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EDITORIAL

Pancreatic fluid collections: What is the ideal imaging technique?

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Abstract

Pancreatic fluid collections (PFCs) are seen in up to 50% of cases of acute pancreatitis. The Revised Atlanta classification categorized these collections on the basis of duration of disease and contents, whether liquid alone or a mixture of fluid and necrotic debris. Management of these different types of collections differs because of the variable quantity of debris; while patients with pseudocysts can be drained by straight-forward stent placement, walled-off necrosis requires multi-disciplinary approach. Differentiating these collections on the basis of clinical severity alone is not reliable, so imaging is primarily performed. Contrast-enhanced computed tomography is the commonly used modality for the diagnosis and assessment of proportion of solid contents in PFCs; however with certain limitations such as use of iodinated contrast material especially in renal failure patients and radiation exposure. Magnetic resonance imaging (MRI) performs better than computed tomography (CT) in characterization of pancreatic/peripancreatic fluid collections especially for quantification of solid debris and fat necrosis (seen as fat density globules), and is an alternative in those situations where CT is contraindicated. Also magnetic resonance cholangiopancreatography is highly sensitive for detecting pancreatic duct disruption and choledocholithiasis. Endoscopic ultrasound is an evolving technique with higher reproducibility for fluid-to-debris component estimation with the added advantage of being a single stage procedure for both diagnosis (solid debris delineation) and management (drainage of collection) in the same sitting. Recently

role of diffusion weighted MRI and positron emission tomography/CT with ^{18}F-FDG labeled autologous leukocytes is also emerging for detection of infection noninvasively. Comparative studies between these imaging modalities are still limited. However we look forward to a time when this gap in literature will be fulfilled.

Key words: Acute pancreatitis; Contrast-enhanced computed tomography; Magnetic resonance imaging; Endoscopic ultrasound; Positron emission tomography scan; Pancreatic fluid collections; Acute necrotic collections; Acute peripancreatic fluid collections; Pseudocysts; Walled-off necrosis

Core tip: Contrast-enhanced computed tomography is widely used imaging modality for the diagnosis and staging of acute pancreatitis due to its excellent capacity to demonstrate early inflammatory changes as well as local complications including fluid collections, However, magnetic resonance imaging may be a better imaging technique due to its, nonionizing nature, higher soft tissue contrast resolution, better safety profile of intravascular contrast media, noninvasive evaluation of pancreatic duct integrity and also has superiority in discrimination of internal consistency of pancreatic collections which is useful in further management plan. Role of endoscopic ultrasound and other newer techniques is still in evolving phase.

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INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory process of the pancreas characterized by auto-digestion of pancreatic parenchyma, vasculitis and fat necrosis[1]. Two of the following three features are required for diagnosis of AP: (1) abdominal pain consistent with pancreatitis (acute onset of a persistent, severe, epigastric pain often radiating to the back); (2) serum lipase activity (or amylase activity) at least three times greater than the upper limit of normal; and (3) characteristic findings of acute pancreatitis on contrast-enhanced computed tomography (CECT) and less commonly magnetic resonance imaging (MRI) or trans-abdominal ultrasonography[2,3].

Morphologically AP can be of two types: interstitial edematous pancreatitis (IEP) and necrotizing pancreatitis (NEP). IEP constitutes a diffuse (or sometimes localized) enlargement of the pancreas due to inflammatory edema and it usually resolves within the first few days[4]. On the other hand necrotizing pancreatitis which is seen in about 5%-10% of patients, commonly manifests as necrosis involving both pancreatic and peripancreatic tissues and less commonly involving only the peripancreatic tissue, and rarely of the pancreatic parenchyma alone. Necrosis develops early in the course of severe pancreatitis and usually well establishes by 96 h after onset of clinical symptoms[5].

The basis of defining morphological classification and local complications in Revised Atlanta classification is CECT. Recent studies have shown that MRI and EUS are better imaging for quantification of solid debris which is the basis for deciding management strategies, and may replace CT in future. Role of the newer technique such as diffusion-weighted MRI (DWI-MRI) and positron emission tomography (PET) CT in the severity assessment and detection of infection noninvasively is still to be established. This editorial is to review the role of all available imaging modalities in differentiating PFCs in patients of AP.

LOCAL COMPLICATIONS OF AP

As per the Revised Atlanta classification[6] local complications of AP comprises of acute peri-pancreatic fluid collections (APFCs), pancreatic pseudocysts, acute necrotic collections (ANCs) and walled-off necrosis (WON). Other local complications include gastric outlet obstruction, splenic and portal vein thrombosis and colonic necrosis.

APFCs

They usually develop in early stage of disease, and establish its borders with retroperitoneum and adjacent organs[6,7]. They can be single or multiple, but their contents are typically homogenous, sterile and lack wall of inflammatory or granulation tissue. Patients with APFCs are asymptomatic and treatment is usually unnecessary. Most of these collections resolve on their own[7,8]. If they do not resolve within a month, they evolve in to pancreatic pseudocysts (which is rarely seen during disease course).

Pancreatic pseudocysts

Pseudocyst is a fluid collection with homogenous internal fluid contents, but without any solid material and enclosed by a clear wall of fibrous tissue. It usually arises from main pancreatic duct or its intra-pancreatic branch disruption resulting in leakage of pancreatic juice; hence high amylase levels are seen in aspirated fluid from these cysts[6]. It is rarely seen following acute pancreatitis except in setting of a disconnected duct syndrome[9] and following surgical necrosectomy.

ANCs

These collections are seen in the setting of necrotizing pancreatitis (within first 4 wk) and consist of inhomogeneous mixture of liquefied, necrotic fatty tissue along with solid pancreatic and extra-pancreatic debris. They can be single or multiple, and are sometimes multiloculated. Differentiating ANC from an APFC may be difficult in the first week of the disease. With passage of time, parenchymal necrosis becomes more obvious, which aids in the distinction between these two. They may gradually resolve, persist as wall-off necrosis or may get infected in course of disease.

WON

Necrotic tissues surrounded by enhancing inflammatory walls are referred as WONs and evolve from ANCs after 4 wk of necrotizing pancreatitis. They can be confined to the pancreatic tissue or at times be away from the pancreas. WONs can be sterile or infected as well as solitary or multiple[6].

ROLE OF IMAGING

Imaging helps in the diagnosis of clinically suspected AP or suggesting alternative diagnosis. It also helps in determination of the cause of pancreatitis like biliary duct obstruction or structural abnormalities. Additionally, imaging can be utilized for assessment of the severity of the disease by identifying pancreatic or peripancreatic necrosis and complications. It has an essential role in classifying fluid collections especially in differentiating APFCs from ANCs and pseudocysts from WON, as presence or absence of solid or necrotic debris in a fluid collection has direct bearing on the outcome as well as on the choice of management strategy.

The choice of appropriate imaging modality depends on the reason for investigation, clinical symptoms, time of onset of symptoms and lab findings.

Role of transabdominal ultrasonogram

Ultrasonogram (USG) is a widely available, cheap, non-invasive investigation and can be repeated as often as necessary. Pancreas visualization using USG is feasible in 75%-93% of patients irrespective of weight or overlying bowel gas[10], It also helpful in identification of biliary lithiasis, exclusion of other causes of acute abdominal pain (medical or surgical) and for separation of severe AP from mild or moderate which is interpreted along with clinical and biochemical parameters. American College of Gastroenterology recommended that trans-abdominal ultrasound should be performed in all patients with AP (strong recommendation, low quality of evidence)[11].

USG is also helpful in monitoring the evolution of fluid collections, which occur as a result of AP, and in guiding diagnostic and therapeutic interventions. A recently published study showed that USG accuracy is comparable to that of endoscopic ultrasound and magnetic resonance imaging in patients with WON for delineation of solid debris but with its limitations in presence of air or high solid content[12].

Advantages of this method are its portable character, high accessibility (cheap equipment, lack of invasion) and dynamic character in real time. Limitations are operator dependence and inability to reproduce images. It cannot replace more efficient examination methods such as CT/MRI especially in case when parenchymal necrosis has to be detected or when the patient suffers from high meteorism.

Role of CECT

CECT is the widely used imaging modality in AP for the diagnosis, severity assessment and morphological classification. It also provides information on presence of collections and their size along with its wall thickness and internal debris. CT is an ideal technique to guide percutaneous aspiration and drainage procedures[13].

Revised Atlanta classification recommended CECT to be done at 5 to 7 d of pancreatitis for more reliably establishing the necrosis which is easily underestimated by immediate CT[9,14]. CECT criteria are used to subdivide AP into two types: IEP and NEP. The criteria for identification of the IEP are relatively homogeneous enhancement of pancreatic parenchyma with mild haziness or peripancreatic fat stranding, whereas lack of pancreatic parenchymal enhancement or necrosis of peripancreatic tissue suggest NEP[6]. Distinction between these two types is important as studies have shown an significant relationship of necrosis with local or systemic complications, hospital stay and death[8,15,16]. The role of recently developed radiological scoring systems based on organ dysfunction and SIRS are also promising in determination of severity and early stratification[17-19].

Additionally local complications of AP are also defined based on CECT[6]. APFCs appear as homogenous collections with low attenuation value without well-defined walls, whereas ANC are heterogeneous collections with varying degree of non-liquid density and without well defined walls. Both these entities usually occur within 4 wk. Later on with progressive liquefaction of pancreatic and/or peripancreatic necrosis, ANC becomes organized and walled-off and termed as WON. The latter appears as heterogeneous collection with both solid and liquid density on CECT[16,20]. On the other hand pseudocyst appears as encapsulated collection of homogeneous fluid density with only liquid component.

Role of CECT in predicting local complications in patients with pancreatic necrosis was evaluated in a prospective study, in which multivariate analysis identified that the degree of pancreatic necrosis and presence of peripancreatic necrosis predicted the development of infected pancreatic necrosis;

whereas transparenchymal necrosis with upstream viable pancreas and no peripancreatic necrosis were associated with pseudocyst development[21]. Heiss *et al*[22] studied the correlation between various morphologic features on CECT with the outcome in a retrospective study of 80 patients with severe AP requiring percutaneous drainage therapy and found that the pancreatic parts exhibiting necrosis (head, body, tail) and the presence of distant fluid collections (posterior pararenal space and/or paracolic gutter) had a significant correlation with mortality. Mortality was 42% if two or all three parts had necrosis whereas it was 20% when none or only one part of the pancreas exhibited necrosis. On basis of presence or absence of distant fluid collections it was 46% and 22%, respectively.

Differentiating WON from pancreatic pseudocyst when visible pancreatic necrosis seen in the initial CECT, is usually not difficult. It is important to note that the necrosis of peripancreatic tissue alone however with normal enhancing pancreas can also develop into WON. Since management of these different types of collections differs, distinguishing between these is vital. Pancreatic pseudocysts can be managed easily by endoscopic methods of simple drainage; however patients with WON require more aggressive endoscopic techniques such as larger tract dilation, placement of multiple stents, aggressive irrigation, and debridement of necrotic tissue by direct endoscopic necrosectomy (DEN) or surgical necrosectomy[23,24]. Recent Studies have shown that EUS-guided drainage using a large-bore fully covered biliary self-expandable tubular metal stent or biflanged metal stent can also provide sufficient drainage, and quick fistula formation[25-27]. The factors determining outcome of standard endoscopic drainage in patients with WON are proportion of solid debris and size of collection. Patients with less than 10% solid debris usually require a one-time endoscopic drainage; multiple sessions are required in patients with 10%-40% solid debris. Patients with > 40% solid debris either need direct endoscopic or surgical necrosectomy[28].

Thus CT imaging helps in delineation of morphology of fluid collections and quantification of the presence of solid debris and fat necrosis (seen as fat density globules) to assess the presence of drainable fluid before intervention.

Although there is no consensus on which imaging modality should be preferred for assessment of organized PFCs, CECT is commonly used in symptomatic collections and for planning therapeutic interventions. Takahashi *et al*[29] retrospectively studied CT of 73 patients with PFCs (45 WON, 28 pseudocysts) to differentiate WON from pancreatic pseudocysts. CT score was also calculated for each PFC. Radiographic features that favored WON included larger size, extension to paracolic or retrocolic space, an irregular border, presence of fat attenuation and debris in the PFC, presence of pancreatic parenchymal deformity or discontinuity, and the absence of dilation of the main pancreatic duct. CT could differentiate WON from pseudocysts, using a CT score of $\geqslant$ 2, with an accuracy of 79.5%-83.6%.

The main limiting factors for CECT are ionizing radiation, use of iodinated contrast material especially in patients with renal failure or contrast allergy and moderate sensitivity in identifying gallstones and biliary stones[30]. The above limiting factors can be overcome by using MRI.

Role of MRI

Similar to CT, MRI can be also used for the diagnosis and severity grading in AP. MR severity index (MRSI) significantly correlated with Ransons score, CTSI, C-reactive protein levels, duration of hospitalization and clinical outcome[31]. It may represent a better imaging technique due to nonionizing nature, higher soft tissue contrast resolution, and better safety profile of intravascular contrast media. Newer innovations in MRI such as the use of phased-array coils, parallel imaging, triggering techniques[32] or motion resistant sequences allow for improved spatial resolution and faster acquisition times making it more practical[33]. MRI also has a role in noninvasive evaluation of peripancreatic soft tissue, pancreatic ductal system and vascular network in a single examination. The concurrent use of secretin improved the diagnostic yield of MRCP in the evaluation of the PD integrity[34].

Acute fluid collections on MR examination are hypointense on T1WI and homogeneously hyperintense on T2WI if the contents are serous; if bleed occurs it appears as hyperintense on T1WI (more evident with fat-suppressed sequences). Simple pseudocysts are hypointense on T1WI and homogeneously hyperintense on T2WI. Their wall enhances slightly in early phases and progressively increases in subsequent phases due to its fibrotic nature; multiplanar MR acquisition improves the visualization of its relationship with surrounding organs. WONs are heterogeneous on T2WI (due to the presence of necrotic debris, bleeding or infection), with proteinaceous fluid contents arranged in layers (liquid-liquid level): the necrotic debris may appear as irregularly shaped regions of low signal intensity within the necrotic collections. Breathing independent T2-weighted sequences such as single-shot echo-train spin echo are useful to evaluate these necrotic collections.

The main advantage of MRI relative to CECT in the evaluation of peripancreatic fluid collections is easier appreciation of solid debris with MRI[35]. A prospective, blinded study compared MR findings with CT and USG to depict solid debris within pancreatic collections prior to intervention. The sensitivity and specificity values, for the prediction of actual drainability were: MR imaging, 100% and 100%; CT, 25% and 100%; US, 88% and 54% respectively[36]. Another recently

published study assessed the reproducibility of CT and MRI findings for debris assessment and presence of ductal communication in patients with symptomatic organized PFCs and found that MRI was superior to CECT for the inter-reader agreement on complexity of pancreatic collections, Also pancreatic duct disruption exclusion can be done more confidently on MRI[37].

Diffusion weighted MRI (DWI-MRI) is a new MRI technique based on diffusion of water protons *in vivo* which is related to the Brownian motion of water molecules within the tissues. DW-MRI yields apparent diffusion coefficient (ADC) as a quantitative parameter. In the literature, limited number of studies have demonstrated successful application of DW-MRI in pancreatic diseases. Yencilek *et al*[38] reported that DWI-MRI and ADC values are helpful in the diagnosis of all subgroups of acute pancreatitis even grade A patients in whom usually there is lack of CT findings. DWI-MRI in AP has been recently evaluated in two studies. One study compared CECT with DWI-MRI in detection of infection and found that sensitivity and accuracy of latter were higher than CT for detection of infection[39]. Another study on use of DW-MRI to differentiate different degrees of severity of AP, showed that DW-MRI is a compatible and safe image option to differentiate tissue image patterns between patients with normal pancreas, mild AP and necrotizing AP, particularly in those with contraindications to contrast-enhanced MRI (which is classically required for determining the presence of necrosis) or CT[40].

Another advantage of MRI over CT is identification of PD disruption which is commonly associated with central gland necrosis. Endoscopic retrograde cholangiopancreatography (ERCP) remains the gold standard for detection of PD disruption but is limited by its invasive nature and potential complications such as post-ERCP pancreatitis. MRI/MRCP may serve as a first line of investigation for treatment planning of symptomatic PFCs to assess drainability and pancreatic duct integrity[34]. Drake *et al*[41] showed that MRCP achieved 95% accuracy in detecting pancreatic duct disruption; thus helps in identifying patients who might benefit from early treatment by bridging the duct. MRI also helps in differentiating fluid collections secondary to pancreatitis from other cystic neoplasms.

Therefore, the logic of using MRI over CT in AP hinges on the following points: (1) this imaging method is without radiation hazard so safe in patients with AP requiring repeated imaging; (2) comparable to CT in demonstrating the presence and extent of necrosis, the presence, site, size and extent of PFCs, but better than CT in assessment of the debris content and drainability of these collections; (3) although the definitive evidence of aggravation of pancreatic injury with the use of iodinated contrast used in CT is debatable, yet evidence of a similar injury from use of Gd-DTPA for MRI does not exist; thus MRI appears to be a safer option in this respect; (4) PD integrity can better appreciated in MRCP; and (5) upcoming role of DWI-MRI has given hope for early and noninvasive detection of infected collections in future. The main limiting factor for MRI and its advanced techniques is high cost.

Role of EUS

With a close propinquity of the EUS probe to the pancreas and better spatial resolution than CT or MRI, EUS has emerged as an invaluable tool for assessment of pancreatobiliary diseases. Moreover, EUS is a minimally invasive procedure with relatively less complication rate compared to ERCP. However data regarding the role of EUS in AP is limited.

The increasing usage of EUS for drainage of PFCs has thrown more light on the important diagnostic role which it could define prior to the drainage. Solid debris in collections can be better delineated on EUS even when CT fails to do so. Apart from identifying the small collections behind the gaseous bowel loops and presence of vascular abnormalities within the wall of fluid collection at the site of drainage, EUS always fares better in defining the solid debris and its proportion as a constituent of fluid collections. This information provided by EUS plays an important role in selecting drainage procedure. MRI also provides similar information on solid debris and the results were comparable to EUS in a recent paper[12]. EUS has the best accuracy in characterizing peripancreatic collections prior to endoscopic intervention which can alter the management decision in up to one third of patients because of alternate diagnosis or by identifying anatomical and vascular factors precluding endoscopic management[42,43]. The largest randomized trial comparing different techniques demonstrated a 91% success rate with employment of EUS, compared with 72% when not used[44]. However the disadvantages of EUS are the requirement of monitored anesthesia care, need for expert endo-sonographer, operator dependence, and interobserver variability, inability to characterize in presence of air and difficult to perform in sicker patients with respiratory distress which these patients usually are.

In our experience with pancreatic fluid collections using EUS and CT, solid debris was detected on CT in only 32% fluid collections, whereas EUS delineated solid debris in 92% fluid collections. The amount of solid debris, graded as minimal (< 10%), moderate (10%-50%) and profound (> 50%), was compared between different types of fluid collections, need for intervention and modality of intervention. While the majority of ANCs (72.2%) had profound solid debris, majority of WONs (62%) had only moderate solid debris (P < 0.001). Of the three pseudocysts labelled on CT, one had moderate (30%) solid debris on EUS (missed on CT). Amongst WONs, need for intervention was present in all patients with profound solid debris, in 40% with moderate solid debris and in none with

minimal solid debris[45]. This delineates the importance of EUS in further management planning.

Role of PET scan

Recently, we have in a pilot study evaluated role of ammonia PET-CT (PET images after intravenous injection of $^{13}NH_3$) in diagnosing and quantifying pancreatic necrosis. We found good agreement with CECT and good interobserver acceptability and concluded that ammonia PET can be an alternative to CECT with minimal radiation burden especially in patients with renal failure[46]. However the main limiting factors are limited availability and cost as compared to other imaging techniques.

Role of radionuclide-labeled leukocyte scintigraphy PET scan as noninvasive modality for diagnosing infected collection has also been evaluated in recent studies as an alternative to image guided FNAC. Earlier these studies were limited to gamma camera scintigraphy with leukocytes labeled with 111 In or ^{99m}Tc in which image quality and resolution were unsatisfactory. However feasibility of labeling leukocytes in vitro with ^{18}F-FDG has given a possible way to overcome these limitations. We have used PET/CT with ^{18}F-FDG labeled autologous leukocytes to detect infection in pancreatic or peripancreatic fluid collections in 41 patients with AP and compared with microbiologic culture of aspirated fluid from the collection and showed 100% sensitivity, specificity, and accuracy of the scan (in 35 patients in whom fluid culture reports were available). We feel that that this technique is a reliable, accurate and noninvasive imaging modality for detection and localization of infection in patients with fluid collections[47].

CONCLUSION

CECT has traditionally been accepted as the method of choice for imaging PFCs in clinical practice, however recent studies have reported a higher accuracy rate with MRI and EUS as compared to CECT especially in quantification of solid debris. This has led better understanding of the natural history of PFCs. Comparative data between these different modalities are still lacking and require further studies.

Furthermore advances in cross-sectional imaging technique such as DWI-MRI and PET/CT with ^{18}F-FDG labeled autologous leukocytes may have promising role in early detection of fluid infection by noninvasive means in near future.

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On deaf ears, *Mycobacterium avium paratuberculosis* in pathogenesis Crohn's and other diseases

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Abstract

The historic suggestion that *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) might be a zoonotic pathogen was based on the apparent similarity of lesions in the intestine of patients with Crohn's disease (CD) with those present in cattle infected with *Map*, the etiological agent of Johne's disease. Reluctance to fully explore this possibility has been attributed to the difficulty in demonstrating the presence of *Map* in tissues from patients with CD. Advances in technology have resolved this problem and revealed the presence of *Map* in a significant proportion of patients with CD and other diseases. The seminal finding from recent investigations, however, is the detection of *Map* in healthy individuals with no clinical signs of disease. The latter observation indicates all humans are susceptible to infection with *Map* and lends support to the thesis that *Map* is zoonotic, with a latent stage of infection similar to tuberculosis, where infection leads to the development of an immune response that controls but does not eliminate the pathogen. This clarifies one of the reasons why it has been so difficult to document that *Map* is zoonotic and associated with the pathogenesis of CD and other diseases. As discussed in the present review, a better understanding of the immune response to *Map* is needed to determine how infection is usually kept under immune control during the latent stage of infection and elucidate the triggering events that lead to disease progression in the natural host and pathogenesis of CD and immune related diseases in humans.

Key words: Crohn's disease; Johne's disease; *Mycobacterium avium* subsp. *paratuberculosis*; Animal model; Monoclonal antibodies; Flow cytometry; Cytokines

Core tip: The seminal finding from recent investigations

is the detection of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) in healthy individuals with no clinical signs of disease. The latter observation indicates all humans are susceptible to infection with *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and lends support to the thesis that *Map* is zoonotic, with a latent stage of infection similar to *M. tuberculosis*, where infection leads to the development of an immune response that controls but does not eliminate the pathogen. This clarifies one of the reasons why it has been so difficult to document that *Map* is zoonotic and associated with the pathogenesis of Crohn's disease and other diseases.

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INTRODUCTION

A controversy has persisted since the initial suggestion that *Map* may be a zoonotic pathogen and the causative agent of Crohn's disease (CD). *Map* is a mycobacterial pathogen with a broad host range that can infect many species[1]. The primary hosts are ruminants. It was initially diagnosed as the causative agent of Johne's disease (JD), a chronic enteritis in cattle[2]. The similarities in the clinical manifestations of JD with CD led Dalziel *et al*[3] and later Crohn *et al*[4] to suggest *Map* may be the causative agent of CD[1]. As reviewed comprehensively by Naser *et al*[5] and Gitlin *et al*[6], the debate continues. The reluctance to fully explore the issue of whether *Map* is associated with the pathogenesis of CD has been the difficulty in isolating *Map* from all patients with CD. Chiodini *et al*[7-9] were the first investigators to successfully isolate *Map* from 3 patients. Initial attempts by others, however, proved unsuccessful raising questions as to the role of *Map* in the pathogenesis of CD. Even with improvements in technology, success in detection of *Map* has varied with some investigators more successful than others in isolating *Map* from patients with CD. Sufficient evidence, however, has been obtained over recent years to clearly show that many patients with CD are infected with *Map.* Investigations have revealed the presence of *Map* or *Map* DNA in blood or lesions from adults and children with CD[10-16]. Of special interest, *Map* has also been found in patients with other diseases as well as healthy subjects using several methods of detection[17-20]. A study long overdue, has now been conducted by Singh *et al*[21,22]. The study was conducted in India. It was designed to examine the level of infection in humans where *Map* is endemic in livestock and the general environment. They obtained specimens of blood, serum and stool from 42400 patients, submitted to pathology laboratories and hospitals in different areas of India, where *Map* is endemic, for evaluation over a span of two plus years. The samples were from patients presenting with a spectrum of infectious and non-infectious diseases including typhoid, TB, malaria, skin disorders, type 1 diabetes, anemia, thyroid, ion imbalance, abdominal disorders, lipid profile disorders, and intestinal inflammatory illness of different origins. They also collected samples from healthy individuals. *Map* was detected in all categories of patients at different frequencies, including healthy individuals, reflecting the consequence of large scale exposure of the resident populations to *Map* in the environment, infected animals and the food supply. The predominant biotype of *Map* detected in the samples was the bison type prevalent in livestock. The results clearly show, humans like other species, are equally susceptible to infection with *Map* regardless of health status. It can be predicted that any further large scale screening will provide similar results with the frequency of infected individuals dependent on the prevalence of *Map* in the environment and presence in food supplies[23]. The question remaining and reason for the unending debate is, "is *Map* a zoonotic pathogen and if so, what role does it play in the pathogenesis of CD and other diseases?" The answer is yes for at least a subset of patients presenting with the clinical signs of CD[5,24]. Koch's postulates were actually fulfilled in subsequent studies by Chiodini *el al*[25] where he and his associates demonstrated that passage of one of the first human isolates of *Map* in kid goats led to the development of enteritis similar to the enteritis in CD and JD. A later investigation of an enteritis in a colony of stumptail macaque (*Macaca arctoides*) revealed *Map* was the causative agent, the first report showing the susceptibility of non-human primates to infection with *Map*[26]. The study was the first to show that *Map* could be isolated from feces as an acid fast bacillus, extending what was known about the host range of species susceptible to infection with *Map*. The ensuing studies have shown *Map* isolated from patients with CD may be present in affected tissues with or without the mycobacterial cell wall[9], a characteristic of other mycobacterial pathogens[27]. Studies of healthy individuals and patients with clinical signs of CD and other diseases have shown *Map,* with an intact cell wall, can be detected in blood, tissues, and feces[22]. Cell wall deficient forms have also been found in tissues from animals with JD[28].

ROLE OF *Map* IN CROHN'S AND OTHER DISEASES

The difficulty in elucidating the role of *Map* in CD and other diseases has been the lack of understanding of the mechanisms of pathogenesis of JD. *Map*, like

other pathogenic mycobacteria doesn't cause epidemic disease, where all infected animals develop clinical disease. This is similar to tuberculosis in humans where infection leads to the development of an immune response that controls but does not clear infection. Only an estimated 10% of individuals infected with *Mtb* develop clinical disease. Disease progression is associated with a breakdown in protective immunity. Intensive ongoing investigations have not yet identified the triggering events associated with disruption of protective immunity during the latent stage of infection. No comprehensive studies have been conducted on the immune response to *Map* in humans. However, the presence of an immune response to *Map* in patients with clinical manifestations of CD and the presence of antibody to *Map* in healthy individuals and patients with various diseases show exposure leads to development of an immune response to *Map*[22,29,30]. The detection of *Map* in blood and affected tissues shows that infection persists providing a constant stimulus to the immune system.

Map as a model pathogen to study the mechanism of immunopathogenesis of mycobacterial pathogens

There are two ways *Map* could contribute to the pathogenesis of diseases in humans. The first is as the etiologic agent of CD, where there is a breakdown of immunity present during the latent stage of infection, similar to the breakdown that occurs with tuberculosis[5,24,31]. The second is through mimicry where mimics of proteins present in humans are produced by *Map* that interfere with immune function and or elicit development of cross-reactive antibodies associated with autoimmune diseases[32,33].

For ethical reasons much of the information needed to elucidate the role of *Map* in pathogenesis of CD and other diseases can't be obtained through investigations conducted with human subjects. The alternative is the use of cattle, the natural host of *Map*, as a model species to study the role of *Map* in the immunopathogenesis of CD and other diseases. The added advantage is that it affords an opportunity address the broader problem, the need to elucidate the mechanisms of immunopathogenesis of mycobacterial pathogens in general. The similarities in latency suggest the pathogens use the same signaling pathways to dysregulate the immune response and establish a persistent infection that eventually, over time, leads to a complete breakdown of protective immunity and development of clinical disease. The unique advantage of using *Map* to explore this possibility is that it can be used to study the interaction of the pathogen with the host at all stages of infection. Our studies have been based on the premise that *Map* is a zoonotic pathogen that is the etiologic agent of a subset of patients with CD[5,24,31] and that it plays a role in pathogenesis of other disease through mimicry[22,32].

To provide some background and a review of the current status of knowledge on the mechanisms of immunopathogenesis of JD in cattle, we became interested in the potential role of *Map* in CD when limited information was available on the immune response to *Map* in cattle. Progress was limited by the lack of immune reagents needed to characterize the immune system in cattle and study the cell mediated immune response to *Map*. Studies in dairy herds had shown the disease is characterized by a long latency period, where infected animals were difficult to detect by available diagnostic techniques during the initial stages of infection. Some animals could be detected by development of cell mediated immunity using the tuberculin skin test (reviewed in[1]). At later stages of infection, infected animals could be detected by intermittent shedding of *Map* in feces. Persistent shedding was associated with development of clinical disease where the absorptive mucosa of the intestine is destroyed, leading to death by starvation. The long latency suggested that *Map* could be passed through the gastrointestinal system without infecting all animals and constant exposure was needed for infection to occur. The latency also suggested that there was an age related difference in susceptibility to infection, with older animals more resistant to infection than young animals. Development of immune reagents to characterize the immune system of cattle and a flow cytometric assay provided an opportunity to begin analyzing the immune response to *Map ex vivo* and determine whether there was an age difference in susceptibility to infection and whether it was possible for "pass through" of *Map* without infection[34-36]. Studies in neonatal calves revealed exposure through application of *Map* to tonsillar crypts[36] or by ingestion of *Map* in milk supplement, leads to uniform infection in all animals and development of a lymphoproliferative response *ex vivo* to *Map* purified protein derivative (PPD) and soluble *Map* antigens (Ag) dominated by memory $CD45R0^+$ CD4 T cells 5 mo post infection (PI)[34]. Peripheral colonization of tissue was detected in some tissues at necropsy, with some bacteria detectable in feces in the initial study[36]. In the second study, no gross or microscopic lesions were detectable in intestinal tissue at necropsy one year PI, with *Map* detectable in some tissues by PCR[34]. A surgical intervention model used to directly inoculate the ileum showed *Map* is taken up rapidly and disseminated to other tissues within an hour PI. A proliferative response was detected by 9 mo but no humoral response[37]. Development of a cannulated ileum model allowed us to continuously monitor the early stages of infection in experimentally infected calves for up to 18 mo PI (Figure 1)[35]. Use of the model showed bacteria directly introduced into the ileum, once or multiple times over several days, were rapidly cleared with detection of bacteria in feces only during the first weeks PI. No gross or microscopic lesions were found in biopsies during the 18 mo of

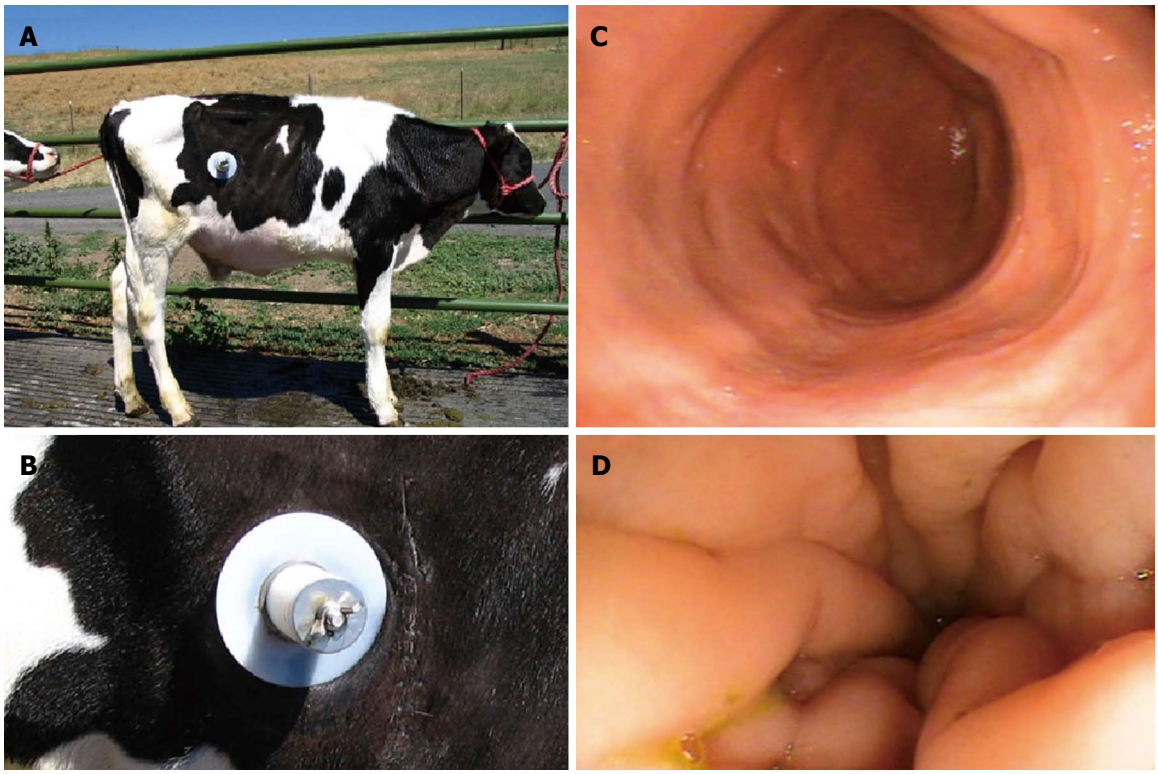

Figure 1 Early stages of infection in experimentally infected calves for up to 18 mo post infection. Pictures showing the cannula used in the studies (A and B); Endoscopic field showing the condition of ileal mucosa 8 mo following infection with *Map* (C); Endoscopic field taken before necropsy of a naturally infected animal showing the characteristic swelling and corrugation of the ileal mucosa that occurs at the clinical stage of infection (D). Modified from[35,38]. *Map*: *Mycobacterium avium* subsp. *paratuberculosis*.

the studies. However, *Map* was detected by PCR with IS900 and culture of some tissues. An immune response was detected using a flow cytometric assay at 3 mo[35]. A subsequent study, with the cannulation model, was focused on determining whether passage of *Map* in humans altered their capacity to infect the natural host and establish a persistent infection. Two of the original isolates obtained from humans by Chiodini *et al*[7] (Linda and Ben) were used to compare the capacity of isolates from humans and cattle to infect calves. The comparison revealed no difference in infectivity or the immune response to the bovine or human isolates of *Map*[38]. Sequence analysis of the human isolate obtained from tissue had identical single sequence repeat as the original isolate, 7g-4ggt, a sequence common in isolates from cattle[39]. This observation indicates it will not be necessary to use human isolates of *Map* to model mechanisms of persistence and pathogenesis in cattle.

MECHANISMS OF IMMUNE EVASION AND IMMUNOPATHOGENESIS

The early events of infection by pathogenic mycobacteria that lead to the development of an immune response that controls but fails to eliminate the pathogen remain to be elucidated. Also, the triggering events that disrupt protective immunity have not been identified. Efforts to breakdown the immune response to *Map* in cattle and accelerate progression to clinical disease have revealed the immune response to *Map* is resilient. Exposure of calves to massive doses of *Map* by different routes or administration of dexamethasone to alter the immune response to *Map* did not modulate the immune response to *Map*[40]. In addition, transient depletion of CD4 T cells in calves with a monoclonal antibody to CD4 did not impair the development of an immune response to *Map* or accelerate progression to clinical disease[41]. Under all conditions tested, including exposure at birth, an immune response develops that controls but does not clear infection. These observations suggest a similar resilient response also occurs in the majority of humans exposed to *Map* that do not develop clinical disease. The challenge that remains is identifying the factors that trigger a breakdown in protective immunity.

Thus far, analysis of the immune response to *Map* has shown the cellular response, under experimental conditions, is characterized by development of an immune response dominated by $CD45R0^+$ memory CD4 T cells using live *Map*, *Map* PPD, and soluble *Map* Ags[34,35,38]. Although a CD8 T cell response also develops, the proliferative response to *Map ex vivo* is less than the proliferative response of CD4 T cells. The response increases over time, however. In a comparative study, the CD8 T cell proliferative response was equivalent to the response observed

with naturally infected animals at the clinical stage of infection[38]. qRT-PCR showed interferon (IFN)-γ, interleukin (IL)-17, and IL-22 gene expression was up-regulated to equivalent extent in experimentally and naturally infected animals[38].

Ongoing efforts to develop a vaccine have provided additional information on the immune response to *Map* and an approach for elucidating how *Map* and mycobacterial pathogens dysregulate the immune response to establish a persistent infection. We used site directed mutation to develop 3 mutants to genes associated with virulence (*relA*, *lsr*2, and *pknG*)[42,43] as part of a multi-institutional endeavor to identify deletion mutants as vaccine candidates[44]. Mutants, made by other investigators, selected for challenge studies in mice and goats based on reduced capacity to survive in macrophages were unsuccessful. All the mutants were able to establish an infection[44]. In contrast, one of the mutants we selected for a challenge study in goats and calves, *ΔMap-relA*, was unable to establish a persistent infection in goats and calves[45]. Further analysis in calves showed no clear difference in the proliferative response to *Map* antigens. The increases in expression IFN-γ, IL-17, and IL22 were comparable indicating more in depth studies are needed to determine how deletion of relA abrogates the capacity of *Map* to establish a persistent infection.

The last observation of interest is on the effect of therapeutic vaccination with BCG on the immune response to *Mtb* in guinea pigs and humans at the clinical stage of infection made by Koch[46]. Vaccination led to a reversal of pathology, showing the triggering events associated with a breakdown in protective immunity, could be reversed by vaccination. Although subsequent studies revealed vaccination only led to a transient reversal, the observation is of interest because studies of vaccination at the clinical stage of infection could reveal which components of the immune response are restored that account for a reversal of pathology. Similar observations have been made more recently by Singh *et al*[47,48] with *Map* in goats and cattle. Although the results looked very promising, reversal of pathology was transient with no improvement even with repeated vaccination (personal communication). The important observation made by Koch *et al*[46] and Singh *et al*[47,48] is that reversal of pathology was associated with an increase in the inflammatory response following vaccination that resolved lesions containing infected cells. *Map* affords an opportunity to explore the cellular and molecular basis for this phenomenon. A preliminary test with a cow at a late clinical stage of infection, with an anergic response to a skin test with *Map* Ag and a diminished proliferative response to Ag *ex vivo*, showed vaccination with *ΔMaprelA* restored the proliferative response to *Map*. With the cannulation model it will be possible to isolate affected tissue before and after vaccination for analysis and characterize the effector cells restored by vaccination and characterize their effector activity.

CONCLUSION

Cumulative studies clearly show humans, like other species, are equally susceptible to infection with *Map*[22]. This places the whole populations at risk of infection, depending on the prevalence of *Map* in the environment and food supply[22,23]. Ongoing studies have shown CD may contribute to the pathogenesis of disease in humans as the etiologic agent of a subset of patients with CD[5] and through antigenic mimicry[32]. Use of the natural host as a model, to understand the immunopathogenesis of CD mediated by *Map*, has provided opportunity to gain insight into the immune response to *Map* in humans during the early, latent, and clinical stages of infection that cannot be obtained through study of *Map* in humans, except at the clinical stage of infection[29]. Analysis has shown the immune response during the latent stage of infection is resilient and not readily disrupted by methods of immunosuppression[40,41]. The components of the immune response to *Map* that control infection during latency remain to be elucidated. The development of a mutant that cannot establish a persistent infection offers 2 opportunities to identify these components, one through analysis of the early stages of infection and the second through analysis of the immune response following restoration by vaccination at the clinical stage of infection.

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Hepatolithiasis and intrahepatic cholangiocarcinoma: A review

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Abstract

Although the incidence of hepatolithiasis is decreasing as the pattern of gallstone disease changes in Asia, the prevalence of hepatolithiasis is persistently high, especially in Far Eastern countries. Hepatolithiasis is an established risk factor for cholangiocarcinoma (CCA), and chronic proliferative inflammation may be involved in biliary carcinogenesis and in inducing the upregulation of cell-proliferating factors. With the use of advanced imaging modalities, there has been much improvement in the management of hepatolithiasis and the diagnosis of hepatolithiasis-associated CCA (HL-CCA). However, there are many problems in managing the strictures in hepatolithiasis and differentiating them from infiltrating types of CCA. Surgical resection is recommended in cases of single lobe hepatolithiasis with atrophy, uncontrolled stricture, symptom duration of more than 10 years, and long history of biliary-enteric anastomosis. Even after resection, patients should be followed with caution for development of HL-CCA, because HL-CCA is an independent prognostic factor for survival. It is not yet clear whether hepatic resection can reduce the occurrence of subsequent HL-CCA. Furthermore, there are no consistent findings regarding prediction of subsequent HL-CCA in patients with hepatolithiasis. In the management of hepatolithiasis, important factors are the reduction of recurrence of cholangitis and suspicion of unrecognized HL-CCA.

Key words: Cholangiocarcinoma; Hepatolithiasis; Intrahepatic; Management

Core tip: In this study, we review recent studies on hepatolithiasis and discuss hepatolithiasis-associated cholangiocarcinoma (HL-CCA). Management of hepatolithiasis requires proper treatment to reduce recurrence and achieve early detection of HL-CCA. It is not clear whether hepatic resection can reduce the occurrence of HL-CCA, and there is no surveillance tool to predict subsequent occurrence. Patients should be followed after treatment because there are no effective measures to prevent HL-CCA and premalignant lesions.

Kim HJ, Kim JS, Joo MK, Lee BJ, Kim JH, Yeon JE, Park JJ, Byun KS, Bak YT. Hepatolithiasis and intrahepatic cholangiocarcinoma: A review. *World J Gastroenterol* 2015; 21(48): 13418-13431 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13418.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13418

INTRODUCTION

Gallstone disease is common in Western and Asian countries. One type of gallstone disease, hepatolithiasis, is characterized by the presence of stones within the intrahepatic bile ducts proximal to the right and left hepatic ducts. Hepatolithiasis is rare in Western countries, and the incidence in East Asian, such as Taiwan, China, Hong Kong, South Korea, and Japan, is higher[1-4].

Hepatolithiasis is benign in nature, but the prognosis is poor due to an association with recurrent cholangitis, biliary strictures, liver abscesses, and atrophy or cirrhosis of the affected liver; however, there have been advances in the management of the stones associated with hepatolithiasis[5-7]. Hepatolithiasis is also a known risk factor for intrahepatic cholangiocarcinoma (CCA)[8,9]. Although mortality due to cholangitis from hepatolithiasis is very low, the occurrence of hepatolithiasis-associated CCA (HL-CCA) is a prognostic factor for poor outcome[10-12]. Early diagnosis of HL-CCA is still challenging even though there have been advances in diagnostic modalities and various efforts to identify it in early stages[13].

Here, we review the epidemiology, pathogenesis, diagnosis, and management of hepatolithiasis and HL-CCA. This review also summarizes the predictive factors for HL-CCA.

EPIDEMIOLOGY

Hepatolithiasis

The incidence of hepatolithiasis varies, but it is fairly common in Asian, such as China, Taiwan, Hong Kong, Korea, and Japan, with incidence rates ranging from 2% to 25%[1-4]. In Western countries hepatolithiasis is rare, with incidences reported between 0.6% and 1.3%[4,14].

The mechanism of intrahepatic stone formation is not well understood, but malnutrition and low socioeconomic status have been suggested to be causatively related to hepatolithiasis. According to epidemiologic surveys, the pattern of gallstone disease is changing, and the incidence of hepatolithiasis is decreasing as people in Eastern countries adopt a Westernized diet. A national survey conducted in Japan reported that the relative proportion of hepatolithiasis was 4.1% in the years from 1970-1977, 3.0% in the years from 1975-1984, 2.3% in the years from 1985-1988, 2.2% in the years from 1989-1992, and 1.7% in the years from 1993-1995[15]. The apparent decrease in the incidence of hepatolithiasis has contributed to this chronological shift, but it may be partly caused by the increase in gallbladder stones due to westernized diet[16]. In Taiwan, the relative proportion of hepatolithiasis slightly decreased from 21.3% in 1981 to 18.7% in 1989[1]. In South Korea, the pattern of gallstone disease has become similar to that seen in Western countries, except for a high prevalence of hepatolithiasis. The proportion of gallbladder stones has gradually increased from 80% to 85% and that of common bile duct stones has decreased from 34% to 19%; however, that of hepatolithiasis remains unchanged, at 11%-15% over a 20 year period from 1980 to 2000[2,3].

