated miRNAs (miR-21# and miR-27a#) in paired tumorous tissues (data not shown).

Gene oncology and Kyoto encyclopedia of genes and genomes pathway analysis of the deregulated miRNAs

The targets of the 21 deregulated miRNAs (Table 2) were predicted by TargetScan. To identify the most significant candidates and investigate the cellular function, the signaling pathway and GOs of the target genes were analyzed. The results showed that a wide variety of cellular processes were featured significantly in signaling pathways (Figure 2A and B). Many of these signaling pathways, such as insulin, MAPK, TGF-β and Wnt signaling pathway, participated in the tumorigenesis[14-17]. However, some other signaling pathways have never been reported to play a role in tumorigenesis, e.g., axon guidance. Among all these differentially regulated signaling pathways, "regulation of actin cytoskeleton" and "pathway in cancer" appeared to be the most enriched one among both up-regulated and down-regulated miRNA groups. A similar phenomenon was observed in GOs analysis. Many cellular functions were featured significantly, of which the "signal transduction" appeared to be the most enriched one (Figure 2C and D). The miRNA-mRNA interaction network analysis integrated these miRNAs and GOs by outlining the interactions of miRNA and GO-related genes (Figure 3A). MiR-519a and miR-199a-5p showed the target genes of 53 (degree 53) in up-regulated miRNAs and target genes of 32 (degree 32) in down-regulated miRNAs. MiR-138 had a degree of 30, which was the second place in down-regulated miRNAs. These results indicated that miR-138 and miR-199a-5p as well as their targets, might be of great importance to the HCC tumorigenesis. Twenty-six target genes, including *RPS6KA3*, *SMAD4*, *ACVR2A*, *CHC*, and *MAPK1*, *etc*., had more than 3 miRNAs (degree > 3) (Figure 3B).

CCND3 is a direct target of miR-138

Among these deregulated miRNAs, miR-138 was most abundant in non-tumor tissues and miR-199a-5p was most significant in paired HCC tissues (Figure 4A). Thus, miR-138 and miR-199a-5p were selected for the further study. CCND3 was predicted as a potential target of miR-138 by TargetScan. The 3'-UTR of CCND3 mRNA contained a complementary site for the seed region of miR-138 (Figure 4B). CCND3 was found to be up-regulated in 4 specimens of HCC tissues compared with adjacent non-tumor liver tissues (Figure 4C), showing a negative correlation with down-regulated miR-138. These results indicated that miR-138 may be associated with CNND3 and both of them may be involved in HCC tumorigenesis.

To validate whether CCND3 is a direct target of miR-138, a human CCND3 3'-UTR fragment containing wild-type or mutant miR-138 binding sequence (Figure 4B) was cloned downstream of the firefly luciferase reporter gene in pGL3. In HEK293 cells cotransfected with the reporter plasmids and miR-138 mimic or NC duplex, the luciferase activity of the reporter that contained wild-type 3'-UTR was significantly suppressed by miR-138 mimic, but the luciferase activity of mutant reporter was unaffected (Figure 4D), indicating that miR-138 may suppress gene expression through miR-138 binding sequence at the 3'-UTR of CCND3. Furthermore, transfection of miR-138 mimic decreased CCND3 expression in HepG2 cells at protein level (Figure 4E). All these results showed that miR-138 could regulate the expression of endogenous human CCND3 by directly

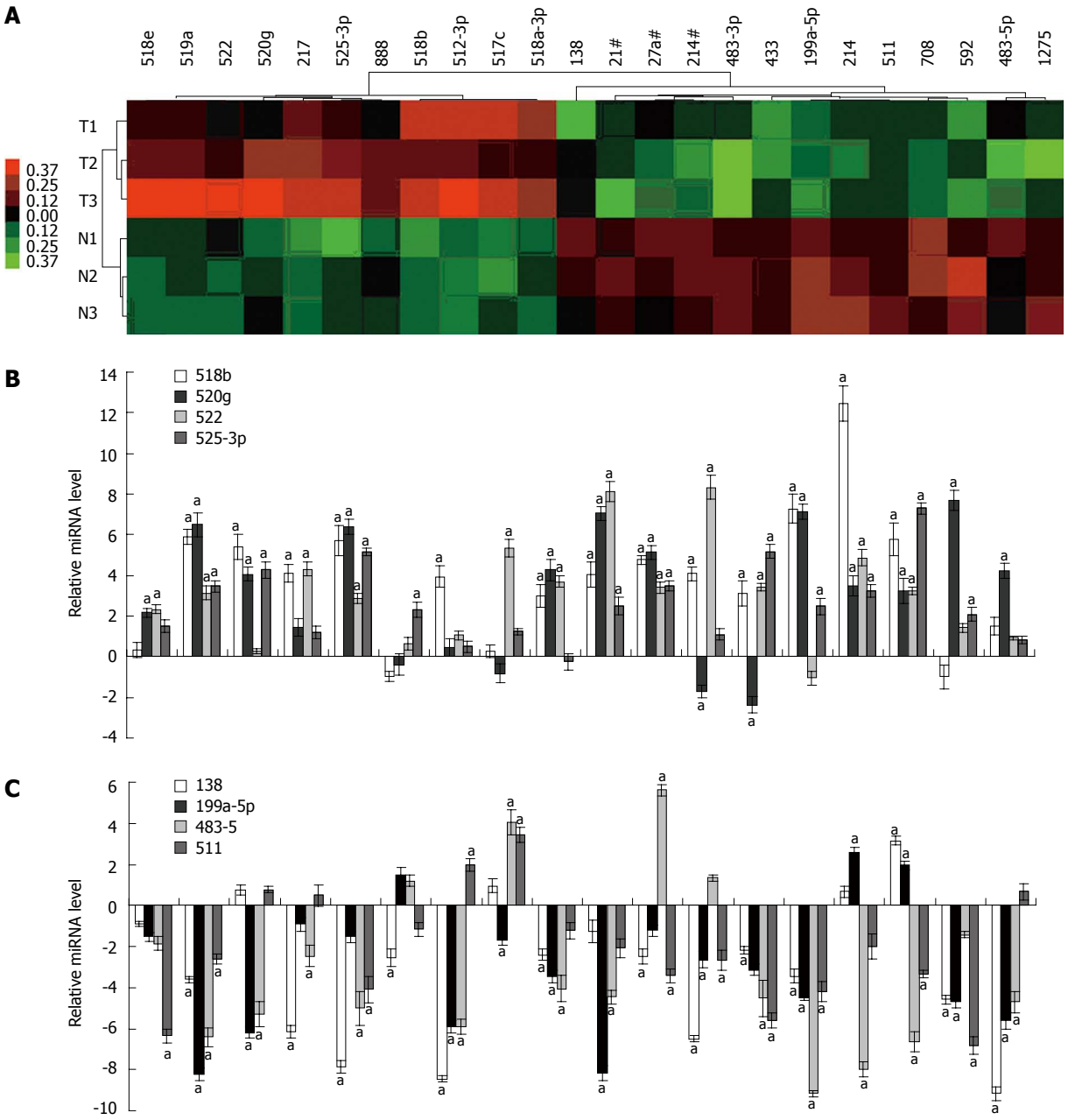


Figure 1 MiRNA profiles differentiate hepatitis B virus-associated hepatocellular carcinoma from adjacent non-tumor tissues. A: The cluster analysis of down-regulated (green) and up-regulated (red) miRNAs identified in hepatocellular carcinoma (hepatitis B virus-hepatocellular carcinoma). Samples consist of paired samples from three patients; B: Validation of Taqman array data using quantitative reverse transcription polymerase chain reaction (RT-PCR) for up-regulated miRNA; C: Validation of Taqman array data using quantitative RT-PCR for down-regulated miRNA. Triplicate assays were done for each RNA sample and the relative amount of each miRNA was normalized to U6 snRNA. $^{a}P < 0.01$ *vs* control group.

targeting the 3'-UTR of CCND3 mRNA and human CCND3 was a new target of miR-138.

Clathrin heavy chain is a direct target of miR-199a-5p

CHC was found to be a direct target of miR-199a-5p. CHC was predicted as a potential target of miR-199a-5p by TargetScan (Figure 4B). CHC was up-regulated in 4 specimens of HCC tissues as compared with adjacent non-tumor liver tissues (Figure 4C). The luciferase activity of the reporter containing wild-type 3'-UTR of CHC was significantly suppressed by miR-199a-5p mimic (Figure 4D). Furthermore, transfection of miR-199a-5p mimic decreased CHC expression in HepG2 cells at protein level (Figure 4E). The results showed that miR-199a-5p could regulate the expression of endogenous human CHC by directly targeting the 3'-UTR of CHC mRNA, and human CHC is a new target of miR-199a-5p.

DISCUSSION

MiRNAs were frequently deregulated in HCC, and some specific miRNAs were associated with the clinicopatho-

Table 2 Deregulated miRNA in hepatitis B virus-associated hepatocellular carcinoma *n* (%)

miRNA	Fold change	*P* value	Validation[1]
Up-regulated miRNAs			
hsa-miR-520g	9475.09	0.00	13 (72.2)
hsa-miR-519a	8204.48	0.00	10 (55.6)
hsa-miR-522	6178.34	0.00	13 (72.2)
hsa-miR-518e	4096.74	0.00	11 (61.1)
hsa-miR-525-3p	1489.49	0.00	14 (77.8)
hsa-miR-217	1080.33	0.00	11 (59.4)
hsa-miR-518b	982.85	0.00	14 (77.8)
hsa-miR-512-3p	197.21	0.00	10 (55.6)
hsa-miR-517c	130.92	0.00	11 (61.1)
hsa-miR-518a-3p	46.84	0.00	10 (55.6)
Down-regulated miRNAs			
hsa-miR-199a-5p	0.00237	0.00050	13 (72.2)
hsa-miR-433	0.00310	0.00270	10 (55.6)
hsa-miR-592	0.01137	0.00350	11 (59.4)
hsa-miR-214#	0.01327	0.00610	11 (59.4)
hsa-miR-483-5p	0.03310	0.00340	14 (77.8)
hsa-miR-483-3p	0.03323	0.00510	12 (72.2)
hsa-miR-138	0.03727	0.00190	14 (77.8)
hsa-miR-214	0.04213	0.00530	11 (59.4)
hsa-miR-511	0.07957	0.00490	13 (72.2)
hsa-miR-708	0.02135	0.00480	10 (55.6)
hsa-miR-1275	0.06296	0.00530	10 (55.6)

[1]Quantitative reverse-transcription polymerase chain reaction was performed in 18 pairs of tumor and non-tumor tissues in hepatitis B virus-associated hepatocellular carcinoma.

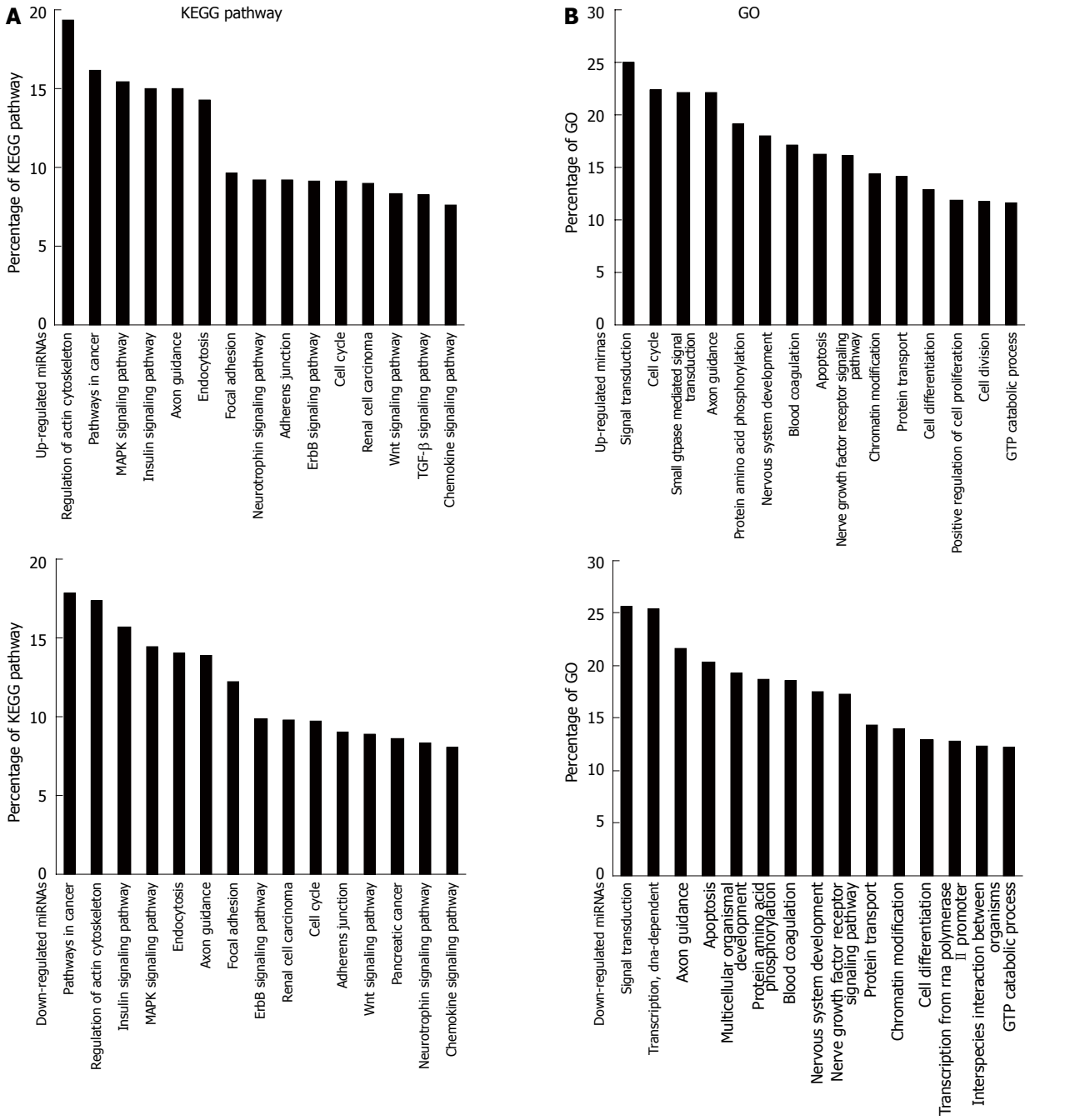
logical features of HCC, such as metastasis, recurrence, and prognosis[18-20]. Moreover, compelling evidence has demonstrated that miRNAs play an important role in HCC progression and directly contribute to the cell proliferation, avoidance of apoptosis, and metastasis of HCC. Identifying the miRNAs and their targets that are essential for HCC progression may provide promising therapeutic opportunities.

In this study, with Taqman miRNAs array and real-time RT-PCR confirmation, 10 up-regulated miRNAs (miR-217, miR-518b, miR-517c, miR-520g, miR-519a, miR-522, miR-518e, miR-525-3p, miR-512-3p, and miR-518a-3p) and 11 down-regulated miRNAs (miR-138, miR-214, miR-214#, miR-199a-5p, miR-433, miR-511, miR-592, miR-483-5p, miR-483-3p, miRNA-708 and miRNA-1275) were identified in HCC. More importantly, of these 21 deregulated miRNAs, only miR-199a-5p was involved in HCC[21,22], and the other 20 deregulated miRNAs were first reported to be involved in HCC tumorigenesis. No report of MiR-214#, miR-518a-3p and miR-518e has been available in the literature. The rest 17 miRNAs were reported in various cancer but not HCC. For example, the up-regulated miR-512-3p and miR-525-3p were associated with a cisplatin resistant phenotype in human germ cell tumors[23]. The up-regulated miR-519a and down-regulated miR-511 and miR-485-5p were associated with histological subtypes in ovarian cancers[24]. MiR-517c and 520 g promote *in vitro* and *in vivo* oncogenicity, modulates cell survival, and robustly enhances growth of untransformed human neural stem cells (hNSCs) in neuroectodermal brain tumors[25]. MiR-433 could regulate tumor-associated proteins GRB2 in gastric carcinoma[26]. MiR-592 was reported to be associated with the stepwise progression for transformation from normal colon to carcinoma[27].

On the contrary, some well known HCC-related miRNAs were not found in this study, possibly due to the very strict criteria of selection defined in TLDA (significant difference in all three pairs of HCC tissues). For example, miR-21[28], miR-122[29,30], miR-16[31] and miR-29[32,33] were excluded for their significantly differential expression only in 2 pairs of HCC tissues, and miR-181[34], miR-221[35,36], miR-125[37] and miR-101[38] were excluded for their differential expression in only 1 pair of HCC tissues or no differential expression at all. Using such strict criteria, we might miss some important candidates, but catch some unique ones. Fortunately, quantitative RT-PCR and follow-up studies proved that our strategy helped find the above new deregulated miRNAs. All of the HCC patients in this study were HBsAg positive. Therefore, the expression patterns of identified miRNAs may mainly represent the alterations in hepatitis B virus (HBV)-positive HCC, which may partially account for the inconsistency between our results and results from other studies.

KEGG pathway and GO enrichment analysis based on the reported and predicted target genes of these deregulated miRNAs, was applied to identify which particular functions and pathways were enriched among the genes controlling distinctive characters between HCC and adjacent non-tumor tissues. As a result, KEGG pathway analysis showed that proliferative (cell cycle, MAPK and Wnt), adhesive (actin cytoskeleton, adherens junction and focal adhesion), survival (TGF-β and ErbB) and oncogenic (renal cell carcinoma and pancreatic cancer) signaling pathways were abundant among the significantly enriched ones. Furthermore, the GOs related to signal transduction (signal transduction, small GTPase-mediated signal transduction, protein amino acid phosphorylation and nerve growth factor receptor signaling pathway) and cell growth (cell differentiation, cell division, positive regulation of cell proliferation, cell cycle and multicellular organism development) represented up to 37% of the significantly enriched GOs. As expected, various cell process and signal pathways were involved in HCC tumorigenesis. To narrow the scope of study and evaluate the most significant candidates, miRNAs and their target genes which were in the intersection of "signal transduction" and "regulation of actin cytoskeleton and pathway in cancer" might be the focus of the future studies.

Although computational analysis indicates that one miRNA may directly modulate hundreds of mRNAs, and a single gene may be regulated by multiple miRNAs, such regulation has not been convincingly demonstrated experimentally. As shown in Figure 3, miR-138 had 30 target genes and miR-199a-5p had 32 target genes. The



C

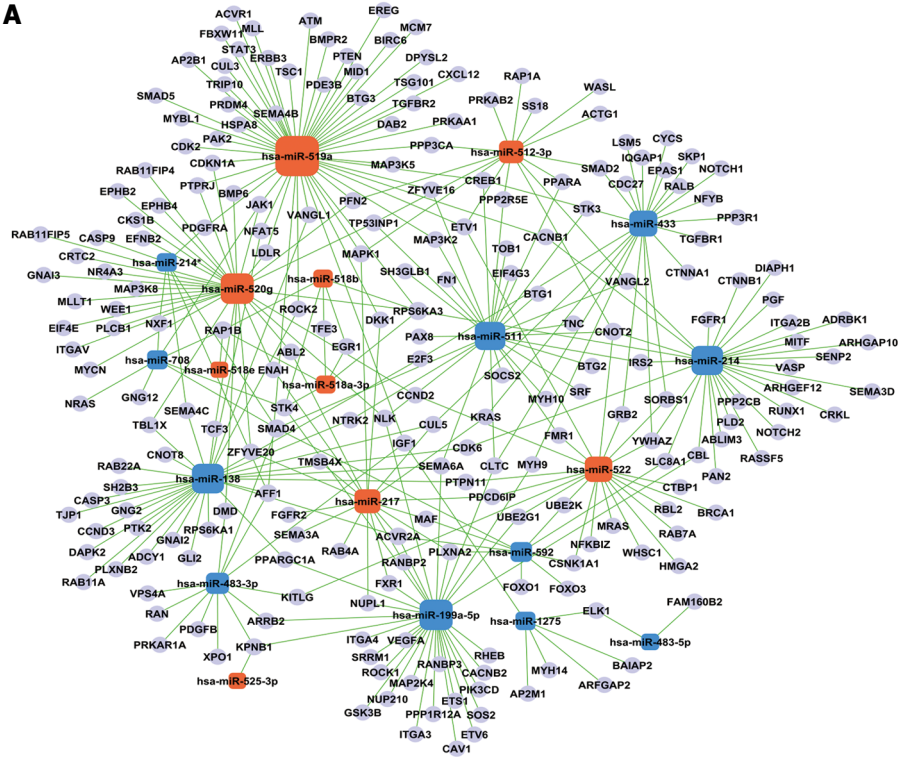
	Significant pathways	Rank	
		Up-regulated	Down-regulated
1	Regulation of cytoskeleton	1	2
2	Pathways in cancer	2	1
3	MAPK signaling pathway	3	4
4	Insulin signaling pathway	4	3
5	Axon guidance	5	6
6	Endocytosis	6	5
7	Focal adhesion	7	7
8	Neurotrophin signaling pathway	8	14
9	Adherens junction	9	11
10	ErbB signaling pathway	10	8
11	Cell cycle	11	10
12	Renal cell carcinoma	12	9
13	Wnt signaling pathway	13	12
14	Chemokine signaling pathway	15	15

D

	Significant GOs	Rank	
		Up-regulated	Down-regulated
1	Signal transduction	1	1
2	Axon guidance	4	3
3	Protein anima acid phosphorylation	5	6
4	Apoptosis	8	4
5	Blood coagulation	7	7
6	Nerve growth factor receptor signaling pathway	9	9
7	Chromatin modification	10	11
8	Protein transport	11	10
9	GTP catabolic process	15	15

Figure 2 Gene oncology and kyoto encyclopedia of genes and genomes pathway analysis based on miRNA targeted genes. A: The upper panel showing significant pathways targeted by up-regulated miRNA and the lower panel showing significant pathways targeted by down-regulated miRNA; B: The upper panel showing significant GOs targeted by up-regulated miRNA and the lower panel showing significant GOs targeted by down-regulated miRNA. The vertical axis is the pathway or GO category, and the horizontal axis is the enrichment of pathways or GOs; C, D: Summary data of A and B respectively. KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene ontology.