Parasitic infestation has often been thought to be a major cause of hepatolithiasis and infestation, as parasites have been detected in up to 30% of patients with hepatolithiasis[17]. In Eastern countries, the persistent prevalence of hepatolithiasis in Korea and the relatively high prevalence in Taiwan may be due to cultural trends of ingesting raw freshwater fish infected with *Clonorchis sinensis* (*C. sinensis*). Clonorchiasis is endemic in the Far East, but heavily endemic areas within individual countries are geographically distributed in parallel with the population of the snail that is an intermediate host for this parasite[18]. Nationwide surveys in Korea of intestinal parasitic infections revealed no drop in the average prevalence of *C. sinensis* infection over time (*i.e.*, 2.6% in 1981 and 2.4% in 2004)[19], and *C. sinensis* infection remains common in Korea. In 1984, the prevalence was reported to be 1.5% in Taiwan[20]. In Japan, the clonorchiasis rate has markedly decreased in parallel with the decreased number of host snails, which is due to various causes, including water pollution[18].

Hepatolithiasis-associated cholangiocarcinoma

Hepatolithiasis is an established risk factor for CCA, similar to clonorchiasis, especially in Asian countries[8,9,21-24]. The association between hepatolithiasis and CCA has been well-documented, and many studies on HL-CCA have been published[25-31]. Cases of HL-CCA are not rare, especially in areas with a high prevalence of hepatolithiasis. The overall incidence of HL-CCA was reported to be 5%-13%[12,32,33]. HL-CCA can be

Table 1 Concomitant cholangiocarcinoma in hepatic resection for hepatolithiasis *n* (%)

Ref.	Year	Nation/region	Case (*n*)	Incidence
Zhu *et al*[34]	2014	China	2056	107 (5.2)
Lin *et al*[32]	2013	Taiwan	211	10 (4.7)
Tabrizian *et al*[35]	2012	Italy	30	7 (23.3)
Cheon *et al*[30]	2009	South Korea	90	8 (9.0)
Uenishi *et al*[12]	2009	Japan	86	10 (11.6)
Lee *et al*[36]	2007	Taiwan	123	4 (3.3)
Catena *et al*[37]	2006	Italy	17	2 (11.7)
Chen *et al*[38]	2004	Hong Kong	103	10 (9.7)

detected at any stage, during evaluation, treatment, or follow up of hepatolithiasis. Detection of concomitant HL-CCA during treatment of hepatolithiasis was reported in 12% of patients in Japan, 5% in Taiwan, 9% in South Korea, and 10% in Hong Kong[12,30,32,34-38] (Table 1). HL-CCA can also develop during follow up for hepatolithiasis. Subsequent HL-CCA following hepatolithiasis has been reported to be 1.6%-9.9% in several studies[6,12,30,32,33,36,38-40]. These studies are summarized in Table 2.

PATHOGENESIS

Hepatolithiasis

The mechanism of intrahepatic stone formation has not yet been fully described; but the presence of brown pigment stones as well as cholesterol stones suggests a complex pathogenesis[41-44]. Factors that may contribute to development of these stones include the precipitation of calcium bilirubinate, the solubility of cholesterol in hepatic bile, gene mutations, and ethnic differences[45-51].

A low fat and low protein diet may increase bile stasis and bacterial infection through relaxation of the sphincter of Oddi and decreased release of cholecystokinin[52,53]. Association with bacterial infection has been implicated in stone formation. Bacteria, such as *Escherichia coli,* and those belonging to the *Clostridium* and *Bacteroides* genera are frequently isolated from the bile of patients with hepatolithiasis. The possible route is ascending infection through the sphincter of Oddi, bacteriobilia through the portal venous system, or transient infection with bile stasis[54,55].

Recent experiments suggest that enhanced inflammatory cytokine-induced phospholipase A_2, cyclooxygenase-2 (COX-2) and COX-2-derived prostaglandin E2 (PGE_2) synthesis in the bile ducts are related to the initiation and propagation of inflammatory changes in hepatolithiasis[56,57].

In terms of the biliary mucin molecules, an increase in acidic mucins, such as sulfomucins and sialomucins, in hepatolithiasis reduces pH in the bile and leads to precipitation of calcium bilirubinate in the bile ducts[56]. The abundance of secretory-type mucins (MUC2, MUC3, MUC5AC, MUC5B, and MUC6) was shown to be significantly higher in the bile ducts of hepatolithiasis patients compared to controls, and gel-forming mucins of MUC2 and 5AC were thought to be more important for the pathogenesis[58,59]. Excessive amounts of mucin secreted into the ducts may provide a microenvironment that initiates a nidus for stones by trapping calcium salts and lipids, which may also cause stones to expand by altering biliary flow in the bile ducts[60,61].

HL-CCA

Known risk factors for CCA are primary sclerosing cholangitis, Caroli's disease, congenital choledochal cysts, parasite infections (*C. sinensis, Opisthorchis viverrini*), hepatolithiasis, and toxins[8,9,19]. Considerable progress has been made in understanding the pathogenesis of CCA[23,62]. It has been proposed to be a multi-step process, involving hyperplasia, dysplasia, and adenocarcinoma *in situ* to invasive adenocarcinoma[63]. Although the process of carcinogenesis from hepatolithiasis is not fully understood, chronic proliferative cholangitis plays a role in biliary carcinogenesis[27]. Recurrent cholangitis, biliary stricture, bile stasis, and chronic bacterial infection are common problems in hepatolithiasis patients, even after multimodal treatment. These recurrent or chronic inflammatory events cause prolonged inflammation of the bile duct epithelium and can lead to the development of CCA[27,62].

The main morphologic feature of stone-containing bile ducts in hepatolithiasis is chronic proliferative cholangitis and peribiliary glands proliferation, in which the epithelial lining becomes hyperplastic[43]. Chronic inflammation can cause epithelial cell proliferation, and this may increase the rate of cellular DNA synthesis and the subsequent production of mutagens coupled with a compromised cellular repair function[64-66]. If these processes are sustained for a long period of time, they may cause the multiple molecular changes necessary to trigger the development of CCA. During histologic exam by choledochoscopy using percutaneous transhepatic cholangioscopic lithotripsy (PTCSL), atypical epithelial hyperplasia and dysplasia are frequently recognized[27]. Chen *et al*[67] reported that intraductal papillary neoplasia was found in 30% of patients with hepatolithiasis and displayed a histologic spectrum from papillary growth with dysplasia to carcinoma. Biliary carcinogenesis associated with hepatolithiasis is thought to be present as precancerous lesions. Intraductal papillary neoplasm of the bile duct (IPNB) and biliary intraepithelial neoplasia (BilIN) are known as precancerous lesions of biliary tract carcinomas[68].

Similar to hepatolithiasis and clonorchiasis, IPNB has mainly been reported in Far Eastern countries. IPNB, including carcinoma and precursor lesions, is known to transform from low-grade dysplasia to invasive carcinoma[69]. BilIN is a flat or micropapillary

Table 2 Subsequent cholangiocarcinoma after initial management for hepatolithiasis

Ref.	Year	Nation/region	Cases (*n*)	Incidence of cholangiocarcinoma		
				Total	Stone removal	
					Complete	Residual
Kim *et al*[33]	2015	South Korea	236	6.8%	3.3%	10.4%
Tsuyuguchi *et al*[39]	2014	Japan	121	9.9%	9.1%	10.4%
Lin *et al*[32]	2013	Taiwan	197	6.1%	4.9%	11.8%
Park *et al*[6]	2013	South Korea	85	2.4%		
Cheon *et al*[30]	2009	South Korea	225	4.9%	4.0%	8.0%
Uenishi *et al*[12]	2009	Japan	76	2.6%		
Lee *et al*[36]	2007	Taiwan	123	1.6%		
Chen *et al*[38]	2004	Hong Kong	91	3.3%		
Huang *et al*[42]	2003	Taiwan	209	2.4%	0.7%	6.6%

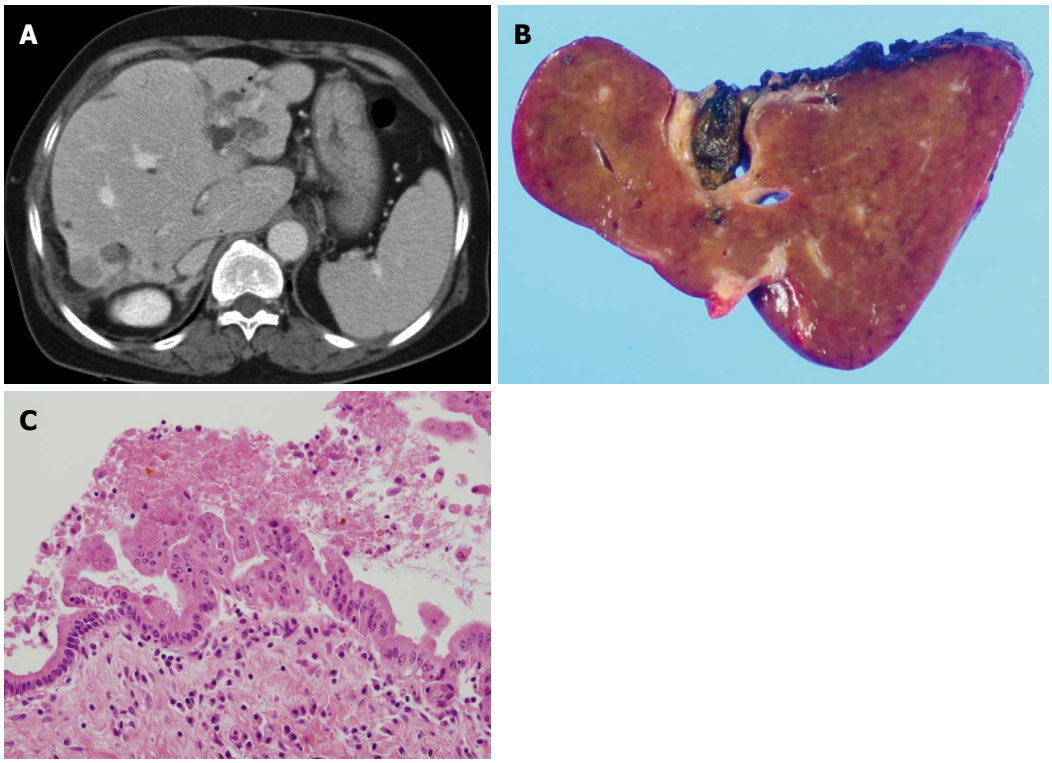

Figure 1 Sixty-seven years old, female was admitted for cholangitis and treated with hepatic resection. She was followed and underwent second resection 1 year later. A: Abdominal computed tomography demonstrated that B2 IHD dilatation with stones and 2 cm sized low density lesion in liver S7; B: Liver, left, lateral, segmentectomy was done; C: BilIN1 and two were shown on dilated duct with glandular proliferation.

dysplastic epithelium in the bile duct and classified as BilIN-1, BilIN-2, and BilIN-3[70]. It is frequently found in the surgical margin of resection specimens of CCA and is also reported in patients with hepatolithiasis[71,72]. Both BilIN and IPNB can be seen at the same time in patients with hepatolithiasis, unlike primary sclerosing cholangitis and parasitic infections, which are only associated with BilIN[70,72] (Figures 1 and 2). A study of BilIN reported that metaplastic changes were more frequently observed in BilIN-2/3 than BilIN-1, and gastric type foveolar metaplasia was the most frequently observed change[70]. In immunohistochemical studies of IPNB, aberrant expression of cytokeratin 20, MUC2, and MUC5AC was frequently shown[73,74]. Another immunohistochemical study showed that decreased expression of β-catenin and E-cadherin occurred early in the carcinogenesis of both BilIN and IPNB[75].

Recent studies have elucidated the molecular mechanism of HL-CCA, which involves epidermal growth factor receptor (EGFR), nuclear factor kappa-B (NF-κB), COX-2, PGE_2, p16, c-met, and deleted in pancreatic cancer 4 (DPC4)/signaling effectors mothers against decapentaplegic protein 4 (Smad4)[76,77]. Increased expression of c-erbB2 and EGFR in both hepatolithiasis and intrahepatic CCA has been reported[78,79]. Zhou *et al*[80] reported that NF-κB and EGFR were more highly expressed in HL-CCA than in patients with hepatolithiasis. These findings demonstrate that prolonged inflammation due to the

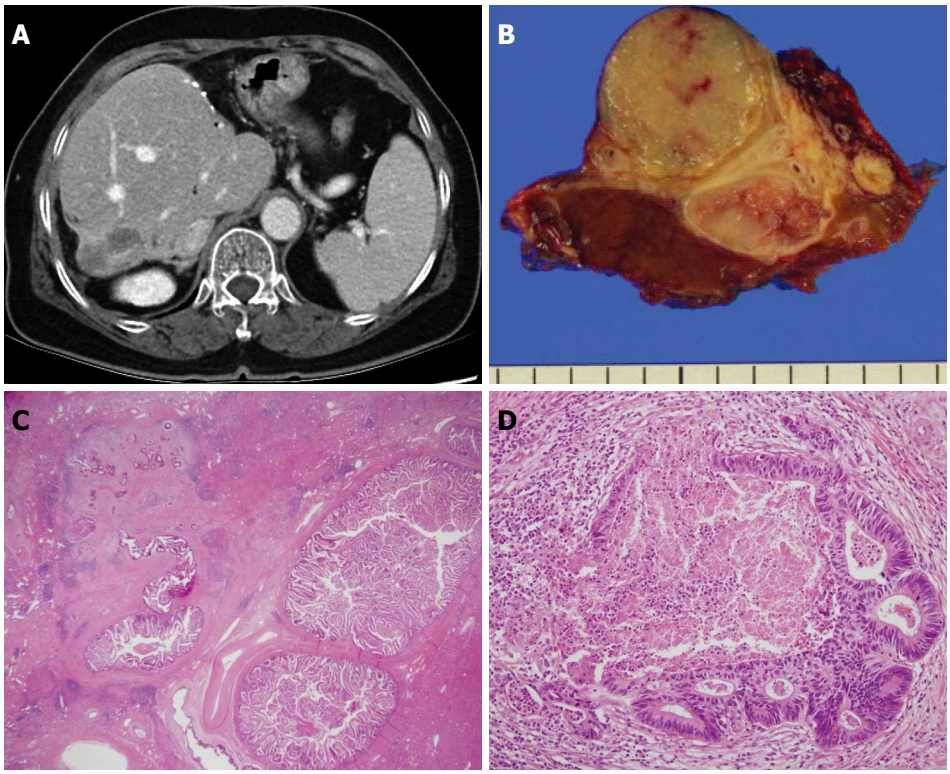

Figure 2 Sixty-seven years old, female was admitted for cholangitis and treated with hepatic resection. She was followed and underwent second resection 1 year later. A: Follow-up abdominal computed tomography. Exophytic hypodense mass in S7 of the liver was more enlarged with peripheral enhancement; B: Liver, S7, segmentectomy was done; C and D: Hematoxylin and eosin (HE) statin (C: × 12.5; D: × 200). Intraductal papillary neoplasm with an associated invasive carcinoma. 6 cm × 3 cm × 3 cm sized intraductal papillary neoplasm including 0.6 cm × 0.5 cm sized invasive tumor.

presence of stones could induce upregulation of these cell-proliferating factors.

COX-2 overexpression correlated with the carcinogenesis of intrahepatic CCA, and it occurred during the early stages of cholangiocarcinogenesis[81,82]. Endo *et al*[83] reported that ErbB2 and COX-2 were overexpressed in the hyperplastic bile ducts of patients with hepatolithiasis.

PGE_2 is known to be increased by upregulated COX activity in inflammatory sites. Shoda *et al*[57] reported that the synthesis of PGE_2 is significantly higher in affected bile ducts with hepatolithiasis, and it can mediate morphologic changes of the intrahepatic bile duct, such as dilatation, stricture, and periductal fibrosis.

It is well known that c-met plays a role in the carcinogenesis of CCA. The *c-met* gene, a proto-oncogene, encodes the membranous tyrosine kinase receptor for hepatocyte growth factor[84]. Terada *et al*[85] reported that c-met protein was overexpressed in proliferated biliary cells of hepatolithiasis and in neoplastic biliary epithelium of intrahepatic CCA.

The cyclin-dependent kinase inhibitor p16 is known as a tumor suppressor gene, and p16 promoter hypermethylation is known to occur in various cancers, including CCA[86]. Ishikawa *et al*[87] reported that inactivation of p16 occurred frequently and at an early stage of IPNB with hepatolithiasis. Sasaki *et al*[88] reported that p16 expression was decreased from BilIN-2,3 to cholangiocarcinogenesis in patients with hepatolithiasis.

In a study of the *DPC4/Smad4* gene, another tumor suppressor gene, Lee *et al*[89] reported that inactivation of *DPC4/Smad4* occurred in both CCA and stone-containing bile ducts, and it was especially pronounced in the dysplastic epithelium of stone-containing bile ducts.

DIAGNOSIS

Hepatolithiasis

Diagnosis of hepatolithiasis is performed mainly through radiologic examinations, and often incidentally without symptoms or laboratory abnormalities. If symptoms occur, they may include fever, fatigue, abdominal pain, and jaundice. Laboratory tests can show leukocytosis, neutrophilia, and abnormal liver biochemistry of an obstructive pattern with raised serum alkaline phosphate and bilirubin.

A hepatolithiasis research group in Japan classified the severity of hepatolithiasis: grade 1 as no symptoms, grade 2 as having abdominal pain, grade 3 as transient jaundice or cholangitis, and grade 4 as continuous jaundice, sepsis, or concurrent CCA. They reported that more than half of 473 new hepatolithiasis cases were classified as grade 3 or 4[90]. Another recent study of 68 hepatolithiasis patients used a new clinical classification system: type 1 primary type (no

previous biliary tract surgery), type 2 inflammatory type (previous biliary tract surgery and cholangitis), type 3 mass-forming type (complicated by hepatic mass-forming lesion), and type 4 terminal type (with secondary biliary cirrhosis and resultant portal hypertension). The authors reported that the incidence of new cases of types 1-4 was 50.1%, 36.8%, 10.3%, and 2.8%, respectively[91].

Abdominal ultrasound (US) and computed tomography (CT) are the primary imaging modalities for hepatolithiasis. The advantages of US are that it is non-invasive, safe, and easily available (even bedside), and can detect dilatations of the biliary tract and stones, as shown by echogenic spots with an acoustic shadow. However, the detectability of stones is dependent on the stone size, shadowing characteristics, echogenicity, and the location in the hepatic lobe[7,90]. Multidetector CT (MDCT) has advanced the diagnosis of hepatolithiasis. MD-CT is very useful for detecting dilated bile ducts, stricture of the bile duct, and calcification stones in the bile duct[7,92]. Therefore, recently, US and CT have become the first choice for examining patients suspected of having hepatolithiasis.

Magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP) can provide realistic bile duct images and can detect stones without exposing patients to radiation[93,94]. The T1-weighted (T1-W) sequence is helpful for depicting stones and demonstrating parenchymal complications, such as an abscess[95]. However, the cost is high, and its spatial resolution is not perfect. MRI and MRCP are usually performed as ancillary investigations to US and CT.

CCA and HL-CCA

The clinical manifestations of intrahepatic CCA are nonspecific, although there may be abdominal pain, cachexia, fatigue, and night sweats[96]. Serum CA 19-9 is known as a tumor biomarker for CCA, but it may be normal or increased in benign diseases, such as bacterial cholangitis or choledocholithiasis[96,97].

Macroscopically, intrahepatic CCA is classified as a mass-forming, periductal-infiltrating, or intraductal-growing carcinoma, where the mass-forming type is most common[98]. MDCT is used to evaluate the location of the tumor and its relationship with adjacent vascularity[99]. Pre-contrast and multiphase CT is useful for the detection and differentiation of an intraductal stone from an intraductal tumor because hypovascular tumors with abundant fibrous stroma show progressive uptake of contrast during the venous phase[100]. Park *et al*[101] suggested the following as specific CT findings for HL-CCA: the presence of periductal soft-tissue density, higher enhancement of the duct than the adjacent bile duct on portal venous phase images, ductal wall thickening or enhancement, portal vein obliteration, and lymph node enlargement.

On MRI, CCA is visible as a hypointense mass on T1-W images and a central hypointensity with irregular hyperintense areas on T2-weighted (T2-W) images[102]. MR imaging with MR cholangiography is superior to CT for the assessment of intraductal lesions, intrahepatic metastasis, and presence of satellite lesions[103]. However, CT may be better for the assessment of vascular encasement, identification of extrahepatic metastasis, and determination of resectability[104].

Positron emission tomography (PET) scan is another useful imaging modality, but the accuracy varies depending on morphologic type[105]. The sensitivity of PET for detection is reported to be 85% for the mass-forming type but 18% for the infiltrating type[105]. Mass forming type intrahepatic CCA shows ring-shaped fluorodeoxyglucose uptake (due to desmoplastic lesions within the tumor) and neovascularity at the periphery, but this finding can be shown in any lesion with central necrosis[103]. Thus, PET scanning is more helpful for detection of distant metastases[106,107].

Difficulty in diagnosis of concomitant CCA in hepatolithiasis

In cases of HL-CCA, there are no specific symptoms other than the clinical manifestation of hepatolithiasis. Laboratory tests can show increased alkaline phosphatase, bilirubin, and CA 19-9[108].

Therefore, detection of CCA in hepatolithiasis is dependent on imaging modalities, such as US, CT, and MRI. However, there are many limitations in differentiating CCA from fibrosis in hepatolithiasis. It is difficult to differentiate strictures, infiltrating types of CCA, mass-forming CCA, and inflammatory pseudotumor because prolonged affected liver segments often become fibrotic and scarred[90,109].

MANAGEMENT STRATEGY FOR HEPATOLITHIASIS

The primary goals of treatment for hepatolithiasis are complete stone removal and the prevention of recurrent cholangitis. Current treatment methods are non-surgical treatments, such as percutaneous transhepatic cholangioscopic lithotripsy (PTCSL), and surgical treatment, such as hepatic resection. PTCSL has been frequently used with a fair success rate[110-113]. The rate of complete stone removal was reported to be similarly high in both treatments, but recurrent cholangitis is quite common in PTCSL[6,11,111]. Generally, hepatic resection has been considered the definite treatment for hepatolithiasis, because it could effectively reduce recurrent cholangitis or stone formation[114-116]. The reported overall success rate of hepatic resection is 95%-98%, and the rates of residual stone and stone recurrence are 15.6% and 7.8%-13.9%, respectively, upon long-term follow-up[5,12,115,116].

However, the incidence of post-hepatectomy

infection was 23.8% higher in hepatolithiasis than in other hepatic malignancies[117]. Additionally, operative intervention is sometimes not acceptable for patients with risky co-morbidities or stones distributed in multiple segments of both hepatic lobes[118,119]. Thus, a tailored multidisciplinary approach combining both approaches is more reasonable.

Meanwhile there are many hepatolithiasis cases with strictures that make it difficult to manage and differentiate CCA. Thus, patients should be screened for concomitant HL-CCA, even though the incidence is low. Choledochoscopy may be indicated in these cases.

Detection and predictive factors of concomitant HL-CCA

Known risk factors for concomitant HL-CCA are older age, bile duct stenosis (stricture), liver atrophy, elevated CA 19-9, left side stone location, residual stone, recurrence of stone, and choledochoenterostomy[120-124] Neither symptoms (abdominal pain, fever, jaundice, and nausea) nor the location of stones (intrahepatic duct only, both intra- and extra-hepatic ducts, right lobe, left lobe, or both lobes) is a significant risk factor[122,125].

In a comparative study of concomitant HL-CCA and hepatolithiasis only, Kim *et al*[120] reported that concomitant HL-CCA should be suspected in patients with a longstanding duration of hepatolithiasis accompanied by weight loss, high levels of serum alkaline phosphatase, low levels of serum albumin, high levels of serum CEA, hepatolithiasis located in either the right or both lobes of the liver, and age > 40 years. Liu *et al*[121] reported that symptom duration of more than 10 years is the most powerful risk factor for ICC in patients with hepatolithiasis, and Lee *et al*[126] reported an association between localized stricture and long-term history (over 10 years) of hepatolithiasis. Additionally, it is suggested that HL-CCA may occur in cases of atypical clinical manifestation of liver abscess, biliary tract infection that is difficult to control, relapsing infection, and abscesses in a more consolidated area[34].

In a Japanese multicenter study, Suzuki *et al*[122] reported that the predictive risk factors were a history of choledocoenterostomy (OR = 3.718) and liver atrophy (OR = 4.424). Association of CCA occurrence with previous surgical biliary-enteric anastomosis may be due to chronic inflammation caused by reflux of bowel contents late after choledocoenterostomy[123,124].

When is hepatic resection advisable for improving prognosis?

Hepatic resection can eliminate both stones and surrounding ductal changes, such as strictures, fibrosis, and micro abscesses. In a long-term follow-up study, operative treatment was reported to reduce recurrence[30,36].

In patients with hepatolithiasis, treatment-related difficulties include the high rates of residual stones and a high recurrence rate even after complete stone removal, especially in patients with biliary strictures[111,112]. Biliary strictures often lead to bile stasis, cholangitis, and stone formation, thus causing stone recurrence. A multivariate logistic regression analysis study reported that risk factors for incomplete stone removal are intrahepatic strictures, nonoperative treatment, and bilateral stones[113]. Thus, hepatectomy is recommended in cases of single lobe hepatolithiasis, atrophy of the affected liver, and stricture[37,118,119].

Furthermore, resection is considered when differential diagnosis is difficult, because detection of HL-CCA is very difficult even during surgical operations, and accurate diagnosis can be proven only through resection. Catena *et al*[37] reported that the rate of unrecognized CCA was quite high at 11.7% and that it might be underestimated.

Can hepatic resection prevent subsequent HL-CCA?

Although mortality due to cholangitis is low, the development of HL-CCA is known to be an independent prognostic factor for survival[34,127]. Hepatolithiasis is a significant risk factor for CCA, and the OR was 40 in a Korean report[128].

Theoretically, hepatectomy for treatment of hepatolithiasis has another advantage for eliminating the risk of developing HL-CCA besides complete removal of stones. In general, hepatectomy seems to reduce the risk of developing of CCA. A cohort study in Japan reported that hepatectomy significantly reduced the risk of developing CCA[15]. A Western study by Tabrizian *et al*[35] reported a high rate (23.3%) of concomitant HL-CCA and excellent long-term results with hepatectomy.

It is not clear whether hepatic resection can reduce the occurrence of HL-CCA. During the follow-up period, the incidence of HL-CCA showed no significant difference between patients with hepatolithiasis with or without previous hepatic resection[30,33]. It is difficult to conclude that hepatic resection definitely prevents the development of HL-CCA in patients with hepatolithiasis.

Meanwhile, incomplete resection is also a problem. Survival outcomes are good only in cases with safe surgical margins, even in incidental HL-CCA found in post-operative pathology[32]. In addition, HL-CCA could develop in the hepatic lobe adjacent to the resection margin (Figures 3 and 4). It is possible that the undetected CCA was present in the remnant liver[32,33]. Therefore, aggressive resection, including neighboring segments, is crucial to achieve sufficient hepatic volume.

MANAGEMENT STRATEGY FOR HEPATOLITHIASIS-ASSOCIATED INTRAHEPATIC CCA

The secondary goal of treatment for hepatolithiasis is to prevent the progression of the disease to cirrhosis or

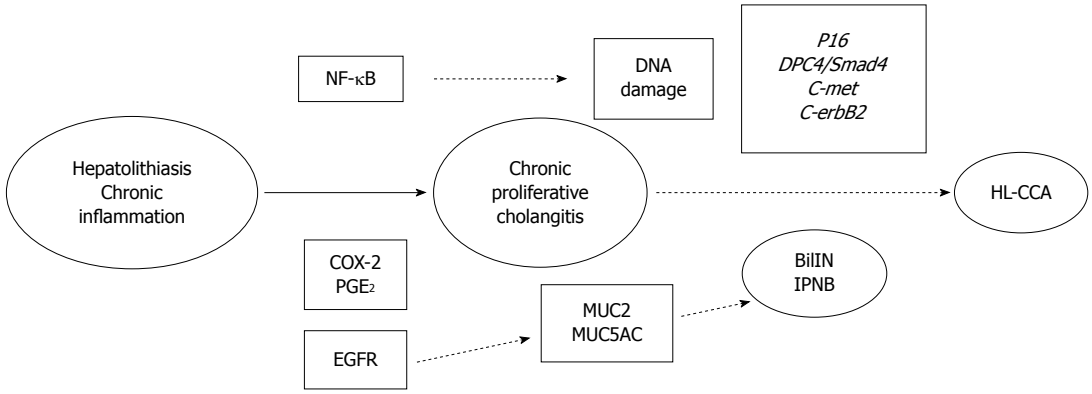

Figure 3 Molecular mechanism of hepatolithiasis-associated cholangiocarcinoma. HL-CCA: hepatolithiasis-associated cholangiocarcinoma; NF-κB: Nuclear factor kappa-B; EGFR: Epidermal growth factor receptor; PGE_2: Prostaglandin E2; COX-2: Cyclooxygenase-2; IPNB: Intraductal papillary neoplasm of the bile duct; BilIN: Biliary intraepithelial neoplasia.

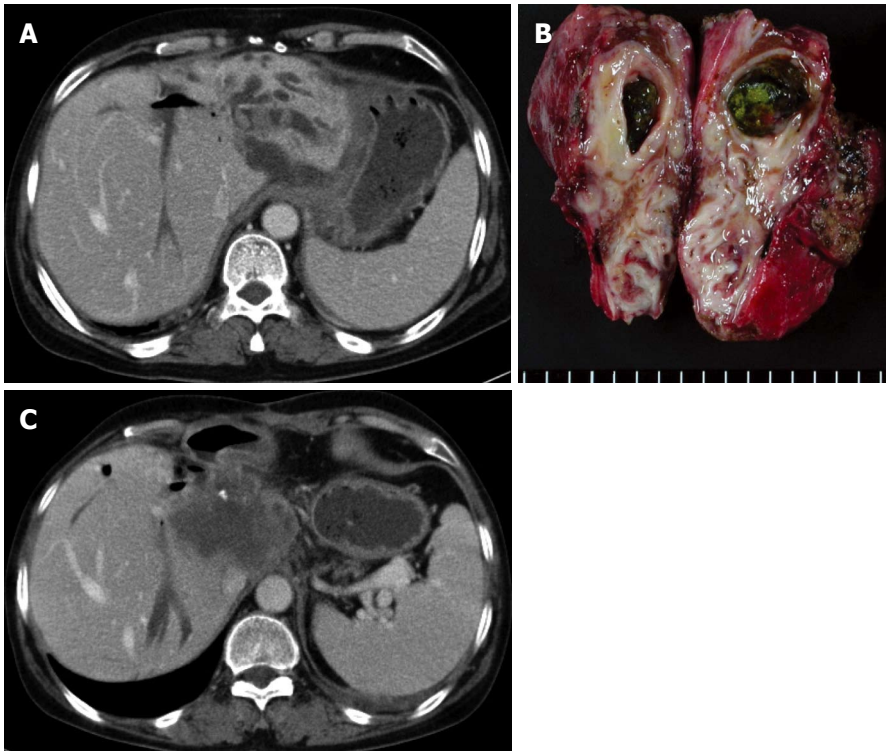

Figure 4 Forty-six years old, female was admitted for cholangitis. A: Abdominal CT demonstrated that multiple calcified stones are present in the lateral segment of the left lobe; B: Liver, left lobectomy was performed, and there was no cancerous lesion; C: Abdominal computed tomography taken 14 mo after resection demonstrated development of cholangiocarcinoma in the caudate lobe of the liver.

cancer. HL-CCA can develop even after complete stone removal through PTCSL or initial hepatectomy[33,129]. Development of HL-CCA is an independent predictor for survival in patients who have undergone hepatic resection with hepatolithiasis[12,38]. Early detection of HL-CCA is very important in follow-up after treatment of hepatolithiasis. However, to date, there is no effective measure to predict subsequent HL-CCA in patients with hepatolithiasis.

Follow-up for early detection of subsequent HL-CCA

Known predictive factors for subsequent HL-CCA after treatment are old age, bile duct stricture, bilioenteric anastomosis, stone recurrence, stones in both hepatic lobes, and no hepatic resection[33,42,122].

Kim *et al*[33] reported that age, gender, CA 19-9, stone location, bile duct stenosis, liver atrophy, stone recurrence, residual stone, and hepatic resection were not significant predictive factors by multivariate analysis. Cheon *et al*[30] reported that there was no significant risk factor for CCA during the follow-up period.

Huang *et al*[42] and Lin *et al*[32] reported that the subsequent HL-CCA incidence was significantly lower after complete stone removal than in cases of residual stones (0.7% *vs* 6.6% and 4.9% *vs* 11.8%,

Table 3 Clinical characteristics of subsequent cholangiocarcinoma patients[33]

Case	Sex/age	Location of stone	Treatment method	Residual stone	Time of CC (mo)	Location of CC	Stage
1	M/65	Lt	ERCP	Yes	14	Lt	ⅣA
2	M/69	Lt	PTCSL	Yes	15	Lt	Ⅲ
3	M/72	Lt	PTCSL	Yes	17	Lt	ⅣA
4	M/66	Lt	ERCP	Yes	21	Lt	Ⅲ
5	M/51	Lt	PTCSL	Yes	13	Lt	ⅣA
6	M/63	Lt	PTCSL	No	53	Lt	ⅣA
7	F/71	Rt	ERCP	Yes	79	Both	Ⅲ
8	F/54	Both	PTCSL	Yes	10	Lt	ⅣA
9	F/56	Rt	PTCSL	No	111	Rt	ⅣA
10	F/65	Both	PTCSL	Yes	72	Lt	ⅣA
11	M/60	Both	Lt. hemi-hepatectomy	Yes	102	Rt	Ⅲ
12	F/67	Both	Lt. lobectomy	Yes	55	Rt	ⅣA
13	F/51	Lt	Lt. hemi- hepatectomy	No	109	Lt	Ⅱ
14	M/52	Both	Lt. lobectomy	Yes	28	Rt	ⅣA
15[1]	F/47	Lt	Lt. hemi-hepatectomy	No	14	Caudate	Ⅲ
16	F/56	Both	Lt. lobectomy	Yes	81	Rt	ⅣB

[1]Described in Figure 3. M: Male; F: Female; CC: Cholangiocarcinoma; Lt: Left; Rt: Right; ERCP: Endoscopic cholangiopancreatography; PTCSL: Percutaneous transhepatic choledochoscopic lithotomy.

respectively). Jo *et al*[108] reported that incomplete removal of stones was a risk factor for subsequent HL-CCA, but complete stone removal was not significant as a good prognostic factor. Furthermore, there are reports that the incidence of subsequent HL-CCA is not significantly different between groups: Kim *et al*[33] reported 3.3% *vs* 10.4% (P = 0.263), Tsuyuguchi *et al*[39] reported 9.1% *vs* 10.4% (P = 0.554), and Cheon *et al*[30] reported 4% *vs* 8% (P = 0.06).

The risk of subsequent HL-CCA was increased in bilateral hepatolithiasis[118,119]. Lin *et al*[32] reported that the incidence of concomitant HL-CCA and subsequent HL-CCA was similar in patients with unilateral hepatolithiasis (4.8% and 4.5%, respectively), but the incidence of subsequent HL-CCA (12.2%) was higher than concomitant HL-CCA (4.7%) in patients with bilateral hepatolithiasis.

Most subsequent HL-CCA has been reported to occur within the same hepatic lobe where treatment was performed[130]. However, Cheon *et al*[30] reported the tumor occurrence in the contralateral hepatic lobe in six out of 11 cases, and Kim *et al*[33] reported it in three out of 12 cases. It is possible that chronic inflammatory conditions play a role in the development of CCA arising from the bile duct without stones[77].

Suzuki *et al*[122] reported that risk factors for HL-CCA were choledochoenterostomy (OR = 3.718), biliary stricture (HR = 4.615), and stone recurrence (HR = 6.264)[122]. Bettschart *et al*[123] reported that patients who underwent bilioenteric anastomosis due to hepatolithiasis should be followed.

Is the prognosis of HL-CCA worse than CCC alone?

Su *et al*[11] reported that the survival of patients with HL-CCA is worse than that of patients with only CCA. In a study of 66 patients with HL-CCA, radical resection was possible in only 38 patients[131].

In contrast, there are studies that HL-CCA is not inferior in resectability and survival. Chen *et al*[132] showed a higher rate of resectability for HL-CCA than for CCC without HL (31.1% *vs* 26.8%). Lee *et al*[133] showed a resectability rate of 52.6% in HL-CCA and 39.2% in CCC only, and Guglielmi *et al*[134] also reported a higher rate of resectability in HL-CCA than CCC alone (91.3% *vs* 68.8%). A possible explanation is that the presence of symptoms due to hepatolithiasis could lead to an earlier diagnosis of CCA[132].

However, despite a higher rate of resectability, there were no differences in overall survival between patients with HL-CCA and patients with CCC alone[133,134]. In 23 patients who underwent resection for HL-CCC, Han *et al*[135] reported that the overall cumulative survival rates were 43.8%, 13.0%, and 4.3% at 1, 3, and 5 years, respectively, even though they were higher at 88.9%, 33.3%, and 11.1%, respectively, in patients with curative resection.

A recent interesting study showed that palliative surgery resulted in a gain in survival. Li *et al*[131] reported that the overall survival rates were 58.3%, 31.7%, and 11.7% at 1, 3, and 5 years, respectively. In the radical resection group, survival rates were 71.1%, 39.4% and 15.8%, respectively, 42.9%, 28.6% and 7.1%, respectively, in the palliative resection group, and 25%, 0%, and 0%, respectively, in the group of abdominal exploration (P < 0.001). Zhang *et al*[136] suggested three reasons for performing palliative resection even in patients classified as Stage IV. First, the indication for operation was not only due to the tumor, but also due to hepatolithiasis, which frequently causes biliary tract infections. Second, significant differences in the median overall survival were found between R1, R2, and non-resection-treated patients (18.2, 14.2, and 9.1 mo, respectively). Third, adjuvant therapy did not significantly prolong the survival. Considering these results, aggressive resection should be performed in practice to prolong

survival even when curative treatment cannot be accomplished for patients with advanced HL-CCA.

Subsequent HL-CCA leads to worse survival than concomitant HL-CCA

Subsequent HL-CCA diagnosed after treatment of hepatolithiasis has a worse prognosis than concomitant HL-CCA. Lin *et al*[32] reported that most patients who developed subsequent HL-CCA after hepatectomy were not eligible for a second hepatectomy due to its locally advanced state, peritoneal seeding, distant metastasis, or insufficient remnant liver volume. Overall, mortality and disease-related mortality of subsequent HL-CCA is significantly higher than that for concomitant HL-CCA. Recently, Tsuyuguchi *et al*[39] and Kim *et al*[33] reported similar poor results (Table 3).

CONCLUSION

Although there have been many advances in the management of hepatolithiasis, there are no consistent results regarding HL-CCA until now. Important factors to consider are the proper treatment to reduce recurrence and early detection of HL-CCA. Patients should be followed after treatment because there is no effective prevention of HL-CCA or premalignant lesions.

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MINIREVIEWS

High antibiotic resistance rate: A difficult issue for *Helicobacter pylori* eradication treatment

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Abstract

Helicobacter pylori (*H. pylori*) infection is associated with a variety of upper gastrointestinal diseases, including gastric cancer. With the wide application of antibiotics in *H. pylori* eradication treatment, drug-resistant strains of *H. pylori* are increasing. *H. pylori* eradication treatment failure affects the outcome of a variety of diseases of the upper gastrointestinal tract. Therefore, antibiotic resistance that affects *H. pylori* eradication treatment is a challenging situation for clinicians. The ideal *H. pylori* eradication therapy should be safe, effective, simple, and economical. The eradication rate of triple antibiotic therapy is currently less than 80% in most parts of the world. Antibiotic resistance is the main reason for treatment failure, therefore the standard triple regimen is no longer suitable as a first-line treatment in most regions. *H. pylori* eradication treatment may fail for a number of reasons, including *H. pylori* strain factors, host factors, environmental factors, and inappropriate treatment.

Key words: *Helicobacter pylori*; Resistance; Eradication treatment; Triple antibiotic therapy; Gastrointestinal disease

Core tip: *Helicobacter pylori* (*H. pylori*) infection is associated with a variety of upper gastrointestinal diseases, including gastric cancer. *H. pylori* eradication treatment failure affects the outcome of these diseases. The eradication rate of triple antibiotic therapy, the worldwide gold standard, is currently less than 80% in most parts of the world. Antibiotic resistance is the main reason for treatment failure, therefore the standard triple regimen is no longer suitable as a first-line treatment for the majority of the world. *H. pylori* eradication treatment may fail for a number of reasons, including *H. pylori* strain factors, host factors, environmental factors, and inappropriate treatment.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is associated with a variety of upper gastrointestinal diseases, including gastric cancer[1]. With the wide application of antibiotics in *H. pylori* eradication treatment, drug-resistant strains of *H. pylori* are increasing. This has attracted the widespread concern of scholars and clinicians throughout the world. *H. pylori* eradication treatment failure affects the outcome of a variety of diseases of the upper gastrointestinal tract. Therefore, antibiotic resistance that affects *H. pylori* eradication treatment is a challenging situation for clinicians. The ideal *H. pylori* eradication therapy should be safe, effective (eradication rate > 90%), simple, and economical. The eradication rate of triple antibiotic therapy, the current worldwide gold standard, is currently less than 80%in most parts of the world[2]. Antibiotic resistance is the main reason for treatment failure, therefore the standard triple regimen is no longer suitable as a first-line treatment in most regions[3]. *H. pylori* eradication treatment may fail for a number of reasons, including *H. pylori* strain factors, host factors, environmental factors, and inappropriate treatment.

H. PYLORI STRAIN FACTORS

Antibiotic resistance and mechanism

Extensive and unreasonable application of antibiotics is the main cause of antibiotic resistance. A European study shows that the resistance rate is related to the dosage of antibiotics used in outpatient wards[4]. Several types of antibiotics were used for *H. pylori* era dication therapy, including macrolides, nitromidazole, lactams, aminoglycosides, quinolones, nitrofurans, and tetracycline. An epidemiological investigation showed that the overall prevalence of *H. pylori* in China still remains high; the adult infection rate being 40%-60%[5]. Forthe sixty pes of antibiotics recommended foreradication treatment, the resistance rates for *H. pylori* are as follows: metronidazole, 60%-70%; clarithromycin, 20%-38%; levofloxacin, 30%-38%; where asamoxicillin, furazolidone, and tetracycline resistance rates are still very low (1%-5%). The drug resistance rate significantly influences the eradication rate.

Clarithromycin is a commonly used drug in *H. pylori* eradication treatment. The resistance rate of *H. pylori* to clarithromycin has increased gradually in recent years and the resistance rate is inversely related to the eradication rate. A study of 910 cases in China showed that the clarithromycin-resistance rate was 27.6% in 2005[6]. Several studies showed that the clarithromycin-resistance rates in American and European populations were 29.3% and 11.1%, respectively, in 2009[7]. The resistance rate in Turkey was 47.5%[8], and in South America, 17.72%[9]. Clarithromycin is a macrolideantibiotic; its pharmacological or antibacterial effect prevents transpeptidation and translocation reactions by binding to domain V of the *H. pylori* 23S ribosomal RNA (23S rRNA), thus inhibiting bacterial protein synthesis. Point mutations in the V functional domain of the 23S rRNA reduces the affinity of clarithromycin for the transpeptidase, inhibiting clarithromycin binding to the 23S ribosomal subunit, resulting in clarithromycin resistance[10]. Mutations in the 23S rRNA gene, including *A2143G*, *A2142G*, and *A2142C*, are closely associated with clarithromycin resistance; the most commonly observed of which is A2143G.Mutations in this gene accounts for 80%-90% of clarithromycin resistance[11].

Metronidazole was the earliest drug used for eradication treatment of *H. pylori*. In the Chinese population, the average *H. pylori* metronidazole-resistance rate is 75.6% and the rate has reached up to 95.4% in some areas[12]. In contrast, Japan has a very low metronidazole-resistance rate of around 3.3%-12.9%[13], which may be associated with the restriction of the use of metronidazole. The *H. pylori* metronidazole-resistance mechanism has the following aspects. First, a number of different *H. pylori* rdxA gene mutations are observed[14]. Detection of rdxA and frxA mutations can not accurately predict drug resistance to metronidazole[15]. Second, the resistance to metronidazole may involve other factors in addition to the nitro-reductase. Finally, most of the research is limited to the detection of mutations at the DNA level, but more studies on the level of transcription and translation are necessary[16]. In general, the mechanism of *H. pylori* resistance to metronidazole is relatively complex and needs further research.