A



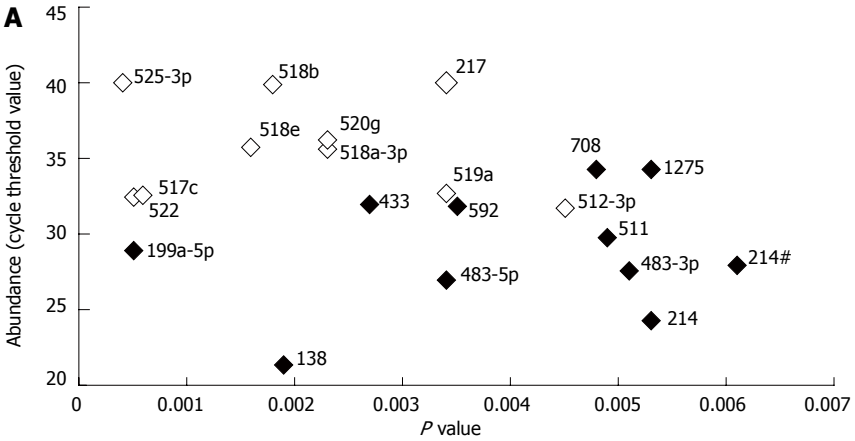
B

Up-regulated miRNA (top 3)	Degree	Down-regulated miRNA (top 3)	Degree
has-miR-519a	53	has-miR-199a-5p	32
has-miR-520g	32	has-miR-138	30
has-miR-522	21	has-miR-214	29

Gene symbol	Degree	Gene symbol	Degree
RPS6KA3	6	LDLR	3
SMAD4	5	MAPK1	3
ACVR2A	4	NLK	3
AFF1	4	PEN2	3
CNOT2	4	PPP2R5E	3
PLXNA2	4	PTPN11	3
RAP1B	4	SEMA6A	3
CHC	3	SLC8A1	3
CREB1	3	STK4	3
CUL5	3	TP53INP1	3
DMD	3	VANGL1	3
ETV1	3	VANGL2	3
KPNB1	3	YWHAZ	3

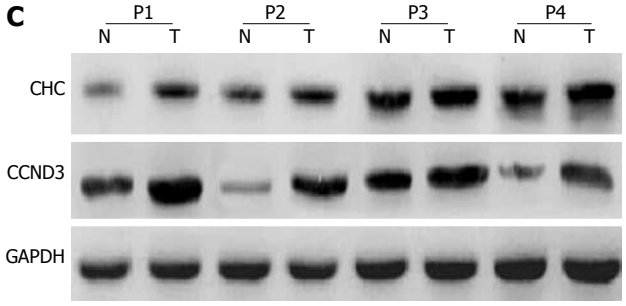
Figure 3 MiRNAs-mRNA network. A: Orange box nodes represent up-regulated miRNAs, blue box nodes represent down-regulated miRNAs, and cyan cycle nodes represent mRNA. Green lines show the inhibitory effect of miRNAs on target mRNAs; B: Summary data of A.

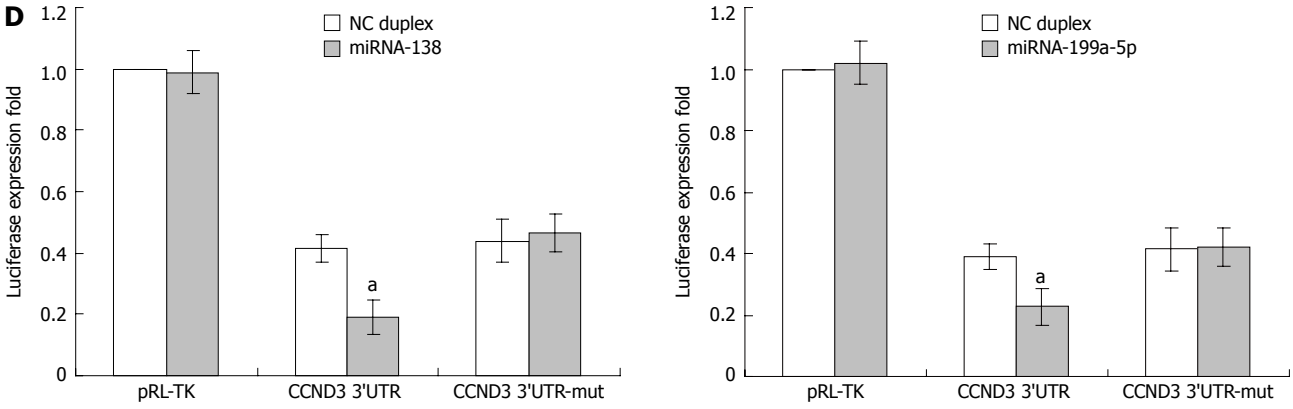
target genes are involved in different cellular processes, thus individual miRNAs play multi-faceted roles in HCC progression. Down-regulated miR-138 has been observed in different types of cancers but not in HCC[39-43]. MiR-138 plays an important role in tongue squamous cell carcinoma cell migration and invasion by concurrently targeting RhoC and ROCK2[39]. MiR-138 could inhibit the expression of HIF-1a and regulate the apoptosis and migration of clear-cell renal cell carcinoma 786-O cells[40]. MiR-138 enhanced cell migration and invasion by targeting enhancer of zeste homologue 2 (EZH2) in squamous cell carcinoma cell lines[41]. MiR-138 may play an important role in cancer initiation and progression by regulating Fos-like antigen 1 in squamous cell carcinoma[42] or G protein alpha inhibiting activity polypeptide 2(GNAI2) in tongue squamous cell carcinoma[43]. Different from miR-138, the down-regulated miR-199a-5p has been reported in HCC[21,22]. MiR-199a-5p contributes to the increase of cell invasion by functional deregulation of discoidin domain receptor-1 (DDR1) activity in HCC[21]. MiR-199a-5p regulates Brm subunit of SWI/SNF in human cancers[22]. We identified CCND3 as a target of miR-138 and CHC as a target of miR-199a-5p in HCC, which may provide new insights into the mechanisms underlying tumorigenesis. CCND3 is expressed in nearly all proliferating cells and can promote the cell cycle progres-



B

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 292-298 of CCND3 3' UTR	5'...AGAAUUGGAUACAUACACCAGCA...
has-miR-138	3' GCCGGACUAAGUGUUGUGGUCGA
Mut CCND3 3'UTR	5'...AGAAUUGGAUACAUAGUGGUCGA...
Position 225-231 of CCND3 3' UTR	5'...UCCUCCCUGCAUCUGACCAGCA...
has-miR-138	3' CCGGACUAAGUGUUGUGGUCGA
Mut CCND3 3'UTR	5'...UCCUCCCUGCAUCUGUGGUCGA...
Position 508-515 of CHC 3' UTR	5'...UAAUCAGCACAUCGUACACUGGA...
has-miR-199a-5p	3' CUUGUCCAUCAGACUUGUGACCC
Mut CCND3 3'UTR	5'...UAAUCAGCACAUCGUUGUGACCA...
Position 444-451 of CHC 3' UTR	5'...AAAGCUAAAGCAAAAACACUGGC...
has-miR-199a-5p	3' CUUGUCCAUCAGACUUGUGACCC
Mut CCND3 3'UTR	5'...AAAGCUAAAGCAAAAUGUGACCC...





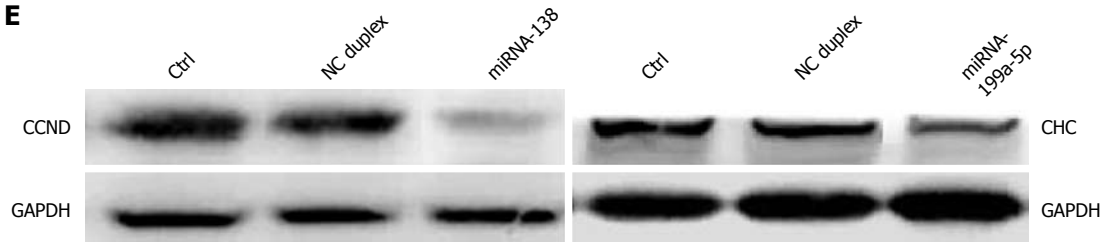


Figure 4 Cyclin D3 and clathrin heavy chain are the direct target of miR-138 and miR-199a-5p. A: Abundance of deregulated miRNAs in hepatitis B virus (HBV)-hepatocellular carcinoma (HCC) non-tumor tissues; B: The putative miR-138 or miR-199a-5p binding sequence in the 3'-UTR of cyclin D3 (CCND3) or clathrin heavy chain (CHC) mRNA; C: The expression of CCND3 and CHC in 4 paired HCCs (T) and adjacent non-tumor tissues (N); D: Suppressed luciferase activity of wild type 3'UTR of CCND3 or CHC by miR-138 or miR-199a-5p mimic. Firefly luciferase activity of each sample was mea sured 48 h after transfection and normalized to Renilla luciferase activity; E: Suppressed expression of endogenous CCND3 or CHC in HepG2 cells by miR-138 or miR-199a-5p mimic, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Column, mean of three independent experiments; bars, SD; $^{a}P < 0.01$ *vs* control group. NC: Non-relative control.

sion[44,45]. Liu Q reported that miR-16 family (including miR-16, miR-195 and miR-424) could induce cell cycle arrest by targeting CCND3, CCNE1 and Cdk6[46]. CHC is a part of clathrin expressed ubiquitously and exerts important functions in endocytosis and mitosis[47]. CHC has been reported to play an important role in inflammation disorder and tumorigenesis[48-51]. CHC functions as a co-activator for p53[48] and contributes to the regulation of basal NF-κB activity in epithelial cells[49]. CHC was identified as immunohistochemical tumor markers for primary HCC[50] and served as a biomarker for early diagnosis of small HCC[51]. Whether CCND3 and CHC regulated by miR-138 and miR-199a-5p respectively are involved in HCC tumorigenesis or other cell functions needs further studies.

In summary, we reported 21 deregulated miRNAs in HCC and CCND3 as the target of miR-138 and CHC as the target of miR-199a-5p. Our findings indicated that the novel miRNAs might be involved in HCC tumorigenesis and provide more evidence for the reliability of integrative bioinformatics analysis.

COMMENTS

Background

MiRNAs are endogenous non-coding RNAs (20-22 nucleotides) that have been identified as post-transcriptional regulators of gene expression. The miRNAs mainly bind to the 3' untranslated regions (UTRs) of target mRNAs, resulting in mRNA degradation or the blockade of mRNA translation. Increasing evidence has demonstrated that miRNAs play an important role in hepatocellular carcinoma (HCC) progression and directly contribute to the cell proliferation, avoidance of apoptosis, and metastasis of HCC. Identifying the miRNAs and their targets that are essential for HCC progression may provide promising therapeutic opportunities.

Research frontiers

Most recently identified miRNAs were found to be frequently deregulated in HCC, and some specific miRNAs were found to be associated with the clinicopathological features of HCC, such as metastasis, recurrence, and prognosis. Moreover, compelling evidence has demonstrated that miRNAs play an important role in HCC progression and directly contribute to the cell proliferation, avoidance of apoptosis, and metastasis of HCC.

Innovations and breakthroughs

In this study, 10 up-regulated miRNAs (miR-217, miR-518b, miR-517c, miR-520g, miR-519a, miR-522, miR-518e, miR-525-3p, miR-512-3p, and miR-518a-3p) and 10 down-regulated miRNAs (miR-138, miR-214, miR-214#, miR-27a#, miR-199a-5p, miR-433, miR-511, miR-592, miR-483-5p and miR-483-3p) were identified in HCC. Of the 20 deregulated miRNAs, only miR-199a-5p was reported to contribute to the increase of cell invasion by functional deregulation of discoidin domain receptor-1 activity in HCC and regulate Brm subunit of SWI/SNF in human cancers, and the other 19 deregulated miRNAs were first reported to be involved in HCC tumorigenesis. MiR-27a#, miR-214#, miR-518a-3p and miR-518e have never been reported in literatures. The rest 15 miRNAs were reported in various cancers, but not in HCC. Compared with the previous version, the newly found miRNAs (337 updated miRNAs) were profiled in Taqman low-density miRNA array v2.0, which helped the authors identify many new differentially expressed miRNAs in this study.

Applications

This study provides new insights into the understanding of the molecular mechanisms of hepatic carcinogenesis regulated by miRNA, and helps develop personalized miRNA-based therapeutics against HCC.

Terminology

MiRNAs are endogenous non-coding 20 to 22 nucleotide RNAs that have been identified as post-transcriptional regulators of gene expression. MiRNAs are processed from precursor molecules (pri-miRNAs), which are either transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase Ⅱ transcripts. A single pri-miRNA often contains sequences of several different miRNAs. Pri-miRNAs fold into hairpin structures containing imperfectly base-paired stems and are processed in two steps, catalyzed by the RNase III type endonucleases Drosha (also known as RN3) and Dicer. The Drosha-DGCR8 complex processes pri-miRNAs to ~70-nucleotide hairpins known as pre-miRNAs. In animals, premiRNAs are transported to the cytoplasm by exportin5, where they are cleaved by Dicer to yield ~20-bp miRNA duplexes. One strand is then selected to function as a mature miRNA, while the other strand is degraded. Occasionally, both arms of the pre-miRNA hairpin give rise to mature miRNAs.

Peer review

This article aimed to reveal microRNAs and target genes associated with hepatocellular carcinogenesis using miRNA array and network/pathway analyses combined with integrative bioinformatical analysis. The topic is of significant clinical importance as HCC is a very common and usually lethal liver tumor, and all researches that may reveal some potential target genes/miRNAs that offer new therapeutic possibilities are useful for the better understanding of the disease and related molecular biological mechanisms.

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BRIEF ARTICLE

Correlation between circulating myeloid-derived suppressor cells and Th17 cells in esophageal cancer

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Abstract

AIM: To perform a comprehensive investigation into the potential correlation between circulating myeloid-derived suppressor cells (MDSCs) and Th17 cells in esophageal cancer (ECA).

METHODS: A total of 31 patients newly diagnosed with ECA and 26 healthy subjects were included in the current study. The frequencies of MDSCs and Th17 cells in peripheral blood were determined by flow cytometry. The mRNA expression of cytokines, arginase 1 (Arg1) and inducible NO synthase (iNOS) in peripheral blood mononuclear cells (PBMCs) and plasma Arg1 were assessed by real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively.

RESULTS: There was an increased prevalence of MDSCs in the peripheral blood from ECA patients (15.21% ± 2.25%) when compared with healthy control (HC) (1.10% ± 0.12%, $P < 0.0001$). The plasma levels of Arg1 in ECA patients were significantly higher than those in HC (28.28 ± 4.10 ng/mL *vs* 9.57 ± 1.51 ng/mL, $P = 0.0003$). iNOS mRNA levels in the peripheral blood of ECA patients also showed a threefold increase compared with HC ($P = 0.0162$). The frequencies of Th17 cells ($CD4^+IL\text{-}17A^+$) were significantly elevated in ECA patients versus HC (3.50% ± 0.33% *vs* 1.82% ± 0.19%, $P = 0.0001$). Increased mRNA expression of IL-17 and ROR-γt was also observed in ECA patients compared with HC ($P = 0.0041$ and $P = 0.0004$, respectively), while the mRNA expression of IL-6 and tumor necrosis factor-α (TNF-α) showed significant decreases ($P = 0.0049$ and $P < 0.0001$, respectively). No obvious correlations were found between the frequencies of MDSCs and Th17 cells in the peripheral blood from ECA patients($r = -0.1725$, $P = 0.3534$). Arg1 mRNA levels were positively correlated with levels of IL-6 ($r = 0.6404$, $P = 0.0031$) and TNF-α ($r = 0.7646$, $P = 0.0001$). Similarly, iNOS mRNA levels were also positively correlated with levels of IL-6 ($r = 0.6782$, $P = 0.0007$) and TNF-α ($r = 0.7633$, $P < 0.0001$).

CONCLUSION: This study reveals the relationship between circulating MDSCs and Th17 cells, which may lead to new immunotherapy approaches for ECA based on the associated metabolites and cytokines.

Key words: Myeloid-derived suppressor cells; Th17 cells; Esophageal cancer; Arginase Ⅰ; Peripheral blood mononuclear cells; Inducible NO synthase

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INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of cells comprised of myeloid progenitor cells and immature myeloid cells, which exert suppressive functions, regulating T cell responses through the production of arginase 1 (Arg1), nitric oxide and reactive oxygen species. These cells are suspected to play a crucial role in local and systemic tumor development, providing a beneficial microenvironment in which tumor cells can proliferate, expand, acquire new mutations and escape host immunosurveillance[1]. Elevated numbers of MDSCs in peripheral blood have been demonstrated in a substantial number of studies in different types of cancer, including malignant gliomas[2], head and neck cancer[3,4], invasive breast carcinomas[4], colon carcinoma[5], pancreatic cancer[6] and mesothelioma[7].

In esophageal cancer (ECA), it has recently been shown that there is a significant increase in the levels of circulating MDSCs, which is correlated with elevated numbers of regulatory T cells (Tregs) and associated with an increased expression of Arg1[6]. A recent report has also identified that MDSCs not only modulate the de novo induction of Tregs and Th17 cells from $CD4^+$ T cells but also catalyze the transdifferentiation of $Foxp3^+$ regulatory T cells from monocyte-induced Th17 cells[8]. These findings suggested that the interaction between MDSCs and T cell subsets may play an important role in the balance of anti- and pro-tumor immune responses. As a new member of the $CD4^+$T-cell family, Th17 cells have been characterized as preferential producers of interleukin (IL)-17A, IL-17F, IL-21, IL-22, and tumor necrosis factor-α (TNF-α) and have been found in increased numbers in tumor-bearing hosts, including ECA[9-11]. However, it is still controversial whether these Th17 cells promote or inhibit tumor progression[12-14]. Furthermore, whether there is a correlation between circulating MDSCs and Th17 cells in ECA remains unclear. The present study was designed to evaluate the relationship between circulating MDSCs and Th17 cells by examining the cell frequencies and related cytokines and other associated products in the peripheral blood from patients with ECA.

MATERIALS AND METHODS

Patients and healthy controls

Thirty-one patients (25 male, 6 female) newly diagnosed with ECA were included in the current study. These patients ranged from 50 to 78 years of age (average age, 61.97 ± 1.24 years). Twenty-six healthy subjects, matched for age and sex with the ECA patients, were studied as the controls. No subject was treated preoperatively or had a history of autoimmune disease, and no healthy control had a prior history of cancer. This study was approved by the research ethics committee of the Affiliated Hospital of Jiangsu University, and written informed consent was obtained from all individuals.

Table 1 Primer pairs used in real-time polymerase chain reaction analysis

Target cDNA	Upper/lower	Sequence (5′ to 3′)
IL-17	U	CAGATTACTACAACCGATCC
	L	ATGTGGTAGTCCACGTTCC
ROR-γt	U	GTGCTGGTTAGGATGTGCCG
	L	GTGGGAGAAGTCAAAGATGGA
IL-6	U	AAAGAGGCACTGGCAGAAAA
	L	TTTCACCAGGCAAGTCTCCT
TNF-α	U	TAGCCCATGTTGTAGCAAACC
	L	ATGAGGTACAGGCCCTCTGAT
Arg1	U	CAAGAAGAACGGAAGAATCAGC
	L	TTGTGGTTGTCAGTGGAGTGTT
iNOS	U	CTTTCCAAGACACACTTCACCA
	L	TATCTCCTTTGTTACCGCTTCC
β-actin	U	TGGCACCCAGCACAATGAA
	L	CTAAGTCATAGTCCGCCTAGAAGCA

TNF-α: Tumor necrosis factor-α; IL: Interleukin; iNOS: Inducible NO synthase; Arg: Arginase; U: Upper; L: Lower.

Flow cytometric quantification of myeloid-derived suppressor cells

The MDSC population was defined as $HLA\text{-}DR^-/CD14^-/CD33^+/CD11b^+$. Heparinized venous blood was freshly obtained from either the ECA patients or the healthy donors. One hundred microliters of blood was mixed with 5μl of each antibody (BD Bioscience, San Jose, CA or eBioscience, San Diego, CA, United States), then incubated in a dark room for 15 min. Each sample was then mixed with 1 mL of 1 × lysing buffer (BD Biosciences). After incubation, the samples were washed with phosphate buffered saline (PBS), and the pellets were resuspended in 250 μL of PBS. Labeled cells were washed and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson) using CellQuest software (Becton-Dickinson). In each case, staining was compared with that of the appropriately labeled isotype control antibody.

Flow cytometric quantification of Th17 cells

Following centrifugation, the plasma was removed and stored at -70 ℃, while peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. PBMCs were used in two parts. Some PBMCs were frozen at -70 ℃ after mixing with TRIzol (Invitrogen, Carlsbad, CA, United States) for extracting total RNA, while the remaining cells was used for analysis of Th17 cells. Briefly, PBMCs were stimulated for 5 h using 50 ng/mL of phorbol myristate acetate (PMA, Sigma-Aldrich, MO, United States) and 1 g/mL ionomycin (Sigma-Aldrich, MO, United States) in the presence of 5 g/mL brefeldin A (Sigma-Aldrich, MO, United States) at

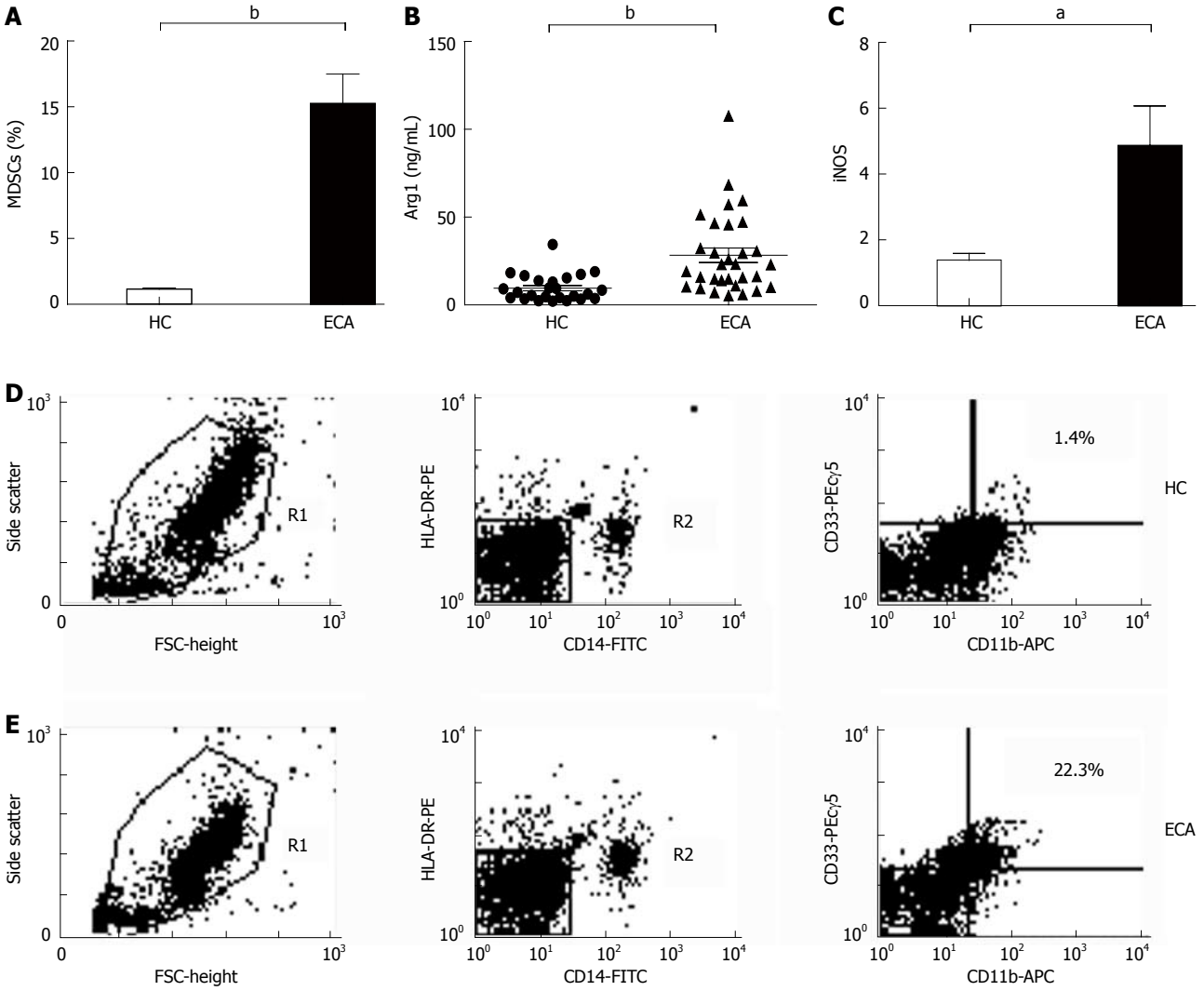

Figure 1 Elevated levels of myeloid-derived suppressor cells and their products, arginase 1 and inducible NO synthase, in esophageal cancer compared with healthy controls. A: Flow cytometric analysis of MDSCs in the peripheral blood from ECA patients and healthy control (HC); B: Plasma arginase 1 (Arg1) level was determined by ELISA; C: The mRNA level of iNOS in the peripheral blood from ECA patients and HC were assessed by real-time PCR; D: Representative flow diagrams of a healthy volunteer with marked elevation of circulating MDSCs; E: Representative flow diagrams of an ECA patient with marked elevation of circulating MDSCs are shown. Data were analyzed by the Student's *t*-test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* HC group. ECA: Esophageal cancer; MDSCs: Myeloid-derived suppressor cells; ELISA: Enzyme linked immunosorbent assay; iNOS: Inducible NO synthase; PCR: Polymerase chain reaction; FITC: Fluorescein isothiocyanate; FSC: Forward scatter; HLA-DR-PE: HLA-DR-phycoerythrin; APC: Allophycocyanin.