With the decreasing eradication rate of classical triple therapy, levofloxacin is becoming widely used in eradication therapy. Although it is a second-line treatment, a high rate of drug resistance has arisen against it. A study in China showed that the levofloxacin-resistance rate was 20.6%[12], and a study in Peru showed the resistance rate at 36.9%[17]. Fluoroquinolones act on the DNA gyrase to exert a bactericidal effect. The gyrase enzyme is necessary to maintain the helical structure of DNA. It is a tetrameric enzyme composed of two Asubunitsen coded by the *gyrA* gene and two Bsubunitsen coded by the *gyrB* gene. Point mutations in the quinolone resistance determining regions can prevent antibiotic binding togyrase, causing antibiotic resistance[18].

At present, amoxicillin resistance rates are relatively low in China, about 1%-5%[5], and even lower in more developed countries. However, penicillin-allergic patients cannot use amoxicillin. Amoxicillin

resistance can arise by different mechanisms. Among them, mutations in the bacterial penicillin binding protein gene (*PBP1A*) is a commonmechanism, which can cause a low or moderatelevel of drug resistance[19]. In recent years, amoxicillin resistance caused by extended spectrum beta-lactamases has also been reported, which can cause high levels of drug resistance[20]. Tetracycline and furazolidone resistant rates are still very low[5,21].

Currently, not only has multi-drug resistance become an increasingly severe problem for clinicians, but different regions and groups have different degrees and patterns of resistance. Research shows the *H. pylori* multi-drug resistance rates as high as 34.5%[22], which is a very serious problem. The multi-drug resistance rate of *H. pylori* to levofloxacin and metronidazole was 16.9%, to clarithromycin and metronidazole was 7%, and the resistance rate to all three of the drugs together was nearly 10%. For eradication therapy of *H. pylori*, patients with penicillin allergy have to choose furazolidone or gentamicin, which are drugs with severe adverse reactions, or are left with no treatment alternatives. With the drug-resistance rate peaking, it is difficult to achievea high eradication rate with the traditional triple therapy. Quadruple therapy can be used as an alternative treatment; however, the effect is limited against multi-drug-resistant strains. An individualized treatment planis the most effective way to evade the problem of multi-drug-resistant bacteria. In regions with high rates of resistance to clarithromycin, the eradication rate based on culture-guided triple therapy for clarithromycin-sensitive cultures was significantly higher than that for empirical treatment[23]. Research shows that sequential therapy and the modified bismuth-containing quadruple therapy has a very high efficacy rate in the Hong Kong area[24]. The mechanism of multi-drug resistance in *H. pylori* is closely related to the efflux pump system. Studies on macrolide resistance found that *H. pylori* contains four genes encoding the resistance nodulation cell division (RND) superfamily efflux pump system. Injection of specific efflux pump inhibitors, such as Phe-arg-β-naphthylamide (PAβN), can inhibit the RND efflux pump system, to reduce the antibiotic minimum inhibitory concentration[25]. Proton pump inhibitors (PPIs) and efflux pump inhibitor shave a similar structure. In addition to inhibiting gastric acid secretion, PPIs can also inhibit the *H. pylori* RND efflux pump system, which can improve the sensitivity of *H. pylori* to antibiotics[26]. A recent comparative analysis of sarcosine-insoluble outer membrane proteins (OMPs) between clarithromycin-resistant and-sensitive strains found that the iron regulation membrane protein, ureaseB, EF-Tu protein complex, and OMPs decreased in resistant strains. At the same time, transmembrane proteins HopT (BabB), HofC, and OMP31 increased in resistant strains. These results were confirmed using western blotand real-time quantitative polymerase chain reaction analyses. Thissuggests that changes in the composition of the OMPs may be an additional mechanism of *H. pylori* resistance to clarithromycin[27].

Genotypeand virulence factors

The *H. pylori* genotype is closely related to the efficacy of antibiotic treatment. Data showed that antibiotic sensitivity of the S1/M1 andS1/M2 strains [mostlycytotoxin-associated protein (CagA)+] is higher than that of S2/M2 strains (mostly CagA-)[28]. The major *H. pylori* virulence factors, vacuolating cytotoxinA VacA) and CagA, not only play an important role in the pathogenesis of *H. pylori*, but also have an important effect on the eradication treatment of *H. pylori*. Some studies suggest that the eradication rate of CagA-positive strains is higher than that of CagA-negative strains, but the results have not been confirmed and this requires further study[29].

Site and density of colonization

H. pylori in cells, gastric fundus, gastric antrum, and gastric body junction are usually thought to be difficult to eradicate and colonization in those areas can contribute to treatment failure. *H. pylori* in the gastric antrum and body junction may escape the effects of antibiotics. Owing to the unique structure of the junction, a unique colonizing environment is created; the *H. pylori* colonization in these sites has a different biological behavior, so that they are not sensitive to antibiotics. In addition, protracted use of antacids can make *H. pylori* colonization in the gastric antrum migrate to the gastric body, making eradication treatment even more difficult. More than 10 years after discovery of *H. pylori*, Stark *et al*[30] found that the *H. pylori* NCTC11637(ATCC43504) strain, when grown in a glass fermenter, can produce a membrane layer structure, insoluble in water and containing polysaccharide, on the surface of a liquid. This confirms that *H. pylori* also has the ability to form bio-films. A large number of *H. pyloric* and produce a "bio-film effect" as a self-protection mechanism and also a high *H. pylori* colonization density directly affects the minimum inhibitory concentration. These are both factors that can contribute to treatment failure.

Conversion to coccoid form

H. pylori in unfavorable growth environments are likely to transform into a coccoid form, which is not sensitive to antibiotics. The coccoid form of *H. pylori* exists in two forms: one is dead or degenerated; the second is a non-active stage *H. pylori* which, although alive, are unable to reproduce. *H. pylori* in the coccoid form, upon termination of the use of antibiotics for 2 or more weeks, will restore their activity. This form of *H. pylori* is not only an important reason for eradication failure, but also is infectious[31], which is an important cause of relapse.

HOST FACTORS

Patient compliance: Patients' poor compliance is gradually coming to the attention of clinicians. Poor compliance may be due to a lack of emphasis on eradication therapy, cumbersome events related to drug therapy, and drug-related adverse effects. The drug side effects mostly consist of diarrhea and un appealing taste of the medication. Although the symptoms are relatively mild and do not affect normal life, they can lead to patients' withdrawal owing to the fear of more severe side effects[32]. Therefore, physicians should not only strengthen clinical work on the treatment of *H. pylori* patients, but also provide education and reasonable treatment options to the patients. They should stress the need for optimal treatment and the impact of poor compliance on the efficacy of treatment, and explain the side effects of the drugs, thereby encouraging the patients to comply with their treatment regimen and reducing the likelihood of eradication failure because of early withdrawal of medication.

Gene polymorphisms in patients

PPIs are the most common drugs used for the treatment of digestive diseases. Through efficient and rapid inhibition of gastric acid secretion, PPIs provides a fast cure for gastrointestinal injury, and *H. pylori* eradication regimens established on this basis rely on their potent acid suppression ability. Polymorphisms in the cytochrome P450 (*CYP*) *2C19* gene affect the efficacy of a PPI-containing regimen, as PPI is mainly metabolized through a CYP2C19 channel. Patients of the strong metabolic type (homozygous wild type, wt/wt) with a high PPI clearance rate, had serum drug concentrations that were significantly lower than in poor metabolizers (homozygous mutants, mt/mt). Therefore, patients with the wild-type allele of *CYP2C19* are less likely to be able to eradicate *H. pylori*[33]. In addition, polymorphisms in the P-glycoprotein (*MDR1*) gene also have an effect on the treatment efficacy with PPI regimens[34,35]. Studies have shown that for triple eradication therapy based on CYP2C19 metabolic pathway-dependent PPIs such as omeprazole or lansoprazole, the rate of eradication is higher in poor metabolizers than in strong metabolizers[36]. Selection of PPIs such as esomeprazole or rabeprazole for use in eradication therapy may help to increase the eradication rate, as the CYP2C19 genotype does not seem to affect the eradication rate when using these PPIs.

Effect of various types of disease with eradication therapy

A French meta-analysis study of 2751 cases, with an overall eradication failure of 25.8% sought to determine whether eradication therapy failure was associated with particular types of gastrointestinal diseases. Duodenal ulcer (DU) patients exhibited an *H. pylori* eradication failure rate of21.9%, which was significantly lower than patients with non-ulcer dyspepsia (NUD) with a failure rate of 33.7% ($P < 10^{-6}$). Drug susceptibility testing implied that the clarithromycin resistance rate of *H. pylori* strains in patients with NUD was significantly higher than DU patients, which also suggests an explanation for the lower *H. pylori* eradication rate in NUD patients[37].

Smoking

Suzuki *et al*[38] suggested that the success rate of *H. pylori* eradication in smokers was lower (8.4%) than that of non-smokers, and among smokers with non-ulcerative dyspepsia, the eradication failure rate was higher, as smoking can reduce gastric mucosal blood flow, can stimulate gastric acid secretion, and also affect the body's metabolism of PPIs.

ENVIRONMENTAL FACTORS

At least 4 wk after eradication treatment is completed, a follow-up *H. pylori* detection test is performed, to determine whether a relapse or re-infection has occurred. The oro-oral and feco-oral routes are considered to be the most likely routes of *H. pylori* transmission. Studies have found that in patients who have undergone periodontal therapy and an oral cleansing process, the *H. pylori* eradication rate was significantly higher than that of patients who did not, suggesting that *H. pylori* may be spread through saliva[39]. In addition to oral *H. pylori* infection, in rural areas and places with poor sanitary conditions, the population has a higher rate of *H. pylori* infection[40].

OTHER

There is a reduction in the eradication rate if PPIs are used prior to radical *H. pylori* eradication therapy. This may be because protracted use of PPI results in *H. pylori* metastases from the gastric antrum to the body, transforming into the coccoid form. Some studies have also shown that *H. pylori* treatment failure is associated with higher body mass index[21].

CONCLUSION

In summary, *H. pylori* eradication therapy is influenced by multiple factors. Currently, the mechanisms of resistance in *H. pylori* strains, hosts, and environmental and other factors are not completely understood; therefore, it is a continuing challenge for clinicians to improve the success rate of *H. pylori* eradication. Clinicians should have an in-depth knowledge of *H. pylori* resistance mechanisms and mechanisms of drug action, and raise patients' awareness of their own condition. Depending on the situation in different regions and of different patients, the appropriate individualized treatment

programs should be selected, thereby improving the success rate of *H. pylori* eradication treatment.

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ORIGINAL ARTICLE

Basic Study

Sphingosine kinase 1 dependent protein kinase C-δ activation plays an important role in acute liver failure in mice

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Abstract

AIM: To investigate the role of protein kinase C (PKC)-δ activation in the pathogenesis of acute liver failure (ALF) in a well-characterized mouse model of D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced ALF.

METHODS: BALB/c mice were randomly assigned to five groups, and ALF was induced in mice by intraperitoneal injection of D-GalN (600 mg/kg) and LPS (10 μg/kg). Kaplan-Meier method was used for survival analysis. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels at different time points within one week were determined using a multiparameteric analyzer. Serum levels of high-mobility group box 1 (HMGB1), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 as well as nuclear factor (NF)-κB activity were determined by enzyme-linked immunosorbent assay. Hepatic morphological changes at 36 h after ALF induction were assessed by hematoxylin and eosin staining. Expression of PKC-δ in liver tissue and peripheral blood mononuclear cells (PBMCs) was analyzed by Western blot.

RESULTS: The expression and activation of PKC-δ were up-regulated in liver tissue and PBMCs of mice with D-GalN/LPS-induced ALF. Inhibition of PKC-δ activation with rottlerin significantly increased the survival rates and decreased serum ALT/AST levels at

6, 12 and 24 h compared with the control group (P < 0.001). Rottlerin treatment also significantly decreased serum levels of HMGB1 at 6, 12, and 24 h, TNF-α, IL-6 and IL-1 β at 12 h compared with the control group (P < 0.01). The inflammatory cell infiltration and necrosis in liver tissue were also decreased in the rottlerin treatment group. Furthermore, sphingosine kinase 1 (SphK1) dependent PKC-δ activation played an important role in promoting NF-κB activation and inflammatory cytokine production in ALF.

CONCLUSION: SphK1 dependent PKC-δ activation plays an important role in promoting NF-κB activation and inflammatory response in ALF, and inhibition of PKC-δ activation might be a potential therapeutic strategy for this disease.

Key words: Acute liver failure; Protein kinase C-δ; Sphingosine kinase 1; Nuclear factor-κB

Core tip: In this study, we found protein kinase C (PKC)-δ expression and activation in liver tissue or peripheral blood mononuclear cells of mice with acute liver failure (ALF). Inhibition of PKC-δ activation attenuated ALF in this animal model. Furthermore, sphingosine kinase 1 (SphK1) was required for PKC-δ activation in LPS-stimulated macrophages and the ALF mouse model. Our findings suggest that SphK1 dependent PKC-δ activation plays an important role in ALF, and inhibition of PKC-δ activation might be a potential therapeutic strategy for this disease.

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INTRODUCTION

Acute liver failure (ALF) is a severe clinical syndrome characterized by sudden and massive death of liver cells resulting from a variety of hepatic diseases, and can lead to hepatic encephalopathy, coagulopathy and multi-organ failure[1]. Despite advances in the development of new treatments for ALF, little progress has been made in seeking efficient interventions that can improve the outcome of this disease. Currently, ALF is still associated with an extremely high mortality and often demands urgent liver transplantation due to limited therapeutic options[1-3]. Although liver cells are the major target cells in ALF, inflammatory cells, especially macrophages and neutrophils, dominate the manifestation of liver injury. Increasing the level of bacterial lipopolysaccharide (LPS) in the blood may induce inflammatory cell activation. There is increasing evidence suggesting that the predominant mechanism responsible for the development of ALF is activation of the systemic immune response, through release of proinflammatory cytokines and damage-associated molecular patterns (DAMPs) as a result of massive hepatocyte necrosis, which in turn plays a pivotal role in the clinical course and outcome in ALF patients[4]. Large studies have demonstrated that the presence of systemic inflammatory response syndrome (SIRS) in ALF is associated with a poor prognosis[5]. Patients with ALF have high circulating concentrations of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6[6,7].

Protein kinase C (PKC)-δ has been identified as a critical inflammatory regulator and is instrumental in neutrophil recruitment, sequestration, and activation. In neutrophils, PKC-δ controls proinflammatory events and regulates cytokine-elicited oxygen radical production, degranulation, and activation of nuclear factor-κB (NF-κB)[8,9]. PKC-δ inhibition prevented neutrophil adherence and migration[10]. In PKC-δ null mice, neutrophil adhesion, migration, oxygen radical generation, and degranulation are limited[11]. Thus, PKC-δ may have an important regulatory role in the inflammatory response. PKC-δ is activated by multiple proinflammatory stimuli, including cytokines, such as TNF-α and IL-1, and PAMPs, such as LPS[12,13]. PKC-δ is an important component of proinflammatory signaling pathways that regulate activation of the transcription factor NF-κB. NF-κB regulates gene expression of chemokines, adhesion molecules, and cytokines, which can initiate and perpetuate inflammation and thus function in a positive-feedback loop.

Sphingosine kinases are intracellular signaling enzymes that catalyze the formation of the lipid mediator sphingosine-1-phosphate[14]. Several proinflammatory stimuli, including LPS, TNF-α, anaphylatoxin C5a and immune complexes, activate sphingosine kinase 1 (SphK1) on human neutrophils and macrophages and promote several proinflammatory responses[15-17]. A recent study demonstrated that SphK1 plays a critical role in endotoxin signaling and sepsis-induced inflammatory responses by activating NF-κB[18]. SphK1 expression was strongly up-regulated and rapidly activated in macrophages stimulated with LPS. SphK1 was required to activate NF-κB and to induce the secretion of proinflammatory cytokines or high mobility group box 1 (HMGB1) in LPS-stimulated macrophages, and SphK1 mediated TLR-triggered NF-κB activation in macrophages through PKC-δ activation[18]. The ability of PKC-δ to mediate the secretion of proinflammatory mediators prompted us to investigate its role in systemic inflammatory response in ALF. Based on the fact that NF-κB activation is the common pathway in proinflammatory cytokine secretion, we speculated that SphK1-mediated PKC-δ activation may play an important role in ALF.

In this study, we demonstrated PKC-δ expression and activation in liver tissue or peripheral blood mononuclear cells (PBMCs) of mice with D-galactosamine (D-GalN)/LPS-induced ALF. Inhibition of PKC-δ with rottlerin ameliorated ALF in this animal model. Furthermore, SphK1 was required for PKC-δ activation in LPS-stimulated macrophages and the ALF mouse model. These results indicate that SphK1 dependent PKC-δ activation plays an important role in ALF.

MATERIALS AND METHODS

Animal model of ALF and treatments

Male BALB/c mice aged 8 wk and weighing 20 ± 0.5 g were obtained from the Experimental Animal Center of Nanchang University, Nanchang, China. The mice were handled and treated in accordance with the strict guiding principles of the National Institution of Health for experimental care and use of animals. ALF was induced in mice by intraperitoneal injection of D-GalN (600 mg/kg; Sigma-Aldrich) and LPS (10 μg/kg; Sigma-Aldrich) as previously described. At 36 h following ALF induction, a portion of the animals were sacrificed to harvest liver tissue for hematoxylin and eosin (HE) staining. N,N-dimethylsphingosine (DMS; Sigma-Aldrich), a PKC-δ specific chemical inhibitor, and rottlerin (Sigma-Aldrich), a SphK1 specific chemical inhibitor, were intraperitoneally injected 0.5 h prior to ALF induction.

Measurement of serum ALT/AST and cytokines

Serum samples were stored at -80 ℃ until analysis. Serum ALT and AST levels were measured using a multiparameteric analyzer (AU 5400, Olympus, Japan). Serum levels of TNF-α, IL-1β, IL-6, IL-10 and HMGB1 were determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, United States).

Western blot analysis

Proteins (40 μg) from total tissue or cell lysates/samples were resolved on 10% polyacrylamide gels under denaturing conditions and then transferred to 0.45 μm nitrocellulose membranes. The blots were probed using a polyclonal anti-PKC-δ antibody (Santa Cruz Biotechnology, United States), and an anti-β-actin antibody (Santa Cruz Biotechnology) was used for confirming equal protein loading. Bands were visualized using a horseradish peroxidase-conjugated anti-IgG secondary antibody and the ECL Western Blotting Detection System (GE Healthcare, United Kingdom).

Determination of PKC-δ phosphorylation

Frozen lung tissue (0.15 g) was homogenized in 3 mL buffer containing 10 mmol/L Hepes (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L Na-orthovanadate, 20 μmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1% Triton X-100, 5 μg/mL leupeptin, phosphatase inhibitor cocktail and protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, United States). Protein concentrations of the cell lysates were determined using a bicinchoninic acid protein assay kit, according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, United States). Proteins (30 μg/lane) were separated on 4%-12% SDS-PAGE gels and transferred to nitrocellulose membranes. PKC-δ (Thr^{505}) phosphorylation was determined by immunoblotting using a phospho-specific PKC-δ (Thr^{505}) antibody (Cell Signaling Technology, Beverly, MA, United States) as described previously. Equal protein loading was confirmed by reprobing membranes using a PKC-δ antibody that recognizes both phosphorylated and nonphosphorylated forms of PKC-δ (Santa Cruz Biotechnology).

Detection of NF-κB activity

Following LPS stimulation, NF-κB activity was analyzed using the Mercury TransFactor Profiling Kit-Inflammation kit (BD), following the manufacturer's instructions. The enzymatic product was analyzed with a standard plate reader.

Histological assays

For morphological investigation, the livers of mice were carefully dissected out at 36 h following ALF induction and immersed in 10% phosphate-buffered formalin for 1 d. The specimens were then dehydrated through an ascending series of ethanol and cleared in toluene, before being embedded in paraffin. The tissue blocks were cut at 4 μm thickness using a Leica rotary microtome (Model 2165). Paraffin sections were mounted on albuminized glass slides by floating and flattening the sections in a water bath at 45 ℃. The mounted sections were drained until dry and kept in an incubator at 30 ℃. Paraffin sections were first dewaxed in two changes of xylene, passed through a descending series of alcohol, and were finally washed in deionized water, before staining with hematoxylin and eosin for 1-2 min at room temperature. The sections were rinsed three times in deionized water, dehydrated quickly through an ascending series of ethanol, and passed through xylene before being mounted with Permount. Slides were viewed with an Olympus microscope using 10 ×, 40 × and 100 × Uplan Apo lenses.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Statistical significance was determined by a two-tailed Student's *t*-test or one-way analysis of variance (ANOVA), and, specifically, a log-rank test for survival analysis. A P-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 13.0 for Windows.

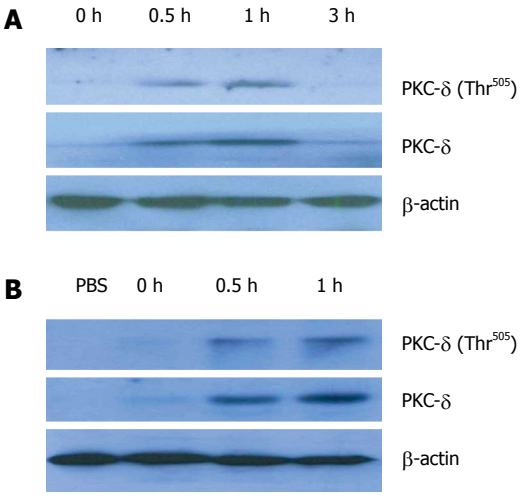

Figure 1 Protein kinase C-δ expression and activation (Thr^{505} phosphorylation) in liver tissue and peripheral blood mononuclear cells of mice with D-galactosamine/lipopolysaccharide-induced acute liver failure. Acute liver failure was induced in BALB/c mice using D-galactosamine (600 mg/kg) and lipopolysaccharide (10 μg/kg). A: PKC-δ expression and Thr^{505} phosphorylation in liver tissue; B: PKC-δ expression and Thr^{505} phosphorylation in peripheral blood mononuclear cells. PKC: Protein kinase C.

RESULTS

PKC-δ expression and activation in liver tissue and PBMCs of mice with D-GalN/LPS-induced ALF

To dissect the role of PKC-δ in ALF, we first detected whether PKC-δ expression is triggered in GalN/LPS-induced ALF *in vivo*. Liver tissue samples were harvested at 0, 0.5, 1 and 3 h, respectively, after D-GalN/LPS injection. Compared with the negative control, the expression of PKC-δ in liver tissue was promptly increased at 0.5 h and 1 h, and then declined to the basal level after 3 h (Figure 1A). Activation of PKC-δ is a multistep process that can be assessed by quantifying phosphorylation of Thr^{505} in the PKC-δ activation loop. There was little phosphorylation of PKC-δ in liver tissue homogenates obtained at 0 and 3 h after ALF induction in mice. In contrast, liver tissues collected at 0.5 h and 1 h after GalN/LPS administration had a significant increase in PKC-δ phosphorylation. As expected, PKC-δ expression and phosphorylation in PBMCs of mice were also increased at 0.5 h and 1 h after ALF induction (Figure 1B). These results suggest that PKC-δ expression and activation in liver tissue and PBMCs are an early event in the acute phase of ALF in mice.

Inhibition of PKC-δ activity improves survival and attenuates serum ALT/AST levels in mice with ALF

For survival analysis, five treatment groups of mice were used; group Ⅰ received PBS only, and groups Ⅱ to Ⅴ were pretreated with four doses (0, 1, 2, and 5 μmol/L) of rottlerin (a PKC-δ specific chemical inhibitor), respectively, 0.5 h after D-GalN/LPS challenge. In group Ⅱ, the mice began to die from 6 h, and only 25.0% (3/12) survived at 48 h. However, pretreatment with rottlerin increased the survival rate in a dose-dependent manner. The survival rates were 41.7% (5/12), 58.3% (7/12), and 75.0% (9/12) within 48 h in mice pretreated with 1, 2, and 5 μmol/L rottlerin, respectively. In group Ⅴ, the survival rates were significantly increased compared with those of group Ⅱ (P = 0.003) (Figure 2A). Since rottlerin at 5 μmol/L significantly protected the liver from ALF, we selected 5 μmol/L rottlerin for the following study.

We next investigated whether rottlerin decreases serum ALT/AST levels. In the surviving mice, the peak levels of ALT/AST were detected at 12 h, both in the control and rottlerin treatment groups. However, the maximum ALT/AST levels were reduced significantly in the rottlerin treatment group than in the control group (Figure 2B and C). The maximum ALT/AST levels at 12 h were reduced by 63.8% (P < 0.001) and 63.2% (P < 0.001), respectively, in the rottlerin treated group. The ALT/AST levels at 6 h and 24 h were also decreased significantly in the rottlerin treated group compared with the control group (P < 0.01). No significant differences were observed at any of other time points from 24 h to one week.

Inhibition of PKC-δ activity down-regulates inflammatory cytokines and reduces liver inflammation and necrosis in mice with ALF

Analysis of serum cytokine levels revealed a significant decrease for TNF-α, IL-1β and IL-6 after rottlerin treatment (P < 0.01). However, level of the anti-inflammatory cytokine IL-10 was not increased in the rottlerin treatment group (Figure 3A). HMGB1 released by macrophages and damaged or necrotic cells functions as a DAMP and contributes to the pathogenesis of ALF. In the control group, serum HMGB1 level increased at 6 h after ALF induction, reached the peak at 12 h, and then fall down rapidly. Serum concentrations of HMGB1 were decreased at above three time points after rottlerin treatment (Figure 3B). These data demonstrate that inhibition of PKC-δ activity with rottlerin reduced liver inflammation and necrosis and decreased proinflammatory cytokine and HMGB1 levels.

Liver histology was normal in the PBS treated normal mice (Figure 3C). D-GalN/LPS treatment alone caused significant hepatic injury in mice at 36 h, where the necrosis areas were more than 50% of almost all lobules examined, and panlobular mononuclear leukocyte infiltration with cytoplasmic vacuolization and severe distortion of tissue architecture was observed (Figure 3D). However, rottlerin treated mice showed only small areas of necrotic and inflammatory cell infiltration (Figure 3E).

SphK1 dependent PKCδ activation plays an important role in ALF

Sphk1 plays a critical role in LPS-induced proinflammatory cytokine release through NF-κB activation as well as PKC-δ. We speculated that SphK1 is the

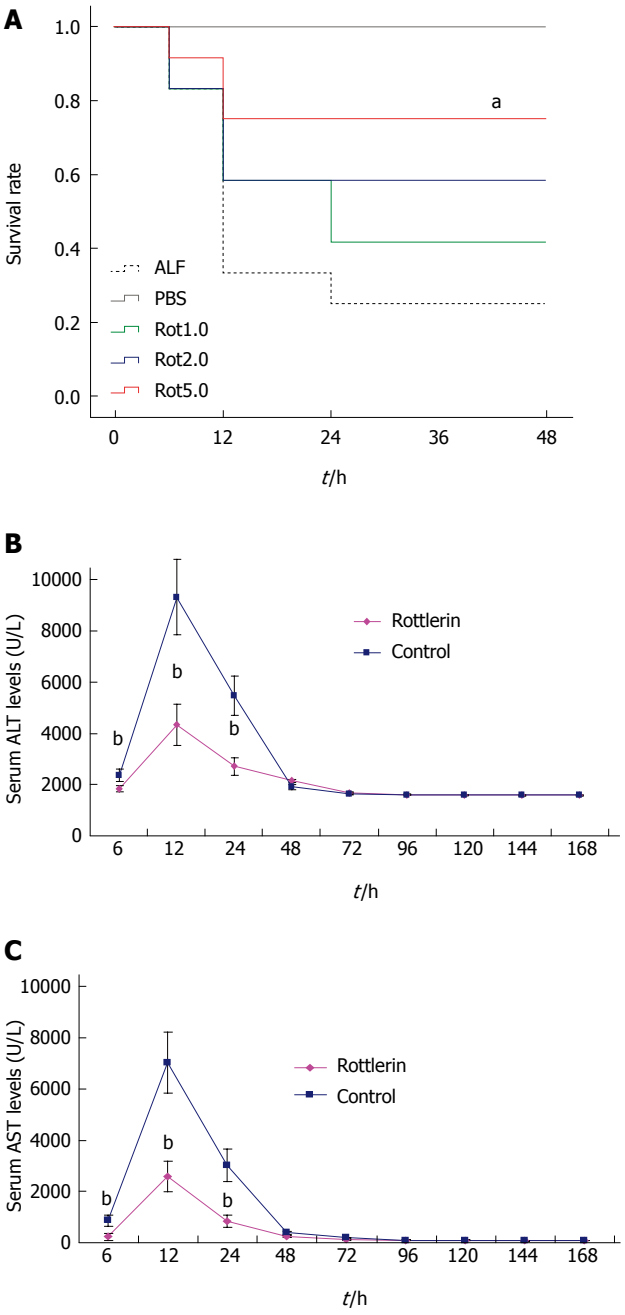

Figure 2 Inhibition of protein kinase C-δ activity with rottlerin improves survival and decreases alanine aminotransferase/aspartate aminotransferase levels in a mouse model of acute liver failure. Animals were treated with PBS or rottlerin (0, 1, 2 and 5 μmol/L) 30 min before the induction of acute liver failure with D-galactosamine/lipopolysaccharide. A: Rottlerin increased the survival rate in a dose-dependent manner. Kaplan-Meier method was used to analyze the effect of rottlerin (5 μmol/L) on the survival rates of the animals. $^{a}P = 0.003$ (log-rank test, $F = 16.023$); B and C: ALT /AST levels in peripheral blood samples collected at 6, 12, 24, 48, 72, 120, 144 and 168 h after treatment. Rottlerin treatment significantly decreased the levels of serum ALT at 6, 12 and 24 h (317.3 U/L ± 119.3 U/L, 2823.7 U/L ± 799.3 U/L, 1195.4 U/L ± 351.9 U/L *vs* 851.5 U/L ± 233.1 U/L, 7789.8 U/L ± 1473.7 U/L, 3943.8 U/L ± 760.2 U/L, $t = 4.17$, 10.26 and 11.37, respectively, $^{b}P < 0.01$) and AST at 6, 12 and 24 h (221.1 U/L ± 124.3 U/L, 2579.1 U/L ± 596.4 U/L, 827.9 U/L ± 242.6 U/L *vs* 853.7 U/L ± 212.7 U/L, 7003.2 U/L ± 1179.6 U/L, 2991.2 U/L ± 629.1 U/L, $t = 8.89$, 11.59 and 11.11, respectively, $^{b}P < 0.01$) *vs* the control group. The mean ± SE of three independent experiments is shown (error bar indicates SE). Rot: Rottlerin; ALT: Alanine aminotransferase, AST: Aspartate aminotransferase.

upstream regulator of PKC-δ activation. In order to verify this hypothesis, we first examined whether inhibition of SphK1 down-regulates PKC-δ expression and activation in liver tissue of mice with ALF. As expected, PKC-δ expression and activation were indeed inhibited in liver tissue of mice pretreated with DMS, a SphK1 specific inhibitor (Figure 4A). We next decided to examine whether SphK1 dependent PKC-δ activation results in NF-κB activation in macrophages. In LPS-stimulated macrophages, DMS as well as PKC-δ inhibitor, rottlerin, largely inhibited LPS-triggered NF-κB activation (Figure 4B). These data suggest that LPS sequentially activate SphK1 and PKC-δ, which then leads to the activation of NF-κB and subsequent generation and release of proinflammatory molecules. Finally, we examined whether SphK1 dependent PKC-δ activation plays an important role in ALF in mice. Blockade of SphK1 with 50 μmol/L DMS significantly decreased mortality (Figure 4C) and serum ALT level (Figure 4D) compared with the control group ($P < 0.01$). Taken together, these results indicate that PKC-δ activation is dependent on SphK1 activation in the ALF mouse model.

DISCUSSION

ALF is an acute inflammatory process of the liver and may lead to SIRS[15]. It can be caused by hepatotoxic drugs and diseases such as hepatitis associated cirrhosis, and complicated with other diseases[19,20]. PKC-δ has been implicated in the inflammatory response and regulates macrophage cytokine production through NF-κB activation. However, the potential role for PKC-δ in the systemic inflammatory response in ALF has not been explored. In *in vivo* experiments, compared with PBS treated normal mice, administration of LPS together with a sub-lethal dose of D-GalN induced more severe hepatic damage accompanied by necrotic changes and sever inflammation in the liver, which is similar to the manifestation of human liver failure[14]. Therefore, it is of interest to further investigate the hepatoprotective potential and mechanism of PKC-δ inhibition in D-GalN/LPS-induced ALF.

PKC-δ is mainly expressed in neutrophils and macrophages. Kupffer cells (KCs) are the liver resident macrophages and constitute most of tissue macrophages. During ALF, there is a remarkable increase of activated hepatic macrophages, and partial deletion of KCs with GdCL3 attenuates D-GalN/LPS-induced ALF[21].

PKC-δ has been identified as a critical inflammatory regulator and it controls proinflammatory response through NF-κB activation[8,9]. PKC-δ inhibition prevented neutrophil adherence and migration[10]. Thus, PKC-δ may have an important regulatory role in the inflammatory response. Our current study demonstrates that expression and activation (Thr^{505}

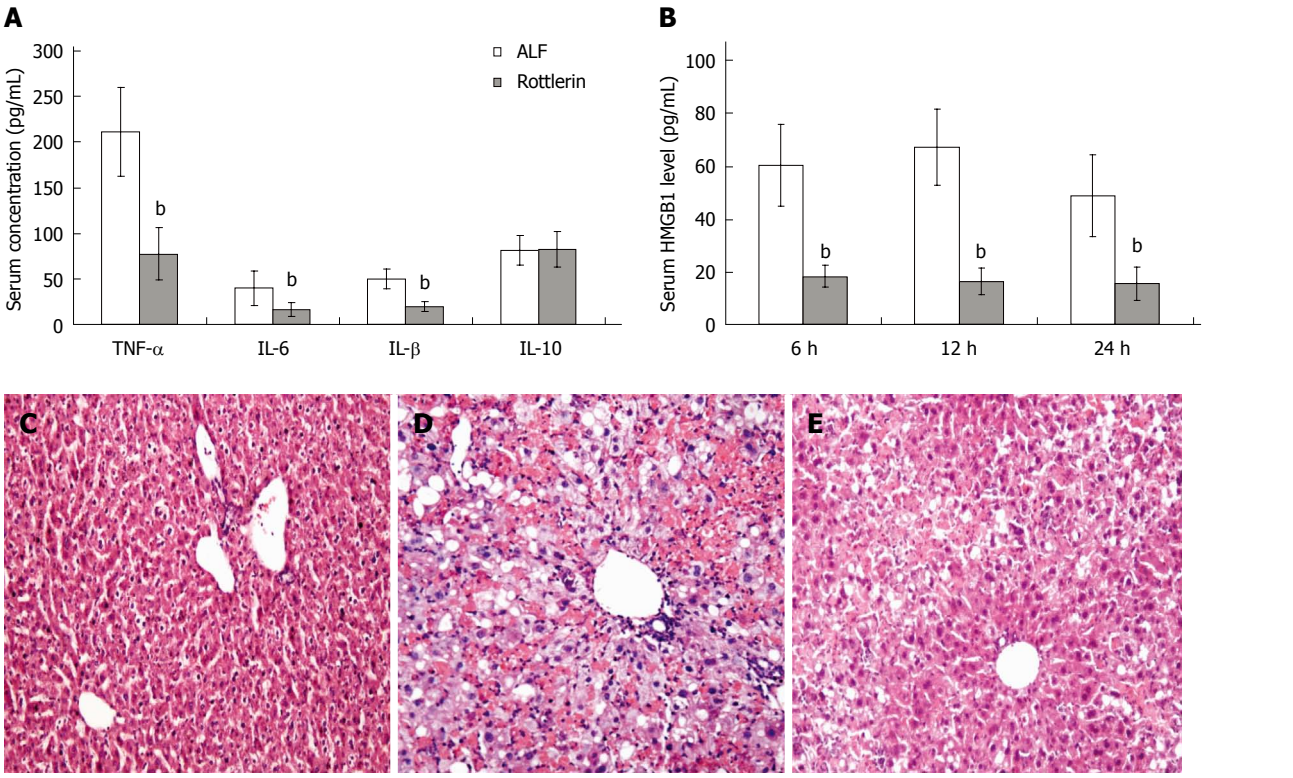

Figure 3 Inhibition of protein kinase C-δ activity with rottlerin down-regulates serum levels of inflammatory cytokines and reduces immune cell infiltration and tissue damage in mice with acute liver failure. A: Rottlerin reduced serum levels of TNF-α, IL-1β and IL-6 significantly (77.22 pg/mL ± 28.56 pg/mL *vs* 211.21 pg/mL ± 49.13 pg/mL; 15.79 pg/mL ± 7.66 pg/mL *vs* 39.83 pg/mL ± 18.78 pg/mL; 19.25 pg/mL ± 5.23 pg/mL *vs* 49.44 pg/mL ± 10.77 pg/mL, t = 8.16, 4.10 and 8.73, respectively, $^{b}P < 0.01$), but did not increase the anti-inflammatory cytokine IL-10 level at 12 h; B: Rottlerin reduced HMGB1 levels at 6, 12 and 24 h in mice (18.14 ng/mL ± 4.08 ng/mL *vs* 60.23 ng/mL ± 5.47 ng/mL; 16.21 ng/mL ± 5.11 ng/mL *vs* 67.14 ng/mL ± 14.27 ng/mL; 15.42 ng/mL ± 6.23 ng/mL *vs* 48.71 ng/mL ± 15.6 ng/mL, t = 9.13, 11.64 and 6.85, respectively, $^{b}P < 0.01$); C: Normal mice; D: Acute liver failure mice; E: Rottlerin treated mice. The mean ± SE of three independent experiments is shown (error bar indicates SE). Immune-cell infiltration and tissue damage at 36 h after induction of acute liver failure were detected by H&E staining (magnification, × 100). TNF: Tumor necrosis factor; IL: Interleukin.

phosphorylation) of PKC-δ in the liver tissue and PBMCs were promptly increased at 0.5 and 1 h after ALF induction. These results suggest that PKC-δ expression and activation in the liver are an early event in the acute phase of ALF.

To address the functional significance of PKC-δ in ALF, we treated mice with rottlerin, a PKC-δ specific chemical inhibitor. Pretreatment with rottlerin increased the survival rate in a dose-dependent manner in the D-GalN/LPS-induced ALF model. In the surviving mice, peak liver enzyme levels were detected at 12 h, and the maximum ALT/AST levels were reduced significantly in the rottlerin treatment group. Furthermore, rottlerin treated mice showed only small areas of necrotic and inflammatory cell infiltration at 36 h. However, mice treated with D-GalN/LPS alone had significant hepatic injury at the same time points, where the necrosis areas were more than 50% of almost all lobules examined, and panlobular mononuclear leukocyte infiltration with cytoplasmic vacuolization and severe distortion of tissue architecture was observed. These data demonstrate that inhibition of PKC-δ activity with rottlerin improved the survival rate, attenuated liver enzyme release, and reduced liver inflammation and necrosis in ALF mice. To the best of our knowledge, this is the first time that PKC-δ activation was found to be critical for the development of ALF.

Although etiology of ALF varies in different areas, the resulting clinical manifestations are remarkably similar, and this reflects common patterns of innate immune responses[22]. Among many others, proinflammatory cytokines (TNF-α, IL-1β and IL-6) may play a common role in the pathophysiology of ALF. In this study, inhibition of PKC-δ decreased proinflammatory cytokine levels, indicating that PKC-δ may positively participates in innate immune responses in liver injury. HMGB1 is a non-histone nuclear protein ubiquitously expressed in eukaryotes and exerts distinct functions at different subcellular localizations. Within the nucleus, HMGB1 plays an important role in the regulation of gene transcription[23]. Upon release by phagocytes and damaged/necrotic cells[24-27], extracellular HMGB1 functions as a DAMP and contributes to the pathogenesis of various inflammatory diseases[28,29]. HMGB1 exerts its effects through a number of the Toll-like receptors (TLR2/4)[27] and leads to the activation

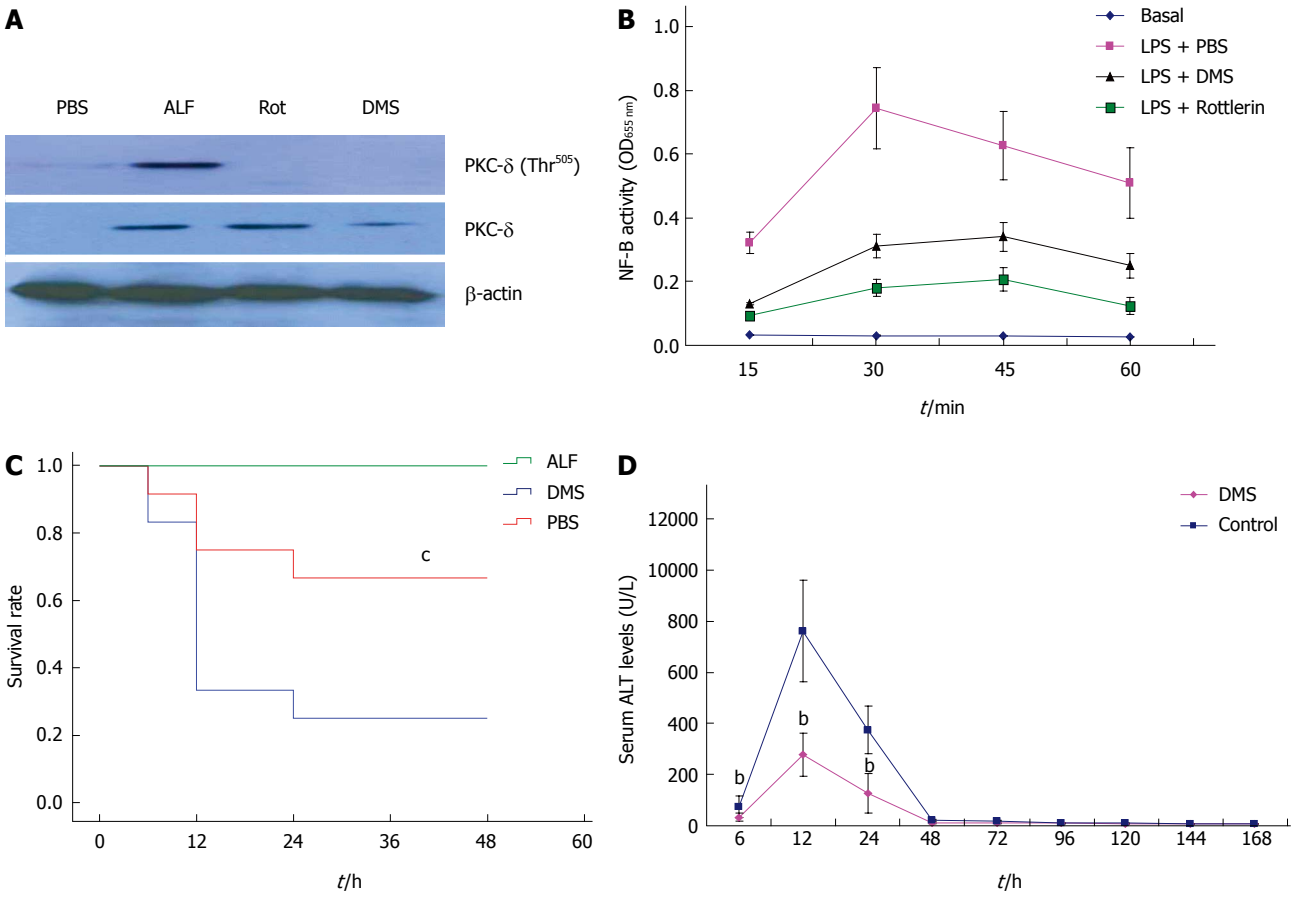

Figure 4 Sphingosine kinase 1 dependent protein kinase C-δ activation in lipopolysaccharide-stimulated macrophages and in the acute liver failure mouse model. Animals were treated with vehicle or DMS (50 μmol/L) 30 min before the induction of acute liver failure. A: Inhibition of Sphk1 activity with DMS down-regulated PKC-δ expression and phosphorylation of Thr^{505} in liver tissue of mice; B: DMS and rottlerin down-regulated NF-κB activity in LPS treated macrophages; C: Kaplan-Meier analysis of the effect of DMS on the survival rate of the animals. ^{c}P = 0.001 (log-rank test, F = 14.841); D: Serum ALT levels at 6, 12, 24, 48, 72, 120, 144 and 168 h after treatment. DMS treatment decreased the levels of serum ALT at 6, 12 and 24 h (203.1 U/L ± 56.9 U/L, 2196.3 U/L ± 676.3 U/L, 982.3 U/L ± 255.9 U/L *vs* 453.7 U/L ± 102.3 U/L, 6993.6 U/L ± 1209.5 U/L, 2991.1 U/L ± 678.5 U/L, t = 3.79, 9.52 and 8.66, respectively, ^{b}P < 0.01). The mean ± SE of three independent experiments is shown (error bar indicates SE). DMS: N,N-dimethylsphingosine; SphK1: Sphingosine kinase 1; PKC: Protein kinase C; ALF: Acute liver failure; LPS: Lipopolysaccharide.

of immune cells and consequent release of multiple proinflammatory cytokines[30]. In animal models of infection or local tissue injury, HMGB1 functions as a critical mediator of systemic or local inflammatory injury[31]. As a result, HMGB1 has been established as a late mediator of lethal systemic inflammatory disease. In the clinical setting, elevated serum HMGB1 levels have been described in patients with sepsis, pneumonia and acute pancreatitis[24,32-35], as well as ALF[36]. In this study, PKC-δ inhibitor attenuated serum HMGB1 level, indicating that HMGB1 positively participates in inflammatory responses in ALF.

Sphk1 play a critical role in LPS-induced release of proinflammatory cytokines through NF-κB activation as well as PKC-δ. This led us to speculate that SphK1 is the upstream regulator of PKC-δ activation. In our study, inhibition of SphK1 indeed down-regulated PKC-δ expression and activation in liver tissue of mice with ALF. Furthermore, DMS, a SphK1 inhibitor, as well as rottlerin, a PKC-δ inhibitor, largely inhibited LPS-triggered NF-κB activation in LPS-stimulated macrophages. These data suggest that LPS sequentially activates SphK1 and PKC-δ, which then leads to NF-κB activation and subsequently promotes proinflammatory cytokine production. Finally, blockade of SphK1 with 50 μmol/L DMS significantly decreased mortality and serum ALT level in ALF mice. Taken together, these results indicate that PKC-δ activation is dependent on SphK1 in the ALF mouse model.

In summary, PKC-δ expression and activation are up-regulated in the liver of mice with ALF and are an early event in the acute phase of ALF. PKC-δ inhibition represents a potent strategy for inhibiting the inflammatory response in ALF. This approach may attenuate liver enzyme release, reduce liver inflammation and necrosis, decrease proinflammatory cytokine levels, and ultimately improves survival in ALF. Furthermore, SphK1 dependent PKC-δ activation may play an important role in ALF. Further preclinical studies with PKC-δ inhibitors may result in the development of a new clinically applicable therapeutic strategy for ALF in the future.

COMMENTS

Background

Acute liver failure is an acute inflammatory process of the liver and may lead to systemic inflammatory response syndrome. Protein kinase C (PKC)-δ and sphingosine kinase 1 (SphK1) have been implicated in the inflammatory response and regulate macrophage cytokine production through nuclear factor (NF)-κB activation.

Research frontiers

Activation of the systemic immune response is the predominant mechanism responsible for the development of acute liver failure. PKC-δ is an important component of proinflammatory signaling pathways that regulate activation of NF-κB, which initiates inflammation in a positive-feedback loop. SphK1 is required to activate NF-κB and to induce the secretion of proinflammatory cytokines through PKC-δ activation.

Innovations and breakthroughs

PKC-δ activation was observed in liver tissue or PBMCs of mice with acute liver failure, and inhibition of PKC-δ ameliorated acute liver failure in mice. Furthermore, SphK1 was required for PKC-δ activation in acute liver failure.

Applications

Further preclinical studies with PKC-δ inhibitors may result in the development of a new clinically applicable therapeutic strategy for acute liver failure in the future.

Peer-review

In this study, the authors demonstrated PKC-δ activation in liver tissue or PBMCs and that inhibition of PKC-δ ameliorated acute liver failure in a mouse model of acute liver failure. Furthermore, SphK1 was found to be required for PKC-δ activation in acute liver failure. It is the first time that the authors demonstrated that SphK1 dependent PKC-δ activation plays an important role in acute liver failure. PKC-δ and SphK1 inhibitors may represent a new clinically applicable therapeutic strategy for acute liver failure in the future.

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ORIGINAL ARTICLE

Basic Study

Hypoxia-inducible factor-1 modulates upregulation of mutT homolog-1 in colorectal cancer

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Author contributions: Qiu Y and Zheng H contributed equally to this work; Yang H designed the study; Qiu Y and Zheng H contributed to tissue collection and review of patients' histopathology; Qiu Y, Zheng H, Sun LH, and Peng K contributed to conduction of experiments and data analysis; Zheng H and Xiao WD provided vital reagents and analytical tools and revised the manuscript.

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Conflict-of-interest statement: The authors declared that there is no conflict of interest related to this study.

Data sharing statement: No additional unpublished data are available.

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Abstract

AIM: To investigate the roles and interactions of mutT homolog (MTH)-1 and hypoxia-inducible factor (HIF)-1α in human colorectal cancer (CRC).

METHODS: The expression and distribution of HIF-1α and MTH-1 proteins were detected in human CRC tissues by immunohistochemistry and quantitative real-time polymerase chain reaction (qRT-PCR). SW480 and HT-29 cells were exposed to normoxia or hypoxia. Protein and mRNA levels of HIF-1α and MTH-1 were analyzed by western blotting and qRT-PCR, respectively. In order to determine the effect of HIF-1α on the expression of MTH-1 and the amount of 8-oxo-deoxyguanosine triphosphate (dGTP) in SW480 and HT-29 cells, HIF-1α was silenced with small interfering RNA (siRNA). Growth studies were conducted on cells with HIF-1α inhibition using a xenograft tumor model. Finally, MTH-1 protein was detected by western blotting *in vivo*.

RESULTS: High MTH-1 mRNA expression was detected

in 64.2% of cases (54/84), and this was significantly correlated with tumor stage (P = 0.023) and size (P = 0.043). HIF-1α protein expression was correlated significantly with MTH-1 expression (R = 0.640; P < 0.01) in human CRC tissues. Hypoxic stress induced mRNA and protein expression of MTH-1 in SW480 and HT-29 cells. Inhibition of HIF-1α by siRNA decreased the expression of MTH-1 and led to the accumulation of 8-oxo-dGTP in SW480 and HT-29 cells. In the *in vivo* xenograft tumor model, expression of MTH-1 was decreased in the HIF-1α siRNA group, and the tumor volume was much smaller than that in the mock siRNA group.

CONCLUSION: MTH-1 expression in CRC cells was upregulated *via* HIF-1α in response to hypoxic stress, emphasizing the crucial role of HIF-1α-induced MTH-1 in tumor growth.

Key words: Hypoxia-inducible factor-1α; Colorectal cancer; MutT homolog-1; 8-oxo-dGTP; Hypoxia

Core tip: Hypoxia is a common characteristic of solid tumors. However, the relationship between hypoxia-inducible factor (HIF)-1α and the human mutT homolog (MTH)-1 had not been clearly investigated. The present study revealed a new mechanism through which HIF-1α upregulates MTH-1 expression in colorectal cancer and provided evidence that hypoxia enhances the expression of MTH-1, likely by modulating HIF-1α protein level. These results emphasize the important role of HIF-1α-induced MTH-1 in tumor progression.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most lethal solid tumors of the gastrointestinal tract and is especially common in elderly populations. In 2008, there were an estimated 1.23 million new cases of CRC and 608700 deaths[1]. Early diagnosis results in a favorable prognosis, such that stage Ⅰ and stage Ⅱ disease have an 80%-90% 5-year survival. In contrast, 5-year survival is only 8.1% for stage Ⅳ[2]. The propensity for tumors to progress and metastasize reflects not only the oncogenic mutations in the cancer cells but also dynamic interactions between tumor cells and their local microenvironment[3]. Understanding these processes has advanced both our understanding of the underlying causes of CRC and our ability to innovate novel cancer treatment strategies.

Hypoxia is a common feature in most solid human tumors. It has been hypothesized that exposure to hypoxia is associated with oxidative stress and elevated levels of oxidatively damaged DNA [*e.g.*, 8-oxo-deoxyguanosine triphosphate (dGTP)][4]. Accumulation of oxidized bases in either nuclear or mitochondrial DNA triggers various cellular dysfunctions, including mutagenesis and programmed cell death or senescence[5]. To offset oxidative damage to nucleic acids, tumor cells are equipped with antioxidant enzymes like human mutT homolog (MTH)-1. MTH-1 is able to eliminate oxidized deoxynucleotide triphosphates (dNTPs) before they are incorporated into DNA by hydrolyzing 8-oxo-dGTP and 2-hydroxy-dATP to the monophosphate[6]. Correlation between MTH-1 expression and clinical stage was previously discussed with regard to non-small cell lung carcinomas and breast tumors[7,8]. Furthermore, it has been suggested that MTH-1 represents a molecular marker of oxidative stress and can be used to explore the relationship between oxidative stress and genomic instability[9].

The search for factors affecting the progression and behavior of tumors has revealed the importance of the microenvironment surrounding the tumor cells. Hypoxia-inducible factor (HIF)-1 is a major transcriptional factor for tumor cells growing in a low-oxygen environment[10]. HIF-1 is a heterodimer composed of an inducible HIF-1α subunit and a HIF-1β subunit. HIF-1β is a nuclear protein that is constitutively expressed and functions independently of oxygen tension. HIF-1α, in contrast to HIF-1β, is a cytoplasmic protein responsive to oxygen levels. Therefore, all HIF-1 activity is determined by the intracellular level of HIF-1α[11]. Hypoxia induces HIF-1α, which binds to the hypoxia-response elements present in target genes, controlling glucose transport, angiogenesis, erythropoiesis, and intracellular homeostasis and potentially increasing the survival of tumor cells[12]. DNA microarray analysis suggests that more than 2% of all human genes in endothelial cells are regulated by HIF-1α[13]. It is well accepted that hypoxia in the depth of solid tumors and cell survival often coexist during tumor growth and that experimental hypoxia provokes base excision repair (BER) changes in CRC cells[14]. However, little is known about hypoxic status *in vivo* and its functional relationship with the expression of MTH-1 in CRCs. Therefore, we first examined the expression and localization of HIF-1α immunohistochemically in relation to MTH-1 in CRC. Based on the topological correlation between the two molecules, we hypothesized that MTH-1 expression may be upregulated by hypoxic conditions to facilitate colorectal tumor growth. In this case, regulation of HIF-1α-induced MTH-1 expression might represent a novel therapeutic target in CRC.

MATERIALS AND METHODS

Cell culture

SW480 and HT-29 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone; Thermo Fisher Scientific, Inc., Pittsburgh, PA, United States) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, United States) and antibiotics (1% penicillin and 1% streptomycin) at 37 ℃ with 95% air and 5% CO_2. To expose cells to a hypoxic environment, cells were placed in an airtight chamber with inflow and outflow valves infused with a gas mixture (1% O_2, 5% CO_2, and 94% N_2).

Patients and tissues

Overall, 84 patients (58 males, 26 females) diagnosed with CRC at the Department of Pathology, Xinqiao Hospital, Third Military Medical University, China, were enrolled in the study. All specimens were resected surgically between 2012 and 2014, and the diagnoses were confirmed pathologically. No patient had received preoperative chemotherapy or radiotherapy. None of the patients had a known history of familial polyposis syndrome or hereditary nonpolyposis colorectal cancer syndrome. Tumor stage was defined according to the CRC staging standard by the International Union Against Cancer. All specimens were classified according to the differentiation degree: 15 cases were well differentiated, 39 were moderately well differentiated, and 30 were poorly differentiated. Each tissue was used with the approval of the Ethics Committee of the Xinqiao Hospital, Third Military Medical University, after obtaining written informed consent from the patients.

Immunohistochemical staining

The tissues were fixed in 4% paraformaldehyde, cut into 4 μm sections, treated with 0.5% hydrogen peroxide in methanol, blocked for 45 min, and subsequently incubated with anti-hMTH-1 (1:250; Abcam, Cambridge, United Kingdom), anti-HIF-1α (1:350; Abcam), or purified rabbit immunoglobulin G (IgG) (10 mg/mL; negative control) overnight at 4 ℃. Following incubation with biotinylated secondary goat anti-rabbit antibody (Zhongshan Golden Bridge, Beijing, China) and an avidin-biotin-peroxidase complex (Zhongshan Golden Bridge) for 45 min at 37 ℃, respectively, slides were colored using diaminobenzidine, and nuclei were counterstained with Mayer's modified hematoxylin and mounted with polyvinylpyrrolidone. The histological examination was performed under a light microscope (400 ×).

Transfection

Human-specific HIF-1α small interfering RNA (siRNA) and a nontargeting control siRNA were synthesized and purified by Sangon Biotech (Shanghai, China). Target sequence for human HIF-1α siRNA was 5′-GGAAATGAGAGAAATGCTTAC-3′, and target sequence for nonsilencing siRNA (mock) was 5′-AATTCTCCGAACGTGTCACGT-3′. SW480 and HT-29 cells were plated at a concentration of 8×10^5 cells per well in six-well plates on the day before siRNA transfection. After 24 h, the cells were transfected with siRNA in Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) reagent. After incubation for 6 h, medium was replaced with fresh RPMI-1640. According to the indicated time, the cells were incubated for subsequent studies.

Quantitative real-time polymerase chain reaction

Samples of tumor and normal tissue were immediately immersed in RNA later solution (Qiagen, Hilden, Germany) and stored at -80 ℃ until processed. Total RNA from cells or tissues was extracted using the commercial RNeasy mini kit (Qiagen). Total RNA was transcribed with a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) assays were performed using the Thermal Cycler Dice Real Time System Ⅱ (Takara Bio) and SYBR Premix Ex Taq Ⅱ (Takara Bio). Primer sequences used in this study were designed as follows: HIF-1α (forward 5′-GCCGCTGGAGACACAATCATA-3′ and reverse 5′-GGTGAGGGGAGCATTACATCAT-3′), MTH-1 (forward 5′-TAGTCAGCTGTTAGACTCCCTGC-3′, reverse 5′-GTGGAAAGCACACCAACAGG-3′), β-actin (forward 5′-ATCATGTTTGAGACCTTCAA-3′, reverse 5′-CATCTCTTGCTCGAAGTCCA-3′). PCR denaturing was set at 94 ℃ for 5 min, and annealing/extending was set at 59 ℃ for 1 min, for a total of 40 cycles. The specificity of the amplified PCR products was assessed by a melting curve analysis. The expression of each gene was normalized to β-actin expression in the individual samples.

Western blotting

The cells were washed twice with phosphate-buffered saline (PBS) before lysis in cold RIPA buffer (PBS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mg/mL (p-amidinophenyl) methanesulfonyl fluoride hydrochloride, 1.0 mmol/L sodium orthovandate, 1 × mammalian protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, United States). The protein concentration was determined according to the Bradford method using bicinchoninic acid (BCA) assay reagent (Beyotime, Beijing, China). Samples (25 μg protein) were resolved on 8%-12% SDS-polyacrylamide gel electrophoresis (PAGE) and then electrophoretically transferred to a polyvinylidene fluoride membranes (Millipore, Bedford, MA, United States). Membranes were blocked in 5% bovine serum albumin for 1 h at room temperature and then incubated with antibodies overnight at 4 ℃: anti-MTH-1 (1:500), anti-HIF-α (1:1000), and anti-β-actin (1:1000). The membranes were then washed three times in Tris-buffered saline with Tween

Table 1 Associations of tumor characteristics with mutT homolog-1 mRNA relative expression in colorectal cancer

Characteristics	*n* (%)	MTH-1	
		means ± SD	*P* value
Total	84 (100)		
Age (yr)			
≤ 60	39 (46.43)	3.782 ± 4.017	0.392[1]
> 60	45 (53.57)	2.720 ± 1.993	
Gender			
Male	58 (69.05)	3.002 ± 2.609	0.298[1]
Female	26 (30.95)	4.146 ± 3.449	
Differentiation degree			
Well and moderate	52 (61.90)	3.549 ± 2.971	0.209[1]
Poor	32 (39.10)	2.666 ± 3.302	
Tumor size (cm)			
≤ 3	32 (38.10)	1.673 ± 1.342	0.043[1]
> 3	52 (61.90)	4.159 ± 3.593	
Stage			
Ⅰ / Ⅱ	51 (60.71)	2.098 ± 1.627	0.023[2]
Ⅲ / Ⅳ	33 (29.29)	4.935 ± 4.028	

[1]One-way ANOVA test; [2]Mann–Whitney *U* test. MTH-1: MutT homolog-1.

(TBST) (50 mmol/L Tris-HCl pH 7.5, 140 mmol/L NaCl, 0.1% Tween) and incubated with secondary antibody at room temperature for 1 h. An enhanced chemiluminescence (ECL) reagent, ECL western blotting detection reagent (Amersham Life Sciences, Chalfont St. Giles, United Kingdom), was used to enable the labeled protein bands to be detected with Image Station 4000R (Kodak, New Haven, CT, United States).

8-oxo-dG assay

The DNA hydrolysates were dissolved in high performance liquid chromatography (HPLC) grade water and filtered through a 0.2-μm syringe filter before applying the samples to a Waters ODS HPLC column (4.6 mm × 250 mm), 5-μm particle size (Milford, MA, United States). The running buffer for 8-oxo-dGTP from nuclear and mitochondrial DNA was 50 mmol/L potassium phosphate (pH 5.1) in 5% acetonitrile, and the retention time was 7.5 min. Detection of 8-oxo-dG required an electrochemical detector (Coulochem 5100H, Interscience, Breda, The Netherlands) with a 5021 conditioning cell and 5010 analytical cell. Standard samples of dGTP and 8-oxo-dGTP were analyzed to ensure their correct separation and to allow identification of those derived from cellular DNA. Three determinations were made on each hydrolyzed pooled sample, one of them including a spike of standard 8-oxo-dGTP, and the average of the two unspiked replicates was used in the calculation of mean values shown in the Tables.

Animal study

Five-week-old female BALB/C nude mice were purchased from the Laboratory Animal Center of Third Military Medical University and were maintained in specific pathogen-free units under isothermal conditions. All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Third Military Medical University Animal Care Committee. SW480 and HT-29 cells (5×10^5) suspended in 0.1 mL serum-free medium were implanted subcutaneously into nude mice. After the tumor volume reached 50-70 mm^3, which was calculated according to the formula $V = 0.5 \times L \times S^2$ (L: long diameter; S: short diameter), the animals were divided into interference and mock groups. According to the method of Filleur *et al*[15], the mice were intraperitoneally injected with 3 μL siRNA or mock siRNA suspended in 50 μL saline three times per week. Tumors were monitored by measuring their volume with a caliper. After 48 d, animals were killed, and the tumor was dissected for further study.

Statistical analysis

All the data were repeated three times and analyzed by Graphpad Prism version 6.0 (La Jolla, CA, United States). Data of immunohistochemical staining were analyzed by using Fisher's exact probability test. One-way analysis of variance (ANOVA) and the Mann-Whitney *U* test were used to analyze quantitative data, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Relationship between transcription level of MTH-1 and clinicopathological features of CRC

MTH-1 mRNA level was measured in 84 matched pairs of CRC/adjacent histologically normal mucosa tissue samples by qRT-PCR. The tumor/normal ratio of MTH-1 expression (T/N ratio) in each case was calculated. All of the normal colorectal mucosa expressed MTH-1 mRNA in low amounts. Relative overexpression of MTH-1 mRNA (T/N ratio ⩾ 1.5) was observed in 54 of 84 (64.2%) CRCs (Table 1). The relationship between expression of the *MTH-1* gene and clinicopathological features of the CRC was examined. Stage Ⅲ/Ⅳ tumors exhibited significantly higher levels of MTH-1 mRNA relative to early-stage tumors. A significant difference was also observed for tumor size ($P = 0.043$, Table 1). No significant differences were observed in age, gender, and histological differentiation.

Correlation of MTH-1 and HIF-1α expression in CRC tissues

To investigate the association between HIF-1α and MTH-1 expression in CRC, we performed immunohistochemical staining for these proteins in tissue specimens from 84 CRC patients. Staining for HIF-1α and MTH-1 in representative clinical samples is shown in Figure 1. Among the 84 CRC specimens, 66.7% (56/84) and 60.0% (47/84) were positive for HIF-1α and MTH-1 expression, respectively. HIF-1α

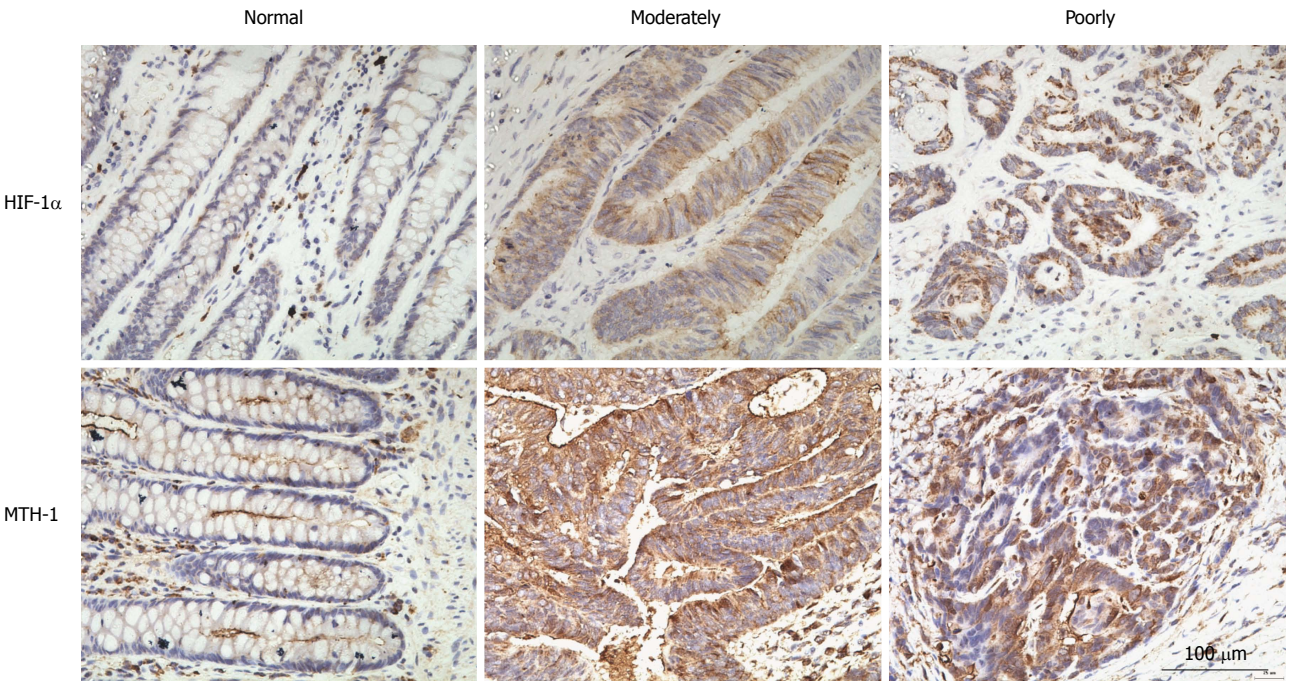

Figure 1 Images of three groups defined by immunohistochemical staining for hypoxia-inducible factor-1α and mutT homolog-1 in normally, moderately, and poorly differentiated colorectal cancer. No HIF-1α immunoreactivity was detected in normal colorectal mucosa (A). Moderate to strong HIF-1α immunoreactivity was detected in the cytoplasm of tumor cells in moderately or poorly differentiated colorectal adenocarcinoma (B and C). MTH-1 immunoreactivity of normal colon mucosa was faint in the cytoplasm, and strong cytoplasmic immunostaining was observed in lymphoid cells (D). Moderately differentiated and poorly differentiated colorectal adenocarcinoma exhibited strong cytoplasmic staining for MTH-1 protein (E and F). Original magnification, × 400. HIF-1α: Hypoxia-inducible factor-1α; MTH-1: MutT homolog-1.

Table 2 Association analysis between mutT homolog-1 and hypoxia-inducible factor-1 expression in human colorectal cancer tissue

MTH-1	HIF-1α (*n*)		Total
	Positive	Negative	
Positive	43	4	47
Negative	13	24	37
Total	56	28	84

$R = 0.640$; $P < 0.001$. MTH-1: MutT homolog-1; HIF-1α: Hypoxia-inducible factor-1α.

was predominantly expressed in the cytoplasm and nucleus of the tumor cells, while MTH-1 was largely expressed in the cytoplasm. However, there was no expression or only weak expression of both proteins in the corresponding normal tissue. The expression level of HIF-1α and MTH-1 was significantly higher in CRC tissues compared with corresponding normal tissues. Coexpression of HIF-1α and MTH-1 was detected in 43 (51.2%) patients. Spearman analysis showed that the expression level of HIF1-α was significantly associated with MTH-1 expression ($R = 0.640$, $P < 0.01$; Table 2).

Expression of MTH-1 in colon carcinoma cells after normoxic and hypoxic incubation

SW480 and HT-29 cells were incubated under normoxic and hypoxic conditions. mRNA levels of MTH-1 were determined by qRT-PCR from cells exposed to hypoxia for 0, 48, and 72 h. Hypoxia enhanced the levels of MTH-1 mRNA in a time-dependent manner (Figure 2A and C). Protein levels of MTH-1 were markedly increased after exposure to hypoxia for 48 h and 72 h (Figure 2B and D).

Effects of HIF-1α on expression of MTH-1 in colon cancer cell lines under hypoxic conditions

A major increase in MTH-1 was observed after 72 h of hypoxia, therefore, this time point was chosen for the following experiments. As shown in Figure 3A-D, transfection with HIF-1α siRNA markedly reduced the mRNA expression level of HIF-1α ($P < 0.01$) and expression of HIF-1α protein ($P < 0.01$). Upregulation of MTH-1 mRNA, induced by hypoxia, was significantly attenuated when HIF-1α expression was knocked down by siRNA (Figure 3E). Consistent with this observation, the HIF-1α siRNA also decreased MTH-1 protein expression in response to hypoxia (Figure 3F). To determine whether the downregulated expression of MTH-1 was involved in BER, levels of 8-oxo-dGTP were measured by HPLC/electrochemical detection in nucleoside mixtures prepared from DNA of two strains of CRC cells (SW480 and HT-29). These cells displayed elevated residual 8-oxo-dGTP base lesions at 48 and 72 h following hypoxia treatment. As shown in Figure 3G, transfection with HIF-1α siRNA markedly increased the level of 8-oxo-dGTP.

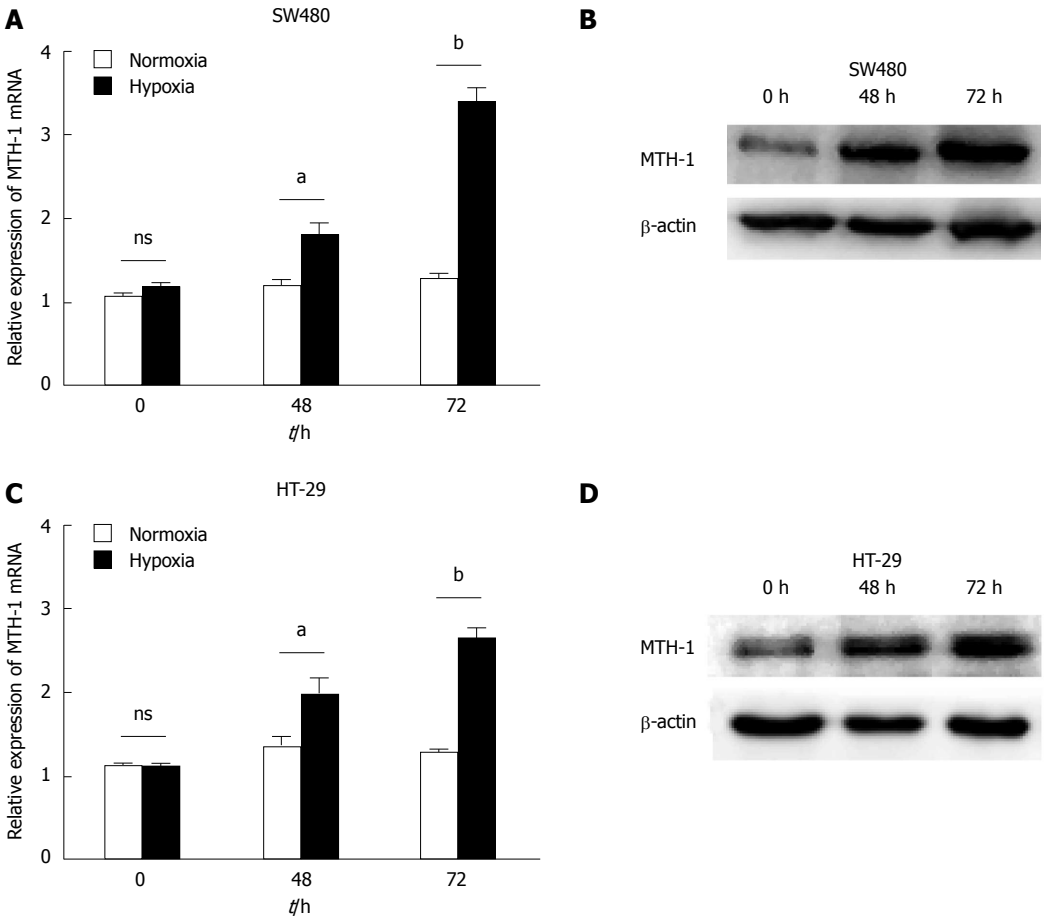

Figure 2 Hypoxic stress-induced mutT homolog-1 expression in colon carcinoma cell lines. SW480 and HT-29 cells were incubated under normal or hypoxic conditions. mRNA relative expression of MTH-1 was evaluated by qRT-PCR, as shown in A and C (48 h: $P = 0.013$, $P = 0.041$; 72 h: $P = 0.000$, $P = 0.000$). Protein levels of MTH-1 were evaluated by western blotting, as shown in B and D. $^{a}P < 0.05$, $^{b}P < 0.01$, normoxia *vs* hypoxia. MTH-1: MutT homolog-1; qRT-PCR: Quantitative real-time polymerase chain reaction.

Effects of HIF-1α on MTH-1 in the in vivo xenograft tumor model

To determine the effect of HIF-1α on MTH-1 expression and CRC tumor growth *in vivo*, SW480 and HT-29 cells suspended in serum-free medium were subcutaneously implanted into nude mice, and siRNA was intraperitoneally injected. Growth of the xenograft tumor was studied by tumor growth curve. There was a significant difference in tumor growth between the HIF-1α interference group and the mock group after 20 d (Figure 4A and B). MTH-1 protein expression was analyzed in the xenograft tumor by western blotting. As shown in Figure 4C, MTH-1 proteins showed a marked reduction in the siHIF-1α group as compared with the mock group.

DISCUSSION

Regions of acute and chronic hypoxia exist within solid tumors and can lead to increased rates of mutagenesis and/or altered DNA damage and repair protein expression. The MTH-1 protein sanitizes oxidized dNTP pools to prevent incorporation of damaged bases during DNA replication[16]. Recent studies showed that MTH-1 protein may be a potent promoter of tumorigenic growth and that MTH-1 catalytic activity is markedly increased in many cancers[17,18], which is an example of non-oncogene addiction and probably a survival response to prevent cell death induced by oxidative DNA damage. In the present study, we analyzed the expression patterns of MTH-1 mRNA in CRC. We found a relative overexpression of MTH-1 mRNA (T/N ratio $\geqslant$ 1.5) in 64.2% of CRCs. In addition, we observed that advanced-stage tumors exhibited significantly higher levels of MTH-1 mRNA relative to early-stage tumors. Although the number of cases analyzed in this study was not sufficient to draw any definitive conclusion, these results suggest that the level of oxidative stress in CRC increases with the stage of the disease. Our data are consistent with previous reports in which expression of MTH-1 showed significant correlation with aggressive features of CRC, such as tumor size and advanced stage[6]. Thus, MTH-1 seems to be a highly valuable marker that is closely associated with survival and dissemination of CRC cells, conferring a survival advantage through the inhibition of oxidative-stress-induced DNA damage.

A recent study demonstrated that expression of

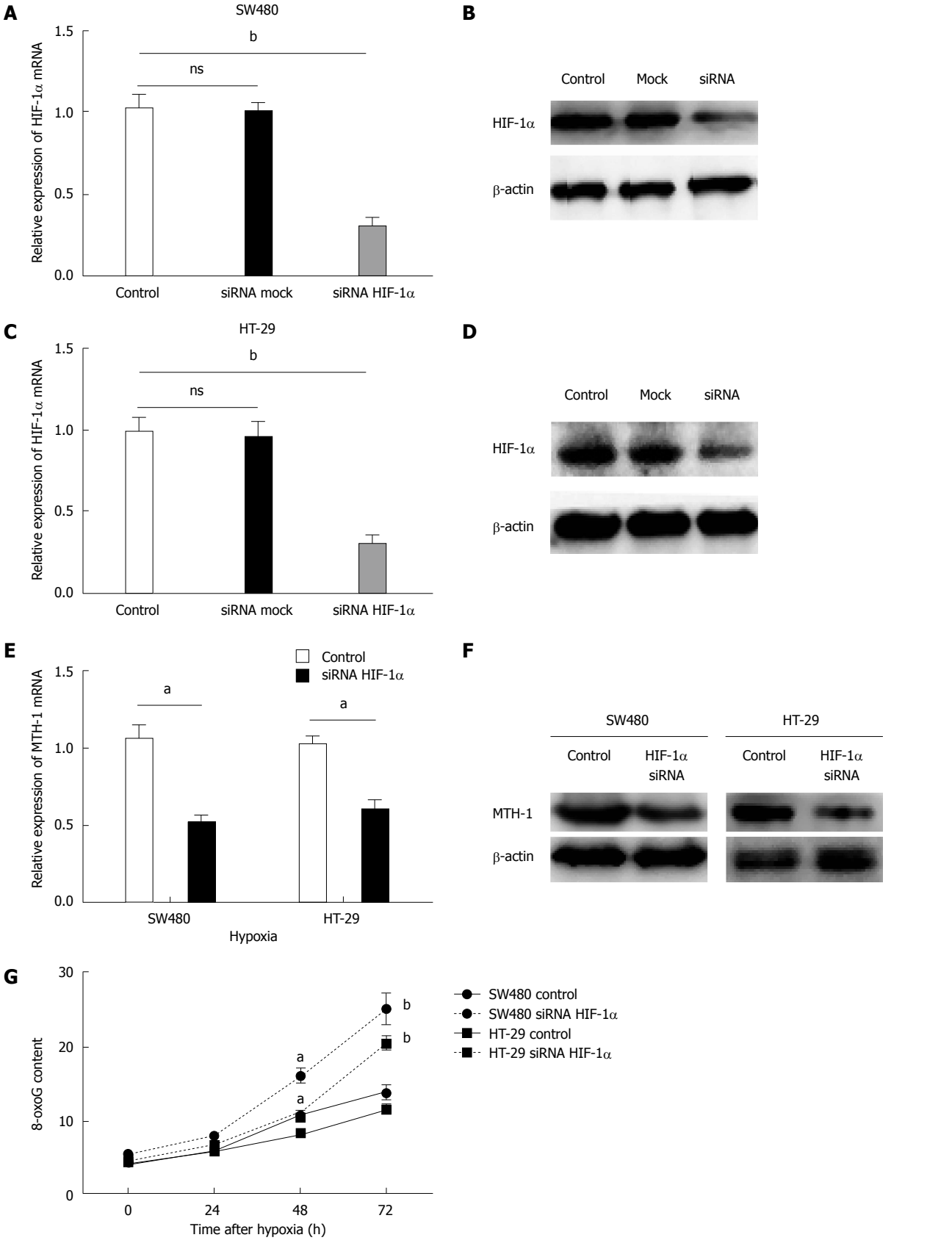

Figure 3 Hypoxia-inducible factor-1α is involved in hypoxia-enhanced expression of mutT homolog-1. SW480 and HT-29 cells transfected with either siRNA targeting HIF-1α or mock siRNA were exposed to hypoxia for 72 h or normoxia. qRT-PCR showed that siRNA targeting HIF-1α significantly inhibited expression of HIF-1α mRNA in colon cancer cells as compared to cells treated with mock siRNA(P = 0.0003, P = 0.0007) (A and C). Western blotting showed that siRNA targeting HIF-1α significantly suppressed expression of HIF-1α protein under hypoxia, but the mock siRNA group was unaffected (B and D). MTH-1 mRNA expression and MTH-1 protein were decreased by silencing of HIF-1α at 72 h (P = 0.002, P = 0.003) (E and F). The residual 8-oxo-dGTP base lesions were markedly higher in the HIF-1α siRNA groups compared to the mock siRNA groups of SW480 and HT-29 cells (G) (48 h: P = 0.011, P = 0.016; 72 h: P = 0.003, P = 0.000). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. NS: Non-significant; siRNA: Small interfering RNA; HIF-1α: Hypoxia-inducible factor-1α; MTH-1: MutT homolog-1; qRT-PCR: Quantitative real-time polymerase chain reaction.

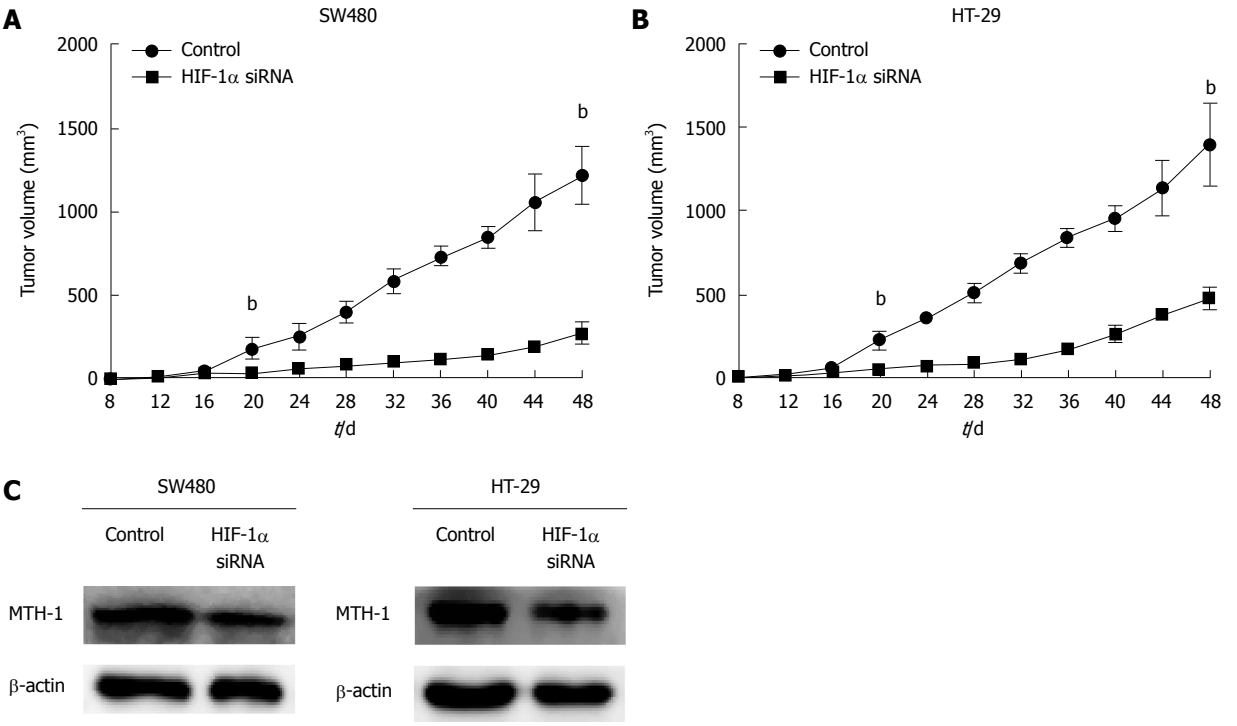

Figure 4 Effects of siRNA of hypoxia-inducible factor-1α on tumor growth and mutT homolog-1 expression in a nude mouse xenograft tumor model. Tumor volume in the mouse interference group measured by calipers was significantly lower than that in the mock group of SW480 and HT-29 cells (A and B) (20 d: $P = 0.004$, $P = 0.001$; 48 d: $P = 0.000$, $P = 0.000$). Western blotting revealed that expression of MTH-1 protein was inhibited by silencing of HIF-1α (C). $^{b}P < 0.01$, *vs* control. HIF-1α: Hypoxia-inducible factor-1α; siRNA: Small interfering RNA; MTH-1: MutT homolog-1.

BER proteins in CRC cells was adaptable and strongly dependent on the tumor microenvironment[14]. Thus, we focused on hypoxia, which has been documented in colorectal tissues, ranging from normal mucosa to benign adenoma and carcinoma[19,20]. In colonic adenocarcinoma, HIF-1α overexpression was associated with angiogenesis, invasion, and metastasis[21]. We found HIF-1α expression in 56 (66.7%) of 84 CRCs. Moreover, correlation analysis showed that expression of HIF-1α was positively correlated with that of MTH-1. In our study, expression of HIF-1α was mainly localized to the cytoplasm of tumor cells and was observed diffusely throughout the entire tumor tissue. MTH-1 immunoreactivity of normal colon mucosa was faint in the cytoplasm, and strong cytoplasmic immunostaining was observed in lymphoid cells. In contrast, MTH-1 immunoreactivity was markedly increased in the cytoplasm of tumor cells. Its immunoreactivity was stronger than that of normal mucosal epithelium. With respect to tumor biology, our results suggest that tumor cells that experience hypoxia may express MTH-1, leading to acquired genetic stability and contributing to tumor progression.

Based on the above findings, we hypothesized that hypoxia may directly or indirectly upregulate the expression of MTH-1, resulting in the tumor progression observed in CRC. In this study, we demonstrated that expression of MTH-1 was upregulated in SW480 and HT-29 cells that were exposed to hypoxic stress. Upregulation of MTH-1 mRNA and protein in SW480 and HT-29 cells was observed after exposure to 48 and 72 h of hypoxic stress. However, Chan *et al*[14] showed no significant changes in mRNA and protein levels of MTH-1 in RKO human CRC cell line after exposure to 72 h hypoxia of 0.2% O_2. They also demonstrated that the expression of *MTH-1* and other *BER* genes (*MYH* and *OGG1*) was independent of the *p53* gene in the RKO cell line. Although difference in the types of cells may account for the different cellular responses in MTH-1 expression, these results suggest that expression of MTH-1 is dynamically regulated by hypoxic stress. To confirm that MTH-1 is induced by HIF-1α, siRNA was used to decrease the expression of HIF-1α. We found that hypoxia-induced MTH-1 expression was HIF-1α dependent, as demonstrated by the ability of HIF-1α siRNA to inhibit hypoxia-induced MTH-1 upregulation in SW480 and HT-29 cells. The effect of siHIF-1α on suppression of MTH-1 protein level was limited. This may have been due to the long half-life of the MTH-1 protein[22]. However, even a minor increase in BER protein levels may have important implications for carcinogenesis and tumor progression in the colon[23]. Notably, we found that the cells in the siHIF-1α groups displayed increased residual base damage at 72 h following hypoxia. This result suggests that hypoxic stress induces the corresponding defensive mechanisms, producing higher levels of defensive mechanisms against 8-oxo-dGTP accumulation in tumor cells, as previously suggested by Kennedy *et al*[24].

In the *in vivo* xenograft tumor model, siRNA was intraperitoneally injected. Twenty days later, the tumor volume of the siHIF-1α group was smaller than that in the mock group. We also found that the reduction in HIF-1α significantly reduced the expression of MTH-1 in CRC cells *in vivo*. These results support a role for HIF-1α in mediating MTH-1 expression in CRC and imply that MTH-1 and HIF-1α together play a role in the growth of CRC. Silencing of HIF-1α facilitated residual base damage and inhibited tumor growth in this study. However, whether or not HIF-1α is a good target for the development of cancer therapeutics requires further investigation. Since it has a large number of downstream genes, HIF-1α may play a complex role in cancer biology[25,26]. Results of a recent paper validated MTH-1 as an anti-cancer target *in vivo* and described certain small molecules as first-in-class nudix hydrolase family inhibitors that potently and selectively engage and inhibit the MTH-1 protein in tumor cells[27,28]. Together, these results exemplify the non-oncogene addiction concept for anti-cancer treatment and validate MTH-1 as a lethal cancer phenotype.

The present study revealed a new mechanism through which HIF-1α upregulates MTH-1 expression in CRC and provides evidence that hypoxia increases expression of MTH-1, likely by modulating HIF-1α protein level. These results emphasize the important role of HIF-1α-induced MTH-1 in tumor progression. The dissection of the multistep regulation of *MTH-1* gene under hypoxic conditions is just beginning. Further studies are required to elucidate the mechanism by which HIF-1α drives MTH-1 upregulation.

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COMMENTS

Background

Hypoxia-inducible factor (HIF)-1 is a major transcriptional factor for tumor cells. To offset oxidative damage to nucleic acids, tumor cells are equipped with mutT homolog (MTH)-1. Hypoxia in solid tumors and cell survival often coexist during tumor growth, and hypoxia provokes base excision repair changes in colorectal cancer (CRC) cells.