37 ℃ and 5% CO_2. The cells were then washed in PBS and surface-labeled with CD4-FITC (eBioscience, San Diego, CA, United States). Following surface staining, the cells were fixed and permeabilized using IntraPrep Permeabilization Reagent (Beckman Coulter Inc., Fullerton, CA, United States) and then stained with IL-17A-PE (eBioscience, San Diego, CA, United States)[11]. Labeled cells were washed and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson) using the CellQuest software (Becton-Dickinson).

RNA extraction, cDNA synthesis and polymerase chain reaction

Total RNA was extracted from individual PBMC preparations using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States). cDNA was prepared by reverse transcription with oligo (dT) from total RNA extraction. Real-time polymerase chain reaction (PCR) for IL-17, IL-6, TNF-α, Arg1, iNOS and a reference gene (β-actin) was performed in a LightCycler instrument (Roche Molecular Diagnostics, Mannheim, Germany) using the SYBRgreen mastermix kit (TaKaRa, Ohtsu, Japan). The expression data for each target gene were then normalized relative to β-actin. All primer sequences are shown in Table 1.

Enzyme-linked immunosorbent assay

Plasma levels of Arg1 were measured using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols (eBioscience, San Diego, CA, United States). Hemolyzed samples were excluded. All samples were assayed in triplicate, and the mean absorbance was calculated from the standard curve.

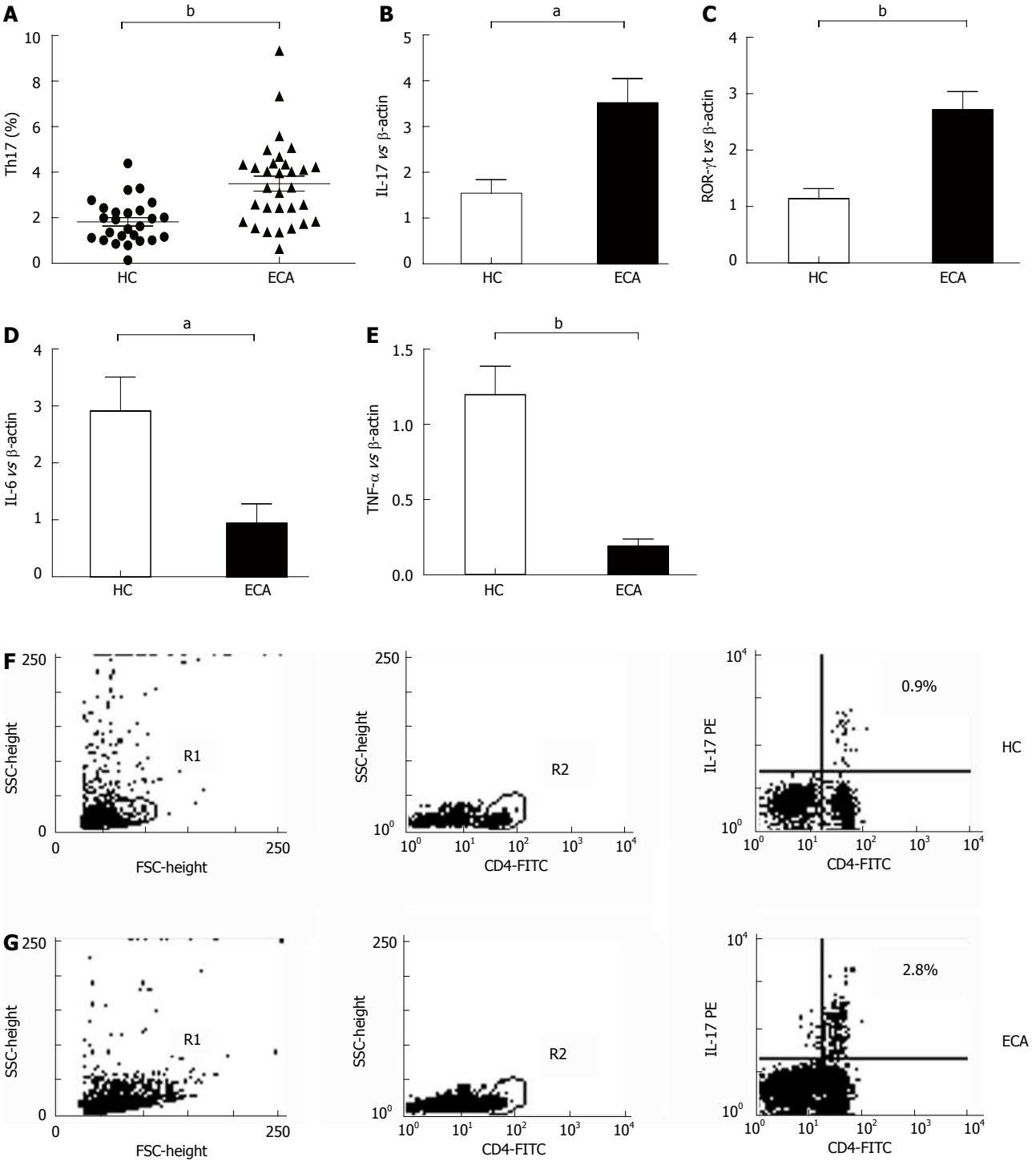

Figure 2 Levels of Th17 cells and Th17-related cytokines and transcriptional factor in the peripheral blood of esophageal cancer compared with healthy controls. A: Flow cytometric analysis of Th17 cells in the peripheral blood from ECA patients and healthy control (HC); B: The mRNA levels of IL-17 was determined by real-time PCR; C: The mRNA levels of ROR-γt was determined by real-time PCR; D: The mRNA levels of IL-6 was determined by real-time PCR; E: The mRNA levels of TNF-α was determined by real-time PCR; F: Representative flow diagrams of a healthy volunteer with marked elevation of circulating Th17 cells; G: Representative flow diagrams of an ECA patient with marked elevation of circulating Th17 cells are shown. Data were analyzed by the Student's *t*-test. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* HC group. ECA: Esophageal cancer; TNF-α: Tumor necrosis factor-α; IL: Interleukin; PCR: Polymerase chain reaction; FITC: Fluorescein isothiocyanate; FSC: Forward scatter; SSC: Side scatter.

Statistical analysis

Statistical comparisons between groups used the appropriate Student's *t*-test. Statistically significant correlation between two continuous variables was analyzed by the Spearman test. A *P*-value < 0.05 was considered significant. Calculations were performed using GraphPad Prism,

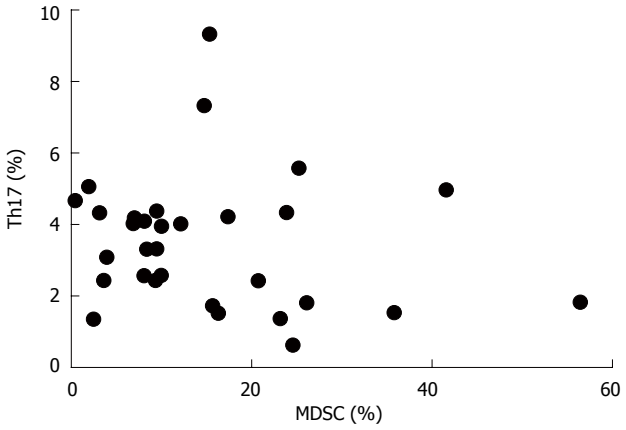

Figure 3 Correlation between the frequencies of myeloid-derived suppressor cells and Th17 cells in the peripheral blood of esophageal cancer patients. Data were analyzed by the Spearman test. MDSCs: Myeloid-derived suppressor cells.

Version 5.0, software (San Diego, CA, United States).

RESULTS

Increased frequencies of myeloid-derived suppressor cells in the peripheral blood of esophageal cancer patients

In the present study, MDSCs was defined as HLA-DR$^-$/CD14$^-$/CD11b$^+$/CD33$^+$ cells. The frequencies of MDSCs were determined by multicolor flow cytometry and calculated as the percent (%) of total nucleated cells in whole blood samples. As shown in Figure 1A, D and E, there was an increased prevalence of MDSCs in the peripheral blood from ECA patients (15.21% ± 2.25%) when compared with healthy control (HC) (1.10% ± 0.12%, $P < 0.0001$).

Increased plasma arginase 1 and inducible NO synthase mRNA expression in the peripheral blood from esophageal cancer patients

To investigate whether the products of MDSCs were also elevated in ECA patients, we determined the plasma levels of Arg1 by ELISA and the mRNA levels of iNOS by real-time PCR. As shown in Figure 1B, the plasma levels of Arg1 in ECA patients were significantly higher than those in HC (28.28 ± 4.10 ng/mL *vs* 9.57 ± 1.51 ng/mL, $P = 0.0003$). iNOS mRNA levels in the peripheral blood of ECA patients also showed a threefold increase compared with HC ($P = 0.0162$, Figure 1C).

Increased frequency of Th17 cells in the peripheral blood mononuclear cells of esophageal cancer patients

The frequencies of Th17 cells in PBMCs of ECA patients and HC were also determined by flow cytometry. As shown in Figure 2A, C and D, the frequencies of Th17 cells (CD4$^+$IL-17A$^+$) were significantly elevated in ECA patients when compared with HC (3.50% ± 0.33% *vs* 1.82% ± 0.19%, $P = 0.0001$).

mRNA expression analyses in peripheral blood mononuclear cells from esophageal cancer patients showed increased interleukin-17 and ROR-γt and decreased interleukin-6 and tumor necrosis factor-α

We also determined the mRNA levels of the Th17-related molecules IL-17, ROR-γt, IL-6 and TNF-α by real-time PCR. As shown in Figure 2B, increased mRNA expression of IL-17 and ROR-γt was observed in ECA patients compared with HC ($P = 0.0041$ and $P = 0.0004$, respectively), while the mRNA expression of IL-6 and TNF-α showed significant decreases ($P = 0.0049$ and $P < 0.0001$, respectively).

Correlation between myeloid-derived suppressor cells and Th17 cells in the peripheral blood from esophageal cancer patients and HC

We next analyzed the possible correlation between the frequencies of MDSCs and Th17 cells in the peripheral blood from ECA patients and HC. As shown in Figure 3, no obvious difference was found between the two groups ($r = -0.1725$, $P = 0.3534$).

Correlation of arginase 1, inducible NO synthase and Th17 cell-related molecules in the peripheral blood from HC and esophageal cancer patients

We examined the possible correlations between the mRNA levels of Arg1, iNOS and Th17-related molecules (IL-17, ROR-γt, IL-6 and TNF-α) in ECA patients. As shown in Figure 4D and E, Arg1 mRNA levels were positively correlated with levels of IL-6 ($r = 0.6404$, $P = 0.0031$) and TNF-α ($r = 0.7646$, $P = 0.0001$). Similarly, as shown in Figure 4I and J, iNOS mRNA levels were also positively correlated with levels of IL-6 ($r = 0.6782$, $P = 0.0007$) and TNF-α ($r = 0.7633$, $P < 0.0001$). However, no obvious correlations were observed with Arg1 or iNOS with IL-17 or ROR-γt (Figure 4A-C and 4F-G).

DISCUSSION

MDSCs are a heterogeneous cell population that was recently identified as a pivotal factor in the immunosuppressive network described in cancer, autoimmune disease, sepsis, infectious disease and trauma[1,15,16]. In cancer, MDSCs are responsible for T cell defects as well as angiogenesis and tumor cell motility. In this study, we evaluated the frequencies of MDSCs in the peripheral blood in ECA patients. Our study confirms previous findings about the significant increase of circulating MDSCs in a variety of cancers. An early study reported the accumulation of immature cells, including early stage myeloid cells and immature monocytes and DCs, in the blood of patients with head and neck, breast, and lung cancer[4]. Myeloid cells with immunosuppressive properties can also be found among monocytes and neutrophils circulating in the peripheral blood of patients with colon cancer and melanoma[5]. Patients with glioblastoma have increased MDSC counts (CD33$^+$HLADR$^-$) in their blood that are composed of neutrophilic (CD15$^+$, > 60%), lineage-

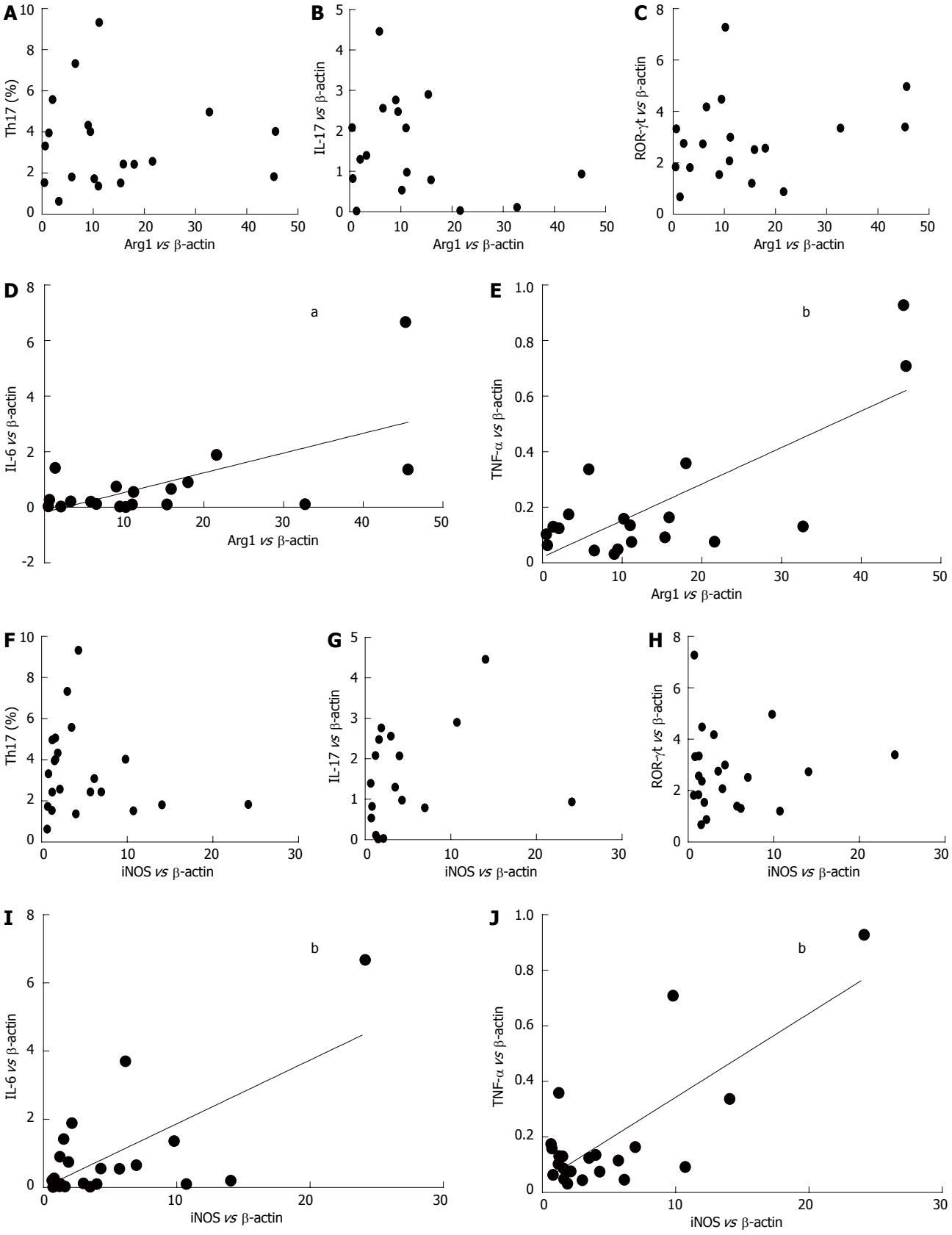

Figure 4 Correlations between the mRNA level of arginase 1 or inducible NO synthase and Th17-related molecules (interleukin-17, ROR-γt, interleukin-6 and tumor necrosis factor-α) in the peripheral blood of esophageal cancer patients. Data were analyzed by the Spearman test. [a]$P < 0.05$, [b]$P < 0.01$ *vs* β-actin. TNF-α: Tumor necrosis factor-α; IL: Interleukin; iNOS: Inducible NO synthase; Arg1: Arginase 1.

negative ($CD15^-CD14^-$, 31%), and monocytic ($CD14^+$, 6%) subsets[2]. It has also been reported that there was an accumulation and persistence of long-lived immature granulocytic MDSC with T cell-suppressive function and impaired migratory properties in the peripheral blood of squamous cell carcinoma of the head and neck (HNSCC), lung cancer and cancers of bladder and ureter[3]. Overall, these studies demonstrated the increase of circulating MDSCs in cancers, although the levels of MDSCs in peripheral blood varied across studies. These varying levels may partly be the result of differences in the type of cancers. Another explanation for these discrepancies may be methodological differences in which different markers and cell preparations were used to identify MDSCs.

In human, MDSCs are most prevalently considered as cells expressing CD11b but lacking the expression of CD14 or more narrowly defined as $HLA\text{-}DR^-CD33^+$ cells[4,17]. In this study, we used $CD14^-HLA\text{-}DR^-CD33^+CD11b^+$ as specific markers for human MDSCs in lysed whole blood. A recent study also reported elevated MDSCs in pancreatic, esophageal and gastric cancer and demonstrated that these cells were an independent prognostic factor and associated with the significant elevation of the Th2 cytokine interleukin-13[6]. The authors of such study focused on circulating MDSCs defined as $HLADR^-Lin1^{low/-}CD33^+CD11b^+$ and used a cell preparation of PBMCs rather than lysed whole blood, as was used in our study. However, both studies demonstrated elevations of plasma Arg1, which is the functional product of MDSCs, in ECA patients, compared with the controls. In addition to Arg1, we also found that iNOS mRNA levels in the peripheral blood of ECA patients showed a threefold increase compared with HC ($P = 0.0162$).

It is generally believed that Th17 cells and their associated cytokines IL-6, TNF, IL-1β, IL-23 and TGF-β may play important roles in promoting the growth and survival of cancer. Consistent with other previous reports, our study demonstrated that ECA patients exhibited a remarkable increase in the frequency of circulated Th17 cells. Furthermore, the mRNA levels of Th17-related cytokines, IL-17 and ROR-γt, have also been shown to be significantly increased[18]. Deans *et al*[19]reported that proinflammatory cytokines IL-6 and TNF-α were significantly overexpressed both at the mRNA and protein levels in cancer specimens compared with the mucosa from controls. However, in contrast to Deans's study, we found that the mRNA levels of IL-6 and TNF-α were obviously decreased in ECA patients. One possible explanation for this discrepancy may result partly from the limited scale of ECA subjects and determination methods. Another explanation is that, as is the case for most cancers, changes in peripheral blood could reflect partly or rather poorly the changes in tumor microenvironment. Further investigation should be performed to confirm the above inferences.

Recent data demonstrated that *in vivo* transfer of G-MDSCs inhibited Th17 immune responses and ameliorated experimental autoimmune encephalomyelitis (EAE)[20]. *In vitro*, $CD14^+HLA\text{-}DR^+$ monocytes promote the generation of IL-17-secreting $RORc^+$ Th17 cells when cocultured with naive CD4+ T cells[8]. These results suggest that the interaction of MDSC with Th17 cells would help to explain the pathogenesis of autoimmune diseases and cancers. In fact, the initial question that motivated our study was to determine whether there would be a positive correlation between circulating MDSCs and Th17 cells in individual ECA patient. As expected, there were high correlations between the mRNA levels of Arg1 or iNOS and IL-6 or TNF-α, which represent the products of MDSCs and Th17 cells, respectively. Although both the frequencies of MDSCs and Th17 cells in ECA were much higher than those in healthy controls, there was surprisingly no obvious correlation between the frequency of circulation MDSCs and Th17 cells. Consistently, there were no significant correlations between Arg1 or iNOS and Th17 cells, IL-17 or ROR-γt. There is no clear explanation for such unexpected results. Further studies are necessary to explore whether the peripheral increase of these cells could be found in local cancer tissue and whether a correlation between MDSCs and Th17 cells exist.

In conclusion, we have demonstrated a significant increase in circulating MDSCs and Th17 cells in ECA patients. The frequency of MDSCs is not correlated with Th17 cells. Arg1 and iNOS, the products of MDSCs, are consistently not associated with Th17 cells, IL-17 or ROR-γt but are positively correlated with Th17-related cytokines, including IL-6 and TNF-α. These results may bring new lines of investigation on the role of MDSCs and Th17 cells in ECA, possibly leading to new immunotherapy approaches based on the regulation of metabolites and cytokines.

COMMENTS

Background

Esophageal cancer (ECA) occurs when the tissue that lines the esophagus becomes malignant. Cancer of the esophagus is the sixth leading cause of cancer mortality worldwide, constituting approximately 2% of all malignant tumors. The precise mechanisms of initiation and progression of this disease are unclear. Recently, a suppressor cell population of myeloid lineage was identified, named myeloid-derived suppressor cells (MDSCs), which is capable of reducing antitumor as well as inflammatory immune responses. However, the characterization of MDSCs and their correlation with the newly identified pro-inflammatory Th17 cells in esophageal cancer remains unclear.

Research frontiers

MDSCs have gained much attention in recent years, mainly in the tumor immunology community. Because MDSCs are still a very poorly defined cell population, it will be difficult to specifically target these cells in cancer patients with the aim of engaging tumor-specific immune responses. The current research priority is how to identify better markers and the interaction of MDSCs with other cell types in different clinical settings, including in esophageal cancer.

Innovations and breakthroughs

Although the distribution of MDSCs and Th17 cells in esophageal cancer has been previously reported, a comprehensive correlation between circulating MDSCs and Th17 cells in ECA remains unclear. The present study was designed to evaluate the relationship between circulating MDSCs and Th17 cells by examining the cell frequencies and related cytokines and other products in the peripheral blood from patients with ECA. The authors confirmed previous findings about significant increases in circulating MDSCs and Th17 cells in ECA patients. However, the frequency of MDSCs is not correlated with Th17 cells.

Arginase I (Arg1) and inducible NO synthase (iNOS), the products of MDSCs, are consistently not associated with Th17 cells, IL-17 or ROR-γt but are positively correlated with Th17-related cytokines, including interleukin (IL)-6 and tumor necrosis factor-α (TNF-α). The results reveal the association between circulating MDSCs and Th17 cells, which may lead to new immunotherapy approaches for ECA based on the regulation of metabolites and cytokines.

Applications

Although it is difficult to specifically target these cells for immunotherapy, the results suggest that the regulation of metabolites and cytokines would be a potential therapeutic approach for esophageal cancer.

Terminology

MDSCs: Myeloid-derived suppressor cells (MDSCs), a heterogeneous population of cells that consists of immature myeloid cells, immature granulocytes, monocytes-macrophages, dendritic cells and myeloid progenitor cells, is capable of reducing anti-tumor as well as inflammatory immune responses; Th17 cells: a new member of the $CD4^+$T cell family that have been characterized as preferential producers of interleukin-17A (IL-17A), IL-17F, IL-21, IL-22, and TNF-α and have been found in increased numbers in tumor-bearing hosts.

Peer review

This paper reported on the relationship between MDSCs and Th17 cells in ECA patients. The presented data are preliminary but novel.

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BRIEF ARTICLE

Effect of the ginsenoside Rb1 on the spontaneous contraction of intestinal smooth muscle in mice

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Abstract

AIM: To investigate the effect and the possible mechanism of ginsenoside Rb1 on small intestinal smooth muscle motility in mice.

METHODS: Intestinal smooth muscle strips were isolated from male ICR mice (5 wk old), and the effect of ginsenoside Rb1 on spontaneous contraction was recorded with an electrophysiolograph. The effect of ginsenoside Rb1 on ion channel currents, including the voltage-gated K^+ channel current (IK_V), calcium-activated potassium channel currents (IK_{Ca}), spontaneous transient outward currents and ATP-sensitive potassium channel current (IK_{ATP}), was recorded on freshly isolated single cells using the whole-cell patch clamp technique.

RESULTS: Ginsenoside Rb1 dose-dependently inhibited the spontaneous contraction of intestinal smooth muscle by 21.15% ± 3.31%, 42.03% ± 8.23% and 67.23% ± 5.63% at concentrations of 25 μmol/L, 50 μmol/L and 100 μmol/L, respectively (n = 5, P < 0.05). The inhibitory effect of ginsenoside Rb1 on spontaneous contraction was significantly but incompletely blocked by 10 mmol/L tetraethylammonium or 0.5 mmol/L 4-aminopyridine, respectively (n = 5, P < 0.05). However, the inhibitory effect of ginsenoside Rb1 on spontaneous contraction was not affected by 10 μmol/L glibenclamide or 0.4 μmol/L tetrodotoxin. At the cell level, ginsenoside Rb1 increased outward potassium currents, and IK_V was enhanced from 1137.71 ± 171.62 pA to 1449.73 ± 162.39 pA by 50 μmol/L Rb1 at +60 mV (n = 6, P < 0.05). Ginsenoside Rb1 increased IK_{Ca} and enhanced the amplitudes of spontaneous transient outward currents from 582.77 ± 179.09 mV to 788.12 ± 278.34 mV (n = 5, P < 0.05). However, ginsenoside Rb1 (50 μmol/L) had no significant effect on IK_{ATP} (n = 3, P < 0.05).

CONCLUSION: These results suggest that ginsenoside Rb1 has an inhibitory effect on the spontaneous contraction of mouse intestinal smooth muscle mediated by the activation of IK_V and IK_{Ca}, but the K_{ATP} channel was not involved in this effect.

Key words: Ginsenoside Rb1; Intestinal smooth muscle; Intestinal smooth muscle cell; Potassium channel; Spontaneous contraction; Whole-cell patch clamp technique

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INTRODUCTION

Sijunzi decoction (SJZD) is one of the most famous and widely used traditional prescriptions. This prescription contains four common herbs, including Panax ginseng, Poria cocos, Atractylodes macrocephala and Glycyrrhiza uralensis, and it has been used either alone to replenish or invigorate intestinal and stomach function or as a complement to other herbs during treatment of other diseases, such as poor health and cancer[1,2]. Ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), is a principal component of SJZD. Ginsenoside, a component of ginseng, has a four-ring steroid-like structure with attached sugar moieties. Recently, ginseng's chemical and pharmacological properties have been reported by many investigators[3,4]. Approximately 30 ginsenosides have been isolated and identified from the Panax ginseng root. These ginsenosides appear to be responsible for most of the pharmacological effects of ginseng. Many reports made it evident that ginseng saponins, or ginsenosides, have various effects on gastrointestinal motility. Ginsenosides modulate the pacemaker activities of the interstitial cells of Cajal (ICCs), making the ICCs targets for ginsenosides, and their interaction can affect intestinal motility[5]. The aqueous extract of Ginseng Radix possesses ameliorative properties and improves carbachol-induced accelerated small intestinal transit, and Rb1 contributed to the suppressive effects of Ginseng Radix on intestinal motility. Rb1 is one representative of the compounds contained in Ginseng Radix that is capable of ameliorating the accelerated transit of the small intestine[6]. However, the mechanism of Rb1 modulation of gastrointestinal motility has not been clearly demonstrated. Based on the studies cited above, it could be deduced that ICCs and gastrointestinal smooth muscle cells might be targets for Rb1. In this study, we attempted to determine the effect of ginsenoside Rb1 on the motility of intestinal smooth muscle and determine its mechanism.

MATERIALS AND METHODS

Preparation of intestinal smooth muscle and isometric measurement

Five-week-old male ICR mice (provided by the Experimental Animal Centre of the Chinese Academy of Sciences, Shanghai) weighing approximately 30 g were sacrificed by cervical dislocation. The small intestines were removed and kept in Krebs solution. After removing the mucosal and submucosal layers, single circular muscle bundles with the attached longitudinal muscle layer were prepared. Approximately 2 mm × 6 mm muscle strips were needed and were fixed in a vertical chamber (5-mL capacity containing 5 mL CO_2/bicarbonate-buffered Krebs solution bubbled with 5% CO_2/95% O_2). The chamber was maintained at 37 ℃ using a water jacket. One end of the chamber was attached to an isometric force transducer (RM6240C, Chengdu Instrument Factory, China) to record the contraction. The muscle strip was incubated at the appropriate tension[7].

Cell preparation and electrophysiological recording

Intestinal smooth muscle cells were freshly isolated from mice. The intestine was rapidly cut, and the mucosal layer was separated from the muscle layers in a Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS). The circular muscle layer was dissected from the longitudinal layer using fine scissors and was cut into small segments (2 mm × 3 mm). These segments were incubated in a medium modified from Kraft-Bruhe (K-B) solution for 30 min at 4 ℃. The segments were subsequently incubated for 10-12 min at 36 ℃ in Ca^{2+}-free PSS digestion medium containing collagenase (0.5 mg/mL, Worthington), DTT [0.5 mg/mL, Sigma Aldrich (St. Louis, MO, United States)], papain [1.5 mg/mL, Sigma Aldrich (St. Louis, MO, United States)] and bovine serum albumin (4 mg/mL, Biotech Grade)[7,8]. After digestion, the supernatant was discarded, and the softened muscle segments were transferred into the modified K-B solution. The single cells were dispersed by gentle trituration using a wide-bore fire-polished glass pipette. The isolated intestinal smooth muscle cells were incubated in a modified K-B solution at 4 ℃ until use on the same day. Several drops of the cell suspension were dropped into a perfusion bath, which was fixed on the stage of an inverted phase-contrast microscope for 15-20 min before the experiments. Next, the cells were perfused with PSS at a rate of 1-1.5 mL/min. A single 4-channel perfusion system (BPS-4, ALA, United States) was used to exchange the solution.

A conventional whole-cell patch clamp configuration was used to record the K_{ATP} channel current (IK_{ATP}), the spontaneous transient outward currents (STOC) and the voltage-gated K^+ channel current (IK_V). To record IK_{ATP}, the membrane potential was clamped at -60 mV. The pipette solution consisted of the following (mmol/L): KCl 107, KOH 33, Hepes 10, $MgCl_2$ 1, Na_2ATP 0.1, NaADP 0.1, and GTP 0.3, adjusted to a pH of 7.2 with NaOH. To observe the effect of Rb1 on IKv, we applied a depolarising step pulse to the cells, and the membrane potential was clamped at -60 mV. The pipettes were filled with solution containing the following (mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, disodium creatine phosphate 2.5, MgATP 5, Hepes 5, $MgCl_2$ 1.0, and EGTA 10, adjusted to a pH of 7.3 with KOH. To record STOC, the holding potential was clamped at -20 mV. The pipettes were filled with a solution containing the following (mmol/L): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, disodium salt 2.5, Hepes 5, and EGTA 0.1, adjusted to a pH of 7.2 with Tris. The patch pipettes were pulled from borosilicate glass capillaries using a pipette puller (PC-10, Narishige Group, Japan). The current was amplified with an EPC-10 patch

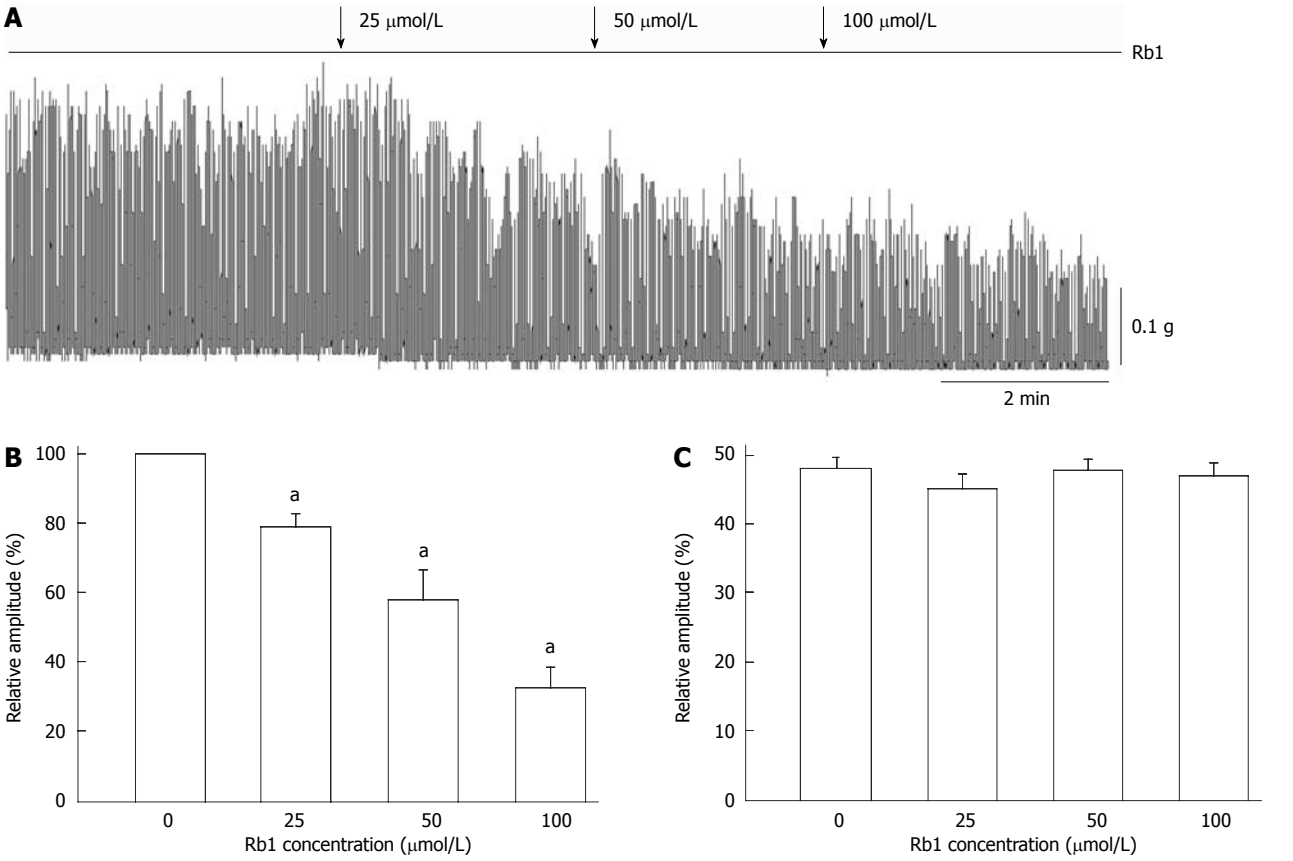

Figure 1 Effect of Rb1 on spontaneous contraction of intestinal smooth muscle. A: The representative effects of Rb1 on spontaneous contraction of intestinal smooth muscle in a dose-dependent manner; B, C: The amplitude of spontaneous contraction was decreased after Rb1 administration, but the frequency was unaffected. Values are expressed as means ± SE. $n = 5$, $^aP < 0.05$ *vs* control group.

clamp amplifier (HEKA Instruments, Germany) and digitised with a PCI-16 A/D converter (HEKA Instrument). All pipettes had a resistance of 3-5 MΩ[7,9,10].

All experimental protocols included in this manuscript were approved by the local animal care committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of the PRC (STCC Publication No. 2, revised 1988).

Solutions and drugs

Chemicals used included Ginsenoside Rb1 (purchased from Sichuan Weiqi Biological Technology CO., Ltd.), Glibenclamide [a K_{ATP} channel blocker, purchased from Tocris (Ellisville, Misso6yuri, United States)], tetraethylammonium (TEA, a non-selective potassium channel blocker), 4-aminopyridine (4-AP, a voltage-gated K^+ channel blocker), and tetrodotoxin (TTX, a blocker of voltage-dependent Na^+ channels) purchased from Sigma Aldrich (St. Louis, MO, United States). Ginsenoside Rb1 was dissolved first in dimethyl sulphoxide (DMSO) at a concentration of 200 mmol. For the intestinal smooth muscle isometric measurements, all chemicals were further diluted with Krebs solution to prepare the desired concentrations before use. In the electrophysiological recording experiment, Ginsenoside Rb1 was diluted with PSS to the final concentration immediately before use.

The ionic composition of the Krebs solution was as follows (in mmol/L): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H2PO_4^-$ 1.2, Cl^- 134, and glucose 11.5. The solution was aerated with O_2 containing 5% CO_2, and the pH was maintained at 7.2-7.3. The composition of Kraft-Bruhe (K-B) solution was as follows (in mmol/L): EGTA 0.5, Hepes 10, $MgCl_2$ 3, KCl 50, glucose 10, KH_2PO_4 20, Taurine 20, and L-Glutamic acid 50, adjusted to a pH of 7.4 with KOH. The composition of Ca^{2+}-free PSS was as follows (in mmol/L): NaCl 134.8, KCl 4.5, Hepes 10, $MgCl_2$ 1, and glucose 10, adjusted to a pH of 7.4 with Tris. The composition of PSS was as follows (in mmol/L): NaCl 134.8, KCl 4.5, Hepes 10, $MgCl_2$ 1, glucose 10, and $CaCl_2$ 2, adjusted to pH 7.4 with Tris. The pipette solution for recording the K_{ATP} channel current contained the following (mmol/L): KCl 107, KOH 33, Hepes 10, $MgCl_2$ 1, Na_2ATP 0.1, NaADP 0.1, and GTP 0.3, adjusted to a pH of 7.2 with NaOH. The pipettes were filled with a solution for IK_{Ca} containing the following (in mmol/L): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, disodium salt 2.5, Hepes 5, and EGTA 0.1, adjusted to a pH of 7.2 with Tris. The pipettes were filled with solution for IK_V containing the following (in mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, disodium-creatine phosphate 2.5, MgATP

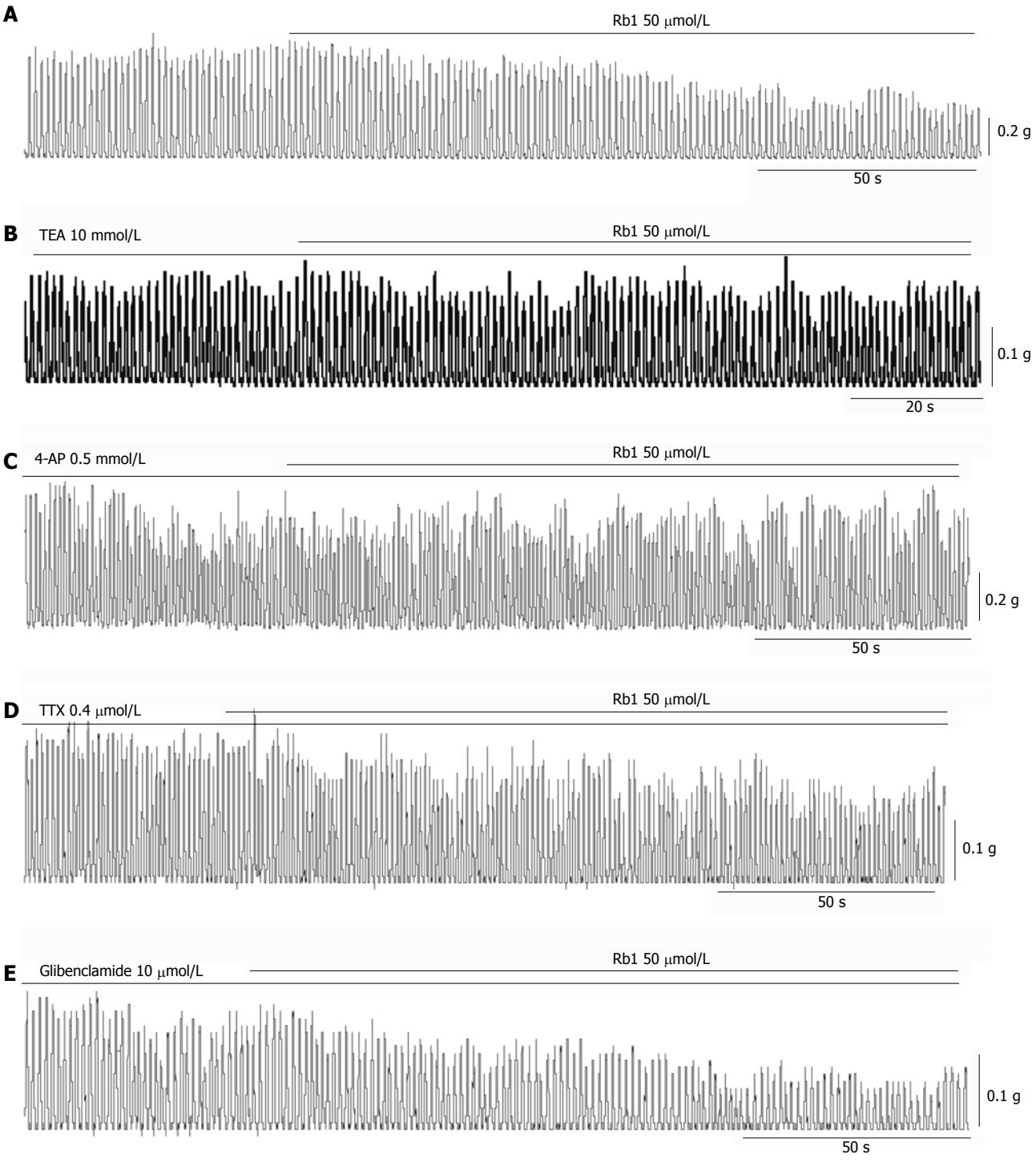

Figure 2 Effect of Rb1 on spontaneous contraction of the intestinal smooth muscle. A: Effect of Rb1 on spontaneous contraction of the intestinal smooth muscle; B-F: Effects of Rb1 (50 μmol/L) on spontaneous contraction of the intestinal smooth muscle pretreated with TEA (10 mmol/L), 4-AP (0.5 mmol/L), TTX (0.4 μmol/L) and Glibenclamide (10 μmol/L) respectively. TEA: Tetraethylammonium; 4-AP: 4-aminopyridine; TTX: Tetrodotoxin.

5, Hepes 5, $MgCl_2$ 1.0, and EGTA 10, adjusted to a pH of 7.3 with KOH.

Statistical analysis

Experimental values were expressed as the mean ± SD. Statistical significance was tested using Student's *t*-test, and probabilities of less than 5% ($P < 0.05$) were considered to be significant.

RESULTS

Effect of Rb1 on the spontaneous contraction of intestinal smooth muscle

In this study, Rb1 exhibited an inhibitory effect on the spontaneous contraction of intestinal smooth muscle strips in a dose-dependent manner (Figure 1). Rb1 suppressed spontaneous contraction by 21.15% ± 3.31%,

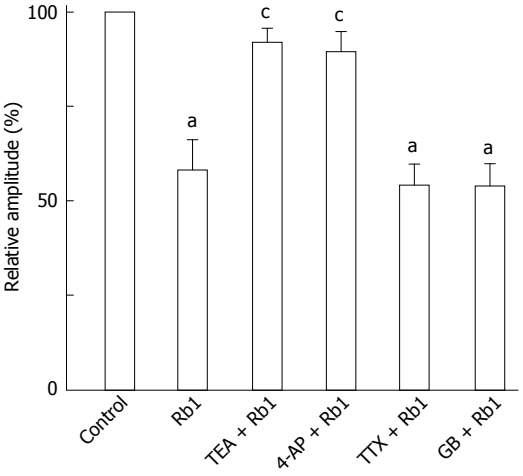

Figure 3 Summary in effect of Rb1on spontaneous contraction. Summary in effect of Rb1on spontaneous contraction of normal intestinal smooth muscles and those pretreated with TEA (10 mmol/L), 4-AP (0.5 mmol/L), Glibenclamide (10 μmol/L) and TTX (0.4 μmol/L) respectively. Values are expressed as means ± SE. $n = 5$, $^aP < 0.05$ *vs* control group; $^cP < 0.05$ TEA + Rb1 or 4-AP + Rb1 *vs* Rb1 group. TEA: Tetraethylammonium; 4-AP: 4-aminopyridine; TTX: Tetrodotoxin.

42.03% ± 8.23% and 67.23% ± 5.63% (Figure 1B, $n = 5$, $P < 0.05$) at concentrations of 25 μmol/L, 50 μmol/L and 100 μmol/L, respectively. Rb1-induced inhibition of spontaneous contraction appeared to decrease the amplitude of spontaneous contractions (Figure 1B), but the frequency was not changed (Figure 1C).

The Rb1-induced inhibitory effect on spontaneous contractions was almost completely abolished by 10 mmol/L TEA (a non-selective potassium channel blocker) and 0.5 mmol/L 4-AP (Figure 2B, C). The inhibitory percentage of Rb1 decreased from 42.03% ± 8.23% to 9.17% ± 3.54%, and the inhibitory percentage decreased from 10.90% ± 5.19% with TEA and 4-AP, respectively (Figure 3, $n = 5$, $P < 0.05$). After pre-treatment with 0.4 μmol/L TTX and 10 μmol/L glibenclamide, the inhibitory effect of Rb1 on spontaneous contraction was stable (Figure 2D, E). The inhibition percentages of Rb1 were 42.03% ± 8.23%, 46.12% ± 5.66% and 47.16% ± 3.99% in the control, TTX and glibenclamide groups, respectively (Figure 3, $n = 5$, $P > 0.05$).