Research frontiers

Recent studies have shown that MTH-1 protein may be a potent promoter of tumorigenic growth and that MTH-1 catalytic activity is markedly increased in many cancers. However, little is known about hypoxic status *in vivo* and its functional relationship with the expression of MTH-1 in CRC.

Innovations and breakthroughs

The present study revealed a new mechanism through which HIF-1α upregulates MTH-1 expression in CRC and provides evidence that hypoxia increases the expression of MTH-1, likely by modulating HIF-1α protein level. These results emphasize the important role of HIF-1α-induced MTH-1 in tumor progression.

Applications

Cancers have dysfunctional redox regulation resulting in reactive oxygen species production, damaging both DNA and free deoxynucleotide triphosphates (dNTPs). These results exemplify the non-oncogene addiction concept for anti-cancer treatment and validate MTH-1 as a lethal cancer phenotype.

Terminology

HIF-1 is a major transcriptional factor for tumor cells growing in a low-oxygen environment. MTH-1 is able to eliminate oxidized dNTPs before they can be incorporated into DNA.

Peer-review

This interesting work demonstrates the upregulation of MTH-1 expression in colorectal cancer cells *via* HIF-1α in response to hypoxic stress. Under hypoxic conditions, the authors utilized small interfering RNA for HIF-1α to show the association between HIF-1α and MTH-1.

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ORIGINAL ARTICLE

Basic Study

Fenugreek lactone attenuates palmitate-induced apoptosis and dysfunction in pancreatic β-cells

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Abstract

AIM: To investigate the effect of fenugreek lactone (FL) on palmitate (PA)-induced apoptosis and dysfunction in insulin secretion in pancreatic NIT-1 β-cells.

METHODS: Cells were cultured in the presence or absence of FL and PA (0.25 mmol/L) for 48 h. Then, lipid droplets in NIT-1 cells were observed by oil red O staining, and the intracellular triglyceride content was measured by colorimetric assay. The insulin content in the supernatant was determined using an insulin radio-immunoassay. Oxidative stress-associated parameters, including total superoxide dismutase, glutathione peroxidase and catalase activity and malondialdehyde levels in the suspensions were also examined. The expression of upstream regulators of oxidative stress, such as protein kinase C-α (PKC-α), phospho-PKC-α and P47phox, were determined by Western blot analysis and real-time PCR. In addition, apoptosis was evaluated in NIT-1 cells by flow cytometry assays and caspase-3 viability assays.

RESULTS: Our results indicated that compared to the control group, PA induced an increase in lipid accumulation and apoptosis and a decrease in insulin

secretion in NIT-1 cells. Oxidative stress in NIT-1 cells was activated after 48 h of exposure to PA. However, FL reversed the above changes. These effects were accompanied by the inhibition of PKC-α, phospho-PKC-α and P47phox expression and the activation of caspase-3.

CONCLUSION: FL attenuates PA-induced apoptosis and insulin secretion dysfunction in NIT-1 pancreatic β-cells. The mechanism for this action may be associated with improvements in levels of oxidative stress.

Key words: Fenugreek lactone; Diabetes; Oxidative stress; Insulin secretion; Apoptosis

Core tip: Fenugreek is a widely used traditional Chinese medicine that can improve hyperglycemia. The hypoglycemic active ingredients and mechanisms of fenugreek remain unclear. We studied an ingredient of fenugreek, fenugreek lactone, and our results suggest that fenugreek lactone attenuates palmitate-induced apoptosis and insulin secretion dysfunction in NIT-1 pancreatic β-cells by improving oxidative stress.

Gong J, Dong H, Jiang SJ, Wang DK, Fang K, Yang DS, Zou X, Xu LJ, Wang KF, Lu FE. Fenugreek lactone attenuates palmitate-induced apoptosis and dysfunction in pancreatic β-cells. *World J Gastroenterol* 2015; 21(48): 13457-13465 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13457.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13457

INTRODUCTION

Diabetes mellitus (DM), a metabolic disease, is characterized mainly by elevated blood glucose. Chronic hyperglycemia may produce organ damage and cause serious harm to human health. Because the worldwide prevalence of DM continues to increase, much attention has been paid to the pathophysiology and treatment of DM. On the basis of the latest reports, the incidence of diabetes among Chinese adults has reached 11.6% and the incidence of prediabetes is 50.1%[1]. Patients who develop type 2 diabetes (T2DM) account for more than 90% of these individuals. The development of T2DM is pertinent to insulin resistance and dysfunctional insulin secretion. Recent research has shown that increased pancreatic β-cell apoptosis and decreased islet β-cell mass contribute to the insulin secretion impairment observed in T2DM[2]. However, the reason for this increased β-cell apoptosis remains unclear. However, an increasing amount of evidence suggests that hyperglycemia (glucotoxicity), elevated fatty acids (lipotoxicity), and oxidative stress are closely associated with enhanced apoptosis in β-cells[3].

The mechanism by which free fatty acids (FFA) induce β-cell impairment is not fully clear. However, FFA-related oxidative stress injury has attracted extensive attention. When β-cells are exposed to FFA, they first convert FFA into triglycerides or cholesterol esters and then store them as lipid droplets inside the cytoplasm. When lipid influx exceeds the cell's storage threshold, excessive FFA may impair insulin secretion and induce apoptosis. It has been demonstrated that diglycerol (DAG), a metabolite of palmitic acid (PA), can induce the activation of the protein kinase C (PKC)/NADPH oxidase pathway, leading to the intracellular accumulation of reactive oxygen species (ROS) products[4]. Because pancreatic β-cells produce few antioxidant substances, they are more susceptible to oxidative stress[5]. Sustained production of ROS inside the cells can trigger *apoptosis via* caspase-dependent pathways and decrease the secretion of insulin, thereby impairing the mass and function of β-cells[6,7]. Therefore, antioxidants may exert a beneficial effect that protects β-cells and thereby play an important role in treating diabetes.

Fenugreek (*Trigonella foenum-graecum* L.) is a widely used traditional Chinese medicine that can improve hyperglycemia. Our previous studies also showed that fenugreek reduced oxidative stress and improved glucose and lipid metabolism by inhibiting the PKC-α/NADPH oxidase pathway in diabetic rats[8]. The hypoglycemic active ingredients and mechanisms of fenugreek remain unclear. Some studies have suggested that trigonelline and 4-hydroxy isoleucine in fenugreek improve hyperglycemia[9-11], but this evidence does not deny that other compounds also display hypoglycemic activity. Fenugreek lactone (FL), a flavor component of many foods[12], exists naturally in fenugreek, wine, Virginia tobacco, and other foods[13]. This spice is widely used in condiments, baked products, wine, caramel and coffee[14]. Some studies have revealed that FL has antioxidant activities[12]; however, its medical effect has been less studied. In the current research, we studied the impact of FL on PA-induced apoptosis and insulin secretion dysfunction in NIT-1 islet β-cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was purchased from Gibco. Sigma-Aldrich Co. provided the reagents 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), FL (purity ⩾ 97%) and PA (purity ⩾ 99%). RPMI-1640 was obtained from Thermo Fisher Scientific Co., Ltd. Trypsin was obtained from Boster Biological Technology Co., Ltd. Penicillin and streptomycin were acquired from HyClone Laboratories. Insulin radio-immunoassay reagents were obtained from HTA Co. Total superoxide dismutase (T-SOD),

glutathione peroxidase (GSH-Px) and catalase (CAT) activity assay kits and malondialdehyde (MDA) level assay kits were bought from Jiancheng Bioengineering Co., Ltd. The triglyceride detection kit was provided by Mind Bioengineering Co., Ltd. Rabbit anti-mouse p47phox, phosphorylated PKC-α, PKC-α and β-actin antibodies were provided by Abcam Company. The Prime Script RT reaction Kit, SYBR Premix Ex Taq and Trizol reagent were obtained from TaKaRa Bio Inc. The fluorescein isothiocyanate (FITC)-labeled annexin V/propidium iodide (PI) apoptosis detection kit was provided by BestBio Co.

Culture and treatment of NIT-1 cells

Mouse insulinoma NIT-1 cells were acquired from Tongji Medical College. NIT-1 islet cells were cultured with 1640 medium (containing 11.1 mmol glucose) replenishing 100 μg/mL streptomycin, 100 units/mL penicillin and 10% FBS in a humid environment with 5% CO_2/95% air at 37 ℃. At approximately 70% confluence, the NIT-1 cells were further incubated with or without FL and 0.25 mmol/L palmitate for 48 h.

Cell viability assays and interventions

NIT-1 cells were seeded at 8 × 10^3 cells per well in 96-well culture plates and incubated for 24 h. After cells had adhered, the medium was replaced by medium containing the experimental drugs at the indicated concentrations for 24 h. The viability of NIT-1 cells in wells containing different concentrations of FL or PA was assessed by MTT assay. After the treatments, 10 μL MTT solution was added to every well and cells were then cultured for 4 h at 37 ℃. The crystals in each well were dissolved in DMSO and the absorbance was measured at 570 nm using a Synergr2 Almighty Microplate Reader. When the survival rate was between 60%-70%, the corresponding PA concentration was chosen for modeling.

Oil red O staining and triglyceride content determination

At a density of 4 × 10^5 cells/well, NIT-1 pancreatic cells were seeded in 6-well plates and cultured for 48 h exposed to 0.25 mmol/L PA in the model group. In the treatment group, the medium also contained FL (1 μmol/L). When the culture period was over, the cells were washed twice with PBS and immobilized with 4% paraformaldehyde for 30 min. After two rinses with double distilled water, the cells were dyed with oil red O at 37 ℃ for 30 min and soon afterwards decolorized in 60% isopropanol for 5 s. Cells were then stained with hematoxylin for 1 min. After washing and the observation of the lipid droplets in NIT-1 cells, pictures were taken using a light microscope. The triglyceride (TG) contents were determined as previously described[15].

Reactive oxygen species measurement

When the treatment period ended, cells were collected following trypsin digestion. Cells were washed twice in PBS, and 2 × 10^7 cells were suspended in 400 μL PBS. An ultrasonic cell disrupter was then used to break the cells. MDA levels, T-SOD, CAT activity and GSH-Px activity in the suspensions were tested according to the manufacturer's protocols in commercial kits. The protein concentrations in the suspension were measured using BCA assays (Kerui Institute of Biotechnology, Wuhan, China).

Insulin content measurement

Cells were seeded into 6-well plates at 6 × 10^5 cells per well. After treatment, the cells were pre-incubated with glucose-free medium for 30 min. Then, high-glucose (25 mmol/L) DMEM medium was substituted for the glucose-free medium and cells were incubated for another 2 h. The supernatant was collected for further measurements. Insulin was determined with an insulin radio-immunoassay kit. Each experiment was repeated five times. The insulin concentration was corrected according to the number of NIT-1 pancreatic cells.

Apoptosis determination

As previously described[16], a fluorescein isothiocyanate (FITC)-labeled annexin V/propidium iodide (PI) apoptosis detection kit was used to analyze apoptosis in cells in accordance with the instructions. The cells were collected after EDTA-free trypsin digestion. After two washes in cold PBS, the cell pellet was collected *via* centrifugation at 1000 *g* for 10 min. The number of cells was adjusted to 1 × 10^6 cells/mL. Then, the NIT-1 cells were resuspended in binding buffer, stained lucifugally with PI for 15 min at 4 ℃ and stained with FITC-labeled annexin V at 4 ℃ for 5 min away from light. FACSCalibur flow cytometer (BD LSR II, San Jose, CA, United States) was used to perform the Flow cytometric analysis. The percentages of apoptotic cells were calculated using CellQuest software (Becton, Dickinson and Co.). The quadrant containing annexin V-positive and PI-negative cells represented the early phase of apoptosis, while the quadrant containing cells positive for both annexin V and PI represented the late phase. The total ratio of apoptotic cells was calculated by the sum of cells in the early and late stages of apoptosis. A caspase-3 viability kit was also used to measure apoptosis in NIT-1 cells, as previously described[17].

Western blot analysis

After centrifugation and determination of total protein concentrations, NIT-1 cell supernatant extracts in RIPA lysis buffer were combined with sample buffer and heated in boiled water for approximately ten minutes. Then, 80 μg of the protein extraction was separated on a 10% SDS-PAGE gel (120 V, 1.5 h), and the proteins on the gel were thereafter transferred onto nitrocellulose (NC) membranes. The NC membranes were blocked using 5% non-fat milk powder in dis-

Table 1 Primers for real-time PCR assay

Gene	Forward	Reverse
β-actin	5'-CATCCGTAAAGACCTCTATGCCAAC-3'	5'-ATGGAGCCACCGATCCACA-3'
p47phox	5'-ATAACGGGCGTAGGGAGTCT-3'	5'-AGCAAAGTCTCCGCATCACT-3'
PKC-α	5'-GTGCAAGGAACACATGATGG -3'	5'-ACGCCCACCAATCTACAGAC-3'

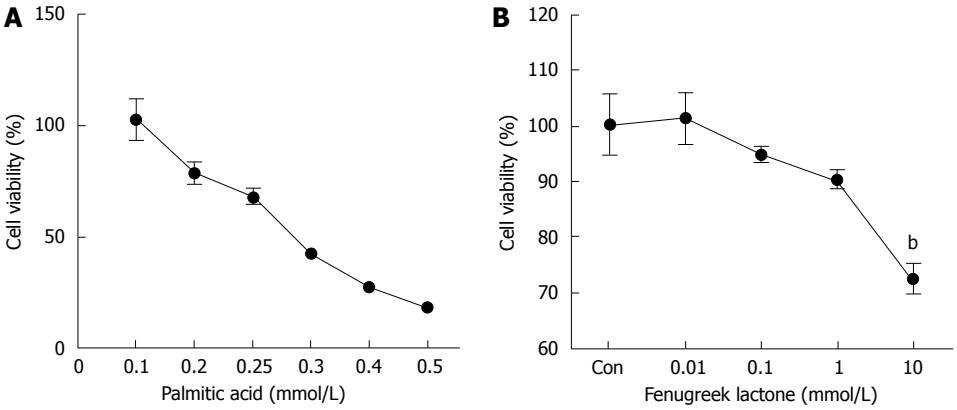


Figure 1 Viability of pancreatic NIT-1 β-cells exposed to various concentrations of palmitic acid (A) and fenugreek lactone (B). To select the appropriate concentration of palmitate (PA), dose-response experiments were conducted (A). When NIT-1 cells were cultured in medium with 0.25 mmol/L PA for 24 h, the survival rate was between 60%-70%. (B) NIT-1 cells were treated with various concentrations of fenugreek lactone (FL) (0.01-10 μmol/L) for 24 h. The maximum non-toxic concentration of FL is 1 μmol/L. Data represent the mean ± SD. $^{b}P < 0.01$ *vs* PA group.

tilled water for 1 h at normal temperature and then incubated at 4 ℃ overnight with primary antibodies (p47phox, phosphorylated PKC-α, PKC-α, and β-actin). After washing the membranes using TBST three times for 10 min each, the membranes were lucifugally incubated with a fluorescent secondary antibody at normal temperature for 1 h. Thereafter the membranes were washed with TBST lucifugally four times. Finally, protein immunoreactivity was measured using Odyssey near-infrared laser imaging apparatus. The ratio between the OD value of target band and that of β-actin was used to quantify the band densities.

Extraction of total RNA and RT-PCR

In the light of the manufacturer's directions, total RNA in NIT-1 cells was extracted using Trizol reagent. A Nucleic Acid/Protein Analyzer was used to determine the purity and concentration of the RNA. The reverse transcription of extracted total RNA (1 μg) was performed in a total reaction volume of 20 μL on a Mastercycler gradient PCR instrument using a PrimeScript RT reaction Kit. Before PCR amplification, the cDNA was stored at -80 ℃. Real-time fluorescence quantitative PCR reactions were manipulated with SYBR Premix Ex Taq enzyme and StepOne Real-Time PCR System. The $2^{-\Delta\Delta CT}$ method was employed to analyze the results. Primer sequences are all listed in Table 1.

Statistical analysis

All outcomes represent at least three independent experiments. All of the results are displayed as the mean ± SD and were analysed with SPSS 20.0 software. The statistical significance was determined by one-way analysis of variance (ANOVA). A LSD test was also employed when analyzing the data, and $P < 0.05$ suggested that a statistical difference exists.

RESULTS

Optimal concentrations of FL and PA in NIT-1 pancreatic β-cells

In this study, PA was used to induce oxidative stress in NIT-1 β-cells. After 24 h of treatment with PA, cell viability decreased in a dose-dependent manner. Compared to the controls (untreated cells), the cells treated with 0.25 mmol/L PA for 24 h showed a 34.68% decrease in cell viability (Figure 1A).

To determine the concentration of FL that could be used without affecting the viability of the NIT-1 pancreatic β-cells, we also performed an MTT assay. The cells were seeded in 96-well plates and therein cultured for 24 h. Different concentrations of FL were then added to the cells followed by incubation for 24 h. FL was well-tolerated up to a concentration of 1 μmol/L. At concentrations higher than this, FL decreased cell viability by 27.49% (Figure 1B). Thus, we concluded that the optimal concentration of FL for use in further experiments was up to 1 μmol/L.

FL decreases PA-induced intracellular lipid accumulation in NIT-1 cells

To measure the influence of FL on lipid accumulation in NIT-1 islet cells, oil Red O dyeing and colorimetric assays

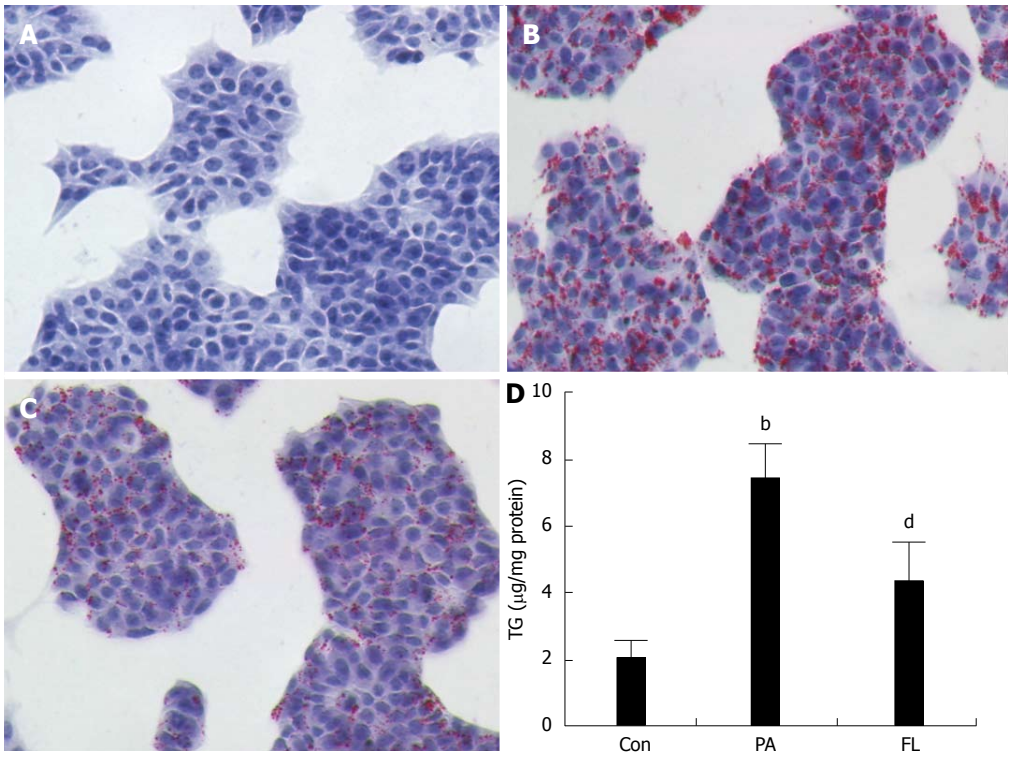


Figure 2 Effects of fenugreek lactone on palmitate-induced lipid accumulation in NIT-1 pancreatic β-cells. A-C: Oil Red O staining of lipid droplets showed that palmitate (PA) induced a palpable increase in lipid accumulation, which was inhibited by the fenugreek lactone (FL) treatment (A: Control group; B: PA group; and C: FL-treated group); D: Intracellular triglyceride (TG) content extracted from NIT-1 cells. FL decreased intracellular TG content in NIT-1 pancreatic β-cells compared with PA group. CON refers to control group, PA refers to palmitic acid intervention group and FL refers to FL-treated group. Data are reported as the mean ± SD (n = 4). ^{b}P < 0.01 *vs* control group; ^{d}P < 0.01 *vs* PA group.

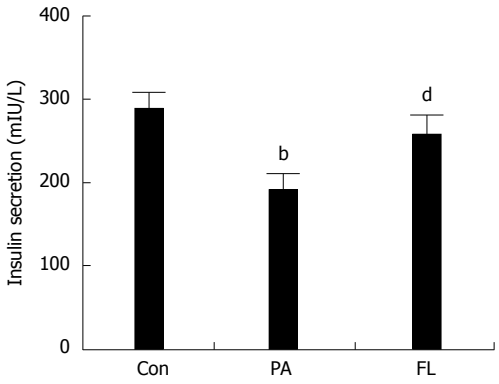


Figure 3 Effects of fenugreek lactone on glucose stimulated insulin secretion in NIT-1 pancreatic β-cells exposed to palmitate. Insulin content in the supernatant was measured using a radio-immunoassay kit after the treatment. Insulin concentration was corrected with cell numbers. Data represent the mean ± SD from six wells. ^{b}P < 0.01 *vs* control group; ^{d}P < 0.01 *vs* PA alone. PA: Palmitate; FL: Fenugreek lactone.

were applied. As shown in Figure 2, cellular lipid droplets in the FL group were diminished significantly compared to the PA group. In addition, intracellular TG contents were also markedly decreased (P < 0.01) in the FL group compared to cells in the PA group (Figure 3).

FL attenuates PA-induced insulin secretion dysfunction in NIT-1 cells

To ascertain whether PA damages insulin secretion in pancreatic islet cells, a radioimmunoassay kit was used to test insulin concentrations in the supernatants of the medium from different cell treatments. As shown in Figure 3, PA-treated NIT-1 cells exhibited decreased insulin levels compared to the control group (P < 0.01). However, FL significantly inhibited this effect (P < 0.01).

FL decreases MDA levels and increases the antioxidants T-SOD, CAT and GSH-PX in NIT-1 pancreatic β-cells

As shown in Table 2, NIT-1 cells treated with PA showed increased MDA levels and decreased T-SOD, GSH-PX and CAT levels compared with levels in the control group (P < 0.01). FL treatment markedly decreased the levels of MDA and increased the levels of SOD, CAT and GSH-PX (P < 0.01 or P < 0.05).

FL inhibits caspase-3 activity and apoptosis in NIT-1 cells after exposure to PA

Caspase-3 is a crucial enzyme for apoptosis and cell survival. The activation of caspase-3 triggers the cell to undergo apoptosis. Therefore, we determined whether FL inhibits caspase-3 activation and PA-induced apoptosis in NIT-1 β-cells. Double staining using FITC-labeled annexin V and PI was performed. The result showed increased caspase-3 activity (Figure 4) and an increased apoptotic rate (Figure 5) in the PA group (P < 0.01). However, co-treatment with 1 μmol/L FL markedly inhibited caspase-3 activity and PA-induced

Table 2 Comparison of intracellular malondialdehyde accumulation and contents of antioxidants (total superoxide dismutase, glutathione peroxidase and catalase) in different groups

	CAT (U/mg protein)	GSH-PX (U/mg protein)	T-SOD (U/mg protein)	MDA (nmol/mg protein)
Control	8.68 ± 0.89	71.67 ± 8.08	3.44 ± 0.11	0.13 ± 0.06
PA	4.33 ± 1.10[b]	22.46 ± 8.49[b]	2.77 ± 0.10[b]	0.91 ± 0.30[b]
FL	7.13 ± 0.58[d]	39.58 ± 4.90[a]	3.03 ± 0.12[d]	0.41 ± 0.10[d]

Data represent mean ± SD. [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* PA group and [a]$P < 0.05$ *vs* PA group. MDA: Malondialdehyde; T-SOD: Total superoxide dismutase; GSH-Px: Glutathione peroxidase; CAT: Catalase.

apoptotic cell death ($P < 0.01$) (Figures 4 and 5).

FL decreases PKC-α, phosphorylated PKC-α and p47phox gene expression in NIT-1 pancreatic β-cells

As shown in Figures 6 and 7, the protein and mRNA levels of PKC-α and p47phox were significantly increased in NIT-1 cells that were treated with PA compared with cells in the control group ($P < 0.01$). But there was a significant decrease in the expression of p47phox and PKC-α at both the protein and mRNA levels in the FL group. The protein levels of phosphorylated PKC-α also increased significantly ($P < 0.01$) following treatment with PA alone but were decreased by co-treatment with FL ($P < 0.01$).

DISCUSSION

T2DM, especially when combined with obesity, is always associated with the excessive release of FFA from proliferative adipose tissue, which leads to an increase in the plasma FFA concentration[18]. According to the Paris Prospective Study, a high plasma FFA concentration is a risk factor for the deterioration of glucose tolerance, with a relative risk of approximately 1.3 per 0.12 mmol/L change in the level of FFA[19].

Circulating FFA can sustain basal pancreatic β-cell function and assure efficient insulin secretion during fasting. FFA in islets has also been viewed as beneficial during the hypersecretion of insulin to protect against glucose intolerance in the early stages of obesity and diabetes[20]. However, under certain circumstances, it has the opposite or even a deleterious effect. When pancreatic beta cells were exposed to excessive quantities of FFA for long periods of time, islet cells malfunctioned and the biosynthesis and secretion of insulin were reduced *in vitro* in both rats and humans[21,22]. In addition to inhibiting insulin secretion, elevated plasma FFA might play a causal role in pancreatic β-cell apoptosis[22,23]. Apoptosis was observed in pancreatic cells that were exposed to 0.5 mmol/L PA for 48 h[6]. The PA concentration was 0.25 mmol/L in our present research, but our results were consistent with previous findings.

Oxidative stress has been considered to be involved

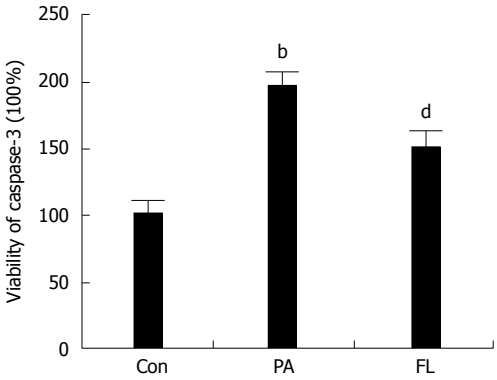
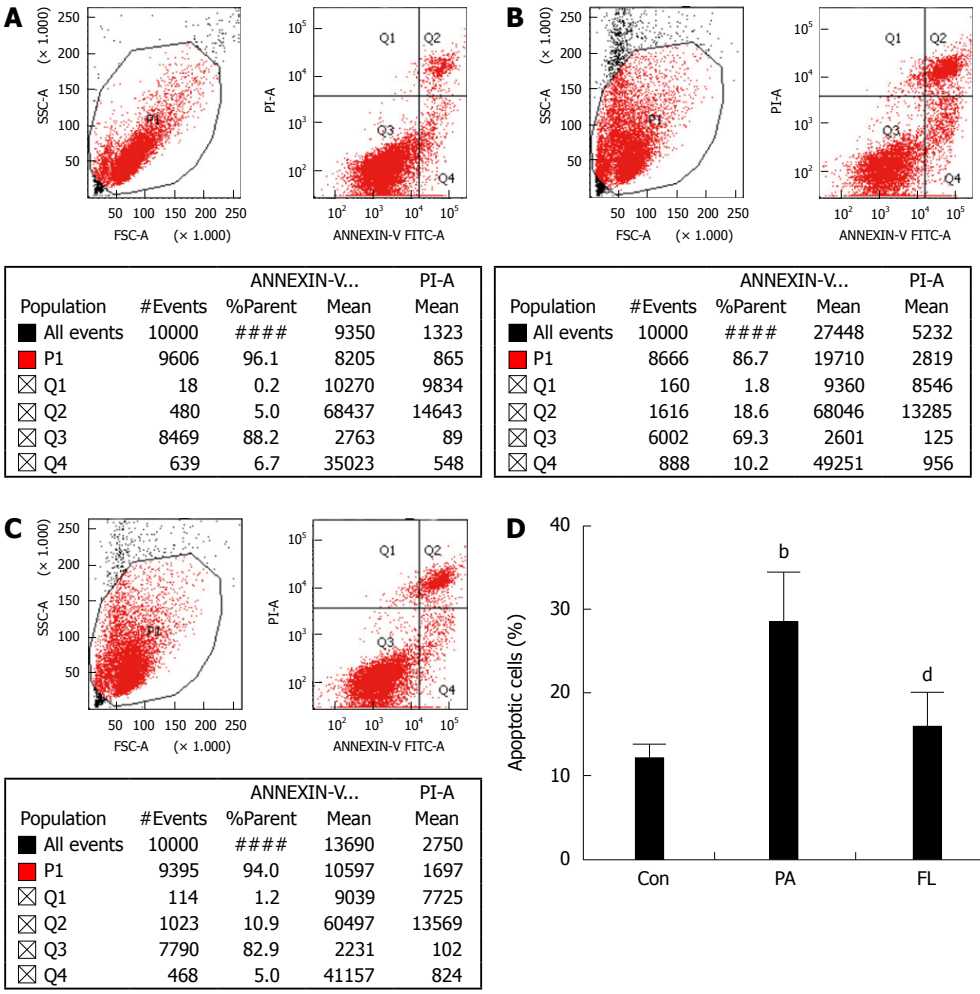


Figure 4 Effects of fenugreek lactone on caspase-3 viability in NIT-1 islet β-cells exposed to palmitate. Each bar represents mean ± SD; [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* PA group. PA: Palmitate; FL: Fenugreek lactone.

in the pathogenesis of islet β-cell dysfunction and apoptosis induced by prolonged exposure to FFA[6]. FFA has been shown to initiate the production of ROS in islet β-cells in a number of studies. PA, at high doses, can pass through the cell membrane and into the mitochondria, where oxidative reactions occur[24]. During metabolic clearance of PA, β-oxidation and oxidative phosphorylation produce large amounts of ROS, and antioxidants are reduced in pancreatic islet β-cells[25]. It is widely accepted that excessive levels of ROS damage cells directly by oxidizing lipids, DNA and proteins. Our study also suggests that MDA, a lipid peroxidation product, showed raised levels and that antioxidant levels were diminished in pancreatic β-cells that were stimulated by PA.

How FFA induces oxidative stress is unknown. DAG is not only a metabolite of FFA but also a potent activator of PKC[26]. PKC-α-dependent NADPH oxidase might serve as a possible critical source of ROS in response to stimulation by PA[27]. The factor p47phox is a subunit that is critical for the activity of NADPH oxidase, which catalyzes the production of many ROS, breaks normal redox homeostasis, and results in cellular dysfunction and damage by oxidative stress[28]. Our results also confirm that PA provokes oxidative stress through the PKC-α/NADPH oxidase pathway. New therapeutic strategies aimed at reducing oxidative stress have attracted wide attention. Several lines of evidence have shown the potential value of antioxidant remedies and their advantageous impacts on islet cell protection[29]. For example, the antioxidant N-acetylcysteine prevented the decrease in insulin content that was induced by oleate in MIN6 islet cells[30].

Fenugreek is a traditional Chinese herb for the treatment of diabetes. Increasing experimental and clinical studies have supported the hypoglycemic effect of fenugreek, and a meta-analysis of human experiments also demonstrated the hypoglycemic effectiveness of fenugreek[31]. The active ingredients in fenugreek are not clear. FL [3-amino-4,5-dimethyl-2(5H)-furanone] is widely used as a flavor in foods due to its strong smell. However, its medical function is less well studied. Several



Population	#Events	%Parent	ANNEXIN-V... Mean	PI-A Mean
All events	10000	####	9350	1323
P1	9606	96.1	8205	865
Q1	18	0.2	10270	9834
Q2	480	5.0	68437	14643
Q3	8469	88.2	2763	89
Q4	639	6.7	35023	548

Population	#Events	%Parent	ANNEXIN-V... Mean	PI-A Mean
All events	10000	####	27448	5232
P1	8666	86.7	19710	2819
Q1	160	1.8	9360	8546
Q2	1616	18.6	68046	13285
Q3	6002	69.3	2601	125
Q4	888	10.2	49251	956

Population	#Events	%Parent	ANNEXIN-V... Mean	PI-A Mean
All events	10000	####	13690	2750
P1	9395	94.0	10597	1697
Q1	114	1.2	9039	7725
Q2	1023	10.9	60497	13569
Q3	7790	82.9	2231	102
Q4	468	5.0	41157	824

Figure 5 Comparison of apoptosis rates of NIT-1 cells by flow cytometry in different groups. A: Control group; B: PA intervention group; C: FL-treated group; D: Data are reported as the mean ± SD of percentages from four wells. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* PA group. PA: Palmitate; FL: Fenugreek lactone.

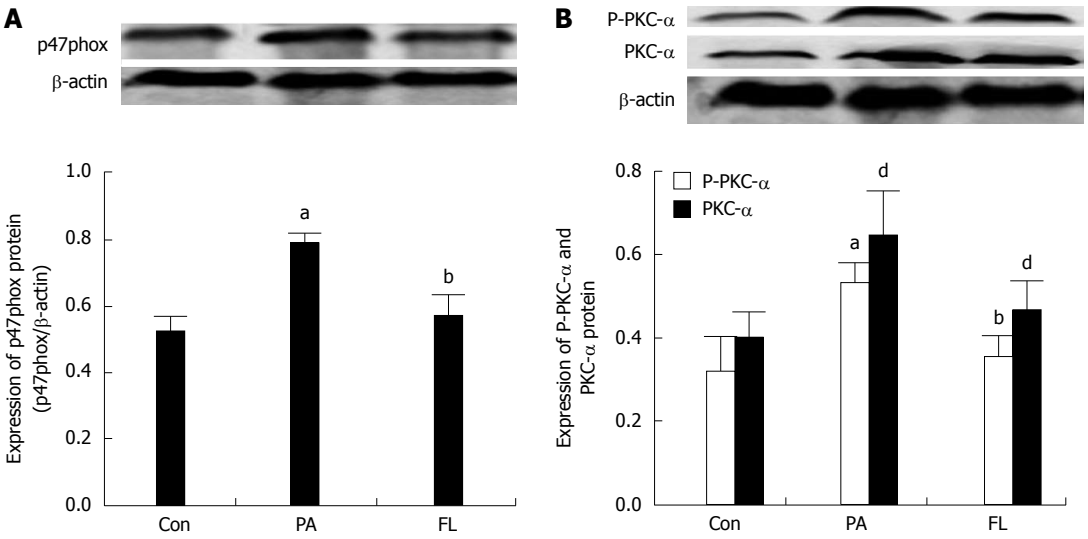


Figure 6 Effects of fenugreek lactone on protein expression of protein kinase C-α, P-protein kinase C-α and p47phox in NIT-1 β-cells exposed to palmitate. Each bar is described as corresponding proteins/β-actin. FL supplementation inhibited the expression of above mentioned proteins. Representative gels and protein levels for (A) p47phox and PKC-α or P-PKC-α (B) were measured by Western blot in NIT-1 pancreatic β-cells. Data are shown as the mean ± SD. $^{a}P < 0.01$ *vs* control group; $^{b}P < 0.01$ *vs* PA group; $^{d}P < 0.01$ *vs* PA group. PA: Palmitate; FL: Fenugreek lactone.

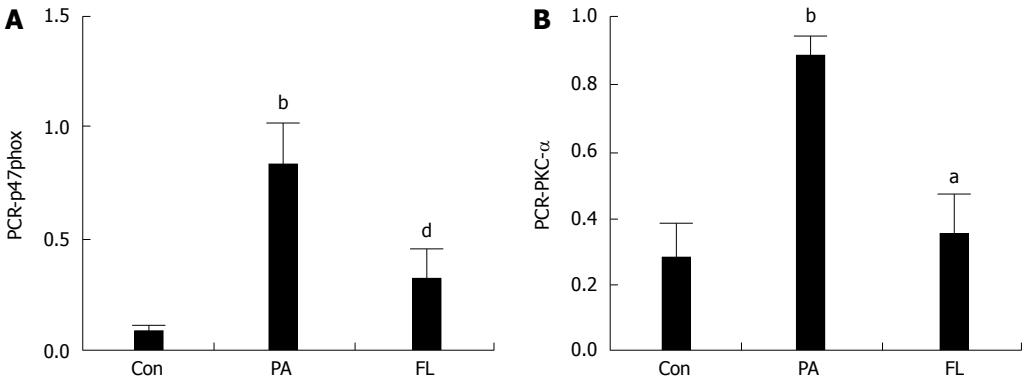


Figure 7 Effects of fenugreek lactone on mRNA levels of protein kinase C (A) and p47phox (B) in NIT-1 β-cells. Each bar represents mean $2^{-\Delta\Delta CT}$ ± SD (n = 3). $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* PA group; $^{a}P < 0.05$ *vs* PA group. PA: Palmitate; FL: Fenugreek lactone.

furanones have been shown to display anticancer activity in mice and antioxidative effects to improve human health[12]. In our prior studies, fenugreek and Hu-Lu-Ba-Wan reduced the accumulation of oxidation products, improved insulin secretion and protected the function of the kidneys and testis in STZ-induced diabetic rats[8]. In these experiments, PA-treated NIT-1 cells were more prone to damage to their anti-oxidative defense systems, and administration of FL significantly augmented decreases in the levels of GSH-PX, CAT and T-SOD. FL also promoted insulin secretion and reduced apoptosis in islet β-cells by inhibiting the PKC-α/NAPDH oxidase pathway. This might be the mechanism for the protective effect of FL in NIT-1 pancreatic cells. Our findings may provide a new perspective on the use of FL as a potential new therapeutic medicine for preserving pancreatic β-cell mass and function.

However, some limitations remain. FFA can damage islet cells indirectly by activating several stress-sensitive signaling pathways, such as the JNK/Akt, NF-κB and p38MAPK pathways[6]. We did not examine other downstream pathways. Tests using DHE staining to show the activity of NADPH might be used to clarify the mechanisms of PA-induced oxidative stress. Some furanone compounds increase the DNA damage caused by oxidative stress, but FL relieves oxidative stress. The mechanism by which FL increases the activity of antioxidant enzymes remains to be further described. Other effective ingredients of fenugreek also need more study.

In brief, we have shown that 48-h treatment with PA impaired NIT-1 pancreatic β-cells *via* oxidative stress. The PA-induced lipid overload, decrease in insulin secretion and increase in cell apoptosis were prevented by FL. FL inhibited the PKC-α/NADPH oxidase pathway and protected islet cells from injury by oxidative stress. FL may be a promising antioxidant for use as a treatment for DM.

COMMENTS

Background

Fenugreek is a widely used traditional Chinese medicine which can improve hyperglycemia. However, the mechanism underlying its hypoglycemic effect remains not fully clarified. In the present study, we investigated the effect of fenugreek lactone (FL), a component of fenugreek, on palmitic acid (PA)-induced apoptosis and insulin secretion dysfunction in NIT-1 pancreatic β cells.

Research frontiers

It has been established that diabetes mellitus is associated with oxidative stress. Previous studies have shown that FL reduced oxidative impairment, but its medical effect has been less studied.

Innovations and breakthroughs

Whether FFA improves or decreases insulin secretion is a controversial question. The present study suggests that 48 h of incubation with a high-dose of FFA restrains insulin secretion. In addition, this is the first study to evaluate the effect of FL on FFA-induced impairment of pancreatic cells. The results showed that FL attenuates PA-induced increased apoptosis and decreased insulin secretion in pancreatic β-cells. The mechanism for these actions may be related to improvements in oxidative stress.

Applications

FL might be promising as an antioxidant for the treatment of diabetes mellitus.

Peer-review

This is a good piece of work in which investigators have demonstrated the effect of fenugreek lactone on PA-induced apoptosis and insulin secretion dysfunction in pancreatic NIT-1 β-cells. This work has been well planned and results properly discussed.

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ORIGINAL ARTICLE

Basic Study

Effect of gingerol on colonic motility *via* inhibition of calcium channel currents in rats

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Author contributions: Cai ZX and Tang XD contributed equally to this work; Guo HS designed the research; Cai ZX, Tang XD and Wang FY performed the research; Duan ZJ, Li YC and Qiu JJ analyzed the data and revised the manuscript; Guo HS and Cai ZX wrote the paper.

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Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate the effect of gingerol on colonic motility and the action of L-type calcium channel currents in this process.

METHODS: The distal colon was cut along the mesenteric border and cleaned with Ca^{2+}-free physiological saline solution. Muscle strips were removed and placed in Ca^{2+}-free physiological saline solution, which was oxygenated continuously. Longitudinal smooth muscle samples were prepared by cutting along the muscle strips and were then placed in a chamber. Mechanical contractile activities of isolated colonic segments in rats were recorded by a 4-channel physiograph. Colon smooth muscle cells were dissociated by enzymatic digestion. L-type calcium currents were recorded using the conventional whole-cell patch-clamp technique.

RESULTS: Gingerol inhibited the spontaneous contraction of colonic longitudinal smooth muscle in a dose-dependent manner with inhibition percentages of 13.3% ± 4.1%, 43.4% ± 3.9%, 78.2% ± 3.6% and 80.5% ± 4.5% at 25 μmol/L, 50 μmol/L, 75 μmol/L

and 100 μmol/L, respectively ($P < 0.01$). Nifedipine, an L-type calcium channel blocker, diminished the inhibition of colonic motility by gingerol. Gingerol inhibited L-type calcium channel currents in colonic longitudinal myocytes of rats. At a 75 μmol/L concentration of gingerol, the percentage of gingerol-induced inhibition was diminished by nifedipine from 77.1% ± 4.2% to 42.6% ± 3.6% ($P < 0.01$). Gingerol suppressed I_{Ba} in a dose-dependent manner, and the inhibition rates were 22.7% ± 2.38%, 35.77% ± 3.14%, 49.78% ± 3.48% and 53.78% ± 4.16% of control at 0 mV, respectively, at concentrations of 25 μmol/L, 50 μmol/L, 75 μmol/L and 100 μmol/L ($P < 0.01$). The steady-state activation curve was shifted to the right by treatment with gingerol. The value of half activation was -14.23 ± 1.12 mV in the control group and -10.56 ± 1.04 mV in the 75 μmol/L group ($P < 0.05$) with slope factors, Ks, of 7.16 ± 0.84 and 7.02 ± 0.93 ($P < 0.05$) in the control and 75 μmol/L groups, respectively. However, the steady-state inactivation curve was not changed, with a half-inactivation voltage, 0.5 V, of -27.43 ± 1.26 mV in the control group and -26.56 ± 1.53 mV in the 75 μmol/L gingerol group ($P > 0.05$), and a slope factor, K, of 13.24 ± 1.62 in the control group and 13.45 ± 1.68 ($P > 0.05$) in the 75 μmol/L gingerol group.

CONCLUSION: Gingerol inhibits colonic motility by preventing Ca^{2+} influx through L-type calcium channels.

Key words: Gingerol; Colonic motility; L-type calcium channel current; Spontaneous contraction; Longitudinal smooth muscle myocytes

Core tip: Gingerol, a non-pungent molecule, has an inhibitory effect on colonic motility. There are many ion channels and second messengers involved in this process; however, no reports have described the effects of gingerol on L-type calcium channel currents. In the present study, we found that 6-gingerol obviously inhibited spontaneous contraction of longitudinal smooth muscle by preventing Ca^{2+} influx through L-type calcium channels.

Cai ZX, Tang XD, Wang FY, Duan ZJ, Li YC, Qiu JJ, Guo HS. Effect of gingerol on colonic motility *via* inhibition of calcium channel currents in rats. *World J Gastroenterol* 2015; 21(48): 13466-13472 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13466.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13466

INTRODUCTION

Ginger, the rhizome of *Zingiber officinale* Roscoe, and its components have various pharmacological actions, including anti-inflammatory, anti-cancer, anti-oxidant, anti-platelet, anti-aggregation, anti-fungal, anti-constipation and anti-diarrheal activities[1-6]. The chemical constituents of ginger rhizomes are volatile and non-volatile pungent phytochemicals and include the biologically active components, gingerols, shogaols, paradols and zingerone[7,8].