Effect of Rb1 on voltage-gated K^+ channel current of intestinal smooth muscle cells

Previous experiments demonstrated that both TEA, a non-specific potassium channel blocker, and 4-AP, a specific delayed potassium channel blocker, significantly suppressed the inhibitory effect of RB1 on the spontaneous contraction of intestinal smooth muscle strips. These results indicate that Rb1-induced inhibition might be mediated by calcium-activated potassium channels and delayed repolarisation of the potassium channel. The effect of Rb1 on the IKv in intestinal smooth cells was observed in succession using the conventional whole-cell patch clamp technique. IKv was elicited by a step voltage command pulse from -40 mV to +100 mV at 20-mV increments for 400 ms at 10 s intervals. The membrane potential was clamped at -60 mV. Rb1 significantly increased IKv elicited by the step voltage command pulse (Figure 4A). Furthermore, based on the I-V relation curve, Rb1 increased IKv at all command potentials from +20 mV to +100 mV (Figure 4B). The IKv at +60 mV increased from 1137.71 ± 171.62 pA to 1449.73 ± 162.39 pA, which represented 132.11% ± 7.77% of the level in the control concentration (100%) of 50 μmol/L Rb1 (Figure 4C, $n = 6$, $P < 0.05$).

Effect of Rb1 on the Ca^{2+}-sensitive K^+ channel current of intestinal smooth muscle cells

IK_{Ca} is activated by intracellular Ca^{2+} and can be monitored by spontaneous transient outward currents (STOCs). STOCs are believed to represent the spontaneous, sporadic release of Ca^{2+} from storage sites in the cell in relation to Ca^{2+}-sensitive K^+ channels[8,11]. In this study, we observed that Rb1 (50 μmol/L) enhanced the amplitude of STOCs from 582.77 ± 179.09 mV to 788.12 ± 278.34 mV, which represented a 137.76% ± 11.95% increase from the control level (100%) (Figure 5A, C, $n = 5$, $P < 0.05$) without changing the frequency.

Effect of Rb1 on K_{ATP} channel current of intestinal smooth muscle cells

We investigated the effect of Rb1 on K_{ATP} channels using a whole-cell patch clamp. The inward current was activated at a holding potential of -60 mV following perfusion with a symmetrical 140 mmol/L K^+ solution (140 mmol/L KCl, 10 mmol/L glucose, 10 mmol/L Hepes, 1 mmol/L molgCl_2, and 0.1 mmol/L $CaCl_2$). Rb1 (50 μmol/L) did not change the K_{ATP} current (IK_{ATP}), which increased from 79.04 ± 35.88 pA to 81.32 ± 37.84 pA, representing a 102.29% ± 1.15% increase from the control level (100%) (Figure 5B, D, $n = 3$, $P < 0.05$).

DISCUSSION

Sijunzi decoction (SJZD) is widely used as a regular decoction in Chinese Traditional Medicine that can invigorate Pi viscera and replenish Qi. Conventionally, SJZD is useful for treating hypofunction of the spleen, a symptom that is partially equivalent to that of gastrointestinal motility disorders (e.g., abdominal distension and dyspepsia). The mechanism by which SJZD improves gastrointestinal disorder symptoms may relate to gastrointestinal hormones and motility. SJZD could correct deficiencies of the spleen and stomach, which are caused by digestive dysfunction to some extent[5]. Symptoms of rat models with Pi-deficiency could be significantly corrected to the normal level by SJZD treatment[4]. External nutrition plus SJZD treatment can improve and optimise cellular immune function and nutritional status in post-operative gastric cancer patients[12]. Recently, the major active components of SJZD, including ginsenoside, flavonoid, and triterpenoid, have been identified using LC/MS/MS[13]. Kim *et al*[14] reported that ginsenosides modulate the pace-

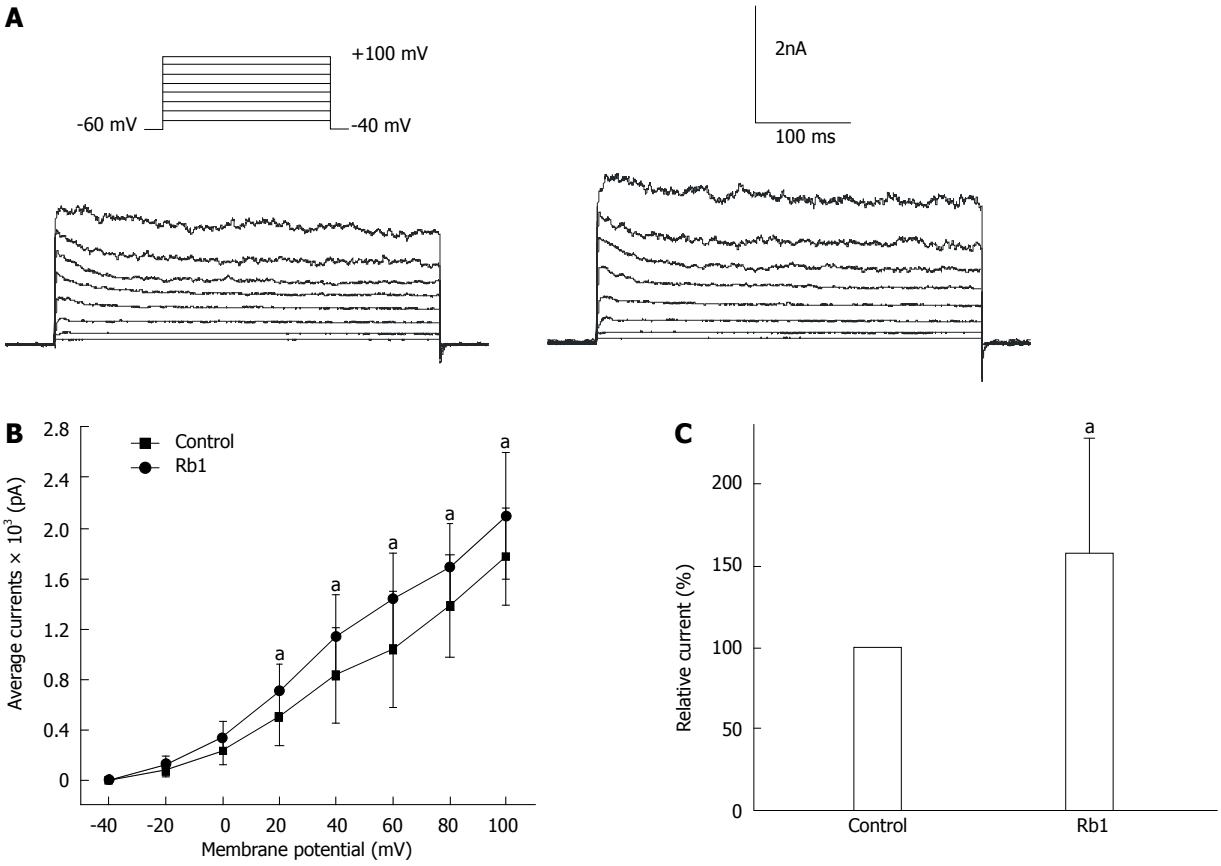

Figure 4 Effect of Rb1 on the voltage-gated K^+ channel current of intestinal smooth muscle cell in mouse. A: The raw trace; B: The I-V relation curve; C: Summary in effect of Rb1 (50 μmol/L) on the voltage-gated K^+ channel current at +60 mV. Values are expressed as means ± SE. $n = 6$, $^aP < 0.05$ *vs* control group.

maker activities of the ICCs. The ICCs can be targets for ginsenosides, and their interaction can affect intestinal motility. The ICCs and smooth muscle cells (SMCs) are coupled electrically, forming a multicellular syncytium. Activation of depolarising or hyperpolarizing ionic conductances in either cell type affects the total input resistance and excitability of the syncytium. For example, activation of K^+ channels in ICCs reduces excitability of coupled SMCs and reduces the likelihood of reaching the action potential threshold. Responses to other stimuli, such as hormones and paracrine substances, are likely to target both ICCs and SMCs, depending upon the expression of appropriate receptors and second-messenger pathways[15]. Hashimoto *et al*[16] reported that Rb1 was one representative of the compounds contained in Ginseng Radix that were capable of ameliorating the accelerated transit of the small intestine. To date, the mechanism of ginsenoside action on gastrointestinal (GI) smooth muscle has not been fully studied.

In this study, we found that ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscles in mice by decreasing the amplitude of spontaneous contractions in a dose-dependent manner (Figure 1). The presence of TEA (10 mmol), a non-selective potassium channel blocker, partially blocked the inhibitory effect of Rb1 on spontaneous contraction (Figure 2B). This finding suggested that the inhibitory effect of ginsenoside Rb1 on the spontaneous contraction of intestinal smooth muscle in mice might be associated with K^+ channels; importantly, at least 20 species of potassium channel types are expressed by SMCs of the GI tract[15,17]. These species include voltage-gated K^+ channels, ATP-dependent K^+ channels, and Ca^{2+}-activated K^+ channels. We evaluated 4-AP, a voltage-gated K^+ channel blocker, which partially blocked the inhibitory effect of Rb1 on spontaneous contraction (Figure 2C). In contrast, glibenclamide, an ATP-dependent K^+ channel blocker, did not influence the inhibitory effect of Rb1 on spontaneous contraction (Figure 2E). In addition, the presence of TTX, a blocker of voltage-dependent Na^+ channels that can block enteric nerves, did not affect the inhibitory effect of Rb1 on spontaneous contraction (Figure 2D). Thus, the results indicated that the inhibitory effect of Rb1 on spontaneous contraction was associated with activation of K^+ channels in intestinal smooth muscle cells. A conventional whole-cell patch clamp configuration showed that Rb1 activated IK_V and IK_{Ca} (Figures 4, 5) without any influence on IK_{ATP} (Figure 5B, D). We concluded that Rb1 inhibited the spontaneous contraction of intestinal smooth muscles *via* increased Ca^{2+}-dependent K^+ channel currents and voltage-dependent K^+ channel currents. However, enteric nerves and K_{ATP} channels were not involved in this process. Next, to determine the Rb1-

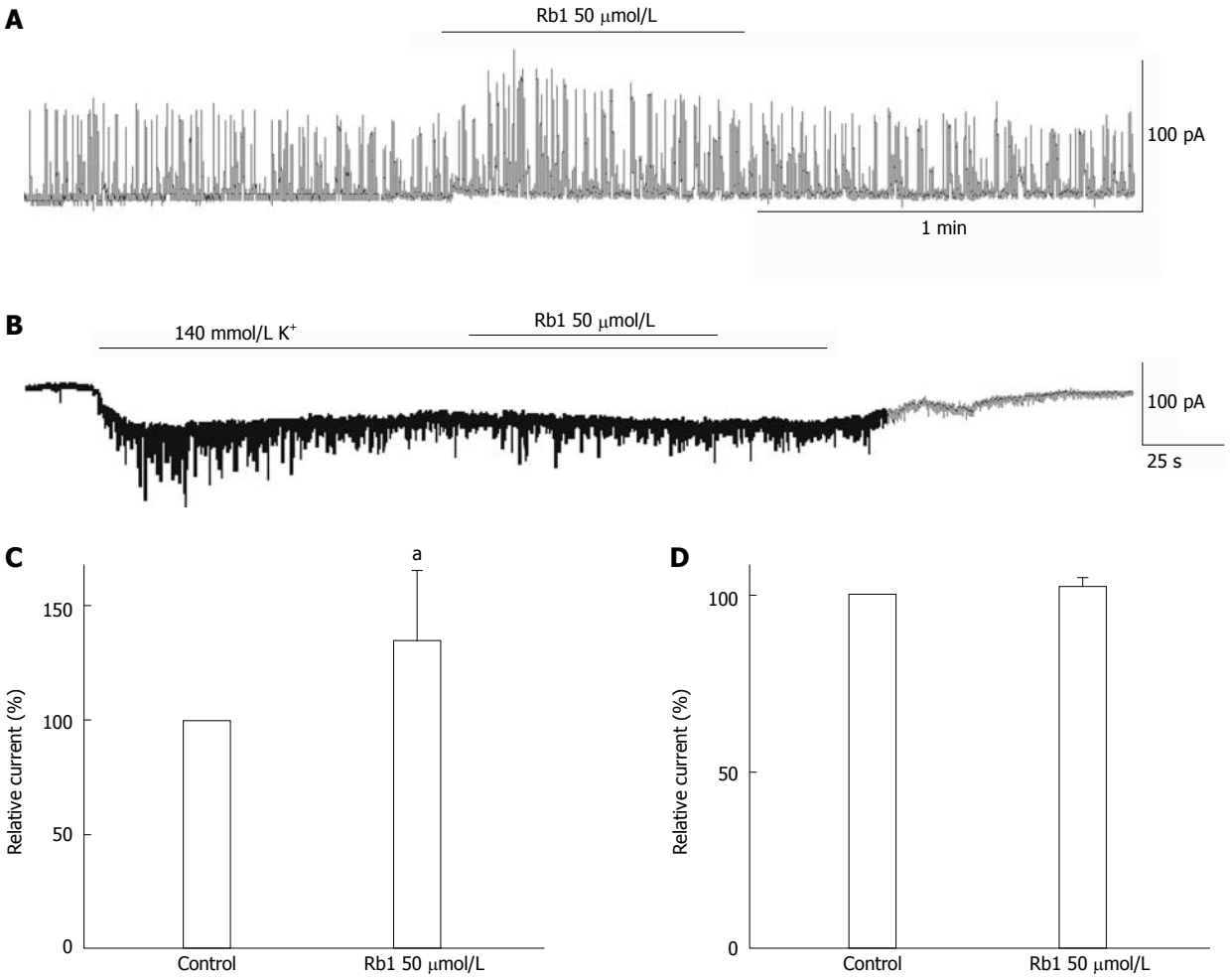

Figure 5 Effect of Rb1 on spontaneous transient outward currents and ATP sensitive potassium channel current of intestinal smooth muscle cell in mouse (*n* = 5). A: The raw trace of Rb1-induced effect on STOC of intestinal smooth muscle cell; B: The raw trace of Rb1-induced effect on IK_{ATP} of intestinal smooth muscle cell; C: Relative current evoked by Rb1 (50 μmol/L) on STOC, $n = 5$, $^aP < 0.05$ *vs* control group; D: Relative current evoked by Rb1 (50 μmol/L) on IK_{ATP} comparing with control group. Values are expressed as means ± SE, $n = 3$. IK_{ATP}: ATP sensitive potassium channel current; STOC: Spontaneous transient outward currents.

induced inhibitory effect on the spontaneous contraction of intestinal smooth muscle, the effect of Rb1 on the slow wave contraction of intestinal smooth muscle was observed. However, the amplitude and frequency of slow wave contraction was not affected by Rb1 (50 μmol/L, 100 μmol/L or 200 μmol/L, data not shown). The results indicated that the inhibitory effect of Rb1 on spontaneous contraction relies on the direct action of the compound with smooth muscle and not the ICCs themselves.

The broad ranges of resting potentials and electrical patterns of GI muscles are partly a function of the variable expression of K^+ channels in SMCs. At least 20 species of K^+ channels are expressed by SMCs in the GI tract[15]. The activation of potassium channels is the main determinant of cell membrane potential. Therefore, potassium channels participate in the regulation of smooth muscle tone. Activation of K^+ channels in the cell membrane allows K^+ efflux, causing a decrease in membrane potential and hyperpolarization. As a consequence, voltage-gated calcium channels in the cell membrane close, and the smooth muscle relaxes[18].

It has been reported that ginsenosides, including Rb1, regulate Ca^{2+} channels in chromaffin cells[19], sensory neurons[20] and ventricular myocytes[21]. Rb1 can alleviate cardiac hypertrophy *in vitro*, mediated by an inhibitive effect on elevated $[Ca^{2+}]i$[3]. The ginsenoside Rb1 suppressed ventricular myocyte shortening and intracellular Ca^{2+} in isolated cardiac myocytes[22]. These results indicate that the primary physiological or pharmacological targets of ginsenosides are Ca^{2+} channels. Li *et al*[23] reported that ginsenosides increased IK_{Ca} activity in endothelial cells. The modulation of IK_{Ca} activity stimulated by ginsenosides was inhibited by 0.5 mmol TEA but not by 0.5 mmol glibenclamide. In our study, we first discovered that potassium channels, especially the Ca^{2+}-dependent K^+ channels and voltage-dependent K^+ channels, were involved with the effects of Rb1 on the spontaneous contraction of intestinal smooth muscles in mice. This result is partially in accordance with the report of the action of ginsenosides by Li *et al*[23] and Kang *et al*[24].

In conclusion, ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscles in mice by decreasing the amplitude of spontaneous contractions in a dose-dependent manner. The inhibitory effect of Rb1 is mediated by potentiating IKv and IK_{Ca} channel currents.

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COMMENTS

Background

Gastrointestinal motility is a prominent research field of traditional Chinese medicine. Chinese herbal compounds, single Chinese erode drugs, Chinese herb extracts, and natural products have been experimentally investigated for their roles in promoting gastrointestinal motility.

Research frontiers

Ginsenosides, which are isolated from the root of Panax ginseng, appear to be responsible for most of the pharmacological effects of ginseng. Ginsenosides modulate the pacemaker activities of the interstitial cells of Cajal (ICCs), and the ICCs can be targets for ginsenosides, thereby affecting intestinal motility. Rb1 was one representative of the compounds contained in Ginseng Radix that was capable of ameliorating accelerated transit in the small intestine. Until now, the mechanism by which ginsenosides affect gastrointestinal smooth muscle had not been fully studied. This study focused on the mechanism by which ginsenosides affect gastrointestinal smooth muscle.

Innovations and breakthroughs

The results suggested that the ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscle in mice in a dose-dependent manner. The inhibitory effect of Rb1 is mediated by current potentiation in the voltage-gated K^+ channel current (IK_V), calcium-activated potassium channel currents (IK_{Ca}) channels. This effect may be involved in the mechanism by which ginseng mediates gastrointestinal motility.

Applications

This study illustrates the mechanism by which Rb1 affects spontaneous contraction of intestinal smooth muscle in mice. These findings may clarify the pharmacological action of ginseng.

Peer review

This is a good descriptive study in which authors analyze the effect and the possible mechanism of ginsenoside Rb1 on small intestinal smooth muscle motility in mice. The results are interesting and suggest that ginsenoside Rb1 has an inhibitory effect on the spontaneous contraction of mouse intestinal smooth muscle mediated by the activation of IK_V and IK_{Ca}, but the ATP-sensitive potassium channel current channel was not involved in this effect.

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BRIEF ARTICLE

Tumor size as a prognostic factor in patients with advanced gastric cancer in the lower third of the stomach

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Abstract

AIM: To explore the impact of tumor size on outcomes in patients with advanced gastric cancer in the lower third of the stomach.

METHODS: We retrospectively analyzed the clinical records of 430 patients with advanced gastric cancer in the lower third of the stomach who underwent distal subtotal gastrectomy and D2 lymphadenectomy in our hospital from January 1998 to June 2004. Receiver-operating characteristic (ROC) curve analysis was used to determine the appropriate cutoff value for tumor size, which was measured as maximum tumor diameter. Based on this cutoff value, patients were divided into two groups: those with large-sized tumors (LSTs) and those with small-sized tumors (SSTs). The correlations between other clinicopathologic factors and tumor size were investigated, and the 5-year overall survival (OS) rate was compared between the two groups. Potential prognostic factors were evaluated by univariate Kaplan-Meier survival analysis and multivariate Cox's proportional hazard model analysis. The 5-year OS rates in the two groups were compared according to pT stage and pN stage.

RESULTS: The 5-year OS rate in the 430 patients with advanced gastric cancer in the lower third of the stomach was 53.7%. The mean ± SD tumor size was 4.9 ± 1.9 cm, and the median tumor size was 5.0 cm. ROC analysis indicated that the sensitivity and specificity results for the appropriate tumor size cutoff value of 4.8 cm were 80.0% and 68.2%, respectively (AUC = 0.795, 95%CI: 0.751-0.839, P = 0.000). Using this cutoff value, 222 patients (51.6%) had LSTs (tumor size ⩾ 4.8 cm) and 208 (48.4%) had SSTs (tumor size < 4.8 cm). Tumor size was significantly correlated with histological type (P = 0.039), Borrmann type (P = 0.000), depth of tumor invasion (P = 0.000), lymph node metastasis (P = 0.000), tumor-nodes metastasis stage (P = 0.000), mean number of metastatic lymph nodes (P = 0.000) and metastatic lymph node ratio (P = 0.000). Patients with LSTs had a significantly lower 5-year OS rate than those with SSTs (37.1% *vs* 63.3%, P = 0.000). Univariate analysis showed that depth of tumor invasion (χ^2 = 69.581, P = 0.000), lymph node metastasis (χ^2 = 138.815, P = 0.000), tumor size (χ^2 = 78.184, P = 0.000) and metastatic lymph node ratio (χ^2 = 139.034, P = 0.000) were significantly associated with 5-year OS rate. Multivariate analysis revealed that depth of tumor invasion (P = 0.000), lymph node metastasis (P = 0.019) and tumor size (P = 0.000) were independent prognostic factors. Gastric cancers were divided into 12 subgroups: pT2N0; pT2N1; pT2N2; pT2N3; pT3N0; pT3N1; pT3N2; pT3N3; pT4aN0; pT4aN1; pT4aN2; and pT4aN3. In patients with pT2-3N3 stage tumors and patients with pT4a stage tumors, 5-year OS rates were significantly lower for LSTs than for SSTs (P < 0.05 each), but there were no significant differences in the 5-year OS rates in LST and SST patients with pT2-3N0-2 stage tumors (P > 0.05).

CONCLUSION: Using a tumor size cutoff value of 4.8

cm, tumor size is a prognostic factor in patients with pN3 stage or pT4a stage advanced gastric cancer located in the lower third of the stomach.

Key words: Gastric carcinoma; Prognosis; Receiver operating characteristic curve; The lower third of stomach; Tumor size

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INTRODUCTION

Gastric cancer is a common gastrointestinal malignancy in China and is the second most common cause of cancer-related deaths worldwide[1,2]. The identification of prognostic factors may be helpful in predicting and improving outcomes in patients with gastric cancer. Lymph node metastasis[3-5] and depth of tumor invasion[6-8] are the most important prognostic factors and are included in the Japanese Classification of Gastric Carcinoma (JCGC) and the American Joint Committee on Cancer tumor-nodes metastasis classification (AJCC TNM). Tumor size is another valuable clinicopathological feature because it can be measured easily before or during surgery and may be prognostic for survival in patients with gastric cancer[9-11]. Although tumor size is included in staging systems for lung and breast cancer, it has not been considered prognostic in gastric cancer. We therefore, retrospectively, analyzed the impact of tumor size on the prognosis of patients with advanced gastric cancer located in the lower third of the stomach.

MATERIALS AND METHODS

Patients

Patients undergoing curative resection (distal subtotal gastrectomy and D2 lymphadenectomy) for advanced gastric carcinoma (pT2-T4a stage) in the lower third of the stomach at the Department of Gastric Surgery, Affiliated Union Hospital of Fujian Medical University, Fuzhou, China, between January 1998 and June 2004 were included. Patients with gastric stump cancer, infiltration of surrounding organs (T4b) or distant metastases (hepatic, lung, peritoneal dissemination, or extraregional lymph nodes such as retropancreatic, mesenteric, and para-aortic lymph nodes) were excluded. After applying these criteria, 430 patients were included.

A surgical procedure was defined as curative if no grossly visible tumor tissue remained after the resection and the resection margins were histologically normal. Dissected lymph nodes were classified according to JCGC[12] criteria by specialist surgeons who reviewed the excised specimens after surgery. A total of 10 400 lymph nodes were dissected. The median number of dissected lymph nodes per patient was 24 (range, 6-61; mean 24.3 ± 8.8). Depth of tumor invasion, lymph node metastasis and tumor-nodes metastasis (TNM) stage were classified with respect to the seventh edition of AJCC TNM classification[13]. The metastatic lymph node ratio (MLR) was defined as the ratio of metastatic lymph nodes to the total number of dissected lymph nodes and categorized as MLR 0 (0%), MLR 1 (1%-9%), MLR 2 (10%-25%), MLR 3 (> 25%).

Routine follow-up consisted of physical examination, laboratory tests (including measurements of CEA, CA19-9 and CA125 concentrations), chest radiography, abdominopelvic ultrasonography and computed tomography (CT). Patients were followed-up every 3 mo during the first year, and every 6 mo or 12 mo thereafter, for a total of 5 years. Endoscopy was performed every 6 mo or 12 mo. All surviving patients were followed for more than five years. Survival was calculated from the date of diagnosis to last contact, date of death, or date when the survival information was collected. Of the 430 patients, 394 (93.0%) were followed-up.

Measurement of tumor diameter

In accordance with JCGC criteria[12], the resected stomach was opened along the greater curvature so that the whole mucosa could be observed. If the tumor was located on the greater curvature, the stomach was opened along the lesser curvature. The opened stomach was placed on a flat board with the mucosal side up and examined macroscopically. The lengths of the greater and lesser curvatures, as well as the attached portion of the esophagus and/or the duodenum and the size and thickness of the tumor, were recorded. Tumor size was measured as maximum tumor diameter (Figure 1). If tumor margins were unclear, the resected stomach was fixed in formalin for 1 h to make the margins clearer.

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 16.0 for Windows. The appropriate cutoff value for tumor size predicting 5-year survival was determined using the receiver-operating characteristic (ROC) curve, from which the area under the curve (AUC) was determined and the Youden index corresponding to each size was calculated. χ^2 tests were used to evaluate differences in proportions, and Student's *t*-tests were used to evaluate continuous variables. Five-year overall survival (OS) rates were calculated by the Kaplan-Meier method, with

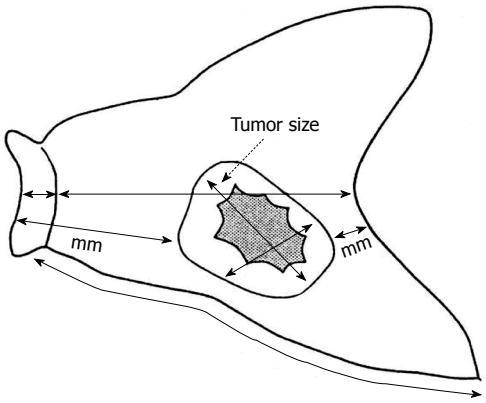

Figure 1 Resected stomach (mucosal side).

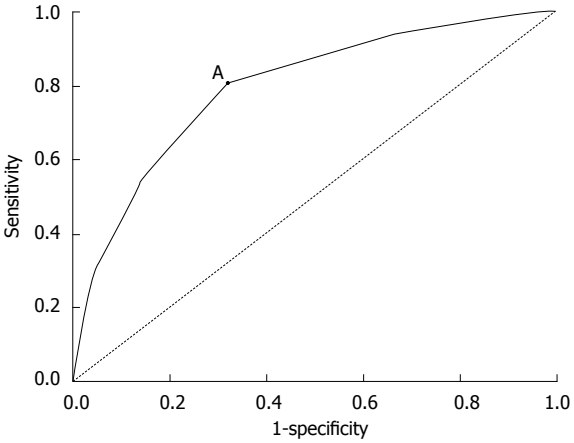

Figure 2 Receiver-operating characteristic curve. A: Shows sensitivity and specificity for a tumor size cutoff value of 4.8 cm were 80.0% and 68.2%, respectively (area under the curve = 0.795, *P* = 0.000).

groups compared by log-rank tests. Multivariate analysis was performed using a Cox's proportional hazard model. *P* values < 0.05 were considered statistically significant.

RESULTS

Clinicopathological characteristics of patients

We retrospectively analyzed a total of 430 patients with pT2-T4a gastric cancer located in the lower third of the stomach. Of these, 132 patients (30.7%) were women and 298 (69.3%) were men, with 187 patients (43.5%) ⩾ 60 years old. Postoperatively, 45 patients (10.5%) were classified as stage ⅠB, 49 (11.4%) as stage ⅡA, 78 (18.1%) as stage ⅡB, 65 (15.1%) as stage ⅢA, 82 (19.1%) as stage ⅢB and 111 (25.8%) as stage ⅢC. In addition, 117 patients (27.2%) were classified as stage pT2, 40 (9.3%) as pT3 and 273 (63.5%) as pT4a, while 105 patients (24.4%) were stage pN0, 92 (21.4%) were pN1, 94 (21.9%) were pN2, and 139 (32.3%) were pN3. Of the 430 patients, 325 (75.6%) had undifferentiated tumors. Based on the MLR classification, 70 patients (16.3%) were classified as MLR 0, 118 (27.4%) as MLR 1, 93 (21.6%) as MLR 2, and 149 (34.7%) as MLR 3.

Table 1 Correlation between other clinicopathologic characteristics and tumor size *n* (%)

Factors	LST	SST	*P*
Gender			0.221
Male	148 (66.7)	150 (72.1)	
Female	74 (33.3)	58 (27.9)	
Age (yr)			0.209
< 60	119 (53.6)	124 (59.6)	
⩾ 60	103 (46.4)	84 (40.4)	
Histological type			0.039
Differentiated	45 (20.3)	60 (28.8)	
Undifferentiated	177 (79.7)	148 (71.2)	
Borrmann type			0.000
Ⅰ / Ⅱ	50 (22.5)	111 (53.4)	
Ⅲ / Ⅳ	172 (77.5)	97 (46.6)	
Lymph node metastasis			0.000
pN0	35 (15.8)	70 (33.7)	
pN1	41 (18.5)	51 (24.5)	
pN2	52 (23.4)	42 (20.2)	
pN3	94 (42.3)	45 (21.6)	
Depth of invasion			0.000
pT2	28 (12.6)	89 (42.8)	
pT3	19 (8.6)	21 (10.1)	
pT4a	175 (78.8)	98 (47.1)	
TNM stage			0.000
ⅠB	9 (4.1)	36 (17.3)	
ⅡA	14 (6.3)	35 (16.8)	
ⅡB	33 (14.9)	45 (21.6)	
ⅢA	30 (13.5)	35 (16.8)	
ⅢB	54 (24.3)	28 (13.5)	
ⅢC	82 (36.9)	29 (14.0)	
Number of lymph nodes	8.02 ± 8.66	3.98 ± 5.65	0.000
MLR			0.000
0	35 (15.8)	70 (33.6)	
1	37 (16.7)	46 (22.1)	
2	41 (18.5)	43 (20.7)	
3	109 (49.0)	49 (23.6)	

LST: Large-sized tumor; SST: Small-sized tumor; MLR: Metastatic lymph node ratio; TNM: Tumor-nodes metastasis.

Cutoff value of tumor size

The mean ± SD tumor size was 4.9 ± 1.9 cm, and the median tumor size was 5.0 cm (range, 1.0-12.0 cm). ROC analysis indicated that a cutoff value of 4.8 cm yielded a sensitivity of 80.0% and a specificity of 68.2% in predicting survival after gastric surgery (AUC = 0.795, 95%CI: 0.751-0.839, *P* = 0.000) (Figure 2). Based on this cutoff value, the patients were divided into 2 groups, with 222 (51.6%) having large-sized tumors [large-sized tumors (LSTs), ⩾ 4.8 cm] and 208 (48.4%) having small-sized tumors [small-sized tumors (SSTs), < 4.8 cm].

Correlation analysis

When we analyzed the correlation between other clinicopathologic factors and tumor size (Table 1), we found that tumor size was significantly correlated with histological type (*P* = 0.039), Borrmann type (*P* = 0.000), depth of tumor invasion (*P* = 0.000), lymph node metastasis (*P* = 0.000), TNM stage (*P* = 0.000), mean number of metastatic lymph nodes (*P* = 0.000) and metastatic lymph node ratio (*P* = 0.000). SSTs were associated with dif-

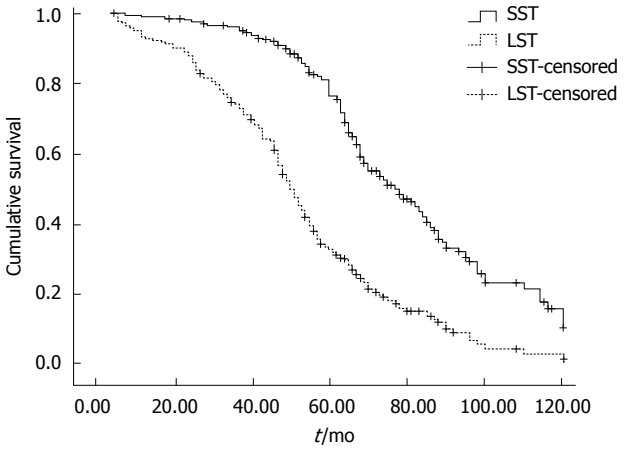

Figure 3 Overall survival curves of patients with large-sized tumor were significantly lower than those of patients with small-sized tumor. SST: Small-sized tumor; LST: Large-sized tumor.

ferentiated and Borrmann Ⅰ / Ⅱ types ($P < 0.05$ each), whereas LSTs were deeper and were associated with more extensive lymph node metastasis ($P < 0.05$). The mean number of metastatic lymph nodes was greater in patients with LSTs than with SSTs ($P < 0.05$).

Survival

The 5-year OS rate of all patients was 53.7%, being significantly lower in patients with LSTs than with SSTs (32.9% *vs* 76.7%; $\chi^2 = 78.184$, $P = 0.000$; Figure 3).

Univariate and multivariate survival analysis for all patients

Univariate analysis showed that depth of tumor invasion ($\chi^2 = 69.581$, $P = 0.000$), lymph node metastasis ($\chi^2 = 138.815$, $P = 0.000$), tumor size ($\chi^2 = 78.184$, $P = 0.000$) and metastatic lymph node ratio ($\chi^2 = 139.034$, $P = 0.000$) were significantly associated with 5-year OS rate, whereas patient age ($P = 0.613$), gender ($P = 0.240$) and histological type ($P = 0.361$) were not (Table 2). Multivariate analysis using a Cox's proportional hazards model revealed that depth of tumor invasion ($P = 0.000$), lymph node metastasis ($P = 0.019$) and tumor size ($P = 0.000$) independently predicted poor prognosis (Table 3).

Survival based on pT and pN stages in the LST and SST groups

Gastric cancers were divided into 12 subgroups: pT2N0; pT2N1; pT2N2; pT2N3; pT3N0; pT3N1; pT3N2; pT3N3; pT4aN0; pT4aN1; pT4aN2; and pT4aN3. The 5-year OS rates in these subgroups were compared in patients with LSTs and SSTs. We found that the 5-year survival rates of patients with pT2-3N3 stage and pT4a stage tumors were significantly lower in the LST than in the SST group ($P < 0.05$ each), but did not differ significantly in patients with pT2-3N0-2 stage tumors in the LST and SST groups ($P > 0.05$) (Table 4).

DISCUSSION

The prognostic value of tumor size in gastric cancer patients has recently received greater attention because tumor size can be measured easily before or during surgery; however, there is as yet no consensus formula to calculate the appropriate cutoff value for gastric tumor size. For example, tumor size of gastric cancer patients has been stratified into four subgroups (≤ 2 cm, ≤ 3 cm, ≤ 5 cm, and > 5 cm) by minimizing the estimated average expected distance (AED) objective function[14]. In another study of gastric cancer patients that used Cox's proportional hazards model to compare survival rates, a significant difference in survival was observed in patients with tumors < 10 cm and ≥ 10 cm[15]. We utilized ROC curve analysis to determine the appropriate tumor size cutoff value predicting 5-year OS rate in patients with advanced gastric cancer in the lower third of the stomach. In clinical and epidemiological fields, ROC analysis is frequently used to determine the cutoff values and reflect the correctness of a method of evaluation. According to the basic principle of ROC curves[16], when the Youden index is maximum, and the sensitivity is maximum, the corresponding tumor size is the appropriate cutoff value; besides, the method of evaluation can reflect predicted efficiency only when AUCs range between 0.7 and 0.9. From our ROC curves, a maximum sensitivity (0.800) at a tumor size cutoff value of 4.8 cm; our finding, of an AUC of 0.795 ($P = 0.000$), suggests that tumor size can reliably predict postoperative outcomes in patients with gastric cancer. Tumor size has shown positive associations with histological type, depth of tumor invasion, lymph node metastasis, peritoneal metastasis, blood vessel invasion and perineural inva-

Table 2 Univariate analysis of the patients' clinicopathological characteristics for overall survival

Factors	*n*	5-year OS (%)	χ^2	*P* value
Gender			1.655	0.198
Male	298	54.8		
Female	132	51.3		
Age (yr)			0.012	0.911
< 60	242	52.6		
≥ 60	188	55.1		
Tumor size (cm)			78.184	0.000
< 4.8	208	76.7		
≥ 4.8	222	32.9		
Depth of invasion			69.581	0.000
pT2	117	82.5		
pT3	40	56.5		
pT4a	273	41.2		
Histological type			0.835	0.361
Differentiated	105	66.5		
Undifferentiated	325	49.8		
Lymph node metastasis			138.815	0.000
pN0	105	85.9		
pN1	92	73.0		
pN2	94	40.4		
pN3	139	21.7		
MLR			139.034	0.000
0	70	85.9		
1	118	70.1		
2	93	54.1		
3	149	23.4		

OS: Overall 5-year survival rate; MLR: Metastatic lymph node ratio.

Table 3 Multiple stepwise regression analysis with the Cox proportional hazards model

Characteristics	B	SE	Wald	*P* value	RR	95%CI	
Depth of invasion			23.143	0.000			
pT3/pT2	0.604	0.228	7.032	0.008	1.829	1.171	2.857
pT4a/pT2	0.775	0.161	23.135	0.000	2.171	1.583	2.977
Lymph node metastasis			9.923	0.019			
pN1/pN0	0.578	0.280	4.268	0.039	1.783	1.030	3.085
pN2/pN0	1.130	0.420	7.224	0.007	3.095	1.358	7.055
pN3/pN0	1.601	0.528	9.204	0.002	4.959	1.763	13.954
MLR	0.061	0.170	0.130	0.719	1.063	0.762	1.483
Tumor size (cm)	0.762	0.123	38.524	0.000	2.143	1.684	2.726

MLR: Metastatic lymph node ratio; CI: Confidence interval; RR: Relative risk; B: Borrmann.

sion[17-20]. We found that LSTs were highly aggressive and malignant, with high disease stages. Compared with SSTs, LSTs showed deeper infiltration and were associated with more extensive lymph node metastasis, as well as having a significantly lower 5-year OS rate (37.1% *vs* 63.3%, $P < 0.05$), indicating that a cutoff of 4.8 cm could be used as a size criterion for gastric cancers.

The prognostic role of tumor size in gastric cancer remains unclear. An examination of 697 patients with gastric cancer who had undergone gastrectomy with curative intent found that tumor size was a predictor of survival in univariate analysis, but not in multivariate analysis[21]. In contrast, other researchers found that tumor size was an independent predictor of prognosis. For example, when patients were divided by tumor size into three subgroups, ⩽ 4 cm, ⩽ 10 cm, and > 10 cm in diameter, tumor size independently predicted patient survival[20]. Similarly, using a cutoff of 8 cm, tumor size was independently prognostic of survival[22]. We found that tumor size was significantly correlated with patient prognosis in both univariate and multivariate analysis, as were depth of tumor invasion and lymph node metastasis. Another study hypothesized that it was difficult to identify the most important variables associated with prognosis, and that the precise evaluation of the impact of tumor size on prognosis was feasible only when depth of invasion was specified[23]. Therefore, that study evaluated survival in patients with pT3 stage gastric cancer relative to pN stage in patients with LSTs and SSTs, finding that tumor size significantly influenced prognosis in pT3N2-3 stage tumors ($P = 0.004$). To eliminate depth of tumor invasion and lymph node metastasis as factors, we compared survival in patients in the LST and SST groups according to pT and pN stages. We observed no significant differences in 5-year OS of patients with pT2-3N0-2 stage tumors classified as LSTs and SSTs. In contrast, the 5-year OS rates were significantly lower in LST than in SST patients with pN3 stage or pT4a stage tumors ($P < 0.05$). In patients with pT2-3N0-2 stage tumors, the tumors likely did not infiltrate the serosa and had less extensive lymph node metastasis, reducing the likelihood of free cancer cells in the peritoneal cavity and decreasing the possibility of peritoneal recurrence. Tumor size, therefore, did not significantly affect postoperative survival in these patients. In patients with pN3 stage or pT4a stage LSTs, however, the interactions between tumors and lymphatic tissue were enhanced, thus increasing the likelihood of lymph node micrometastasis and diffusion to lymphatic vessels; the larger the area of the serosa invaded by tumor, the greater the likelihood for intraperitoneal dissemination, and the poorer the prognosis[24-26]. Tumor size was therefore correlated with survival of patients with pN3 stage or pT4a stage gastric cancer.

In conclusion, using a cutoff value of 4.8 cm, tumor size may be a prognostic factor in patients with pN3 stage or pT4a stage advanced gastric cancer located in the lower third of the stomach.

Table 4 Survival based on pT and pN stages in the large-sized tumor and small-sized tumor groups

	n (5-yr OS, %)		χ^2	*P* value
	LST	SST		
pT2				
pN0	9 (88.9)	36 (96.7)	0.260	0.610
pN1	11 (72.7)	26 (91.1)	0.000	0.986
pN2	4 (50.0)	16 (87.1)	0.066	0.797
pN3	4 (0.00)	11 (63.6)	7.661	0.006
pT3				
pN0	3 (66.7)	9 (100.0)	1.634	0.201
pN1	6 (66.7)	4 (75.0)	0.348	0.555
pN2	2 (0.0)	3 (66.7)	0.825	0.364
pN3	8 (12.5)	5 (40.0)	3.940	0.047
pT4a				
pN0	23 (68.7)	25 (83.1)	5.108	0.024
pN1	24 (54.2)	21 (75.4)	4.743	0.029
pN2	46 (27.2)	23 (61.9)	7.682	0.006
pN3	83 (6.9)	28 (48.6)	23.138	0.000

LST: Large-sized tumor; SST: Small-sized tumor; OS: Overall 5-year survival rate.

COMMENTS

Background

In addition to lymph node metastasis and depth of tumor invasion, tumor size is an important clinicopathological feature of gastric cancer because it can be measured easily before or during surgery. To date, however, the prognostic role of tumor size on survival in patients with gastric cancer remains unclear, with no consensus formula to calculate an appropriate cutoff value.

Research frontiers

Tumor size is included in the staging systems of many malignant diseases such as lung and breast cancer. Although tumor size has been reported to independently influence prognosis in patients with gastric cancer, an appropriate cutoff size has not been determined, especially for advanced gastric cancers located in the lower third of the stomach.

Innovations and breakthroughs

The authors utilized receiver-operating characteristic (ROC) curve analysis to determine the appropriate tumor size cutoff value and assessed the relationship between tumor size and overall survival rate.

Applications

The authors found that, using a cutoff of 4.8 cm, tumor size was a prognostic factor in patients with pN3 stage or pT4a stage advanced gastric cancers located in the lower third of the stomach. That is, patients with pN3 stage or pT4a stage tumors ⩾ 4.8 cm had a poorer prognosis than those with tumors < 4.8 cm.

Peer review

In assessing the effect of tumor size in a large number of patients (430) with advanced gastric cancer located in the lower third of the stomach, the authors

found that tumor size, using a cutoff of 4.8 cm, was prognostic in patients with pN3 stage or pT4a stage tumors. This finding has important clinical implications for gastrointestinal surgeons and for patient prognosis.

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CASE REPORT

Metachronous penile metastasis from rectal cancer after total pelvic exenteration

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Abstract

Despite its abundant vascularization and extensive circulatory communication with neighboring organs, metastases to the penis are a rare event. A 57-year-old male, who had undergone total pelvic exenteration for rectal cancer sixteen months earlier, demonstrated an abnormal uptake within his penis by positron emission tomography/computed tomography. A single elastic nodule of the middle penis shaft was noted deep within Bucks fascia. No other obvious recurrent site was noted except the penile lesion. Total penectomy was performed as a curative resection based on a diagnosis of isolated penile metastasis from rectal cancer. A histopathological examination revealed an increase of well differentiated adenocarcinoma in the corpus spongiosum consistent with his primary rectal tumor. The immunohistochemistry of the tumor cells demonstrated positive staining for cytokeratin 20 and negative staining for cytokeratin 7, which strongly supported a diagnosis of penile metastasis from the rectum. The patient is alive more than two years without any recurrence.

INTRODUCTION

The penis is an uncommon site of metastasis, and only around 300 such cases have been reported in the English-language literature[1,2]. A comprehensive review of the 305 reported cases showed the most frequent primary sites of metastatic penile tumors to be the bladder (34.7%), prostate (29.8%), rectum and sigmoid colon (15.7%), and kidney (6.5%)[1]. Approximately 50 cases of penile metastasis from either rectal or sigmoid colon cancer have been reported[1-3]. Ketata *et al*[4] reported the 20th case of penile metastasis from rectal cancer in 2007, and thereafter five other cases have been reported[5-9]. One-third of all penile metastases are synchronous, whereas the remaining two-thirds are detected a mean of 18 mo after discovery of the primary tumor[7]. About 90% of reported cases of penile metastasis are a part of widespread disease[2], thus isolated metastatic penile carcinomas are exceptionally rare. This report presents a case of metachronous isolated penile metastasis from rectal cancer after total pel-

vic exenteration, which was treated with a curative penile resection.

CASE REPORT

A 57-year-old male had a large rectal cancer which occupied the pelvis minor with massive invasion to both the bladder and prostate. He underwent sigmoid colostomy twenty three months previously. Nine courses of chemotherapy [oxaliplatin 100 mg intravenous (*iv*) on day 1, levofolinate 125 mg *iv* on day 1, 5-fluorouracil (5-FU) 500 mg *iv* bolus on day 1 and 5-FU 750 mg *iv* continuous infusion on days 1 and 2] had been administered after the first operation and the tumor slightly decreased in size without showing any distant metastasis. The patient underwent total pelvic exenteration, ileal conduit and pelvic reconstruction with a gracilis muscular flap sixteen months ago. There was no clinical or radiological evidence of distant metastasis at the time of resection. The histopathological examination revealed a moderately differentiated adenocarcinoma of the rectum invading to the bladder and prostate without lymph node metastasis (none out of 57). The disease was staged as T4 N0 M0 stage Ⅱ (Dukes Classification B). He made an uneventful recovery and was discharged on the nineteenth postoperative day.

No clinical or radiological evidence of recurrence was observed for nine months after total pelvic exenteration until he started complaining of intermittent bloody discharge from his penis. Positron emission tomography/computed tomography (PET/CT) showed high 18-F fluorodeoxyglucose uptake in the penis (Figure 1A). A physical examination at that time showed a single 4 cm elastic nodule of the penis shaft in the middle, deep within Bucks fascia, which had slight tenderness. Penile magnetic resonance imaging (MRI) showed a low intensity lesion on a gadolinium-enhanced fat-suppressed T1-weighted image (Figure 1B). Contrast enhanced computed tomography (CECT) revealed neither other distant metastases nor local recurrence, and the serum tumor marker carcinoembryonic antigen was within normal limits.