Gingerol, a non-pungent component, has an inhibitory effect on colonic motility. Iwami *et al*[9] reported that gingerol can inhibit colonic motility without adverse effects on small intestinal motility and the cardiovascular system. The non-pungent property of gingerol makes it useful as an oral or suppository medicine for treating diarrhea and other gastrointestinal disorders[10,11]. Abnormal facilitation of gastrointestinal motility and excessive fluid secretion of the gastrointestinal tract cause diarrhea. There are many ion channels and second messengers involved in this process; however, no reports have described the effects of gingerol on L-type calcium channel currents. The purpose of the present study was to clarify the effect of gingerol on colonic motility and the role of L-type calcium channel currents in this process.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats (weighing 250 ± 50 g) were purchased from the Experimental Animal Center of Dalian Medical University. They were maintained in plastic cages at 23 ± 2 ℃ and a relative humidity of 55% ± 2% under standard conditions with a 12-h light/dark cycle (light: 7:00 AM to 7:00 PM) for 1 wk prior to performing the experiments. The experiments were approved by the Animal Care and Use Committee of Dalian Medical University.

Preparation and contraction recording of smooth muscle strips

Male Wistar rats bred at the Experimental Animal Center of Dalian Medical University (Dalian, China) were anesthetized with ethyl ether prior to cervical exsanguination. The distal colon was cut along the mesenteric border and cleaned with Ca^{2+}-free physiological saline solution (PSS). Muscle strips (about 3 mm × 10 mm) were removed and placed in Ca^{2+}-free PSS, which was oxygenated continuously. Following the careful removal of the mucosa and submucosa by dissection, smooth muscle strips were obtained. Longitudinal smooth muscle samples were prepared by cutting along the muscle strips and were then placed in a chamber. One end of the strip was fixed on the lid of the chamber by a glass claw, and the other end was attached to an isometric force transducer (TD-112S, Japan) to record contraction. The chamber (5 mL volume) was constantly perfused with pre-Tyrode's solution at 1 mL/min. The temperature of the chamber was maintained at 37.0

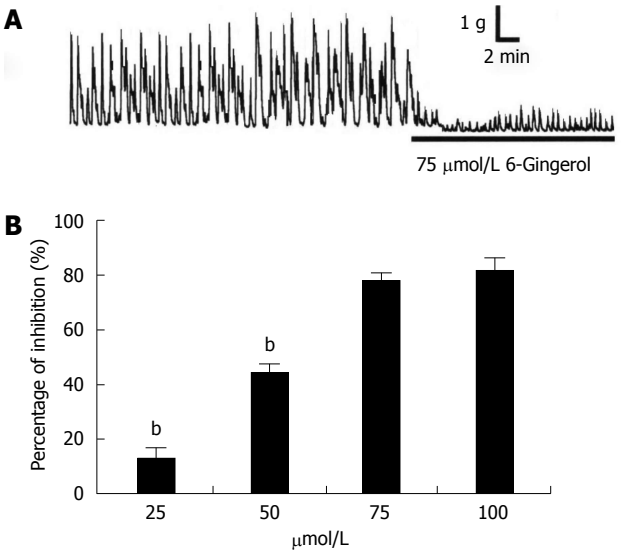

Figure 1 Effect of 6-gingerol on spontaneous contraction of colonic longitudinal smooth muscle in rats. A: The original electrophysiological data of 6-gingerol on spontaneous contraction; B: The inhibitory effect of 6-gingerol on spontaneous contraction at different concentrations. n = 8, $^{b}P < 0.01$ *vs* consecutive high concentration group.

± 0.5 ℃ by a water bath thermostat (WC/09-05, Chongqing, China). The muscle strips were incubated for at least 40 min before beginning the experiments.

Preparation of cells

Colon smooth muscle cells (SMCs) were dissociated by enzymatic digestion. The colon tissue was pinned to the Sylgard surface of a Petri dish, and the mucosa was carefully dissected away under an anatomical microscope. The smooth muscle strips were then cut into small strips (2 mm × 2 mm), placed in 2 mL calcium-free PSS supplemented with 0.12% (w/v) collagenase (type Ⅱ), 0.2% soybean trypsin inhibitor and 0.2% bovine serum albumin, and incubated for 20-30 min at 37 ℃. The tissue pieces were rinsed in Ca^{2+} free PSS solution five times to remove the collagenase enzymes and were maintained at 4 ℃ for 6 h until use. Single SMCs were isolated by several gentle triturations through the tip of a free-polished Pasteur pipette.

Whole-cell patch clamp recordings

L-type calcium currents were recorded using the conventional whole-cell patch-clamp technique. Patch-clamp pipettes were manufactured from borosilicate glass capillaries (GC 150T-7.5, Clark Electromedical Instruments, London, United Kingdom) using a 2-stage puller (PP-83, Narishige, Tokyo, Japan). When filled with pipette solution, the resistance of the patch pipette was 3-5 MΩ. The isolated myocytes were transferred to a small chamber on the stage of an inverted microscope (IX-71 Olympus, Japan) for 10-15 min and were well-attached to the bottom of the chamber. Then, the chamber was continuously superfused with PSS. An 8-channel perfusion system (L/M-sps-8, List Electronics, Germany) was used to change the perfusate. Whole-cell currents were recorded with an EPC-10 amplifier (EPC, Germany). All experiments were performed at room temperature (20-25 ℃).

Drugs and solutions

Tyrode's solution contained the following (in mmol/L): 147 NaCl, 4 KCl, 1.05 $MgCl_2·6H_2O$, 0.42 $CaCl_2·2H_2O$, 1.81 $Na_2PO_4·2H_2O$ and 5.5 glucose. The Ca^{2+}-free PSS contained the following (in mmol/L): 134.8 NaCl, 4.5 KCl, 5 glucose and 10 HEPES; the pH was adjusted to 7.4 with Tris (hydroxymethyl) aminomethane. The modified K-B solution contained the following (in mmol/L): 50 L-glutamate, 50 KCl, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2·6H_2O$, 10 glucose, 10 HEPES, and 0.5 egtazic acid (pH adjusted to 7.40 with KOH). The extracellular solution contained the following (in mmol/L): 127 NaCl, 5 TEA, 4 $NaHCO_3$, 0.33 NaH_2PO_4, 10 HEPES, 1.8 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 5 Na_2-pyruvate (pH adjusted to 7.4 with NaOH). The pipette solution contained the following (in mmol/L): 126 CsCl, 1 $MgCl_2$, 10 HEPES, 3.1 Mg·ATP, 5 Na_2-phosphocreatine, and 0.42 Li_2·GTP; the pH was adjusted to 7.2 with CsOH. Nifedipine and gingerol were purchased from Sigma Chemical Co. (United States).

Statistical analysis

All data are expressed as mean ± SD. The Duncan's multiple range test was used. Differences were considered to be significant at *P*-values of less than 0.05.

RESULTS

Effect of gingerol on spontaneous contraction of colonic smooth muscle

Spontaneous contraction of the muscle strips usually appeared after approximately 40 min of incubation in Tyrode's solution. The effects of 75 μmol/L gingerol on the spontaneous contraction of colonic longitudinal smooth muscle were observed. After administering gingerol, spontaneous contraction was significantly inhibited (Figure 1A, n = 8). Different concentrations of gingerol obviously inhibited the spontaneous contraction of the muscle strips in a dose-dependent manner, with inhibition percentages of 13.3% ± 4.1%, 43.4% ± 3.9%, 78.2% ± 3.6% and 80.5% ± 4.5% at 25 μmol/L, 50 μmol/L, 75 μmol/L and 100 μmol/L, respectively (Figure 1B, n = 8).

Effect of L-type calcium channel blocker on gingerol-induced inhibition of spontaneous contraction of colonic smooth muscle

To further investigate the mechanism of the gingerol-induced inhibition of spontaneous contraction, the effect of gingerol on gastric motility was observed

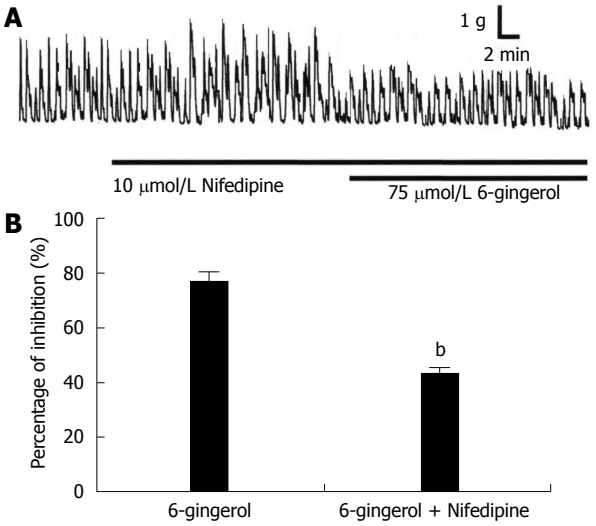

Figure 2 Effect of nifedipine on gingerol-induced inhibition in rat colonic longitudinal smooth muscle. A: The original electrophysiological data of nifedipine on gingerol-induced inhibitory effects in longitudinal smooth muscle contraction; B: The statistical diagram of the inhibition. n = 6, $^{b}P < 0.01$ *vs* gingerol group.

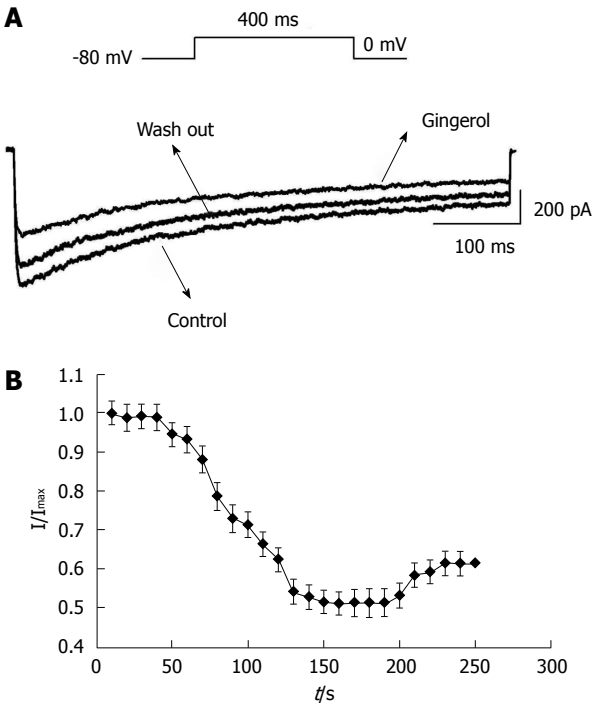

Figure 3 Time dependency of the effect that 6-gingerol acts on I_{Ba} in colonic longitudinal smooth muscle in rats. A: Raw traces of I_{Ba} at 0 mV; B: Peak responses of I_{Ba} at 0 mV normalized and averaged for cells exposed to 6-gingerol.

in the presence of nifedipine (10 μmol/L), an L-type calcium channel blocker. Nifedipine was found to diminish the gingerol-induced colonic motility inhibition. At a 75 μmol/L concentration of gingerol, the percentage of gingerol-induced inhibition was diminished by nifedipine from 77.1% ± 4.2% to 42.6% ± 3.6% ($P < 0.01$) (Figure 2, n = 6).

L-type calcium channel current and I_{Ba} in colonic longitudinal myocytes of rats

The membrane potential was clamped at -80 mV, and an I_{Ca} was elicited by a step voltage pulse from -60 mV to +60 mV for 400 ms at 10 s intervals. With an external solution containing 2 mmol/L $CaCl_2$, an L-type calcium current could be conducted under whole-cell configuration. The peak value of the L-type calcium current appeared at 0 mV, and turnover potential appeared between +50 mV and +60 mV. The amplitude of I_{Ca} was relatively small. After replacing 2 mmol/L Ca^{2+} with 10 mmol/L Ba^{2+} in the external solution, an I_{Ba} was elicited under the same stimulus modality. The shapes of the I_{Ca} and I_{Ba} I-V curves were the same. However, the amplitude of I_{Ba} was much larger than that of I_{Ca}. This indicates that I_{Ba} is a better carrier of Ca^{2+} than I_{Ca}.

Effect of gingerol on I_{Ba}

Under whole-cell configuration, the membrane potential was clamped at -80 mV, and an I_{Ba} was elicited by a single step command pulse from -80 to 0 mV for 400 ms at 10 s intervals. The time-course showed that I_{Ba} was immediately inhibited by the addition of gingerol (50 mmol/L), and within approximately 150 s this inhibitory effect had stabilized. The inhibitory percentage was 51.28% ± 2.12%, and I_{Ba} recovered partially after washout with normal control superfusing solution (Figure 3). Gingerol significantly decreased I_{Ba} in the I-V relation curve at every depolarized command step potential from -20 to +40 mV. Gingerol suppressed I_{Ba} in a dose-dependent manner, and the inhibition rates were 22.7% ± 2.38%, 35.77% ± 3.14%, 49.78% ± 3.48% and 53.78% ± 4.16% of control at 0 mV, respectively, at concentrations of 25 μmol/L, 50 μmol/L, 75 μmol/L and 100 μmol/L (Figure 4).

Effect of gingerol on steady-state inactivation and activation of I_{Ba}

A double-pulse protocol was used to measure the steady state inactivation of I_{Ba} as a function of membrane potential. Prepulse potentials ranging from -100 to +40 mV were applied for a duration of 3.75 s. Following a 7 ms interpulse interval at a potential of -60 mV, the membrane potential was raised to a test potential of 0 mV for 1 s. The currents were then normalized to the current obtained at -100 mV (I/I_{max}) and plotted against each prepulse potential. The plotted data were well fitted by the Boltzmann equation, with a half-inactivation voltage, 0.5 V, of -27.43 ± 1.26 mV in the control group and -26.56 ± 1.53 mV in the 75 μmol/L gingerol group ($P > 0.05$, n = 6), and a slope factor, K, of 13.24 ± 1.62 in the control group and 13.45 ± 1.68 ($P > 0.05$) in the 75 μmol/L gingerol group (Figure 5A). The steady-state activation curves were estimated from the peak

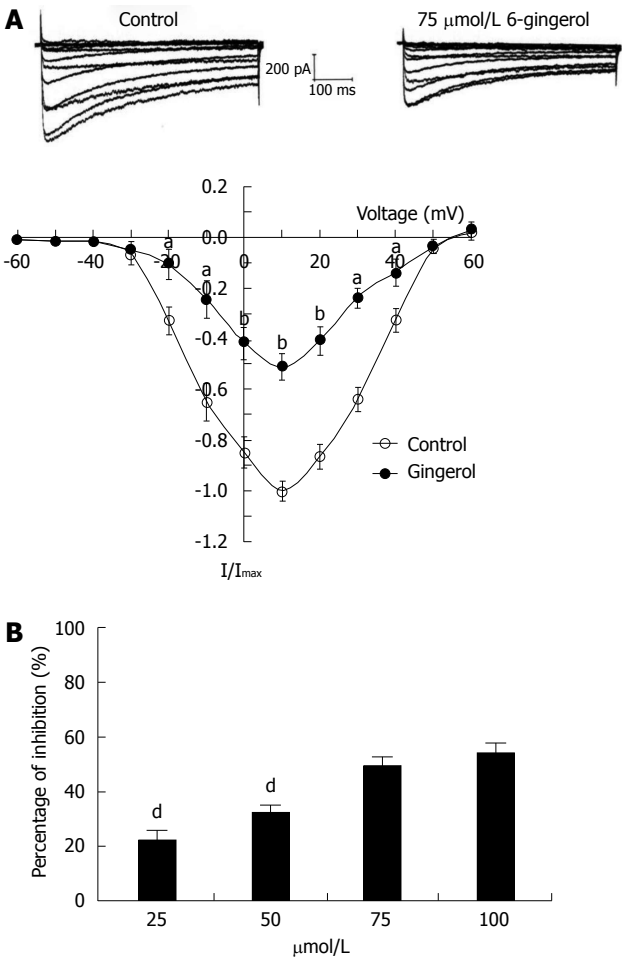

Figure 4 Effect of 6-gingerol on IBa in colonic longitudinal smooth muscle in rats. A: Raw traces of IBa elicited by step pulse and I-V relationship of IBa when cell was exposed to 6-gingerol; B: The inhibitory effect of 6-gingerol at different concentrations. $n = 8$, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* consecutive high concentration group.

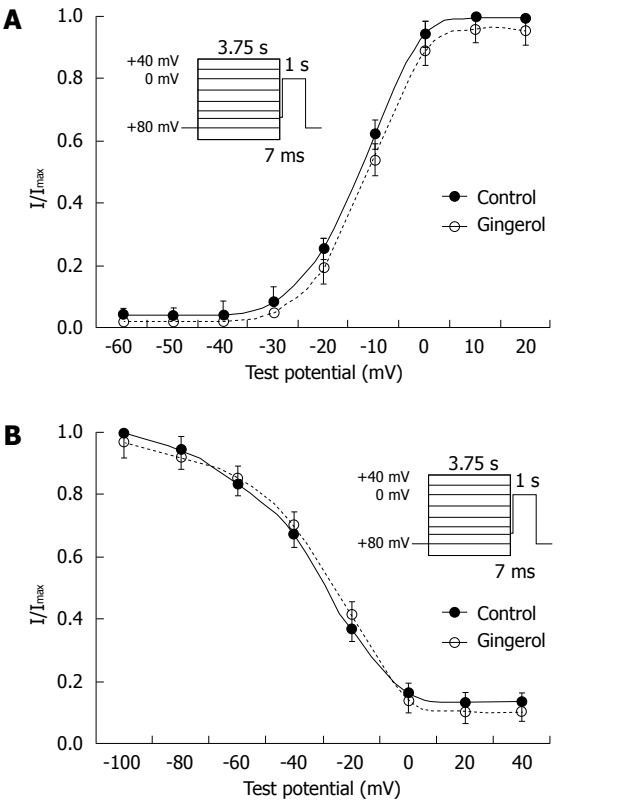

Figure 5 Steady state activation and the steady state inactivation curves. A: Steady state activation for the cells exposed to 6-gingerol, peak conductance was determined from the peak inward currents, corrected for the change in driving force at each of the test potentials and normalized to 1. Driving force was obtained from the difference between the test potential and the observed reversal potential; B: Steady state inactivation relationship. Peak currents were obtained using a two-pulse protocol (3.75 s of prepulse potential from -100 to +40 mV) followed by a 7-ms interpulse interval at -60 mV, and the membrane potential was raised to a test potential of 0 mV for 1 s. The difference between peak current and late current present before and at the end of the test pulse was normalized to 1 and plotted against the prepulse potential.

conductance at each potential using the following equation: $I_{Ba} = g_{Ba}$ (V - Vrev), where g_{Ba}, V and Vrev are peak conductance, test potential and observed reversal potential, respectively. The value of half activation was -14.23 ± 1.12 mV in the control group and -10.56 ± 1.04 mV in the 75 μmol/L group ($P < 0.05$, $n = 6$) with slope factors, Ks, of 7.16 ± 0.84 and 7.02 ± 0.93 ($P < 0.05$) in the control and 75 μmol/L groups, respectively (Figure 5B).

DISCUSSION

In the present study, gingerol inhibited the spontaneous contraction of colonic longitudinal smooth muscle in a dose-dependent manner. Nifedipine (10 μmol/L), an L-type calcium channel blocker, diminished the inhibition of colonic motility by gingerol. Furthermore, gingerol suppressed I_{Ba} in a dose-dependent manner. Gingerol treatment of colonic longitudinal myocytes of rats resulted in the activation curve being shifted to the right, while the inactivation curve did not change.

Ginger has many uses in many of the world's medicinal systems[1,12,13]. More commonly, ginger has been traditionally used in disorders of the gastrointestinal tract, as a stomachic, laxative, sialagogue, antiemetic and anti-dyspeptic agent, gastric emptying enhancer and appetizer, and as an antidiarrheal and anti-colic agent[14]. Gingerol, which is not a natural component of ginger, is a reduced analogue of gingerone. Most previous studies of gingerol have focused on its anti-cancer, anti-oxidant and anti-inflammatory properties[1]. Nigam *et al*[15] reported that the anti-cancer properties of 6-gingerol are mediated by its induction of apoptosis. A previous study indicated that 6-gingerol significantly reduced the DNA strand breaks and micronuclei formation caused by patulin (PAT). Moreover, 6-gingerol effectively suppressed PAT-induced intracellular ROS formation and decreased 8-OHdG level. GSH depletion induced by PAT in HepG2 cells was also attenuated by 6-gingerol pretreatment. These findings suggest that 6-gingerol has a strong protective ability against the genotoxicity caused by PAT and that the antioxidant activity of 6-gingerol may play an important role in attenuating the genotoxicity

of PAT[16]. Nonn *et al*[17] reported that 6-gingerol can up-regulate MKP5 and decrease cytokine-induced p38-dependent pro-inflammatory changes.

Recent studies have examined the effect of a component of ginger on diarrhea. It has been demonstrated that zingerone inhibits enterotoxin-induced fluid secretion in the ileum in mice[18]. Because excessive fluid secretion by the gastrointestinal tract causes diarrhea, zingerone is likely the active constituent of ginger that is responsible for its antidiarrheal activity. In addition to excessive secretion, abnormal facilitation of gastrointestinal motility is another cause of diarrhea. It has been reported that ginger also has suppressive effects on gut motility. Crude ginger extract inhibits rat ileal motility *via* the inhibition of enteric neural excitatory transmission and smooth muscle mechanical activity *in vitro*[19]. Ghayur and Gilani reported inhibitory effects of ginger crude extract on high K^+-induced contractions in isolated guinea pig colons[14]. Furthermore, herbal medicines that include ginger extracts inhibit colonic motility in rats[10,20].

In order to investigate the effect of 6-gingerol on colonic motility and the role of L-type calcium channel in the process, in the present study, we observed the effect of 6-gingerol on spontaneous contraction of colonic longitudinal smooth muscle at different concentrations. The results indicated that spontaneous contraction was significantly inhibited by gingerol. It is in agreement with that of David Banji[21]. Furthermore, 6-gingerol obviously inhibited spontaneous contraction in a dose dependent manner. The inhibition percentage was not significantly different between the 75 μmol/L and 100 μmol/L gingerol treatments, indicating that the gingerol concentration of 100 μmol/L is near to its highest effective dose. As we know, there is a close relationship between calcium and muscle contraction. We therefore utilized nifedipine (10 μmol/L), an L-type calcium channel blocker. At a gingerol concentration of 75 μmol/L, after administering nifedipine, the percentage of gingerol-induced inhibition was diminished from 77.1% ± 4.2% to 42.6% ± 3.6%. This indicates that gingerol inhibited spontaneous contraction by inhibiting calcium influx *via* L-type calcium channels, at least in part. This was also shown at a cellular level. These results indicate that the calcium influx *via* L-type calcium channels was diminished by gingerol to inhibit the spontaneous contraction of the colon in rats. Townsend *et al*[22] reported that 6-gingerol prevented Ca^{2+} influx through L-type Ca^{2+} channels to promote airway smooth muscle relaxation. Furthermore, pretreatment with 20 mmol/L ruthenium red (a ryanodine receptor antagonist) significantly diminished the initial increase of calcium caused by 6-gingerol, suggesting a mechanism of action of 6-gingerol that involves intracellular calcium store depletion. However, we do not know whether intracellular calcium stores participate in the 6-gingerol-induced inhibition of colonic contraction in rats.

Taken together, these findings indicate that 6-gingerol inhibits spontaneous contraction by preventing Ca^{2+} influx through L-type calcium channels. Future studies will investigate if intracellular calcium stores participate in this process.

COMMENTS

Background

Ginger, an herbal medicine, has been traditionally used to treat various kinds of diseases including gastrointestinal symptoms. Gingerol, a non-pungent analogue of gingerone, which is an active constituent of ginger, can effect on diarrhea *via* inhibiting colonic motility. However, the mechanism of gingerol to inhibit colonic motility is not clear.

Research frontiers

Gingerol has been reported to possess a variety of biological properties including anti-cancer, anti-oxidant, anti-inflammation, anti-aggregation, antifungal and anti-diarrhea. But the molecular mechanisms underlying the effects of gingerol on gene expression, the signaling pathway and effectual protein involved are required to elucidate.

Innovations and breakthroughs

Recent reports have highlighted that gingerol will be useful as an oral or suppository medicine for treating diarrhea and other gastrointestinal disorders. Abnormal facilitation of gastrointestinal motility and excessive fluid secretion of the gastrointestinal tract cause diarrhea. There are many ion channels and second messengers involved in this process. However, there is no report in which the effect of gingerol on L-type calcium channel current is described. The purpose of the present study is to clarify the effect of gingerol on colonic motility and the role of L-type calcium channel current in the process.

Applications

By understanding the effect of gingerol on colonic motility and the role of L-type calcium channel current in the process, this study may help us to understand the mechanism of gingerol to treat diarrhea.

Terminology

The L-type calcium channel (also known as the dihydropyridine channel, or DHP channel) is part of the high-voltage activated family of voltage-dependent calcium channel. "L" stands for long-lasting referring to the length of activation. This channel has four subunits (Cav1.1, Cav1.2, Cav1.3, and Cav1.4).

Peer-review

This is a good, solid study with some flaws in data presentation. Part of the discussion, which should refer to newly obtained data, repeats paragraphs from the results section.

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ORIGINAL ARTICLE

Basic Study

Correlational research of Golgi phosphorylation protein 3 expression in colorectal cancer

Yan-Ta Guo, Cheng-Zhi Qiu, Zhong-Xin Huang, Wai-Shi Yu, Xiao-Feng Yang, Ming-Zhen Wang

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Author contributions: Guo YT and Qiu CZ designed research; Guo YT, Yu WS, Yang XF and Wang MZ performed research; Qiu CZ and Huang ZX analyzed data; all authors contributed to the preparation of the manuscript, and approved the final version to be published.

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Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate the effect of Golgi phosphorylation protein 3 (GOLPH3) expression on cell apoptosis, angiogenesis and prognosis in colorectal cancer (CRC).

METHODS: The expression of GOLPH3 in CRC tissues and normal colorectal mucosae was determined by immunohistochemistry in 62 patients. In addition, immunohistochemistry was also carried out to detect the expression of vascular endothelial growth factor (VEGF), CD34 and microvessel density (MVD). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay was used to determine the apoptotic index (AI). The Kaplan-Meier method was used to analyze the relationship between GOLPH3 expression and survival in another 123 CRC cases.

RESULTS: Compared with normal colorectal mucosae, a notably higher level of GOLPH3 protein expression was identified in CRC tissues (53.2% *vs* 24.2%, $P < 0.05$). Positive GOLPH3 expression was significantly associated with tumor invasion depth, TNM stage, and lymph node metastasis ($P = 0.001$; $P = 0.020$; $P = 0.020$; $P < 0.05$, respectively), but not with tumor length, tumor site, and age ($P = 0.363$; $P = 0.819$; $P = 0.599$; $P > 0.05$, respectively). VEGF expression and MVD in GOLPH3-positive CRC was significantly higher than in GOLPH3-negative CRC (VEGF: 69.7% *vs* 31.0%; MVD: 21.45 ± 9.39 *vs* 14.24 ± 8.97; $P < 0.05$).

GOLPH3 expression was negatively correlated with AI in CRC as shown by Spearman correlation analysis (r = -0.320, P < 0.05). The 5-year survival rate in GOLPH3-negative CRC (69.4%) was significantly higher than in GOLPH3-positive CRC (48.6%) (log-rank test, P < 0.05).

CONCLUSION: High expression of GOLPH3 is found in CRC tissues. GOLPH3 expression may be a novel prognostic marker for CRC patients.

Key words: Colorectal cancer; Golgi phosphorylation protein 3; Cell apoptosis; Angiogenesis; Prognosis

Core tip: Golgi phosphorylation protein 3 (GOLPH3) was identified as a Golgi membrane protein initially and as a new oncogene in recent years. This study reports a notably higher level of GOLPH3 expression in colorectal cancer (CRC) tissues compared with normal colorectal mucosae. Positive GOLPH3 expression was significantly associated with tumor invasion depth, TNM stage, and lymph node metastasis. In addition, GOLPH3 expression was correlated with cell apoptosis and angiogenesis. Our data suggest that GOLPH3 expression may be a novel prognostic marker for patients with CRC.

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INTRODUCTION

Colorectal cancer (CRC) is a serious threat to humans, and the incidence and mortality rate of CRC are high in many countries. At present, complete resection is still the main treatment option, however, despite continuing improvements in surgical techniques, radiotherapy and chemotherapy, almost half of patients who receive radical surgery die of tumor metastasis and recurrence. Thus, it is imperative to identify a predictive index of CRC recurrence or metastasis. In addition, an understanding of the molecular regulatory mechanism associated with CRC invasion and metastasis is of great importance to guide clinical diagnosis and treatment[1].

The occurrence and development of CRC requires the participation of multiple genes, but there is still a lack of objective molecular markers to help in the early diagnosis of tumor and prognosis. Thus, it would be of great significance to further explore the pathogenesis of CRC and the resistance mechanism, and identify new targets for tumor targeting therapy.

Golgi phosphorylation protein 3 (GOLPH3), which was identified as a Golgi membrane protein initially, and as a new oncogene in recent years, is found in a number of organisms and plays a decisive part in morphology and Golgi trafficking[2-4]. The mammalian target of rapamycin (mTOR) is a key point in the PI3K/AKT/mTOR signaling pathway, and is associated with tumor growth and proliferation[3,5]. Recent research showed that GOLPH3 can promote the proliferation of cancer cells by activating mTOR and may be a new oncogene[5]. However, research on the *GOLPH3* gene and apoptosis in CRC is relatively rare.

In this study, we measured the expression of GOLPH3 in CRC tissues to investigate its relationship with cell apoptosis and angiogenesis, and explored the effects of GOLPH3 on the development and prognosis of CRC.

MATERIALS AND METHODS

Patients and tissue specimens

Group Ⅰ: This group included 62 CRC patients who underwent surgical resection from February 2012 to July 2012 in Fujian Medical University the Second Affiliated Hospital, and had available tumor samples. A total of 62 CRC tissues were acquired from resected normal mucosae and tumors. The samples were then frozen in liquid nitrogen and stored at -80 ℃. The TNM stages were determined using classification guidelines by the American Joint Committee on Cancer in the 7th edition. The clinicopathological characteristics of the patient cohort are summarized in Table 1. Each patient had a complete medical record and did not receive chemoradiotherapy before surgery.

Group Ⅱ: This group included 123 CRC patients who underwent surgical resection from January 2005 to December 2009 in the Second Affiliated Hospital of Fujian Medical University. All patients (72 males and 51 females) had complete medical records and did not receive chemoradiotherapy before surgery. The age range of the CRC patients was 28-87 years (mean 64.04 ± 12.52 years). All patients were followed up by phone or clinic visit for 1 to 93 mo, and the median follow-up period was 62 mo.

Reagents

Rabbit anti-GOLPH3 (ab98023) polyclonal antibody (Abcam); mouse anti-vascular endothelial growth factor (VEGF) monoclonal antibody (VGI) (Abcam); and mouse anti-CD34 monoclonal antibody (QBEnd/10) (Abcam) were used. The secondary antibody was biotin-labeled Goat anti-rabbit IgG (Abcam). The immunohistochemical kit was from Beijing Zhongshan Biotechnology Co., Ltd (Bejing, China). The *in situ* apoptosis detection kit was from Roche in Shanghai, China.

Immunohistochemistry

Immunohistochemistry was performed according to

Table 1 Relationship between Golgi phosphorylation protein 3 expression in colorectal cancer tissues and clinicopathologic factors

Clinicopathologic parameters	GOLPH3 expression			χ^2	*P* value
	Total	Negative	Positive		
Age (yr)					
≤ 60	30	13	17	0.276	0.599
> 60	32	16	16		
Tumor site					
Right	11	6	5	0.399	0.819
Hemicolon					
Left hemicolon	12	5	7		
Rectum	39	18	21		
Tumor length (cm)					
≤ 5	40	17	23	0.827	0.363
> 5	22	12	10		
Differentiation					
G_1-G_2	51	27	24	4.391	0.036
G_3	11	2	9		
Clinical stage					
Ⅰ-Ⅱ	33	20	13	5.422	0.020
Ⅲ	29	9	20		
Depth of invasion					
Inside serous membrane	14	12	2	11.014	0.001
Outside serous membrane	48	17	31		
Lymph node metastasis					
Yes	29	9	20	5.422	0.020
No	33	20	13		

GOLPH3: Golgi phosphorylation protein 3.

standard protocols, and was used to detect GOLPH3, VEGF and CD34 protein expression in 62 CRC cases, and GOLPH3 protein expression was detected in another 123 CRC cases. Sections from the paraffin-embedded samples were dried overnight at 37 ℃, and then deparaffinized with xylene and rehydrated. The sections were treated with 3% hydrogen peroxide for 20 min to inhibit the activity of endogenous peroxidase and then microwaved for antigenic retrieval using ethylene diamine tetraacetic acid (EDTA) buffer. Nonspecific antibody binding was blocked. Subsequently, the sections were incubated with anti-GOLPH3 antibody (1:100), anti-VEGF antibody and anti-CD34 antibody overnight at 4℃. After rinsing, the sections were incubated with biotin-labeled secondary antibody bound to a streptavidin-horseradish peroxidase complex. The peroxidase reaction was developed in DAB buffer substrate for visualization. The sections were then counterstained, mounted, and observed under microscope. Phosphate buffered saline (PBS) replaced the primary antibody as a negative control.

TUNEL assay

We chose the "no dead zone" of the 62 CRC tissues, and followed the instructions in the apoptosis detection kit.

Evaluation of GOLPH3 and VEGF staining

Immunohistochemical results were evaluated by two pathologists independently who were blinded to patient data, and any disagreement was resolved by consensus. The protein expression of GOLPH3 and VEGF was evaluated by combining the intensity of staining and the proportion of positively stained tumor cells, and the final score was calculated as follows. The scores for the proportion of positively stained tumor cells were: 0, < 5%; 1, 5%-25%; 2, 25%-50%; 3, 50%-75%; and 4, ≥ 75%; and the scores for staining intensity were: 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow brown); and 3, strong staining (brown). The staining index of GOLPH3 and VEGF in CRC tissues was obtained by multiplying the two scores for each sample. The final scores of 0, 1, 2, 3, 4, 6, 9, or 12[6] were obtained. A maximum score of 4 was defined as negative expression.

Calculation of microvessel density

Microvessels were recorded by counting CD34 positively stained endothelial cells. The microvessel density (MVD) was assessed by two pathologists independently, and any disagreements were resolved by consensus. In the few instances of discrepant scoring, two pathologists recounted and an average was obtained. The highest microvascular density areas were selected under low microscope power (100 × magnification) and the vessels in five fields were counted at high microscope power (400 × magnification). MVD measurements were obtained by calculating the average of the counts in five fields. Vessels were excluded if they had thick muscular walls or a large lumen. The calculated results were rounded.

Apoptosis index

The cell nucleus stained yellow-brown was considered positive for the corresponding protein expression. The apoptosis index (AI) was defined as the ratio of positively stained tumor cells to all tumor cells. For each case, 1000 randomly selected tumor cells in 5 areas were counted under 400 × magnification.

Statistical analysis

Statistical analysis was performed using the statistical package for Social Sciences, version 19.0 (SPSS, Inc., Chicago, IL, United States). The relationships between GOLPH3 expression and both clinicopathological features and VEGF were analyzed by the χ^2 test. Pearson correlation analysis was used to determine the relationship between intratumoral MVD and GOLPH3 protein expression, whereas the factors associated with AI were analyzed using the *t* test and Spearman correlation analysis. The Kaplan-Meier method was used to estimate the relationship between GOLPH3 expression and prognosis of CRC, and the differences were compared using the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical data in Group Ⅰ

GOLPH3 expression in CRC tissues and normal colorectal mucosae: Immunohistochemistry results

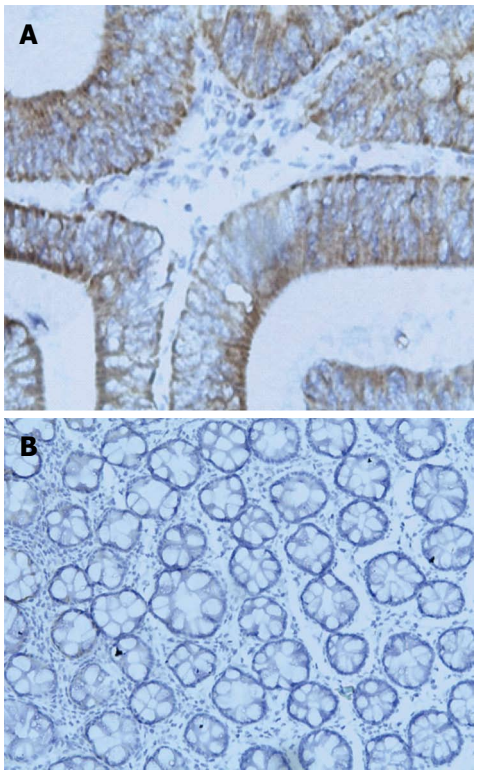

Figure 1 Expression of Golgi phosphorylation protein 3 in colorectal cancer tissues (A) and normal colorectal mucosae (B).

indicated that GOLPH3 expression was mainly located in the cytoplasm of CRC tissues (Figure 1A). The positive expression rate of GOLPH3 was 53.2% (33/62) in CRC tissues, and was markedly higher than that in normal colorectal mucosae [24.2% (15/62); $P < 0.01$] (Figure 1B).

GOLPH3 expression and its association with clinicopathological characteristics in CRC tissues: Compared with moderately-to-well differentiated, no metastatic lymph nodes, and stage Ⅰ-Ⅱ CRCs, GOLPH3 expression level was significantly higher in poorly differentiated, metastatic lymph nodes, and stage Ⅲ CRCs ($P < 0.05$), respectively. However, GOLPH3 expression was not significantly correlated with other clinicopathologic parameters, such as age, site, length of the invasive tumor ($P > 0.05$) (Table 1).

Correlation between GOLPH3 and VEGF expression in CRC tissues: Immunohistochemistry results indicated that VEGF expression was mainly located in the cytoplasm, and showed tan-grains (Figure 2). The rate of VEGF positive expression was 69.7% (23/33) in CRC with GOLPH3 positive expression, and was significantly higher than that in CRC with GOLPH3 negative expression (31.0%, 9/29) ($I^2 = 9.239$, $P = 0.002$). Thus, immunohistochemistry method showed that GOLPH3 expression was associated with VEGF expression ($P = 0.05$).

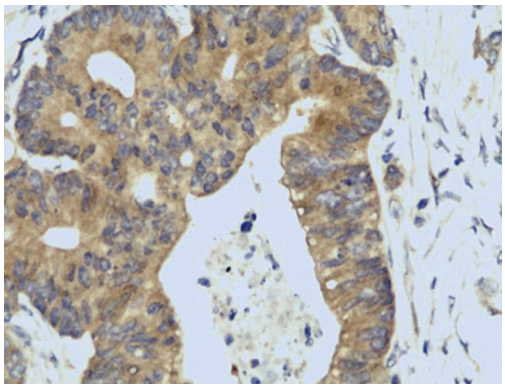

Figure 2 Vascular endothelial growth factor expression in colorectal cancer tissues.

Correlation between GOLPH3 and MVD in CRC tissues: The expression of CD34 was mainly located in vascular endothelial cytoplasm. The MVD in the GOLPH3 negative expression group (29/62) was 14.24 ± 8.97 in CRC tissues, and was significantly lower than that in the GOLPH3 positive expression group (33/62) (MVD = 21.45 ± 9.39) ($t = -3.090$, $P < 0.01$).

Relationship between GOLPH3 expression and apoptosis in CRC tissues. AI was 1.531 ± 0.118 in CRC tissues with GOLPH3 positive expression, and was significantly lower than that in CRC tissues with GOLPH3 negative expression (2.138 ± 0.186) ($t = 2.824$, $P < 0.01$). The staining score for GOLPH3 expression was negatively correlated with AI in CRC tissues using Spearman correlation analysis ($r = -0.320$, $P < 0.05$).

Clinical data in Group Ⅱ

High GOLPH3 expression was related to poor prognosis in CRC. In this group, the overall 5-year survival rate was 56.9%. The CRC patients were divided into two groups according to GOLPH3 expression levels: 5-year survival rate was 48.6% in the GOLPH3 positive expression group, and was 69.4% in the GOLPH3 negative expression group (Figure 3). The log-rank test showed that the survival time in the GOLPH3 positive expression group was significantly lower than that in the GOLPH3 negative expression group ($P = 0.014$).

DISCUSSION

Cancer progression is a very complex process, and includes tumor cell transformation, growth, invasion, angiogenesis, dissemination and survival in the circulation, and subsequent adhesion and colonization in the distant organ or tissue. Among these events, aberrant cell apoptosis, proliferation, and angiogenesis play crucial roles in the growth and dissemination of tumors, and cancer progression[7,8].

GOLPH3, also known as GMx33, is located on

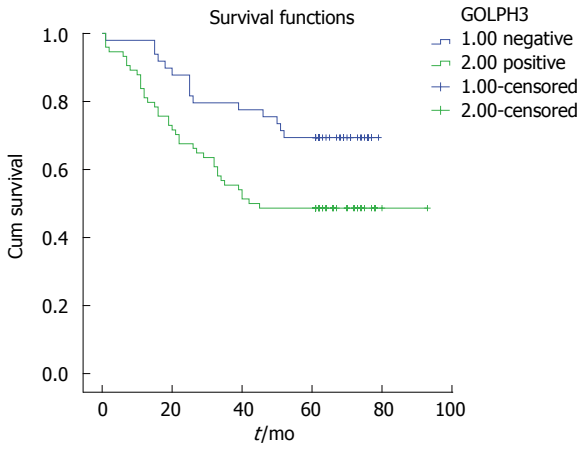

Figure 3 Survival curves of patients with positive and negative Golgi phosphorylation protein 3 expression in colorectal cancer tissues.

chromosome 5p13, and its encoded protein is a highly conserved 34 kDa protein initially identified through proteomic characterization of the Golgi apparatus, and plays a role in protein transmission[9]. In recent years, high expression of GOLPH3 has been shown to be associated with poor prognosis in many cancers, such as breast cancer[10], esophageal squamous cell carcinoma[11], oral tongue cancer[12], gastric cancer[6], prostate cancer[13], glioblastoma multiforme[14], gliomas[15], and rhabdomyosarcoma[16]. In the present study, it was similarly demonstrated that the overexpression of GOLPH3 in CRC tissues could reflect the degree of malignancy of CRC, and was correlated with poor prognosis, which may be a factor in the biological behavior and prognosis of CRC.

At present, great progress has been made in the research on the mechanism of GOLPH3. Phosphatidylinositol-4-phosphate (PI4P) is known to be highly enriched at the trans-Golgi[17] and is required for Golgi-to-plasma membrane trafficking[18-20]. GOLPH3 plays a critical role in Golgi-to-plasma membrane trafficking as a novel effector of PI4P[3]. In addition, GOLPH3 tightly interacts with an unconventional myosin, MYO18A, recruiting it to the Golgi. MYO18A binds to F-actin and the complex applies a tensile force that pulls on the Golgi membrane[3,21,22]. A series of studies indicated that GOLPH3 protein could cause abnormal secretion of glycoprotein by adjusting the function of glycosyltransferases[23,24]. At present, glycosylation has been confirmed to be related to the growth, adhesion, migration, invasion and immune recognition of tumor cells. Abnormal glycosylation can increase the aggressiveness of tumors. Moreover, some authors observed that overexpression of GOLPH3, by driving the Golgi DNA damage response, confers resistance to killing by DNA damaging therapeutic agents, and may explain its role in determining the poor prognosis of a variety of cancers[25].

Angiogenesis is essential for cancer growth and metastasis, and provides tumor cells with enough nutrients and oxygen, which is regulated by various factors. VEGF is an important factor, which induces tumor vessel formation. Research shows that the expression of VEGF is closely related to the development and infiltration of CRC[26,27]. Our results showed that the level of GOLPH3 protein expression in VEGF negative-expression cases was significantly lower than that in VEGF positive-expression cases, and the GOLPH3 expression level was positively correlated with MVD. These findings demonstrated that GOLPH3 expression can upregulate VEGF expression, which may promote tumor angiogenesis and growth in CRC. GOLPH3 overexpression can upregulate HIF-1 expression by activating the PI3K/AKT/mTOR signaling pathway and promote VEGF overexpression, which results in the migration of endothelial cells to generate new blood vessels to increase the blood flow to tumor cells. Moreover, activation of the AKT signaling pathway can also activate endothelial nitric oxide synthase by affecting neural phospholipase, which results in persistent nitric oxide production and the promotion of CRC angiogenesis.

Our results showed that GOLPH3 expression was negatively correlated with AI in CRC tissues and may inhibit cell apoptosis. GOLPH3 protein can activate the AKT signaling pathway[9], and the activated AKT signaling pathway can inhibit the activity of caspase-9 by a phosphorylating reaction, resulting in a cascade reaction, including caspase-2, 3, 6, 8, and 10[28]. This series of reactions plays a role in resistance to apoptosis.

In conclusion, high expression of GOLPH3 was found in CRC. GOLPH3 overexpression may participate in the occurrence and development of CRC by inhibiting apoptosis and promoting angiogenesis, and may be used as a prognostic predictor for CRC patients.