The patient underwent total penectomy based on the diagnosis of penile metastasis from rectal cancer with the resection of all the residual urethra, as a curative resection. The tumor was localized in the corpus spongiosum, and had almost completely replaced it (Figure 2). A histopathological examination of the resected specimen revealed an increase of well differentiated adenocarcinoma in the corpus spongiosum, consistent with his primary rectal tumor. Immunohistochemistry of tumor cells demonstrated positive staining for cytokeratin 20 and negative staining for cytokeratin 7 (Figure 3), which supported the diagnosis because the majority of rectal cancer does not stain for cytokeratin 7, but instead does stain for cytokeratin 20, and also because urothelial cells commonly shows positive staining for cytokeratin 7[4]. He underwent adjuvant chemotherapy, and now is good in health for more than two years since the last surgery with no recurrence.

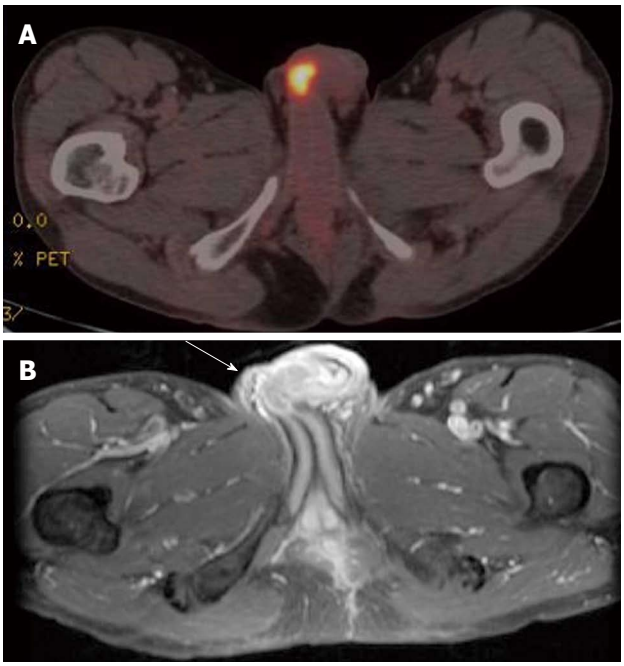

Figure 1 Positron emission tomography and magnetic resonance imaging image. A: Positron emission tomography/computed tomography showing high 18-F fluorodeoxyglucose uptake in the penis; B: Gadolinium-enhanced fat-suppressed T1-weighted image showing a low intensity lesion on the middle penis shaft (white arrow).

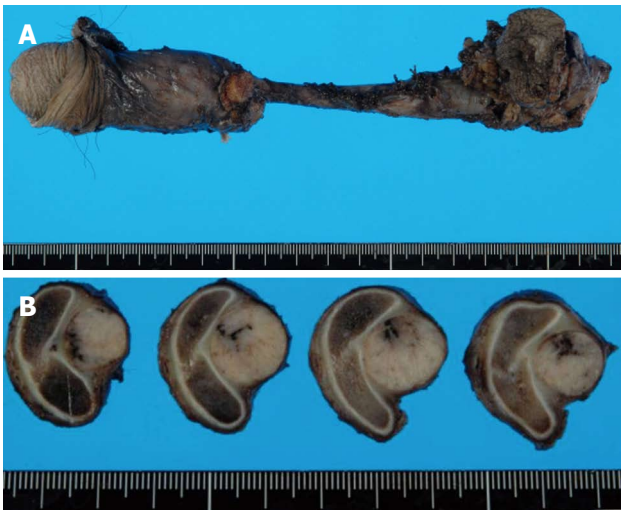

Figure 2 Resected specimen showing penile metastasis. A: Total penectomy with all the residual urethra; B: The tumor was localized in the corpus spongiosum, and had almost completely replaced it.

DISCUSSION

A metastatic penile tumor is rare despite the rich vascularity and the end arterial nature of its supply. Various mechanisms of penile metastasis have been suggested; retrograde venous spread, retrograde lymphatic spread,

Figure 3 Histopathological findings of the tumor. A histopathological examination revealing well differentiated adenocarcinomas in the corpus spongiosum (A) with negative staining for cytokeratin 7 (B) and positive staining for cytokeratin 20 (C) by immunostaining.

arterial spread, implantation and secondary to instrumentation or direct extension[1]. Among these mechanisms, retrograde venous spread from the pudendal venous system into the dorsal venous system of the penis appears to be the most common mode of spread[7]. Retrograde lymphatic spread into penile lymphatic channels after obstruction of inguinal and hypogastric nodes, is also considered[2]. One or both corpora cavernosa is the most common site of penile metastasis, and the glans penis and corpus spongiosum are rarely involved[1,10]. However, the current tumor was localized not in the corpora cavernosa but in the corpus spongiosum without widely disseminated disease, which seems to be extremely rare. These findings suggest that cancer cells in the residual urine at the time of total pelvic exenteration had likely become implanted in the corpus spongiosum.

Penile metastasis tends to show a poor prognosis because metastasis to the penis, in most cases, tends to be part of widely disseminated disease. The majority of patients die within one year[5]. Therefore, many treatment algorithms against penile metastases are described. The treatment is essentially palliative, and the combination of radiotherapy with chemotherapy produces only occasional responses. Some cases are treated by penectomy, an aggressive surgical excision, in order to palliate such symptoms as priapism and pain. Penectomy can offer the possibility of cure in patients with disease localized to the penis, like the current patient, even though such cases may be rare.

In conclusion, this report described a case of penile metastasis from rectal cancer. The patient underwent a complete cure resection with subsequent adjuvant chemotherapy, rather than palliative treatment. Penile involvement is usually a marker of widespread hematogenous dissemination which indicates a poor prognosis, but such a prognosis does not seem applicable in the current case.

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CASE REPORT

Primary hepatic leiomyosarcoma with liver metastasis of rectal cancer

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Abstract

Primary hepatic leiomyosarcoma is a particularly rare tumor with a poor prognosis. Curative resection is currently the only effective treatment, and the efficacy of chemotherapy is unclear. This represents the first case report of a patient with primary hepatic leiomyosarcoma co-existing with metastatic liver carcinoma. We present a 59-year-old man who was diagnosed preoperatively with rectal cancer with multiple liver metastases. He underwent a curative hepatectomy after a series of chemotherapy regimens with modified FOLFOX6 consisting of 5-fluorouracil, leucovorin and oxaliplatin plus bevacizumab, FOLFIRI consisting of 5-fluorouracil, leucovorin and irinotecan plus bevacizumab, and irinotecan plus cetuximab. One of the liver tumors showed a different response to chemotherapy and was diagnosed as a leiomyosarcoma following histopathological examination. This case suggests that irinotecan has the potential to inhibit the growth of hepatic leiomyosarcomas. The possibility of comorbid different histological types of tumors should be suspected when considering the treatment of multiple liver tumors.

Key words: Leiomyosarcoma; Rectal cancer; Metastasis; Chemotherapy; Surgery

Peer reviewers: Dr. Marek Bebenek, Department of Surgical Oncology, Regional Comprehensive Cancer Center, 53-413 Wroclaw, Poland; Dr. Benjamin Perakath, Professor, Department of Colorectal Surgery, Christian Medical College, Vellore 632004, India

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INTRODUCTION

Hepatic sarcomas constitute only 1%-2% of primary malignant tumors of the liver, and primary hepatic leiomyosarcoma is particularly rare[1]. The prognosis of primary hepatic leiomyosarcoma is poor, but long-term survival is possible if complete resection can be achieved[2]. We herein report a case of primary hepatic leiomyosarcoma co-existing with synchronous liver metastases of rectal cancer. The patient was treated by a curative hepatectomy following chemotherapy.

CASE REPORT

A 59-year-old man with a history of hypertension and

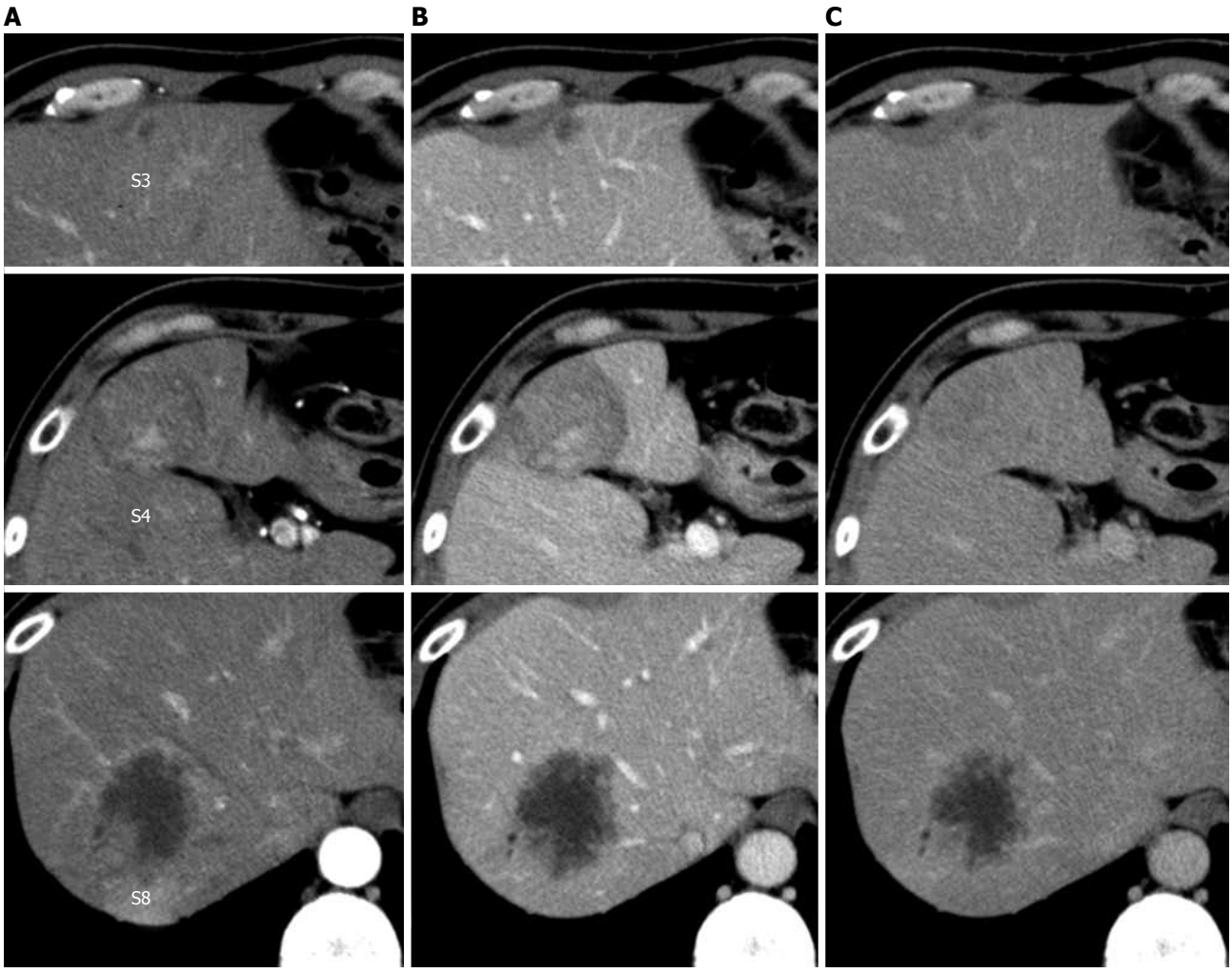

Figure 1 Dynamic computed tomography of the liver. A: Arterial phase; B: Portal phase; C: Venous phase. A hypo- or iso-dense tumor with heterogeneous enhancement in segment 4, a hypodense tumor with peripheral enhancement in segment 8, and a small hypodense tumor in segment 3.

hyperuricemia demonstrated fecal occult blood and multiple liver tumors in a comprehensive medical examination in October 2009 and was observed at our hospital. There were no remarkable physical findings. Laboratory examinations revealed a slight increase in uric acid (7.4 mg/dL). Hepatitis B and C viral markers were negative. Of the tested tumor markers, only carcinoembryonic antigen was elevated (17.9 ng/mL).

Dynamic computed tomography (CT) of the liver revealed a well-defined, hypo- or isodense tumor, 42 mm in diameter, with early enhancement and delayed washout or gradual enhancement in segment 4. Segment 8 contained an ill-defined, hypodense tumor, 52 mm in diameter, with continuous peripheral enhancement, and segment 3 contained a hypodense tumorthat was 11 mm in diameter (Figure 1).

The tumor in segment 4 appeared to be isointense throughout the dynamic phase on gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging (MRI), while the tumors in segments 3 and 8 appeared hypointense. All of the tumors appeared hypointense in pre-enhancement scans and the hepatocellular phase on T1-weighted images (Figure 2). Additionally, all of the tumors appeared hyperintense on T2-weighted images and diffusion-weighted images. Ultrasonography demonstrated that the tumors were hypo- or isoechoic heterogeneous masses without halos (Figure 3).

Colonoscopy revealed a Borrmann type 2 tumor at the rectosigmoid junction. Histology of the biopsy tissue revealed a moderately differentiated adenocarcinoma.

The patient was diagnosed with rectal cancer and multiple liver metastases. Because the simultaneous resection of the primary tumor and liver metastases would be a high-risk procedure, we performed an initial high anterior resection with D3 dissection in December 2009. A histopathological examination revealed extraserosal invasion by a moderately differentiated adenocarcinoma and metastases in 8 of 14 resected lymph nodes. A genetic analysis confirmed wild-type KRAS. The patient received chemotherapy with modified FOLFOX6 (mFOLFOX6) plus bevacizumab after the initial surgery. The liver tumor in segment 3 vanished, and the tumor in segment 8 decreased in size from 52 mm to 25 mm in diameter. However, the tumor in segment 4 increased

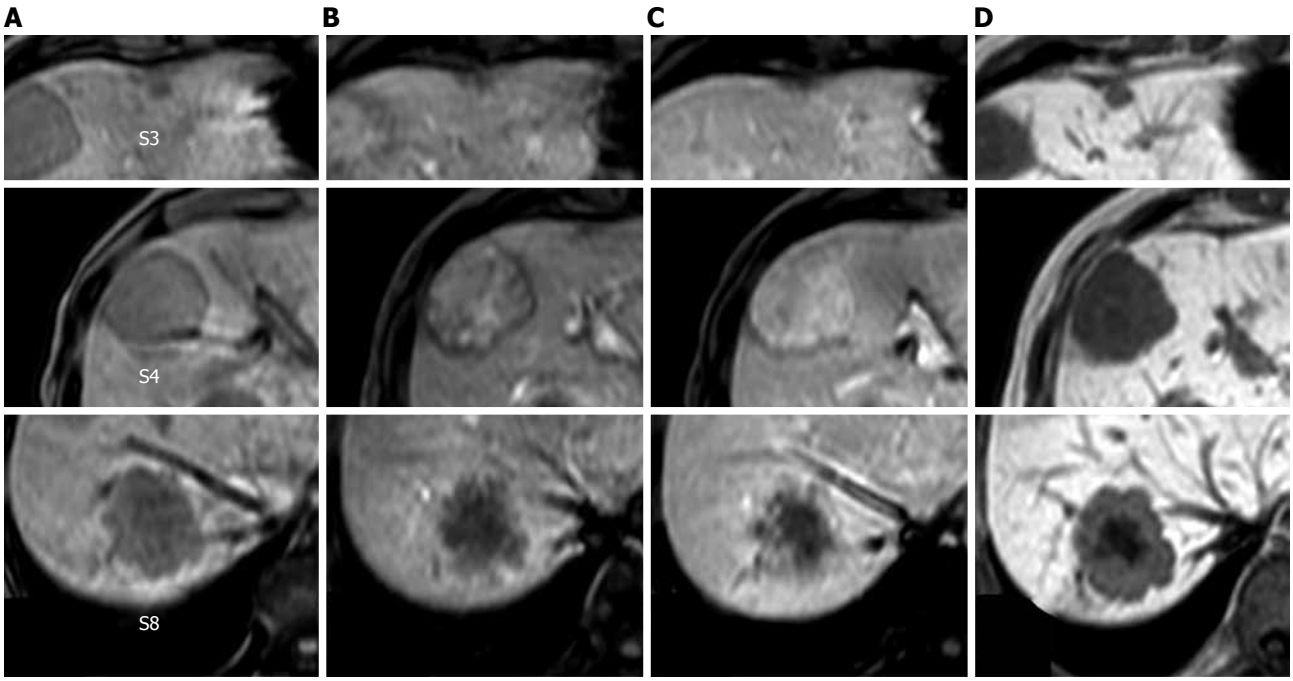

Figure 2 T1-weighted gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging. A: Pre-enhancement; B: 30 s; C: 120 s; D: Hepatocellular phase. The tumor in segment 4 showed heterogeneous enhancement in the dynamic phase. The tumors in segments 3 and 8 showed gradual peripheral enhancement.

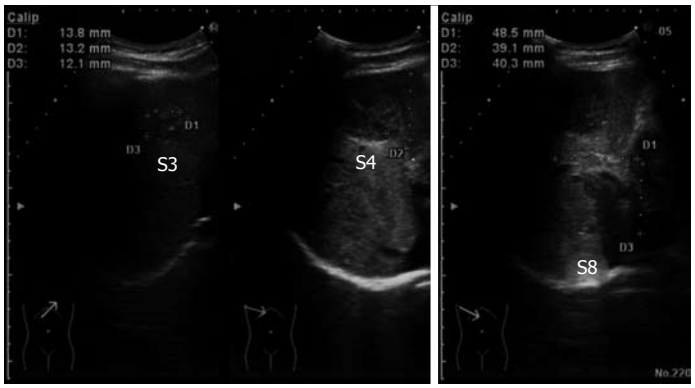

Figure 3 Ultrasonography of the abdomen. All of the tumors showed hypo- or isoechoic heterogeneous masses without halos.

in size from 42 mm to 60 mm in diameter. He then underwent seven courses of FOLFIRI plus bevacizumab, followed by a course of irinotecan plus cetuximab; the tumor in segment 4 decreased in size from 60 mm to 55 mm in diameter, and the tumor in segment 8 decreased in size from 25 mm to 22 mm in diameter (Figure 4). With the exception of the liver tumors, positron emission tomography-CT after the chemotherapy series showed no significant uptake of fluorodeoxyglucose.

The patient then underwent a central two-segment resection and a partial resection of segment 3 in September 2010. The resected specimen from the two central segments weighed 380 g, while that from segment 3 weighed 3.8 g. The tumors from segments 4, 8 and 3 measured 56 mm × 44 mm, 24 mm × 22 mm and 7 mm × 4 mm, respectively (Figure 5). A histopathological examination revealed fibrosis and calcification in the tumors from segments 3 and 8, with a few degenerated residual adenocarcinoma cells, which was compatible with rectal cancer metastasis. In contrast, the tumor from segment 4 consisted of irregular fascicles of spindle-shaped cells with eosinophilic cytoplasms and nuclear atypia (Figure 6). Chemotherapy had no apparent effect histologically. An immunohistochemical examination demonstrated that the tumor cells in segment 4 were positive for α-smooth muscle actin and desmin, and negative for CD34, S-100, c-kit and cytokeratin AE1/3 (Figure 7). Based on these findings, the tumor in segment 4 was diagnosed as a leiomyosarcoma.

The patient underwent six courses of FOLFIRI plus bevacizumab as adjuvant chemotherapy, and had no recurrence of leiomyosarcoma or rectal cancer at 16 mo after the hepatectomy.

DISCUSSION

Primary hepatic leiomyosarcoma is a particularly rare tumor. Most hepatic leiomyosarcomas are metastatic

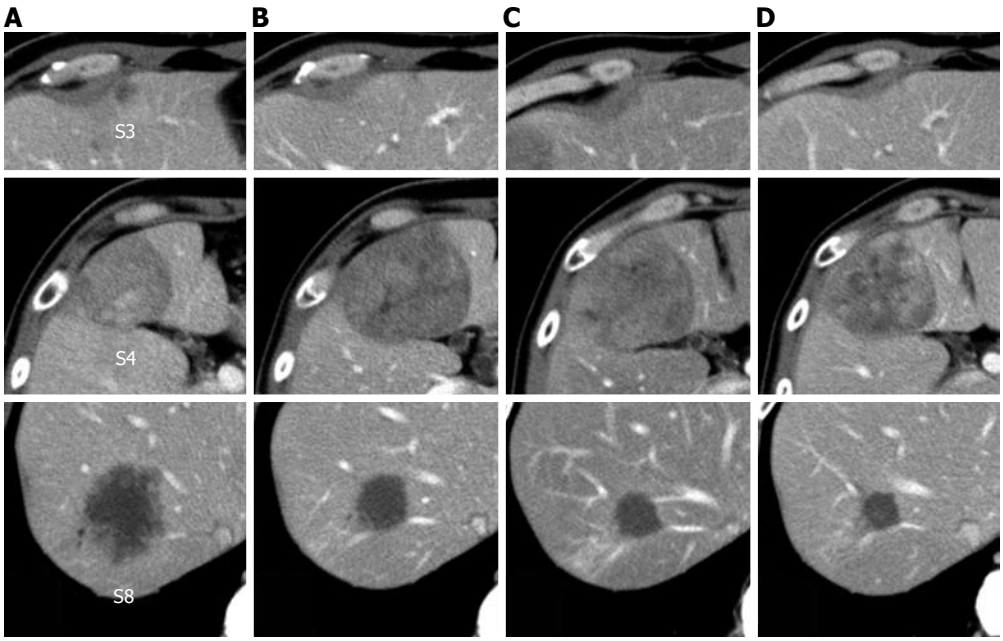

Figure 4 Change in tumor size observed using computed tomography during the clinical course. A: Before treatment; B: After six courses of modified FOLFOX6 plus bevacizumab; C: After seven courses of FOLFIRI plus bevacizumab; D: After one course of irinotecan plus cetuximab.

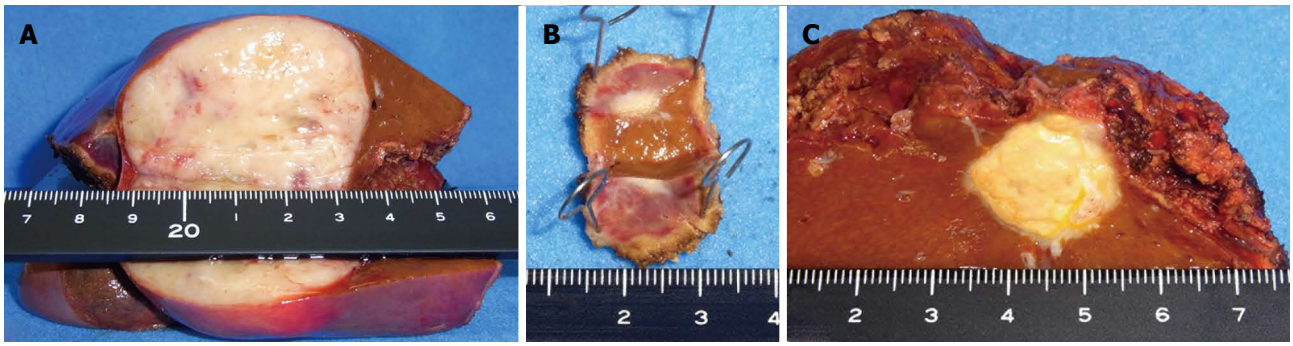

Figure 5 A macroscopic view of the cut-end surfaces of the resected specimens. A: Segment 3; B: Segment 8; C: Segment 4.

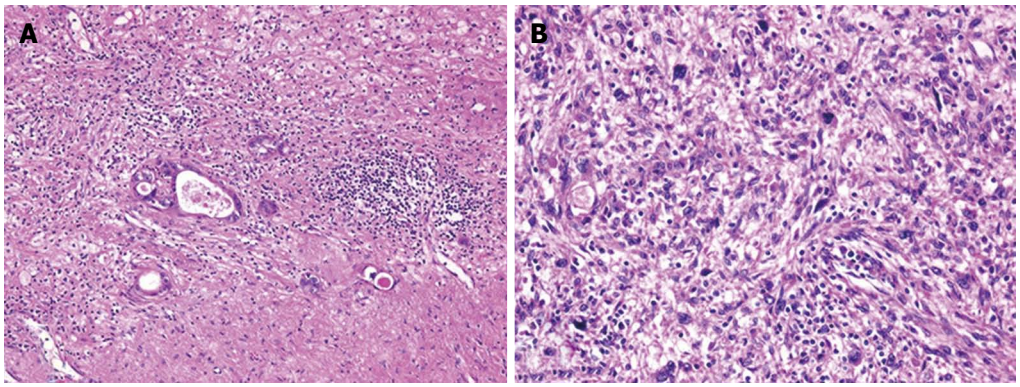

Figure 6 Histological findings of the tumors and hematoxylin and eosin staining. A: The tumor in segment 8 demonstrated fibrosis and calcification, with a few degenerated residual adenocarcinoma cells; B: The tumor in segment 4 had irregular fascicles of spindle-shaped cells with eosinophilic cytoplasm and nuclear atypia.

tumors, and the exclusion of metastatic leiomyosarcoma is therefore essential for an accurate diagnosis[3]. To our knowledge, this represents the first case report in the literature to describe a primary hepatic leiomyosarcoma co-existing with metastatic liver tumors of another origin, but the clinical and histological relevance is unclear. Surgery is considered to be the best treatment for primary hepatic leiomyosarcomas, if an R0 resection can

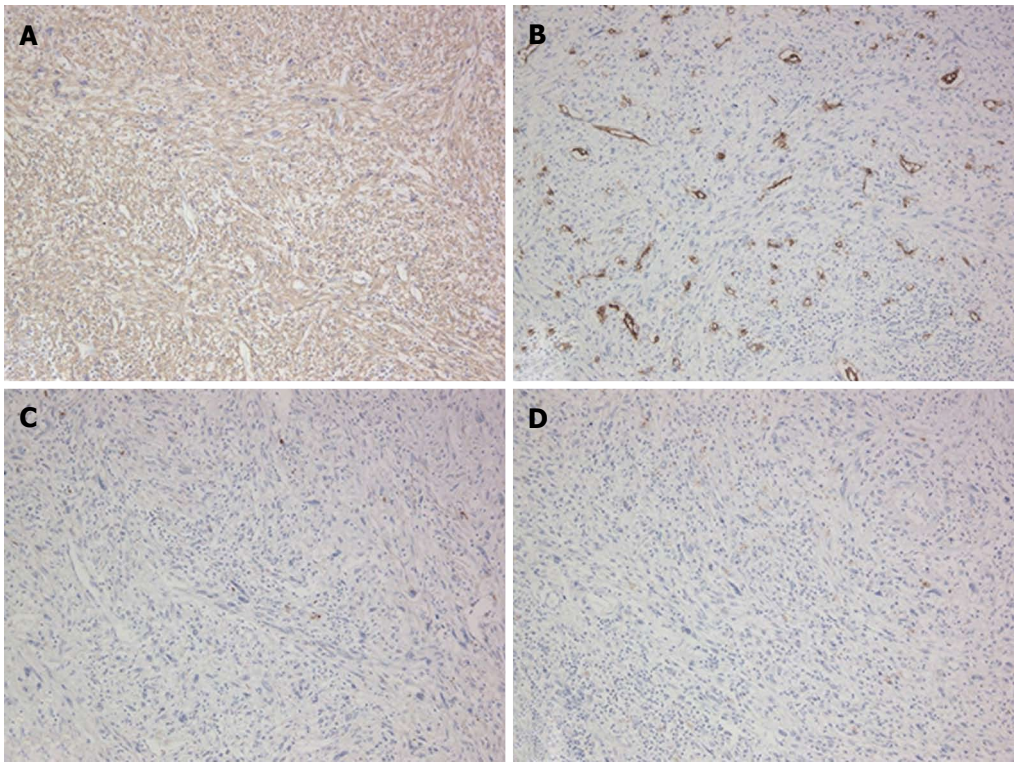

Figure 7 Immunohistochemical features of the tumor in segment 4. A: Positive for α-smooth muscle actin; B: Negative for CD34; C: Negative for S-10; D: Negative for c-kit.

be performed[3,4]. Doxorubicin has been the standard chemotherapy for soft tissue leiomyosarcomas, with gemcitabine and docetaxel or trabectedin as possible alternatives[5,6]. However, the efficacy of chemotherapy for primary hepatic leiomyosarcoma has not been confirmed. In our case, mFOLFOX6 plus bevacizumab did not inhibit the growth of the leiomyosarcoma, while FOLFIRI plus bevacizumab and irinotecan plus cetuximab did. Some studies have demonstrated the efficacy of irinotecan combined with other anticancer agents in some types of sarcomas, such as Ewing's sarcoma[7] or rhabdomyosarcoma[8], but little is known about the effect of irinotecan on leiomyosarcomas. The present case suggests that irinotecan exerted a growth-inhibiting effect on this leiomyosarcoma.

In a randomized controlled trial, Portier *et al*[9] demonstrated a disease-free survival benefit of adjuvant chemotherapy with 5-fluorouracil and leucovorin after liver resection for patients with liver metastases from colorectal cancer compared to surgery alone. In another randomized controlled trial, Nordlinger *et al*[10] demonstrated that perioperative chemotherapy with FOLFOX4 in patients with resectable liver metastases improved progression-free survival compared to surgery alone. However, adjuvant chemotherapy after hepatectomy for patients converted from unresectable liver metastases is still controversial. We selected FOLFIRI plus bevacizumab for adjuvant chemotherapy with the expectation of an effect of irinotecan on the leiomyosarcoma and because the patient developed grade 3 peripheral neuropathy with mFOLFOX6.

The preoperative diagnosis of primary hepatic leiomyosarcoma is challenging because of the non-specific nature of the symptoms and the lack of serological markers[11]. CT usually shows a well-defined hypodense heterogeneous mass with peripheral enhancement. MRI shows hypointensity on T1-weighted images and hyperintensity on T2-weighted images[12]. Some cases of hepatic leiomyosarcoma diagnosed by fine-needle aspiration cytology have been reported[13]. However, the preoperative histological diagnosis of liver tumors, especially hepatocellular carcinoma, is controversial because of the risk of needle-track seeding. In the current case, the complication of liver metastases from rectal cancer made preoperative diagnosis more difficult. In retrospect, however, the leiomyosarcoma lesion showed slightly different CT and MRI findings compared to the metastatic tumors.

In conclusion, this case suggests the potential of irinotecan to inhibit the growth of hepatic leiomyosarcomas. Irinotecan may be a chemotherapy option for hepatic leiomyosarcomas. The possibility of different histological types of tumors should be considered when planning the treatment of multiple liver tumors, especially when the tumors might have heterogeneous responses to chemotherapy.

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CASE REPORT

Duodenal obstruction following acute pancreatitis caused by a large duodenal diverticular bezoar

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Abstract

Bezoars are concretions of indigestible materials in the gastrointestinal tract. It generally develops in patients with previous gastric surgery or patients with delayed gastric emptying. Cases of periampullary duodenal diverticular bezoar are rare. Clinical manifestations by a bezoar vary from no symptom to acute abdominal syndrome depending on the location of the bezoar. Biliary obstruction or acute pancreatitis caused by a bezoar has been rarely reported. Small bowel obstruction by a bezoar is also rare, but it is a complication that requires surgery. This is a case of acute pancreatitis and subsequent duodenal obstruction caused by a large duodenal bezoar migrating from a periampullary diverticulum to the duodenal lumen, which mimicked pancreatic abscess or microperforation on abdominal computerized tomography. The patient underwent surgical removal of the bezoar and recovered completely.

Key words: Bezoar; Diverticulum; Pancreatitis; Duodenal obstruction

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Kim JH, Chang JH, Nam SM, Lee MJ, Maeng IH, Park JY, Im YS, Kim TH, Park IY, Han SW. Duodenal obstruction following acute pancreatitis caused by a large duodenal diverticular bezoar. *World J Gastroenterol* 2012; 18(38): 5485-5488 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i38/5485.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i38.5485

INTRODUCTION

Bezoars are concretions of foreign bodies found in the gastrointestinal tract. They are usually detected after gastric operations because of reduced gastric motor activity and delayed gastric emptying[1]. Bezoars in a periampullary duodenal diverticular without previous surgery are rare. The presence of bezoars may or may not be accompanied by gastrointestinal manifestations, such as epigastric pain and postprandial fullness. Major complications of bezoars include gastritis, gastric ulcer, gastric perforation, and intestinal obstruction[2]. Small bowel obstruction is a rare complication usually due to the migration of gastric bezoars. It could also be caused by formation of primary bezoars in the small bowel in association with underlying diseases such as diverticulum, stricture or tumor[3]. Acute pancreatitis secondary to a bezoar is rare, but can present as a possible complication[4]. However, duodenal obstruction by a diverticular bezoar following acute pancreatitis

has not been reported.

We report a case of acute pancreatitis and subsequent duodenal obstruction caused by a large duodenal bezoar migrating from periampullary diverticulum to duodenal lumen, which was difficult to diagnose by radiological study.

CASE REPORT

A 75-year-old female visited our hospital with epigastric pain, nausea and vomiting for 4 d. She was diagnosed with diabetes 15 years ago and was taking oral hypoglycemic agents for the control of blood sugar level. There were no specific findings from her social history. Her blood pressure on admission was 130/80 mmHg, pulse rate 70/min, respiratory rate 20/min, and body temperature 36.4 ℃. On physical examination, she appeared acutely ill, with a soft abdomen but with tenderness in the epigastric area and right upper quadrant. Complete blood count test showed a leukocyte count of 10 340/mm^3, a hemoglobin level of 13.1 g/dL, and a platelet count of 174 000/mm^3. Blood chemistry showed total protein 7.1 g/dL, albumin 4.3 g/dL, total bilirubin 1.1 mg/dL, aspartate aminotransferase 491 IU/L, alanine aminotransferase 375 IU/L, alkaline phosphatase 154 IU/L, γ-glutamyl transpeptidase 213 IU/L, amylase 1730 IU/L, and lipase over 300 IU/L. Abdominal computerized tomography (CT) taken on the day of admission revealed a 5 cm sized subtle rim enhancing mass with air bubbles in the pancreatic head which raised the suspicion of an air forming abscess of the pancreatic head or a duodenal diverticulum of the second duodenal portion (Figure 1A and B). Following this, magnetic resonance cholangiopancreatography (MRCP) showed no evidence of abnormality in the common bile duct or pancreatic duct. The medical treatments including intravenous fluids, parenteral alimentation, and antibiotics were started, and abdominal pain improved thereafter. Additionally, the elevated serum transaminase and pancreatic enzymes progressively improved. However, nine days after admission, the patient's body temperature rose to 40.4 ℃, her blood pressure was 80/50 mmHg, leukocyte count 47 620/mm^3, erythrocyte sedimentation rate 66 mm/h, and C-reactive protein 166 mg/dL. The follow-up abdominal CT revealed aggravation of duodenal wall thickening and stranding of the surrounding soft tissues with some tiny extraluminal air bubbles indicating possible micro-perforation (Figure 1C). She received fluid resuscitation through rapid intravenous administration of crystalloid fluid and vasopressor support, and antibiotic therapy with meropenem was started for the management of septic shock. After vigorous treatment, the patient's state became stabilized, but more than 1500 cc of dark-colored fluid was drained through the Levin tube per day. Esophagogastroduo- denoscopy (EGD) was performed for suspected duodenal obstruction. On entering the duodenum, a huge dark yellowish mass was found obstructing the lumen of the second portion of the duodenum. This mass was thought

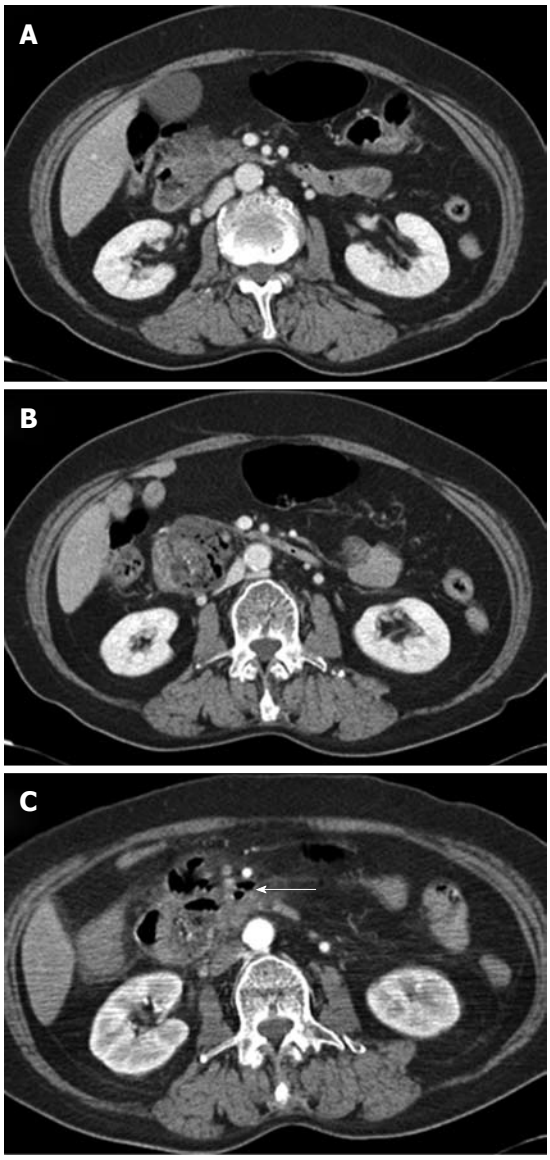

Figure 1 Abdominal computed tomography findings. A: The pancreas head showed swelling with irregular contour of the pancreatic margin and mild peripancreatic infiltration; B: There is a 5 cm size subtle rim enhancing mass with air bubbles, indicating acute diverticulitis in the second duodenal portion or air forming abscess of the pancreatic head; C: After nine days, a few extraluminal air bubbles (arrow) suspicious for microperforation were found.

to be originated from the large periampullary diverticulum and migrated toward the distal portion (Figure 2A). A large broad based inflamed diverticulum was observed, with small secondary diverticula corresponding to the extraluminal air bubbles seen on the follow-up abdominal CT. Endoscope was not able to pass through because of luminal obstruction. We tried to extract the bezoar with the help of a basket and a net, but it was unsuccessful as the bezoar was too hard and big (Figure 2B). On eighteenth hospital day, when the clinical symptoms and the laboratory findings of acute pancreatitis and septic shock had been improved, the patient was transferred to the department of general surgery for operation. A gastrotomy was performed and a huge bezoar (5 cm × 2.5 cm) was removed through the antrum by manually push-

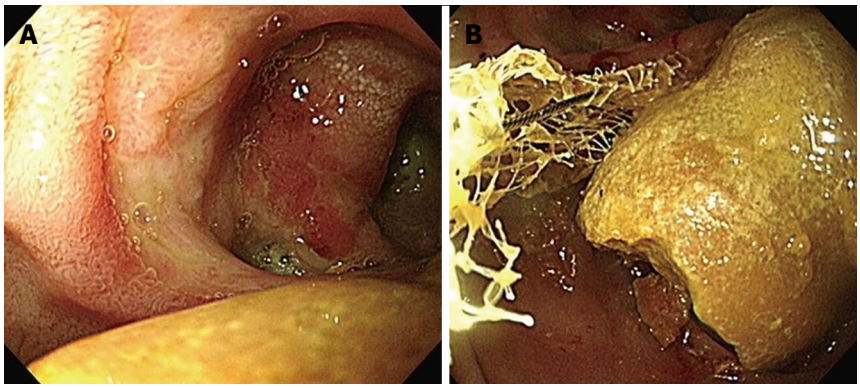

Figure 2 Endoscopic findings. A: There is a huge luminal obstructing bezoar near the duodenal diverticulum; B: The bezoar was too large to be captured and removed by an endoscopic basket or net.

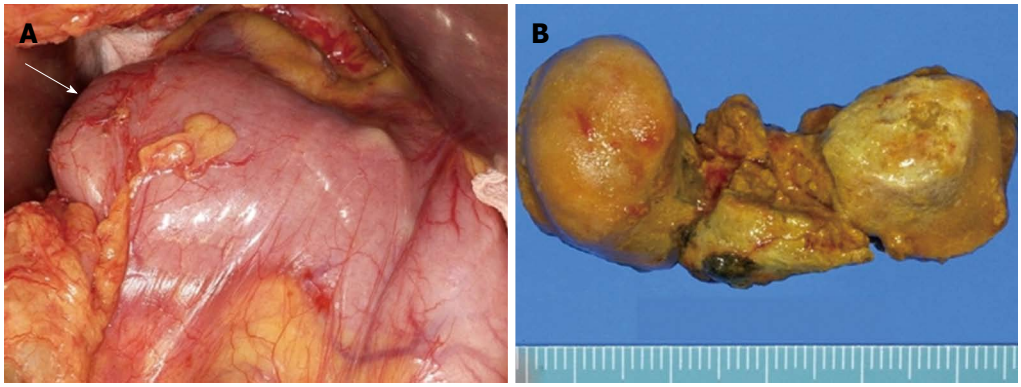

Figure 3 Surgical findings. A: Obstructed duodenum with impacted huge bezoar (arrow); B: Surgical specimen of the divided bezoar, 5 cm long, 2.5 cm in diameter.

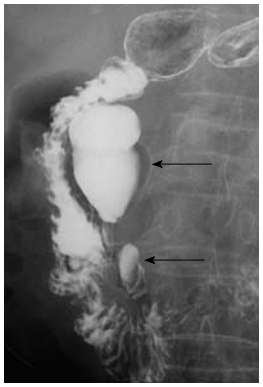

Figure 4 Upper gastrointestinal series show two periampullary diverticula (arrows) in the second duodenal portion.

ing the duodenal bezoar up into the stomach (Figure 3). The patient was discharged on the 6th postoperative day. During the 2-mo follow-up, the patient did not complain of any discomfort and two periampullary diverticula were seen in the upper gastrointestinal series (Figure 4).

DISCUSSION

Bezoars are composed of vegetable matter (phytobezoar), hair (trichobezoar), or more unusual materials[1]. They are usually related to previous gastric operations, such as vagotomy or partial gastrectomy, which reduce gastric motor activity and delay gastric emptying. In one report, 70% of patients with bezoar had undergone previous surgery[2]. Bezoars also can be induced by gastroparesis caused by hypothyroidism or diabetes mellitus, and poor mastication or ingestion of indigestible materials[3].

Most bezoars occur in the stomach, but may be encountered elsewhere, including the small bowel and even the esophagus and rectum. Clinical manifestations vary depending on the location of the bezoar from showing no symptom to acute abdominal syndrome, such as, epigastric distention, abdominal pain, and acid regurgitation. Major complications of bezoars include intestinal obstruction, gastric perforation, gastric ulcer, and gastritis[5]. Small bowel obstruction is a severe complication requiring surgery[6]. Small bowel obstructions were usually due to migration of gastric bezoars, but it could also be caused by primary bezoars formed in the small bowel in association with stricture, tumor or diverticulum[3]. Biliary obstruction or acute pancreatitis caused by bezoars has seldom been reported[4,7,8]. In our case, a large duodenal bezoar had originated from an inflamed periampullary diverticulum, causing acute pancreatitis. This was then followed by a spontaneous migration of the bezoar, resulting in a duodenal obstruction and septic shock. The duodenal obstruction due to large gallstone migrated through a cholecysto-enteric fistula can give a similar

radiological and endoscopic appearance as our case[9]. However, there was no cholecysto-duodenal fistula or air in the gallbladder in the present case.

Although duodenal bezoars are rare, they can develop in patients with previous gastric surgery[8], deformed duodenal bulb[10], superior mesenteric artery syndrome[11], and duodenal diverticulum[12] as in our case. Most cases of pancreatitis caused by duodenal bezoars are trichobezoars also known as the "Rapunzel syndrome"[4]. This was a case of having unusually long hairballs that extend from the stomach into the jejunum or ileum. It may cause extrinsic compression of the ampulla, common bile duct or pancreatic head. It is thought that bezoars in the periampullary diverticulum may also cause acute pancreatitis through similar mechanisms[13].

Several studies suggested that patients with periampullary diverticulum had higher frequencies of pancreatitis. It is still controversial whether or not pancreatitis is induced by periampullary diverticulum per se. Periampullary diverticulum is associated with choledocholithiasis, and it predispose to gallstone pancreatitis[14]. Our patient showed no evidence of stones in the common bile duct on abdominal CT and MRCP, thus acute pancreatitis did not result from bile duct stones. In fact, the large bezoar had caused compression of major ampulla leading to pancreatitis.

Contrast-enhanced abdominal CT is the most useful tool for diagnosis of duodenal diverticulitis, but it can be misinterpreted as acute pancreatitis or its complications (phlegmon, pseudocyst, abscess), cystic pancreatic head neoplasms, and peripancreatic lymphadenopathy[15]. In our case, the enhanced CT showed findings that were suspicious for pancreatic abscess or microperforation of duodenal diverticulum adjacent to the pancreatic head. After EGD, the condition was recognized as periampullary diverticulitis with secondary diverticula inside. Endoscopy is an important diagnostic modality for diagnosis of diverticular diseases. The side-viewing endoscopy can be beneficial compared to the forward-viewing endoscopy, because acute diverticulitis causes mucosal swelling and narrowing of the diverticular orifice that further hampers the diagnostic yield of a forward-viewing endoscopy[16].

Bezoars are treated by endoscopy or surgery. If the bezoar is large, fragmentation is tried with the use of overtube, baskets, lithotripsic equipment, paraffin, cellulose, acetylcysteine, Coca-Cola lavage, and even lasers. If the bezoar is accompanied by complications such as small bowel obstruction, gastric perforation or gastric hemorrhage, the patient needs urgent treatment, such as surgery[3].

In conclusion, we report a case of a large duodenal bezoar, which had originated from the periampullary diverticulum, causing acute pancreatitis, with subsequent duodenal obstruction by migration. Complications caused by duodenal bezoar, in particular, pancreatitis and subsequent duodenal obstructions are rare, but a high index of suspicion is required for the correct diagnosis and proper management.

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua 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]

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

No volume or issue

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

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Personal author(s)

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

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

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

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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

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

Electronic journal (list all authors)

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

Patent (list all authors)

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

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Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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