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COMMENTS

Background

Golgi phosphorylation protein 3 (GOLPH3) was initially identified as a Golgi membrane protein and as a new oncogene in recent years, and plays a crucial role in Golgi trafficking and morphology. Recent research showed that GOLPH3 can promote the proliferation of cancer cells by activating mTOR. Colorectal cancer (CRC) is one of the most common malignant tumors of the human digestive system, thus, it is important to identify a predictive index of CRC recurrence or metastasis.

Research frontiers

High expression of GOLPH3 has been shown to be associated with poor prognosis in breast cancer, esophageal squamous cell carcinoma, oral tongue cancer, gastric cancer, prostate cancer, gliomas, and rhabdomyosarcoma. Some authors observed that overexpression of GOLPH3, by driving the Golgi DNA damage response, confers resistance to killing by DNA damaging

therapeutic agents, and may explain its role in determining the poor prognosis of a variety of cancers.

Innovations and breakthroughs

This study reports, for the first time, the relationship between the expression of GOLPH3 and CRC by detecting cell apoptosis and angiogenesis. A notably higher level of GOLPH3 protein expression was found in CRC tissues compared with normal colorectal mucosae. GOLPH3 expression was correlated with cell apoptosis and angiogenesis. The 5-year survival rate in GOLPH3-negative CRC was significantly higher than that in GOLPH3-positive CRC.

Applications

High expression of GOLPH3 was positively associated with CRC. Therefore, GOLPH3 expression may be a novel prognostic marker for patients with CRC.

Terminology

Vascular endothelial growth factor (VEGF) is secreted by certain tumor cells, inducing the formation of tumor blood vessels and stimulating tumor growth. VEGF is a strong angiogenesis factor. TUNEL is a special technique used to determine cell apoptosis, which is necessary for the growth and dissemination of tumors, and cancer progression.

Peer-review

The authors demonstrate the urgency for discovery of novel molecular markers for CRC; and their work strives to address this need. Notably, the role of GOLPH3 in cancer has only been recognized recently, and there is need to further explore how its expression affects the behavior of different types of cancer, including CRC.

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ORIGINAL ARTICLE

Basic Study

Neural mechanism of gastric motility regulation by electroacupuncture at RN12 and BL21: A paraventricular hypothalamic nucleus-dorsal vagal complex-vagus nerve-gastric channel pathway

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Author contributions: Wang H and Liu WJ contributed equally to this work; they drafted the article and made critical revisions related to the intellectual content of the manuscript, performed the research, and analyzed the data; Shen GM designed the study and critically reviewed the manuscript; Zhang MT, Huang S and He Y assisted with the experiments; all authors approved the final version of the article to be published.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at shengm_66@163.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To study the neural mechanism by which electroacupuncture (EA) at RN12 (Zhongwan) and BL21 (Weishu) regulates gastric motility.

METHODS: One hundred and forty-four adult Sprague Dawley rats were studied in four separate experiments. Intragastric pressure was measured using custom-made rubber balloons, and extracellular neuron firing activity, which is sensitive to gastric distention in the dorsal vagal complex (DVC), was recorded by an electrophysiological technique. The expression levels of *c-fos*, motilin (MTL) and gastrin (GAS) in the paraventricular hypothalamic nucleus (PVN) were assayed by immunohistochemistry, and the expression levels of motilin receptor (MTL-R) and gastrin receptor (GAS-R) in both the PVN and the gastric antrum were assayed by western blotting.

RESULTS: EA at RN12 + BL21 (gastric Shu and Mu points), BL21 (gastric Back-Shu point), RN12 (gastric Front-Mu point), resulted in increased neuron-activating frequency in the DVC (2.08 ± 0.050, 1.17 ± 0.023, 1.55 ± 0.079 *vs* 0.75 ± 0.046, $P < 0.001$) compared with a model group. The expression of *c-fos* (36.24 ± 1.67, 29.41 ± 2.55, 31.79 ± 3.00 *vs* 5.73 ± 2.18, $P < 0.001$), MTL (22.48 ± 2.66, 20.76 ± 2.41, 19.17 ± 1.71 *vs* 11.68 ± 2.52, $P < 0.001$), GAS (24.99 ± 2.95, 21.69 ± 3.24, 23.03 ± 3.09 *vs* 12.53 ± 2.15, $P < 0.001$), MTL-R (1.39 ± 0.05, 1.22 ± 0.05, 1.17 ± 0.12 *vs* 0.84 ± 0.06, $P < 0.001$), and GAS-R (1.07 ± 0.07, 0.91 ± 0.06, 0.78 ± 0.05 *vs* 0.45 ± 0.04, $P < 0.001$) increased in the PVN after EA compared with the model group. The expression of MTL-R (1.46 ± 0.14, 1.26 ± 0.11, 0.99 ± 0.07 *vs* 0.65 ± 0.03, $P < 0.001$), and GAS-R (1.63 ± 0.11, 1.26 ± 0.16, 1.13 ± 0.02 *vs* 0.80 ± 0.11, $P < 0.001$) increased in the gastric antrum after EA compared with the model group. Damaging the PVN resulted in reduced intragastric pressure (13.67 ± 3.72 *vs* 4.27 ± 1.48, $P < 0.001$). These data demonstrate that the signals induced by EA stimulation of acupoints RN12 and BL21 are detectable in the DVC and the PVN, and increase the levels of gastrointestinal hormones and their receptors in the PVN and gastric antrum to regulate gastric motility.

CONCLUSION: EA at RN12 and BL21 regulates gastric motility, which may be achieved through the PVN-DVC-vagus-gastric neural pathway.

Key words: Dorsal vagal complex; Gastrin receptor; Motilin receptor; Neuronal firing activity; Paraventricular hypothalamic nucleus; RN12; BL21

Core tip: This study supports the "targeted convergence" hypothesis: Shu-acu point and Mu-acu point signals gather not only in spinal cord but also evince a 'targeted convergence' in brain stem and hypothalamus. We determined that the signals induced by electro-acupuncture (EA) stimulation of gastric Shu and Mu points gather in the dorsal vagal complex (DVC) and paraventricular hypothalamic nucleus (PVN), increasing levels of gastrointestinal hormones and their receptors in the PVN and gastric antrum to regulate gastric motility. We hypothesize that EA at gastric Shu and Mu points regulates gastric motility, which may be achieved through the PVN-DVC-vagus-gastric pathway.

Wang H, Liu WJ, Shen GM, Zhang MT, Huang S, He Y. Neural mechanism of gastric motility regulation by electroacupuncture at RN12 and BL21: A paraventricular hypothalamic nucleus-dorsal vagal complex-vagus nerve-gastric channel pathway. *World J Gastroenterol* 2015; 21(48): 13480-13489 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13480.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13480

INTRODUCTION

According to the theory of traditional Chinese medicine (TCM), the application of acupuncture at a combination of Back-shu and Front-mu points has a solid theoretical basis in the theories of Yin and Yang and of Qijie. RN12 and BL21 points belong to the gastric Front-Mu point and gastric Back-Shu point, respectively, and it has been demonstrated that the combination of RN12 and BL21 is effective for the regulation of motility in clinical practice. These points regulate the function of the bowel and viscera *via* the closely related neuro-endocrine-immuno network regulatory system. Among these components, the functional activity of the hypothalamus and brainstem is the core foundation of acupuncture-modulated effects.

The dorsal vagal complex (DVC) comprises the nucleus tractus solitari (NTS) and the dorsal motor nucleus of the vagus (DMN). The paraventricular hypothalamic nucleus (PVN) located on the top of the hypothalamus, on the side of the third ventricle, is one of the most prominent nuclei in the anterior hypothalamus. DVC and PVN are considered to be the most important nuclei regulating gastrointestinal function in the higher central nervous system (CNS), a regulated effect that is achieved through neuroendocrine and autonomic function. The hypothalamus can affect some independent activities through direct projections to the DVC. The stimulation of the neurons in the PVN activates the DMN neurons projecting into the gastrointestinal system, namely, the locus coeruleus-dorsal motor nucleus of the vagus (LC-DMN) pathway, which may participate in the regulation of vagal preganglionic neurons in the DMN by the PVN[1]. The NTS sends nerve fibers to the hypothalamus, and also receives the most extensive projections of the PVN[2,3]. The PVN-DVC-vagus nerve pathway is involved in the gastric response to noxious stimuli[4]. Our recent study demonstrated that electroacupuncture (EA) at RN12 and BL21 could induce the up-regulation of *c-fos* in the DVC, which, remarkably, suggests the convergence of acupuncture signals in the medullary DVC, and the participation of gastrointestinal hormones such as motilin and gastrin in modulating the effect of EA

as well[5]. The integrative activity that occurs within the DVC nuclei is the result of inputs originating from higher CNS (PVN) areas as well as from neuro-hormonal signals. All of these signals finely tune the coordinated function of the stomach. As the DVC contains gastric distension-sensitive neurons, the exact acupuncture signals that gather in the DVC remain to be studied by using the electrophysiology method; however, the more important target is to explore whether the higher CNS (PVN) and gastrointestinal hormones play an important role in regulating gastric motility with EA stimulation of RN12 and BL21 (gastric Shu and Mu points).

Therefore, the present study aimed to explore: the exact acupuncture signals that gather in the DVC by the electrophysiology method; the neural mechanisms by which the PVN mediates the regulation of gastric motility by RN12 (Wei Shu) and BL21 (Zhong Wan) stimulation; and the brain-gut peptide receptors, such as motilin-receptor (MTL-R) and gastrin-receptor (GAS-R or CCK-B receptor), through which gastrointestinal hormones can influence gastric functions in the PVN, determined *via* Western blotting and immunohistochemistry.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats (SD, 220-280 g) were obtained from Anhui Medical University (Hefei, Anhui, China) in the present study. All animals were housed under controlled conditions at a temperature of 22 ± 2℃ and a 12-h light/dark cycle. Commercial chow diet and autoclaved water were available to the animals *ad libitum*. Animals were fasted for 12 h, and anesthetized with pentobarbital (40 mg/kg, *ip*) before sacrifice. All experimental procedures were conducted in accordance with Anhui University of Traditional Chinese Medicine guidelines for the care and use of experimental animals and were approved by the Institutional Animal Care and Use Committee at Anhui University of Traditional Chinese Medicine (NO. 201309106).

Experimental design

Four separate experiments were performed, as follows: (1) In experiment A, the intragastric pressure was recorded, while the PVN was damaged. Rats were randomly divided into three groups: the model group (MOD, non-EA while submitted to gastric distention alone), the PVN lesion group (damaged PVN) and the PVN lesion + EA group (damaged PVN + EA at RN12 together with BL21); (2) In experiment B, rats were randomly divided into the following five groups to explore neuron firing activity in the DVC: the model group (MOD), the non-acupoint (the middle points of the line connecting the meridians where RN12 or BL21 belong and their lateral neighbor meridians) group (NA), the EA at RN12 group (RN12), the EA at BL21 group (BL21), and the EA at RN12 plus BL21 (RN12 is located on the abdomen and BL21 is located on the back) group (RN12 + BL21); (3) In experiment C, the grouping was the same as in experiment B, except the hypothalamus was removed to determine the expression of *c-fos*, MTL and GAS by immunohistochemical staining; and (4) In experiment D, the grouping was the same as in experiment B, except both the hypothalamus and gastric antrum were removed to determine the expression of MTL-R and GAS-R by western blotting.

Measurement of intragastric pressure

The method was very similar to our previous experiments[5]. Briefly, a self-made balloon was used to measure the ingastric pressure. It was inserted into the body of the anesthetized rats' stomach through a small incision in the duodenum, which we call the gastric distention model (MOD). Intragastric pressures were recorded by the PowerLab 8/30 system (Australia) and analyzed by the LabChart.

PVN lesion

After the rats were anesthetized, they were placed in a stereotaxic frame. Based on the "rat brain in stereotaxic coordinates" of Paxinos and Watson[6], the electrode was advanced into the PVN (coordinates: AP 1.5 mm, R 0.4 mm, H 7.7-7.8 mm), and the bilateral PVN were destroyed using DC current (2 mA, 10 s) *via* a lesion-making device.

Electroacupuncture procedure

Based on the anatomical localization in rats as compared with that in the human body (Figure 1), EA was performed at RN12 (20 mm above the umbilicus on the midline of the upper abdomen, with a needle inserted at 2 mm), left BL21 (5 mm on side of the twelfth thoracic vertebra, with a needle inserted at 4 mm) and non-points (the middle points of the line connecting the meridians where RN12 or BL21 belonged and their lateral neighbor meridians). These needles were connected to an SDZ-IV type electronic acupuncture instrument (Suzhou, Jiangsu, China), with the frequency set to 20-100 Hz, the current intensity set to 2 mA. In experiments A and B, the EA process was conducted for 20 min each time in each rat when the intragastric pressure measurement instrument in experiment A or the neural data acquisition system in experiment B were ready. The EA process lasted 20 min every day for 7 d to ensure changes in *c-fos*, gastrointestinal hormones, and their receptors in experiment C and D.

Extracellular neuronal recording

Rats were anesthetized with pentobarbital (40 mg/kg, *ip*) and tightly fixed in a stereotaxic apparatus; the bregma and lambda surface of the brain were exposed

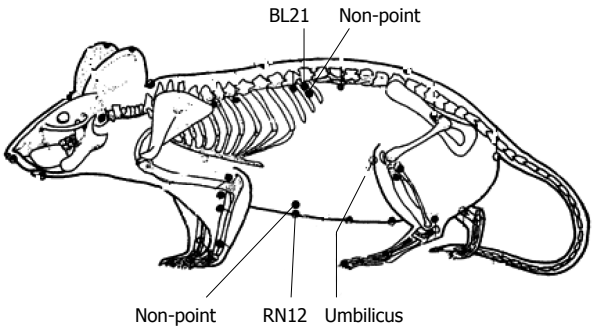

Figure 1 The location of RN12, BL21 and non-points in rat.

and then placed at the same level. An approximately 2 mm × 2 mm hole was drilled into the skull and brain membrane was carefully removed according to the coordinates of Paxinos and Watson: DVC (Ap: 11.3-14.3 mm, L: 0.5-1.7 mm, and H: 7.5-8.7 mm). The electrode was inserted into the coordinates during the experiment, while the wound was covered with a moist cotton ball of 37 ℃ to protect brain tissues from drying out before recording started.

Each recording of extracellular neuron firing activity was made using a pair of 16-channel stainless steel microwire arrays (reference and recording) as an electrode, the ends of which were covered with polyethylene glycol (PEG) to reinforce its strength. The electrode was connected with an electrically isolated digitizing amplifier into an OmniPlex neural data acquisition system (Plexon, United States). These neural firing activity data were analyzed using an Offline Sorter (Plexon, United States).

Before the experiment began, all the instruments for measuring intragastric pressure were prepared. The electrode was then inserted into the DVC, and after the neuronal firing pattern became stable, the neuron was then tested with 37 ℃ water inserted into the balloon to determine whether it was responsive to gastric distention. If the neuron was responsive to gastric distention, we began to record its discharge activity for approximately 30 s, then performed the EA procedure for another 30 s before finally removing the EA process and allowing it to recover to its firing pattern before EA.

Immunohistochemistry

To detect the expression of the assayed proteins *c-fos*, motilin and gastrin in the PVN, immunohistochemistry was performed. The technology was very similar to our previous experiments[5]. Briefly, we prepared the corresponding polyclonal rabbit antibody diluted in PBS (primary antibody), dropping the biotinylated goat anti-rabbit secondary antibody. All the operations were performed according to the immunohistochemical staining procedure (SP kit). *c-fos*, MTL, and GAS immunoreactivity in the PVN can be seen under an electron microscope as a dark brown nuclear staining. Image-Pro Plus 5.1 software analyzed the integrated optical density (IOD) in the PVN.

Western blotting

To detect the expression of the assayed proteins, gastrin/CCK-B receptor and motilin receptor in both hypothalamus and gastric antrum, western blotting was performed. The receptor proteins were extracted from the hypothalamus and gastric antrum (1.5 cm above the pylorus). Samples were mechanically dissociated and lysed in radioimmunoprecipitation assay 37 (RIPA) buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na_2-EDTA, 1% NP-40, 0.25% Na-deoxycholate) containing protease and phosphatase inhibitor cocktails (Roche). After brief sonication and heating, the supernatants were subjected to SDS-PAGE and transferred to PVDF membranes. Blots were incubated overnight at 4 ℃ with primary antibodies (CCK-BR, Beyotime Biotechnology, 1:800; MTL-R, Beyotime Biotechnology, 1:500; β-actin, Beyotime Biotechnology, 1:500) and were then incubated with HRP-conjugated secondary antibodies (GE Healthcare). Blots were visualized with an enhanced chemiluminescence reagent (Millipore) and quantified with Image Lab (Bio-Rad).

Statistical analysis

All experimental values were expressed as the mean ± SD. Statistical analysis was performed with one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test. A difference with a P value < 0.05 was considered statistically significant.

RESULTS

The recording of extracellular neuron firing activity in the DVC

We tested whether there is a direct neuronal link by which DVC could regulate gastric motility that could be activated by EA to explore the neural pathway of EA at gastric Shu and Mu points (Figure 2A). Compared with the MOD group, the neuron-activating frequency of gastric distension sensitive neurons increased significantly in the RN12 + BL21, RN12 and BL21 groups (P < 0.01). EA had no significant effects on the non-acupoint group. Compared with the RN12 + BL21 group, the neuron-activating frequency decreased in the RN12 and BL21 groups (P < 0.01) (Figure 2B). These results suggest that EA at RN12 and BL21 can activate DVC neurons and that acupuncture signals may gather in the DVC.

c-fos expression in the PVN

To establish whether the RN12 and BL21 acupuncture signals gather in the PVN, we used immunohistochemistry to determine *c-fos* expression in the PVN (Figure 3A). Compared with the MOD group, Fos-positive neurons increased significantly in the RN12+BL21, RN12 and BL21 groups (P < 0.01). They

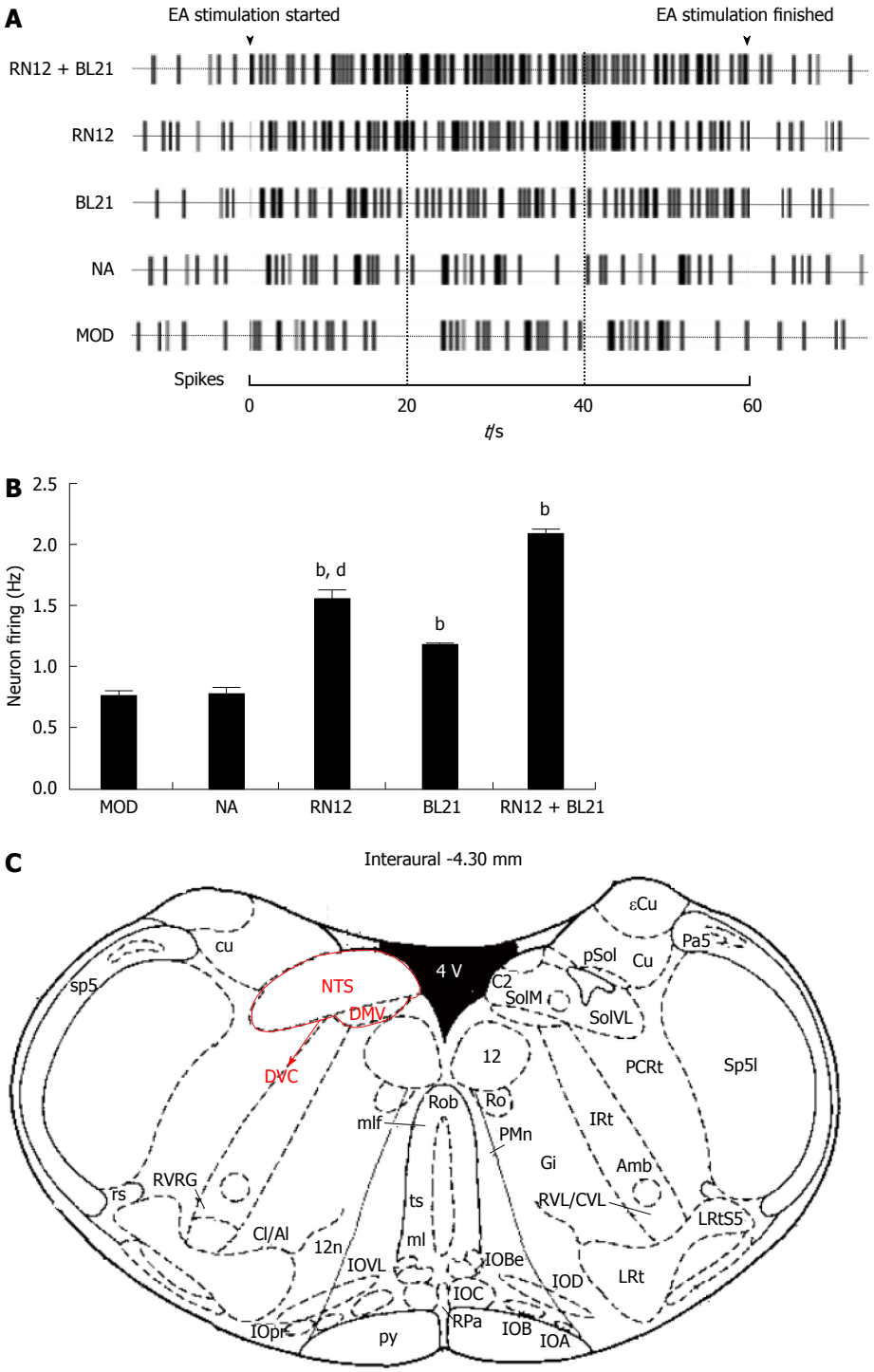

Figure 2 Recording of extracellular neuron firing activity in the dorsal vagal complex. A: Representative rat neuronal firing pattern in the dorsal vagal complex (DVC) induced by stimulating RN12 and BL21; B: Summarized data for neuronal firing activity by electroacupuncture (EA) stimulation at RN12 and BL21 in DVC ($^{b}P < 0.01$ *vs* the MOD group; $^{d}P < 0.01$ *vs* the RN12 + BL21 group); C: DVC coordinates: the anatomic location of the DVC and the rat brain in stereotaxic coordinates were adapted from the atlas of Paxinos and Watson.

were significantly increased in the RN12+BL21 group compared with the RN12 and BL21 groups ($P < 0.05$). EA had no significant effects on the non-acupoint group (Figure 3B). These results suggest that RN12 and BL21 acupuncture signals gather in the PVN.

The recording of intragastric pressure

To demonstrate whether PVN was the central target of EA at RN12 + BL21 that regulated gastric motility, the PVN was damaged stereotaxically with a lesion-making device. The intragastric pressure (IGP) was decreased compared with the MOD group ($P < 0.01$), and the waves did not become disordered (in the DVC lesion, the waves became disordered, however). The decreased IGP induced by the PVN lesion did not increase after EA at RN12 + BL21 (Figure 4A and B),

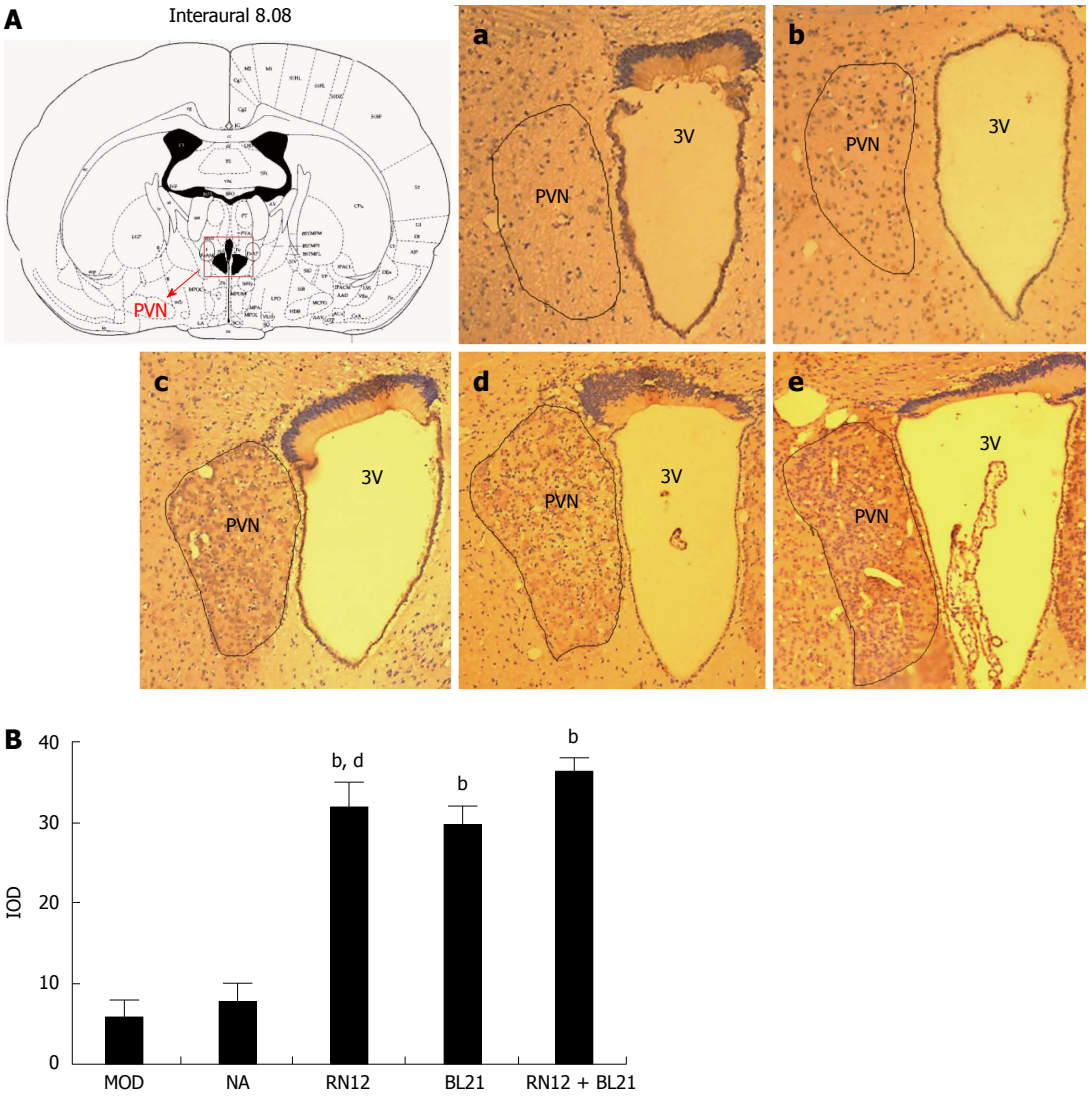

Figure 3 *c-fos* expression in the paraventricular hypothalamic nucleus. A: Photomicrographs of the hypothalamus sections showing Fos immunoreactivity in the paraventricular hypothalamic nucleus (PVN). Lane a: Model group; Lane b: Non-acupoint group; Lane c: BL21 group; Lane d: RN12 group; Lane e: RN12 + BL21 group. The anatomic locations of the photomicrographs are indicated at the top, adapted from the atlas of Paxinos and Watson. Fos-positive neurons are presented as dark brown staining in the cell nuclei; B: Integral optical density (IOD) of Fos-positive neurons in the PVN ($^{b}P < 0.01$ *vs* the MOD group; $^{d}P < 0.01$ *vs* the RN12 + BL21 group). 3 V: Third ventricle.

suggesting that PVN plays critical roles in EA at RN12 + BL21 regulating gastric motility.

MTL and GAS expression in the PVN

To establish whether endogenous MTL and GAS expression may be altered in response to EA in PVN, we used immunohistochemistry to determine the expression of these gastrointestinal hormones in the PVN. Compared with the MOD group, the expression of MTL and GAS in the rat PVN significantly increased in the three EA groups ($P < 0.01$). Additionally, MTL expression levels increased in the RN12 + BL21 group compared with the RN12 group ($P < 0.01$), and GAS expression levels increased in the RN12 + BL21 group compared with the BL21 group ($P < 0.05$). EA had no significant effects in the non-acupoint group (Figure 5). Our data suggest that gastrointestinal hormones in the PVN participate in the modulation of gastric motility by the EA stimulation of RN12 and BL21.

MTL-R and GAS-R expression in the hypothalamus

To establish whether gastrointestinal hormones in the PVN participate in the modulation of gastric motility by EA stimulation of RN12 and BL21 through their receptors, we used western blotting to determine the expression of MTL-R and GAS-R in the hypothalamus (Figure 6A). Compared with the MOD group, the expression of MTL-R and GAS-R in the hypothalamus increased significantly in the RN12 + BL21, RN12 and BL21 groups ($P < 0.01$); MTL-R did not significantly increase in the non-acupoint group, but did significantly increase in the RN12 + BL21 group compared with the RN12 and BL21 groups ($P < 0.01$) (Figure 6B). These results demonstrate that the modulatory effect of EA may be due to the interaction of gastrointestinal hormones and their receptors in the hypothalamus.

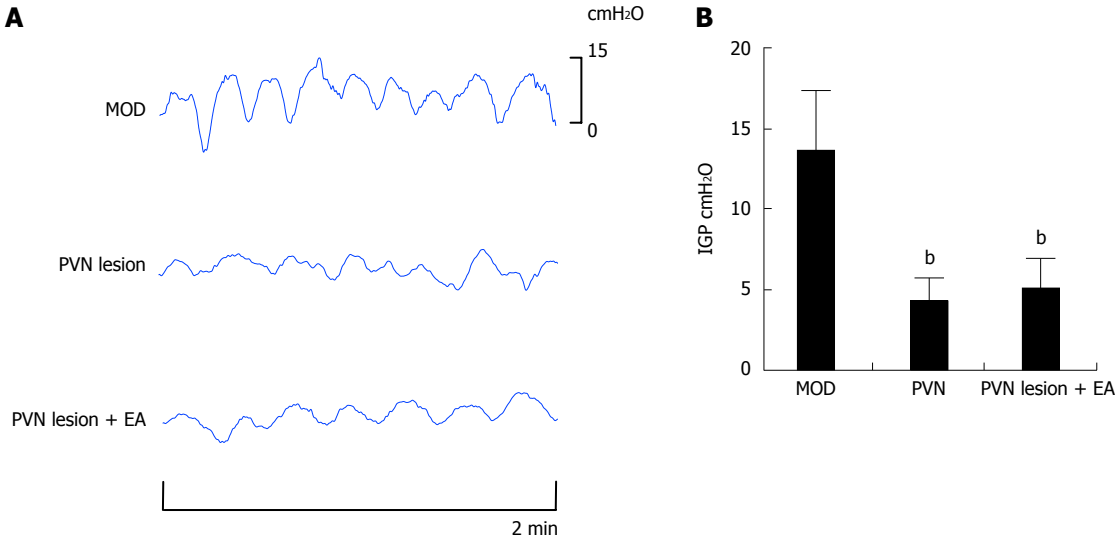

Figure 4 The effects of paraventricular hypothalamic nucleus regulation of intragastric pressure by stimulating RN12 + BL21. A: Representative waves of intragastric pressure (IGP) in rats induced by damaging the paraventricular hypothalamic nucleus (PVN) with or without stimulating RN12 + BL21; B: Summarized data for the effect of stimulation at RN12 + BL21 with PVN lesion on intragastric pressure ($^{b}P < 0.01$ *vs* the MOD group).

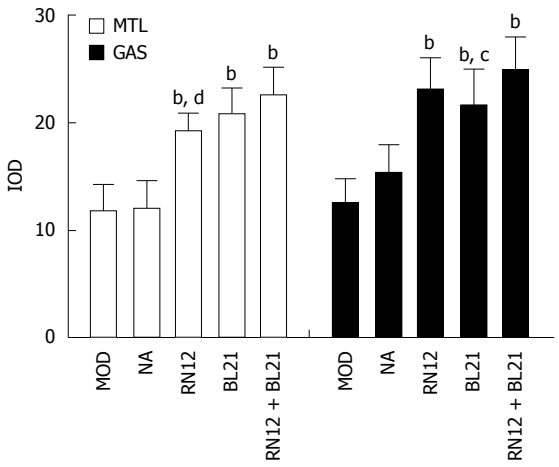

Figure 5 Motilin and gastrin expression in the paraventricular hypothalamic nucleus. Motilin (MTL) and gastrin (GAS) expression in paraventricular hypothalamic nucleus (PVN) is shown by immunohistochemistry. Summarized data for the expression of MTL and GAS by electroacupuncture (EA) stimulation at RN12 and BL21 in the PVN ($^{b}P < 0.01$ *vs* the MOD group; $^{c}P < 0.05$ or $^{d}P < 0.01$ *vs* the RN12 + BL21 group).

MTL-R and GAS-R expression in the gastric antrum

To establish whether endogenous MTL-R and GAS-R expression may be altered in response to EA in the gastric antrum, we used western blotting to determine the expression of these receptors in the gastric antrum (Figure 7A). Compared with the MOD group, the expression of MTL-R and GAS-R in the gastric antrum increased significantly in the RN12 and BL21 groups, as well as in the RN12 + BL21 group ($P < 0.01$). GAS-R expression significantly increased in the RN12 + BL21 group compared to the RN12 and BL21 groups ($P < 0.05$ or $P < 0.01$) but did not significantly increase in

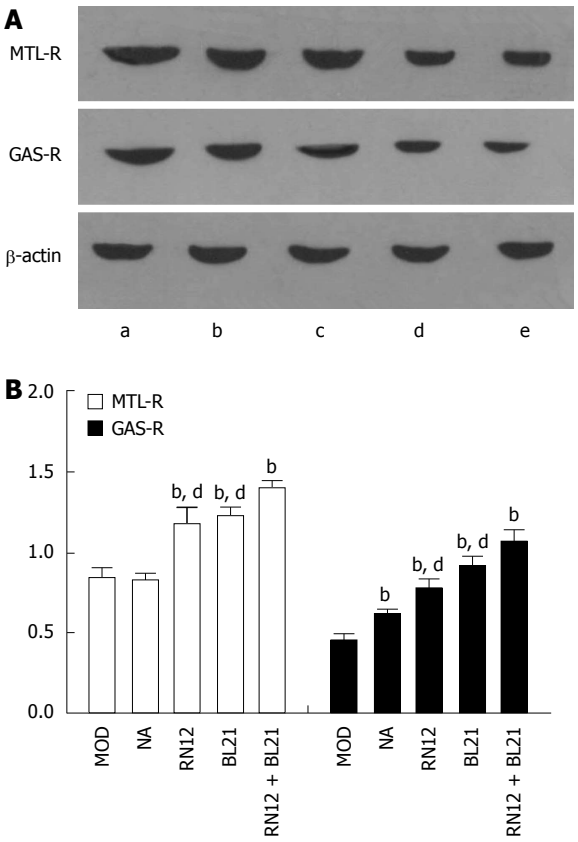

Figure 6 Motilin receptor and gastrin receptor expression in the hypothalamus. A: Motilin receptor (MTL-R) and gastrin receptor (GAS-R) protein expression in the hypothalamus is shown by western blotting. Lane a: RN12 + BL21 group; Lane b: BL21 group; Lane c: RN12 group; Lane d: Non-acupoint group; Lane e: Model group; B: Summarized data for the expression of MTL-R and GAS-R by electroacupuncture (EA) stimulation at RN12 and BL21 in the hypothalamus ($^{b}P < 0.01$ *vs* the MOD group; $^{d}P < 0.01$ *vs* the RN12 + BL21 group.

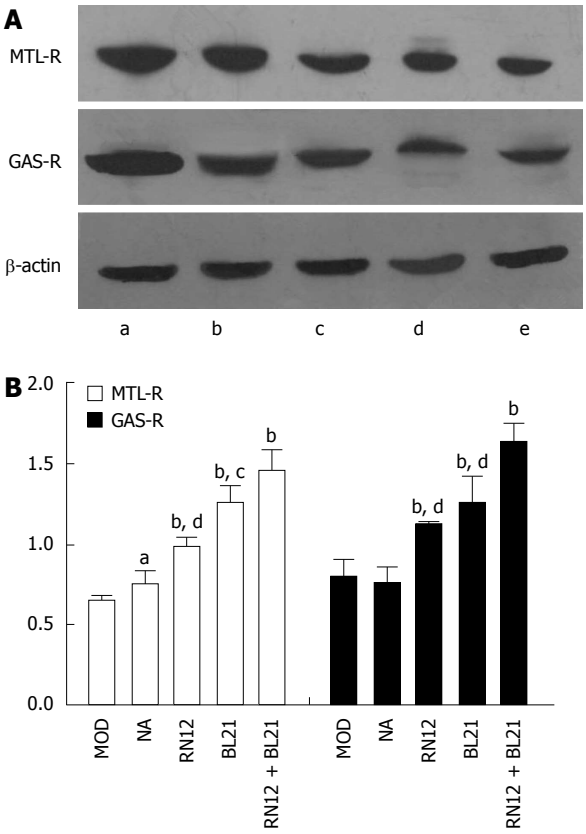

Figure 7 Motilin receptor and gastrin receptor expression in the gastric antrum. A: Motilin receptor (MTL-R) and gastrin receptor (GAS-R) protein expression in the gastric antrum shown by western blotting. Lane a: RN12 + BL21 group; Lane b: BL21 group; Lane c: RN12 group; Lane d: Non-acupoint group; Lane e: Model group; B: Summarized data for the expression of MTL-R and GAS-R by electroacupuncture (EA) stimulation at RN12 and BL21 in the gastric antrum ($^aP < 0.05$ or $^bP < 0.01$ *vs* the MOD group; $^cP < 0.05$ or $^dP < 0.01$ *vs* the RN12 + BL21 group).

the non-acupoint group (Figure 7B). These results, at least in part, demonstrate that the modulatory effect of EA may be due to the increase in some brain-gut-related receptors in the gastric antrum, such as MTL-R and GAS-R.

DISCUSSION

It has been demonstrated that stimulation of the combination of Back-shu points and Front-mu points is effective in clinical practice. However, studies of the mechanisms underlying this phenomenon have been focused at the level of the spinal cord. Advances in the study of Back-Shu and Front-Mu point combinations have focused on whether the superior nerve center involves the regulation of *zang-fu* organs by a combination of Back-shu and Front-mu points. To address whether this convergent effect could extend to the superior nerve center, we put forward a "targeted convergence" hypothesis: gastric Shu and Mu point acupuncture signals gather not only in the spinal cord but also in a targeted way in the brain stem and hypothalamus in the higher central nervous system, achieving an integrative effect through the neural microcircuitry. Likely, gastric Shu and Mu afferent signals primarily convey acupuncture input signals to different levels of the central nervous system, such as the spinal cord, medulla, brain stem, hypothalamus and subcortex.

The DVC in the brainstem plays an important role in autonomic regulation of gastric motility. Based on our previous studies, we found that gastric motility was blocked by DVC lesions or bilateral sub-diaphragmatic vagotomy, and EA at RN12 + BL21 could not restore this depression. DVC was found to play an important role in the regulation of gastric motility by EA at RN12 + BL21, an effect that depended upon the vagus nerve[5]. Electrophysiological experiments in this study further clarified that DVC participated in the regulation of gastric motility by the stimulation of RN12 and BL21, which, in turn, indicated that there was a direct neuronal link in the DVC by which they could participate in the regulation of gastric motility.

The PVN may modulate a range of functions, including ingestive behavior, sodium intake, and glucose metabolism, as well as cardiovascular, gastrointestinal, and respiratory activities. Neurons in these PVN-innervated subnuclei of the lateral PB may play a critical role in relaying ingestive feedback information involving the mechanical distention of the stomach[7]. Some studies have shown that electrical stimulation of the lateral hypothalamic area (LHA) increased the firing activities of GD-responsive neurons in the PVN and promoted gastric motility[8]. The PVN serves as a central nucleus for modulating gastrointestinal activities. The present study demonstrated that repeated electroacupuncture at the bilateral Zusanli and Yanglingquan points has a cumulative analgesic effect by remodeling the synaptic structure of the PVN[9]. In this study, we found that EA at RN12 and BL21 could induce the expression of c-fos in the PVN and that the even more marked upregulation in the RN12 + BL21 group demonstrates that the acupuncture signals of the gastric Shu and Mu points gather in the PVN as well. In addition, we found that PVN lesions caused a decrease in gastric motility, and that EA at RN12 + BL21 was unable to restore this decreased gastric motility. It was demonstrated that the PVN was another central target of the gastric Shu and Mu points that combined to regulate gastric motility. This finding verifies the "targeted convergence" hypothesis as Shu and Mu point acupuncture signals converge not only in the DVC but also in a targeted manner in the PVN.

Emerging evidence indicates that acupuncture treatment not only activates distinct brain regions in different types of diseases but also modulates adaptive neurotransmitters in related brain regions to alleviate autonomic responses[10,11].

In our previous study, motilin and gastrin in the serum, gastric antrum and DVC increased significantly with EA at gastric Shu and Mu points[5,12]. This showed the gastrointestinal hormones react remarkably to

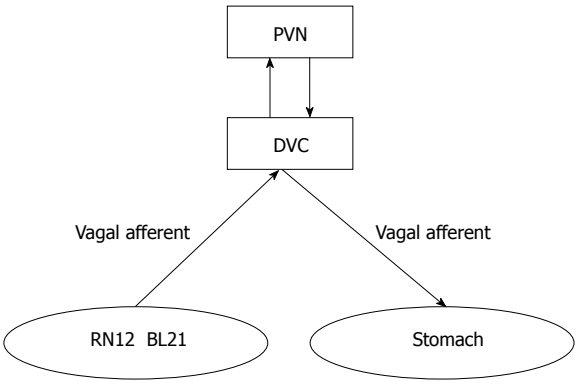

Figure 8 The transmission path of acupuncture signals of the gastric Shu and Mu points.

gastric motility with EA. Neurons with motilin mRNA expression, motilin immunoreactivity, and motilin receptors have been found in various regions of the brain in different species[13,14]. Some studies found that PVN was a central nucleus for modulating gastric motility and motilin expression in the thyroid. PVN excitation was shown to prompt gastric motility, which was partly prevented by the motilin receptor antagonist, GM-109[15]. Furthermore, studies have shown that Motilin/FG-labeled neurons were detected in the PVN, and a motilin receptor antagonist (GM109) could abolish the responses of neurons and excitatory effect of gastric motility induced by motilin[16]. The receptor of gastrin is always called the CCK-B receptor. The majority of CCK-B receptors in the brain are abundantly distributed throughout brain regions such as the cerebral cortex, olfactory bulbs, hippocampus, amygdala, nucleus accumbens, and the nucleus tractus solitary(NTS)[17-19]. The roles of CCK-B receptor in numerous physiologic processes in the gastrointestinal tract and central nervous system have been well documented[20]. Some results showing that antagonists for the CCK-B receptor microinfused into the PVN inhibited colonic motility in non-fasted rats[21] suggest a specific function of the CCK-B receptor in integrative satiety controls *via* the PVN[22]. Furthermore, some studies suggest that the CCK-B receptor might play a role in the tonic inhibition of 100 Hz EA-induced analgesia and in the mediation of chronic tolerance to 100 Hz EA in mice[23]. Results also suggest that the level of CCK-B receptor mRNA expression in the hypothalamus has an important relationship with the individual variations in the response to high frequency EA analgesia in rats[24]. In the present study, we found that EA at RN12 and BL21 could increase the expression of MTL, GAS, MTL receptor and GAS receptor in PVN, as well as MTL receptor and GAS receptor in gastric antrum. This suggests that the increased levels of MTL and GAS (*via* MTL and GAS receptors) within the PVN participate in EA-regulated gastric motility, and that the MTL and GAS receptors in gastric antrum also participate in EA-regulated gastric motility.

Taking into account the principles of traditional Chinese medicine and all the data on gastric motility, DVC neuronal activity, and the expression of brain-gut peptides in both hypothalamus and gastric antrum, this study demonstrated that EA stimulation of the gastric Shu and Mu points could regulate gastric motility, with combined stimulation of these acupoints eliciting a synergistic effect. The effect was closely connected with the DVC and PVN, as acupuncture signals generated by EA at gastric Shu and Mu points gather in the DVC and PVN, elevating the expression of gastrointestinal hormone receptors in the hypothalamus and gastric antrum, which in turn play a role in regulating gastric motility through the vagus nerve. Thus, we propose that the effects of combined EA stimulation of gastric Shu and Mu points may be achieved through the combined efforts of the PVN-DVC-vagus nerve-gastric channel (Figure 8).

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COMMENTS

Background

Clinically, electroacupuncture (EA) at BL21 and RN12 has been frequently utilized as a therapy for patients with gastrointestinal dysfunction in Eastern Asia. Previous studies by the authors demonstrated that EA stimulation of the BL21 and RN12 could synergistically regulate gastric motility *via* elevating gastrointestinal hormones in the dorsal vagal complex (DVC). Similar to the DVC, the paraventricular hypothalamic nucleus (PVN) is also an important nucleus for regulating gastrointestinal function. The present study aims to evaluate if there is a direct neuron link for DVC to regulate gastric motility which can be activated by EA at BL21 and RN12, and further to explore the acupuncture signals of combined BL21 and RN12 not only in the DVC, but also as "targeted convergence" in the PVN.

Research frontiers

Previous experiments have already proved that the acupuncture information of Shu-acu points and Mu-acu points gathers in the spinal cord, while another part gathers at the superior nerve center (brain stem and hypothalamus).

Innovations and breakthroughs

The authors put forward a hypothesis called "targeted convergence": the acupuncture signal of gastric Shu and Mu points gathers not only in the spinal cord, but also have a phenomenon of targeted convergence in the brain stem and hypothalamus of the higher central nervous system, achieving an integration effect through the neural microcircuitry. This is the first study to show the acupuncture signal of gastric Shu and Mu points gathers in superior nerve centers, especially the PVN.

Applications

The present data demonstrate that the signals induced by EA stimulation of acupoints RN12 and BL21 gather in the DVC and the PVN, increasing the levels of gastrointestinal hormone and the receptors in the PVN and gastric antrum to regulate gastric motility. The study indicates the neural biological basis of combination of Back-shu and Front-mu points, to direct the compatibility of acupoints in clinical acupuncture, improving the clinical efficacy.

Terminology

DVC and PVN are two important nuclei regulating gastrointestinal function in the higher central nervous system.

Peer-review

The subject has a very good foundation, and they forward a hypothesis called "targeted convergence", it is stimulating and innovative. The authors attempt to shed a light on the matter of specificity of acupuncture stimuli is welcome, but the article needs some work to be better.

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ORIGINAL ARTICLE

Retrospective Study

Comparison of percutaneous radiofrequency ablation and CyberKnife® for initial solitary hepatocellular carcinoma: A pilot study

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Author contributions: Shiozawa K, Watanabe M and Igarashi Y designed the study; Ikehara T, Matsukiyo Y and Kogame M performed percutaneous radiofrequency ablation; Kishimoto Y, Okubo Y, Makino H and Tsukamoto N performed CyberKnife® therapy; Shiozawa K and Watanabe M analyzed the data and wrote the manuscript; Sumino Y supervised the study; all authors have read and approved the final version to be published.

Institutional review board statement: The study was approved by the Ethical Review Board of Toho University Medical Center, Omori Hospital.

Informed consent statement: Informed consent was obtained from all patients for being included in the study.

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Abstract

AIM: To compare therapeutic outcomes and adverse events in initial solitary hepatocellular carcinoma (HCC) treated with radiofrequency ablation (RFA) and CyberKnife®.

METHODS: Seventy three consecutive patients with initial solitary HCC treated with RFA (38 patients; RFA group) and CyberKnife® (35 patients; CK group) were enrolled in this study. Background factors were compared between the two groups. Local and intrahepatic distant recurrence control, and cumulative survival rates were compared between the two groups. These were determined using the Kaplan-Meier method, and the significance of differences was analyzed by log-

rank test. The presence of more grade 3 on CTCAE ver. 4.0 early and late adverse events was investigated.

RESULTS: In background factors, age was significantly higher (P = 0.005) and the tumor diameter was significantly larger (P = 0.001) in the CK group. The 1-year local recurrence control rates were 97.4% and 97.1% in the RFA and CK groups, respectively (P = 0.71); the 1-year intrahepatic distant recurrence control rates were 85.6% and 86.1%, respectively (P = 0.91); and the 1-year cumulative survival rates were 100% and 95.2%, respectively (P = 0.075), showing no significant difference in any rate between the two groups. There were no late adverse event in the RFA group, but 11.4% in the CK group had late adverse events. In the CK group, the Child-Pugh score at 12 mo after treatment was significantly higher than that in the RFA group (P = 0.003) and significantly higher than the score before treatment (P = 0.034).

CONCLUSION: The occurrence of adverse events is a concern, but CyberKnife® treatment is likely to become an important option for local treatment of early HCC.

Key words: Hepatocellular carcinoma; Radiofrequency ablation; Stereotactic body radiotherapy; CyberKnife®; Adverse event

Core tip: To compare therapeutic outcomes and adverse events in initial solitary hepatocellular carcinoma (HCC) treated with radiofrequency ablation (RFA; 38 patients) or CyberKnife® (35 patients). The 1-year local recurrence control, the 1-year intrahepatic distant recurrence control and the 1-year cumulative survival rates were no significant difference in any rate between the two groups. In the CyberKnife® group, the Child-Pugh score at 12 mo after treatment was significantly higher than that in the RFA group and significantly higher than the score before treatment. The occurrence of adverse events is a concern, but CyberKnife® is likely to become an important option for local treatment of early HCC.

Shiozawa K, Watanabe M, Ikehara T, Matsukiyo Y, Kogame M, Kishimoto Y, Okubo Y, Makino H, Tsukamoto N, Igarashi Y, Sumino Y. Comparison of percutaneous radiofrequency ablation and CyberKnife® for initial solitary hepatocellular carcinoma: A pilot study. *World J Gastroenterol* 2015; 21(48): 13490-13499 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13490.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13490

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, causing more than 500000 deaths every year. The incidence of HCC has increased globally due to the spread of hepatitis B and C virus infections[1,2]. In Japan, therapeutic policy for HCC is mainly decided based on the Evidence-based Clinical Practice Guidelines for HCC developed by the Japan Society of Hepatology (JSH)[3]. For HCC with liver damage A or B and 3 or fewer tumors with a diameter of 3 cm or smaller, liver resection and percutaneous ablation therapy are selected. However, most patients with HCC confined to the liver are not candidates for resection because of the frequent association with cirrhosis and other contraindications. Liver resection is also associated with a recurrence rate of 40%-60%[4,5]. Thus, many HCC patients are treated with percutaneous ablation therapy. Radiofrequency ablation (RFA) was introduced in Japan in 1999 and has been covered by national health insurance since April 2004. RFA is performed at many institutions because it can coagulate a wide area in one session compared to other percutaneous treatments, such as percutaneous ethanol injection therapy, and local control is high[6].

CyberKnife® (Accuray Incorporated, Sunnyvale, CA, United States) stereotactic body radiotherapy (SBRT) is image-guided robotic radiosurgery using a radiation delivery platform that can detect and correct for intrafraction tumor motion, as well as adapt to the patient's breathing pattern by moving the linear accelerator in concert. CyberKnife® was developed in the United States in 1992, first applied clinically in 1994, and introduced in Japan in 1997. CyberKnife® can be used to perform multidirectional irradiation and disperse the dose among normal tissues due to a high degree of freedom in the direction of irradiation. Therefore, irradiation in CyberKnife® treatment is more intensive than that in SBRT using a conventional Liniac system.

The therapeutic indications of CyberKnife® originally included brain tumor and head and neck cancer. Use for cancer in the trunk, including HCC, was begun after approval in June 2008. Tumors in the trunk move with respiration, but CyberKnife® detects minute body movements and fine-tunes the irradiation angle using a seeker. Therefore, this approach has potential as a novel local treatment for HCC due to its low invasiveness and reduced burden on the patient[7,8].

In the 10 years since introduction of RFA for treatment of HCC in Japan, the therapeutic outcome and adverse events in RFA-treated HCC have been widely reported[9,10]. In contrast, there have only been a few studies on the therapeutic outcome of HCC treated with SBRT including CyberKnife®[7,8,11-13] and, to our knowledge, there has been no comparison of therapeutic outcomes and adverse events in HCC between RFA and CyberKnife®. This comparison is important in determining the indication of CyberKnife® treatment for HCC. In this study, we compared therapeutic outcomes and adverse events in patients with initial solitary HCC treated with RFA or CyberKnife®

in almost the same period at our hospital and related institutions, and retrospectively investigated the efficacy of CyberKnife® for HCC treatment.

MATERIALS AND METHODS

Patient characteristics

The subjects were 73 patients with initial solitary HCC without comorbidity like cardiac, pulmonary and celebral diseases, and treated with RFA or CyberKnife® between October 2011 and September 2014 at our hospital and related institutions. There were 38 consecutive patients treated with RFA (RFA group) and 35 consecutive patients treated with CyberKnife® (CK group). All patients were diagnosed with HCC using gray-scale ultrasonography (US), dynamic computed tomography (CT) and Gd-EOB-DTPA-enhanced magnetic resonance imaging (MRI) (EOB-MRI) based on the new guideline of the American Association for the Study of Liver Diseases[14]. Serum α-fetoprotein (AFP), AFP-L3 fraction, and des-γ-carboxyprothrombin (DCP) levels were referred to, as needed. If a diagnosis was difficult based on these examinations, ultrasound-guided percutaneous transhepatic tumor biopsy was performed and the diagnosis was made histopathologically.

We chose treatments for all patients based on the Evidence-based Clinical Practice Guidelines for HCC published by the JSH. The RFA group comprised patients with liver damage A or B, and a solitary tumor with a diameter ≤ 3 cm, and in whom liver resection was not indicated because they were elderly, had other underlying diseases, or did not want liver resection. Among the adaptation cases of RFA, we chose CyberKnife® treatment for elderly patients and patients with respiratory disease for whom breath-holding was difficult, those for whom RFA could not be safely performed because of the location of HCC, and those who requested CyberKnife® treatment. The patients met the following indications established at related institutions based on reports on HCC treated with SBRT: (1) A performance status ≤ 2; (2) Child-Pugh classification A to B (scored 8); (3) serum T-Bil ≤ 3 mg/dL; (4) ICG 15-min ≤ 50%; (5) absence of ascites; (6) solitary tumor ≤ 5 cm; (7) tumor located ≥1 cm from the intestine; (8) tumor not in contact with the gall bladder; and (9) absence of distant metastasis. Patients in both groups were all initial (non-recurrent) cases.

RFA procedure

RFA was performed using a Cool-tip RF System (Covidian, Boulder, CO, United States) or a CelonPower System[15] (Olympus Medical Systems, Tokyo, Japan). All patients underwent ultrasound-guided RFA. Artificial pleural or ascitic fluid (500-1000 mL of 5% glucose solution) was used to facilitate visualization of lesions with a subcapsular location or in the vicinity of the diaphragmatic dome, since these were difficult to visualize by US.

The Cool-tip RF System had a 17-gauge cooled-tip electrode with a 20- or 30-mm exposed tip. For the 20-mm exposed tip, the initial power output was 40 W. This was increased by 10 W/min to a maximum of 60 W. For the 30-mm exposed tip, the initial power output was 60 W, and this was increased by 10 W/min to a maximum of 90 or 100 W. For each tip, RF energy was delivered 1 to 3 times until impedance increased beyond the limit of the generator. After completion of ablation, the RF needle was energized at 60 W and removed while ablating the needle tract.

The CelonPower System generator had needle-type bipolar applicators with electrodes of 3 cm in length. The total energy and output were based on the standard dosimetry table. RF current was generated using automated control of the output with a resistance-controlled power function. Ablation times using 2 and 3 applicators were 17 and 16 min, respectively.

When the ranges ablated using the two RF systems were judged to be insufficient based on US findings during treatment or in dynamic CT performed 2-4 d after treatment, ablation was repeated using the same procedure on the same day or the day after dynamic CT.

CyberKnife® procedure

SBRT was performed with CyberKnife®, a robotic image-guided whole body radiosurgery system equipped with a synchrony system for real-time respiratory tracking of target volumes that move with respiration. Overall accuracy is < 1.5 mm with synchrony for mobile targets, with a treatment accuracy of 0.3 mm. Since CyberKnife® treatment of cancer in the trunk, including HCC, cannot be performed using the skeleton as the focal point (in contrast to the skull in head and neck cancer), a gold fiducial marker was implanted percutaneously around the perimeter of the target volume using an ultrasound-guided procedure prior to acquisition of a planning CT scan. The gold fiducial marker is a coiled device (0.75 mm in diameter and 5 mm in length) that is implanted around the target lesion. For a lesion in the right or left lobe of the liver, the marker could be implanted in the right or left lobe, except for a S6 lesion.

Treatment planning CT was performed at least 7 d after fiducial placement. Patients were immobilized on a vacuum mattress or a self-expanding foam mattress in the treatment position (supine). A spiral CT scan without contrast and a three-phase scan with contrast (arterial, portal, and equilibrium) were acquired for planning. The gross tumor volume (GTV) was contoured on the contrast-enhanced lesion visible on the partial-exhale contrast-enhanced CT scan. Tumor tracking was performed during treatment

Table 1 Patient characteristics

Variable	RFA (n = 38)	CyberKnief® (n = 35)	P value
Observation period	561 (range 222-1223)	379 (range 203-1065)	0.15
Age (yr), mean ± SD	68.7 ± 10.5 (range 42-86)	75.1.7 ± 8.1 (range 55-89)	0.005
Gender			0.82
Male/female	27/11	24/11	
Etiology			0.32
HBV/HCV/alchol/other	9/18/3/8	4/23/1/7	
Child-Push classification, A/B	31/7	28/7	0.86
Tumor size	17.5 ± 6.1 (range 7-29)	28.6 ± 11.5 (range 12-50)	0.001

P < 0.05, statistical significant. RFA: Radiofrequency ablation; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

using the inserted fiducial marker. The clinical target volume (CTV) was defined as the GTV with a 10-mm margin in all directions within the liver. A 1.5-mm margin was applied to the CTV to obtain the planning target volume (PTV). The total dose was 60 Gy and the dose was increased or decreased based upon the tumor size, location and residual liver function to give 95% PTV coverage. Irradiation was divided into 3 to 5 fractions. The irradiation range of the hepatic parenchyma surrounding the tumor was ⩾ 17 Gy and the irradiated site was ⩽ 20% of the whole liver.

Statistical analysis

Outcomes: The course was followed in both groups using changes in tumor markers and liver function in blood tests at 1, 3, 6 and 12 mo after treatment and at appropriate timepoints thereafter; the presence of local and intrahepatic distant recurrence detected on dynamic CT, contrast-enhanced US and EOB-MRI; and adverse events. Background factors of median observation periods, mean age, sex (male/female), Child-Pugh classification (A/B), and mean tumor diameter were compared between the two groups by χ^2 test, Student t-test, and Mann-Whitney U-test.

Local and intrahepatic distant recurrence control, and cumulative survival rates were compared between the two groups. Local recurrence was defined as recurrence of the treated lesion and intrahepatic distant recurrence was defined as recurrence beyond 2 cm from the treated area. For instance, in large segments such as segment Ⅶ, a recurrence within the segment but beyond 2 cm from the previously treated area is considered distant recurrence, although if in the same segment. Only death from HCC or liver failure (*i.e.*, liver disease-related death) was regarded as a fatal case. Local and intrahepatic distant recurrence control, and cumulative survival rates were determined using the Kaplan-Meier method, and the significance of differences was analyzed by log-rank test. Kaplan-Meier curves were also prepared setting the baseline at the day of treatment. Factors involved in local and intrahepatic distant recurrence, including the tumor diameter in the RFA group and the tumor diameter and total dose in the CK group, were analyzed by Student t-test.

Adverse events: The presence of grade 3 or more severe early and late adverse events was investigated based on the Common Terminology Criteria for Adverse Events (CTCAE) ver. 4.0. Changes in the Child-Pugh score from before treatment to 1, 3, 6 and 12 mo after treatment were analyzed by two-way repeated measures ANOVA. Adverse events that developed during treatment and within 3 mo after treatment were defined as early complications, and those that developed 4 mo to one year after treatment were defined as late complications.

P value < 0.05 was regarded as significant in all statistical analyses. The study was approved by the Ethical Review Board of Toho University Medical Center, Omori Hospital.

RESULTS

Background

The median length of observation periods in the RFA and CK groups were 561 and 379 d, respectively, with no significant difference between the groups. In the RFA group, the mean age was 68.7 ± 10.5 years old, and there were 27 males and 11 females. The Child-Pugh classification was A in 31 and B in 7 cases before treatment, and the mean tumor diameter was 17.5 ± 6.1 mm. In the CK group, the mean age was 75.1 ± 8.1 years old, and there were 24 males and 11 females. The Child-Pugh classification before treatment was A in 28 and B in 7 cases, and the mean tumor diameter was 28.6 ± 11.5 mm. Age was significantly higher (P = 0.005) and the tumor diameter was significantly larger (P = 0.001) in the CK group (Table 1).

Outcomes

The 1-year local recurrence control rates were 97.4% and 97.1% in the RFA and CK groups, respectively (P = 0.71) (Figure 1); the 1-year intrahepatic distant recurrence control rates were 85.6% and 86.1%, respectively (P = 0.91) (Figure 2); and the 1-year cumulative survival rates were 100% and 95.2%, respectively (P = 0.075), showing no significant difference in any rate between the two groups (Figure 3).

In the RFA group, local recurrence occurred in 3

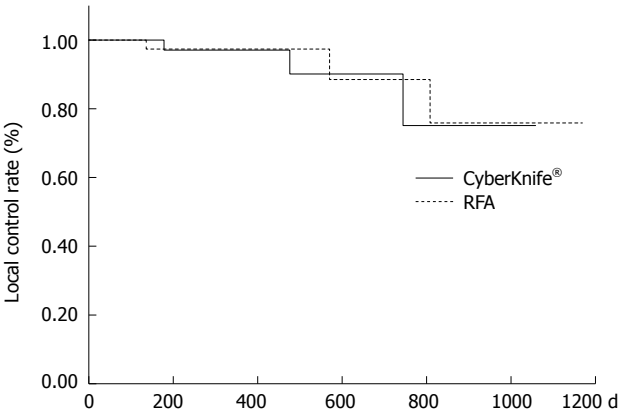

Figure 1 Kaplan-Meier local recurrence curve. Comparison of the 1-year local recurrence control rates between the radiofrequency ablation (RFA) group and the CyberKnife® group (P = 0.71).

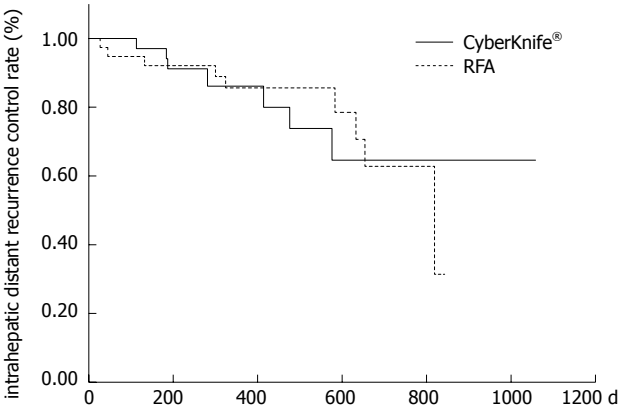

Figure 2 Kaplan-Meier intrahepatic distant recurrence curve. Comparison of the 1-year intrahepatic distant control rates between the radiofrequency ablation (RFA) group and the CyberKnife® group (P = 0.91).

patients and the tumor diameters were 10, 18 and 25 mm, respectively. In the CK group, local recurrence occurred in 3 patients and the tumor diameters and total dose were 18 mm/60 Gy, 33 mm/39 Gy, and 46 mm/36 Gy, respectively. The tumor diameters of 2 of the 3 patients in the CK group were greater than the mean of all patients in the group, and the mean total dose was slightly lower than that for the whole group. Intrahepatic distant recurrence occurred in 9 patients in the RFA group, but there was no significant difference in the tumor diameter in these cases compared to others in the group (P = 0.87) (Table 2). Intrahepatic distant recurrence occurred in 7 patients in the CK group, and the incidence was significantly higher in cases with a large tumor diameter (P = 0.045) and low total dose (P = 0.036) (Table 3). In the RFA group, RFA was performed for all local recurrence (3 cases), and for intrahepatic distant recurrence (9 cases), RFA and transarterial chemoembolization (TACE) were performed for 7 and 1 cases, respectively and untreated case was 1. In the CK group, for local recurrences (3 cases), CyberKnife® treatment and TACE were performed for 2 and 1 cases, and for

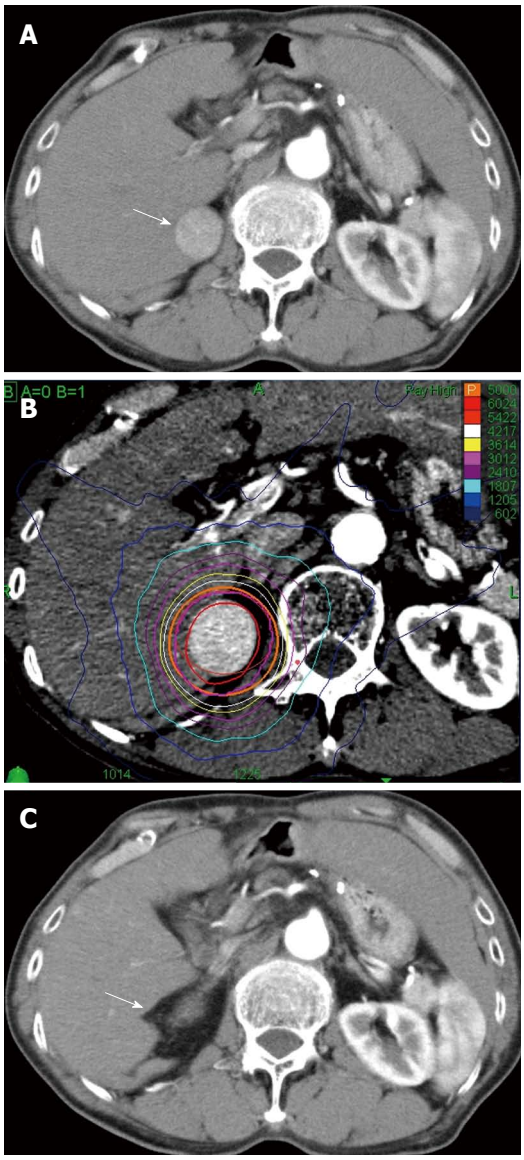

Figure 3 Clinical example of good responder patient by CyberKnife®. A 72-year old male with hepatitis C virus cirrhosis received CyberKnife® therapy at a dose of 50 Gy in 5 fractions for hepatocellular carcinoma sized 28 mm in diameter in S6/7. A: Dynamic computed tomography (CT) scan in arterial phase before the treatment showed a hypervascular lesion in S6/7 (arrow); B: Axial view of radiation dose distribution; C: The lesion did not be distinguished on dynamic CT scan in arterial phase 12 mo after the treatment (arrow). This therapeutic response was described as a complete response.

intrahepatic distant recurrence (7 cases), CyberKnife® treatment and TACE were performed for 3 each and untreated case was 1.

Adverse events

There were no early adverse events in either group. There were also no late adverse event in the RFA group, but 4 of the 35 patients (11.4%) in the CK group had late adverse events. All 4 patients had ascites, and two of these patients were liver disease-related fatal cases, and the Child-Pugh score in both patients was higher at 12 mo after treatment compared to that before treatment (Figure 4). In

Table 2 Risk factor of intrahepatic distant recurrence in the radiofrequency ablation group

	RFA		
	Distant recurrence (-)	Distant recurrence (+)	*P* value
n	29	9	
Tumor size (mm)	17.4 ± 6.2	17.8 ± 6.0	0.87

Data are expressed as the mean ± SD. RFA: Radiofrequency ablation.

Table 3 Risk factor of intrahepatic distant recurrence in the CyberKnife® group

	CyberKnife®		
	Distant recurrence (-)	Distant recurrence (+)	*P* value
n	28	7	
Tumor size (mm)	26.6 ± 11.1	36.3 ± 10.6	0.045
Total dose (Gy)	50.6 ± 7.8	43.6 ± 6.7	0.036

Data are expressed as the mean ± SD. $P < 0.05$, statistical significant.

the CK group, the Child-Pugh score at 12 mo after treatment was significantly higher than that in the RFA group ($P = 0.003$) and significantly higher than the score before treatment ($P = 0.034$) (Figure 5).

DISCUSSION

Chronic hepatitis and liver cirrhosis are present in the background liver as an underlying disease in many HCC cases. In addition to tumor factors such as the tumor diameter and number of tumors, background factors including liver function are important in deciding on a treatment method. Several stage classification systems are used to evaluate hepatic functional reserve, including the Barcelona Clinic Liver Cancer Staging System[16,17], Okuda Staging System[18], and Cancer of the Liver Italian Program Scoring System[19]. In Japan, the therapeutic policy is mainly decided using the Evidence-based Clinical Practice Guidelines for HCC of the JSH[3], although with some differences among institutions. For early HCC with liver damage A or B and ≤ 3 tumors and a tumor diameter ≤ 3 cm, liver resection and percutaneous ablation therapy are recommended. Liver resection is the primary curative treatment for HCC, with a current 5-year survival rate of about 70%, especially for small HCCs < 5 cm in diameter, due to improved surgical techniques and postoperative management[20]. However, liver resection is only an option in 10% to 30% of patients at diagnosis for various clinical reasons[21]. Moreover, intrahepatic distant recurrence occurs after liver resection in many cases due to multicentric carcinogenesis[22], a characteristic of HCC, and some patients do not want liver resection.

For these reasons, many institutions perform RFA as the first choice for treatment of early HCC. RFA is the most common ablation modality worldwide with

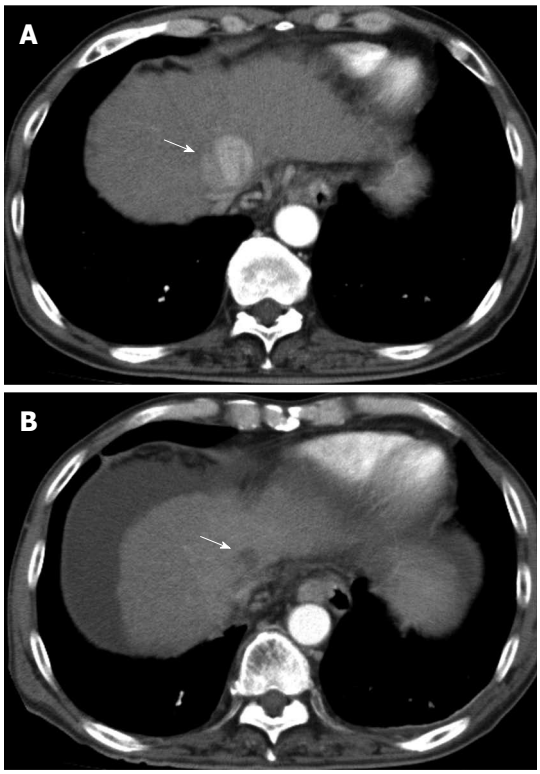

Figure 4 Clinical example of patient had late adverse events by CyberKnife®. A 66-year old male with hepatitis B virus cirrhosis received CyberKnife® therapy at a dose of 42 Gy in 3 fractions for hepatocellular carcinoma sized 40 mm in diameter in S4. The Child-Pugh score before the treatment was 7 and the score rose with 13 when 12 mo after the treatment. After he died of liver failure. A: Dynamic computed tomography (CT) scan in arterial phase before the treatment showed a hypervascular lesion in S4 (arrow); B: The lesion was showed as a hypovasclular lesion on dynamic CT scan in arterial phase 12 mo after the treatment (arrow), but dynamic CT scan showed liver atrophy and massive ascites.

80%-95% complete tumor necrosis and a 33%-57% 5-year survival rate in patients with small HCC[23,24]. A recent retrospective study found that overall survival (OS) and disease-free survival with RFA were significantly better than those with liver resection for central HCC ≤ 2 cm in diameter[25]. With advances in RF systems and techniques, the therapeutic effect of RFA is now comparable to that of liver resection. However, even though the tumor diameter and number of tumors meet the indication, RFA is not applicable in cases with the tumor in a deep region, directly under the liver dome, or near a thick blood vessel or bile duct, or if the tumor cannot be visualized by US including CEUS. CyberKnife® may be a good indication for such cases.

CyberKnife® for cancers of the trunk was approved in Japan relatively recently, and the therapeutic effect and adverse events in application to these cancers, including HCC, remain unclear. For this reason, we compared the utility of CyberKnife® treatment of HCC with that using RFA in the same period in the same institutions. The subjects were limited to patients with an initial solitary HCC. The background factors in the groups were mostly similar, but there were

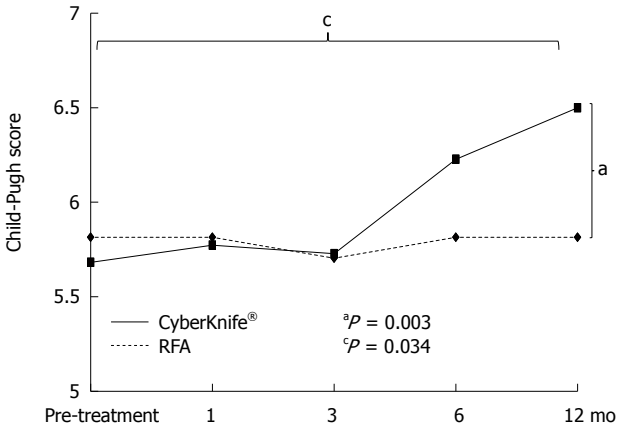

Figure 5 Changes in the Child-Pugh score from before treatment to 12 mo after treatment. In the CyberKnife® group, the Child-Pugh score at 12 mo after treatment was significantly higher than that in the radiofrequency ablation (RFA) group ($^aP = 0.003$) and significantly higher than the score before treatment ($^cP = 0.034$).

significantly more elderly patients and larger tumor diameters in the CK group. Since CyberKnife® can detect and respond to a respiratory fluctuation-induced slight movement of the target lesion, it may be a good indication for elderly patients for whom breath-holding during RFA puncture is difficult. In addition, only a one-day hospital stay is required to place a gold fiducial marker for the irradiation target, and irradiation is applied for 3-5 d at an outpatient clinic, which reduces medical costs and causes less interference with daily life. Because of this low invasiveness, there has been an increase in elderly patients requesting CyberKnife®, which may have been reflected in our results. The tumor diameter was ⩽ 3 cm in the RFA group due to the guidelines for RFA, but a tumor of diameter ⩽ 5 cm may be included in the indication of CyberKnife®.

The local control rate did not differ significantly between the two groups. Several previous studies have examined CyberKnife® for liver tumors, including liver metastasis and cholangiocellular carcinoma (CCC). Bibaut *et al*[12] found 1- and 2-year local control rates of 89.8% in a study on CyberKnife® applied to 96 nodes in 75 HCC patients; and Janoray *et al*[13], Choi *et al*[26], and Cardenes *et al*[27] reported 1-year local control rates of 84% in 21 HCC patients, 71.9% in 32 HCC patients, and 100% in 25 HCC patients, respectively. In contrast, Yoon *et al*[28] found 1- and 3-year local control rates of 94.8% and 92.1%, respectively, in 103 HCC nodes treated with conventional SBRT, indicating that the outcome of CyberKnife® is almost equivalent to conventional SBRT. The 1-year local control rate was 97.1% in the CK group in the current study.

The tumor diameter exceeded 30 mm (33 and 46 mm) in 2 of the 3 cases with local recurrence in the CK group, and the total doses were 39 and 36 Gy, respectively, which were lower than those in the other patients. It has occasionally been reported that there is no correlation between development of local recurrence and total dose in use of conventional SBRT[29]. Using CyberKnife®, Janoray *et al*[13] found a 1-year local control rate of 100% in cases treated with a total dose of 60 Gy, and Dewas *et al*[11] showed that the local control rate was significantly higher in cases with a total dose of 45 Gy compared to < 45 Gy in 99 patients with liver metastasis, 48 with HCC, and 6 with CCC. The reasons for the differences in the association of local recurrence with total dose between conventional SBRT and CyberKnife® are unclear, but it cannot be ruled out that total dose is related to local recurrence after CyberKnife® treatment.

Large tumors, such as those with a diameter of ⩾ 30 mm or volume ⩾ 32 mL, are significantly more likely to show local recurrence after conventional SBRT[28,30]. In this study, the tumor diameter was >30 mm in two cases with local recurrence in the CK group, which suggests that the diameter is also an important factor in local control in treatment with CyberKnife®. Currently, CyberKnife® is indicated for tumors with a diameter ⩾ 30 mm at our hospital and related institutions, but it is uncertain if this tumor diameter cut-off is appropriate. Moreover, local recurrence was also noted in a case with a tumor diameter of 18 mm. Not only the tumor diameter but also the degree of differentiation of HCC, tumor marker[31] and serum ferritin[32] may be involved in local control, and this also applies to RFA. It will be necessary to investigate many cases to establish the indication for CyberKnife® treatment, including the tumor diameter and degree of differentiation.

There was no significant difference in the intrahepatic distant recurrence control rate between the RFA and CK groups. Bibaut *et al*[12] found an incidence of intrahepatic distant recurrence of 24% (18/75 cases) over a median observation periods of 10 mo (30-49 mo). Our study had a similar incidence of 20% (7/35 cases) over a median observation periods of 379 d. There was no significant correlation between the tumor diameter and intrahepatic distant recurrence in the RFA group, but the incidence was significantly higher in cases with a large tumor treated with low total dose in the CK group. Risk factors for intrahepatic distant recurrence after treatment of HCC with SBRT have not been examined in detail, but it is well known that intrahepatic metastasis undetectable by imaging can be present in large HCC. Thus, there is a general risk of intrahepatic distant recurrence in cases of HCC with a large tumor diameter, and the required total dose should be fully investigated before CyberKnife® treatment.

The observation period was short, but there was no significant difference in OS between the RFA and CK groups. The Japanese Nationwide Survey reported 3-year OS rates of HCC patients treated with RFA of 82%-88% and 66%-82% in patients with tumor diameters of ⩽ 20 mm and 21-50 mm, respectively[33]. The 1- and 2-year OS rates were 78.5% and 50.4%, respectively, in Bibaut *et al*[12], and Janoray *et al*[13]

found a 1-year OS of 89% in 56 patients with liver tumors, including liver metastasis. These results show that CyberKnife® achieved OS equivalent to that in other standard local treatment for liver tumors, including HCC. The 1-year OS rate was 95.2% in our study, which is more favorable than those in previous reports. This may have been due to limiting the subjects to those with an initial solitary tumor.

There were two liver disease-related fatal cases in the CK group. Both patients had grade 3 or more severe late adverse events (CTCAE ver.4.0) and the Child-Pugh score increased at 12 mo after treatment compared to that before treatment. In previous reports, grade 3 or more severe hepatotoxicity occurred at rates of 0-25.8% within 3 mo after conventional SBRT[28], and Sanuki *et al*[29] found a rate of grade 3 or more severe early adverse events of 13% after SBRT. In contrast, Bibaut *et al*[12] found grade 1-2 adverse events, such as hepatic pain, nausea, and asthenia, in 15%-17% of cases after CyberKnife® treatment, but no radiation-induced liver disease (RILD)[34] in any patient. In our study, grade 1-2 nausea and malaise occurred in several patients, but the incidences of grade 3 or more severe early and late adverse events were lower than those after conventional SBRT.

In previous studies of SBRT for HCC[27,35], the total dose was significantly correlated with adverse events. The incidence of hepatotoxicity was high in cases with Child-Pugh classification B, and liver cirrhosis progressed with repeated SBRT. Compared to conventional SBRT, the beam direction can be freely set because it can be moved using a robot arm, and the number of beams and the total dose centrality are high with CyberKnife®, which may reduce adverse effects. However, RILD may develop following CyberKnife® treatment, and the Child-Pugh classification changed from A to B in 16.1% of 31 HCC patients[26]. In our study, the Child-Pugh score was significantly higher at 12 mo after treatment in the CK group compared to the RFA group, and in the CK group death occurred in two cases in which the Child-Pugh score was significantly higher than that before treatment. The incidence of serious complications induced by CyberKnife® may be lower than with conventional SBRT, but such complications may affect the outcome. Thus, the hepatic functional reserve should be fully evaluated before CyberKnife® treatment and adverse events should be carefully monitored, as for conventional SBRT.

The limitations of this study include the small number of cases, the different criteria in the both groups, the heterogeneous in two aspects that precisely have an impact in both the relapse and the survival, the short observation period, and the retrospective design. The ideal design for comparison between different techniques is a randomized controlled trial or at least a propensity score analysis. Moreover, less than 2 years of follow up are even less reliable with regard to survival, local and intrahepatic distant recurrence control rate. However, the results suggest that the therapeutic effect of CyberKnife® for HCC was equivalent to that of RFA as a pilot study.

In some cases, RFA is difficult because of the tumor location, and liver resection and the breath-holding during RFA puncture may be a risk in elderly patients with HCC, the number of whom is likely to increase. Including the cost of hospitalization, the costs of RFA and CyberKnife® were 380000 and 630000 yen, respectively and the cost performance of CyberKnife® is higher in approximately 2 times than RFA treatment in Japan. However, CyberKnife® is a low-invasive procedure that is synchronous with respiration and requires no breath holding. Based on the hepatic functional reserve, this procedure may be a good indication for cases with difficulty with RFA.

In conclution, there were many elderly patients and large tumors in the CK group, but the therapeutic outcome was equivalent to that in the RFA group. Tumors with a diameter of $\leqslant$ 30 mm are a good indication for CyberKnife®. Relatively favorable outcomes were achieved in cases with a tumor diameter of 30-50 mm, but a further investigation is needed before these can be included in the indication, in consideration of the patient background. The occurrence of adverse events is a concern, but CyberKnife® treatment is likely to become an important option for local treatment of early HCC.

COMMENTS

Background

Radiofrequency ablation (RFA) for hepatocellular carcinoma (HCC) is performed at many institutions because it can coagulate a wide area in one session, and local control is high. Recently, CyberKnife® became the adaptation in the cancer of the trunk, including HCC. Tumors in the trunk move with respiration, but CyberKnife® detects minute body movements and fine-tunes the irradiation angle using a seeker. Therefore, this approach has potential as a novel local treatment for HCC due to its low invasiveness and reduced burden on the patient.

Research frontiers

The authors compared therapeutic outcomes and adverse events in patients with initial solitary HCC treated with RFA or CyberKnife®, and retrospectively investigated the efficacy of CyberKnife® for HCC treatment. In this study, there were many elderly patients and large tumors in the CyberKnife® group, but the therapeutic outcome was equivalent to that in the RFA group.

Innovations and breakthroughs

CyberKnife® detects minute body movements and fine-tunes the irradiation angle using a seeker. Therefore, this approach has potential as a novel local treatment for HCC due to its low invasiveness and reduced burden on the patient.

Applications

The occurrence of adverse events is a concern, but CyberKnife® treatment is likely to become an important option for local treatment of early HCC.

Terminology

CyberKnife® stereotactic body radiotherapy is image-guided robotic radiosurgery using a radiation delivery platform that can detect and correct for intrafraction tumor motion, as well as adapt to the patient's breathing pattern by

moving the linear accelerator in concert.

Peer-review

The main strength of the paper by Shiozawa *et al* is its novelty as studies directly comparing RFA and CyberKnife® for HCC are lacking.

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ORIGINAL ARTICLE

Retrospective Study

Histological evaluation for chemotherapeutic responses of metastatic lymph nodes in gastric cancer

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Abstract

AIM: To investigate the effect of preoperative chemotherapy (pre-CTx) for metastatic lymph nodes (MLNs) of gastric cancer (GC).

METHODS: A retrospective cohort of patients with advanced GC, who underwent pre-CTx followed by gastrectomy, was reviewed. The histological tumor regression grade (TRG), which considered the percentage of residual cancer in the visible tumor bed, was applied to primary tumors and individual MLNs: G1a (complete response), G1b (< 10%), G2 (10%-50%) and G3 (> 50%). The clinical response to pre-CTx was retrospectively evaluated using only MLNs information, and we compared the histological and clinical evaluations of MLNs.

RESULTS: Twenty-eight patients were enrolled. A total of 438 MLNs were retrieved, and 22 (5%), 48 (11%), 63 (14%) and 305 (70%) LNs were assigned as G1a, G1b, G2 and G3, respectively. Stratification of the residual MLNs based on the TRGs was as follows: 28 G1b MLNs (9%), 48 G2 MLNs (15%), and 253 G3 MLNs (76%) in the D1 region; 20 (23%), 15 (17%), and 52 (60%) in the D2 region, respectively. However, no significant correlation was found between TRGs in MLNs and clinical response in the subgroup for which evaluation of clinical response was available.

CONCLUSION: Pre-CTx does not provide any outstanding histological benefit for MLNs, and an appropriate D2 lymphadenectomy should routinely be performed to offer the chance of curative resection.

Key words: Preoperative chemotherapy; Gastric cancer; Metastatic lymph node; Histological regression grade; Lymphadenectomy

Core tip: Preoperative chemotherapy for gastric cancer does not provide any outstanding histological regression for regional metastatic lymph nodes, and residual metastatic lymph nodes were located irrespective of D1 and D2 region. In addition, no significant correlation was found between the clinical response of metastatic lymph nodes based on RECIST classification and histological response grading. Consequently, an appropriate D2 lymphadenectomy should routinely be performed in order to offer the chance of curative resection of advanced gastric cancer treated with preoperative chemotherapy.

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INTRODUCTION

Gastric cancer (GC) is one of most diagnosed cancers worldwide, and it is estimated to be the third most frequent cause of cancer-related deaths[1]. New incidences of GC have decreased worldwide during recent decades, but the cause-specific mortality remains considerable, even after surgery[2]. Several randomized trials in Western countries have demonstrated that preoperative chemotherapy (pre-CTx) markedly improves the survival rates of patients with resectable GC[3-5]; these results have led to an increasing use of pre-CTx in clinical practice around the world, including Asian countries[6-8].

The surgeon's main purposes in using pre-CTx as an intervention for advanced GC patients are an increased rate of tumor resectability and tumoricidal effects on possible lymph node metastasis[9]. Some retrospective studies have suggested that pre-CTx would improve rates of radical resection in locally advanced GC patients[10]; however, there is no detailed previous report concerning the effects of pre-CTx on lymph node metastasis in GC patients. In a meta-analysis of randomized controlled trials, Xu *et al*[11] reported that N0 status was more frequently achieved in GC patients treated with pre-CTx than those treated with surgery alone. This finding demonstrates the possible effectiveness of pre-CTx on micrometastasis. These findings prompted us to examine the effects of pre-CTx on the metastatic lymph nodes (MLNs) of GC patients. In the present study, we retrospectively examined the histological response to pre-CTx in primary tumors and the MLNs of advanced GC. We also compared the findings with clinical evaluations in order to determine whether limited lymph node dissection is possible for GC patients treated with pre-CTx.

MATERIALS AND METHODS

Patients

Of the patients with gastric cancer treated at Kyoto Prefectural University of Medicine between January 2001 and January 2013, those who had undergone pre-CTx followed by gastrectomy were enrolled in the retrospective study. All the pre-CTx protocols included the anticancer drug S-1 (TS-1, Taiho Pharmaceutical, Tokyo, Japan), an orally active combination of tegafur, gimeracil, and oteracil potassium, which were accepted as S-1 alone[12] (80 mg/m^2 orally every 28 d), S-1 plus cisplatin[13] (S-1: 80 mg/m^2 orally every 21 d; cisplatin: 60 mg/m^2 intravenously on days 8 and 15), or S-1 plus docetaxel[14] (S-1: 80 mg/m^2 every 21 d; docetaxel: 60 mg/m^2 intravenously on days 1, 6, and 15) were administered in two to four identical courses, and open distal or total gastrectomy with Japanese-style D2 lymphadenectomy[15] was performed afterward. Written informed consent was obtained from all of the patients prior to the initiation of this study.

In general, patients underwent a double-contrast barium examination, endoscopy, and multidetector-row computed tomography (MDCT). They were diagnosed preoperatively based on their results in our hospital. Staging laparoscopy was performed to determine whether peritoneal dissemination was present prior to pre-CTx, although this procedure was not mandatory in this study.

Evaluation of clinical response for MLNs

Based on the new Response Evaluation Criteria in Solid Tumors (RECIST) guidelines[16], the clinical

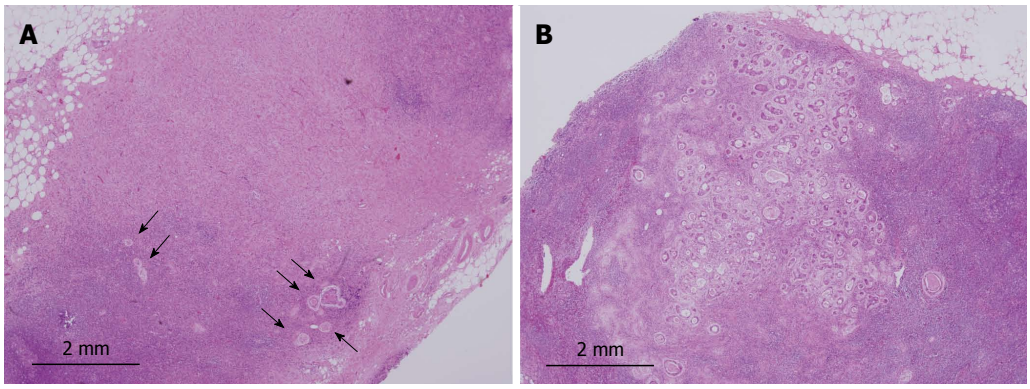

Figure 1 Representative slides of tumor regression grades in metastatic lymph nodes from the same primary tumor. A: G1b, slight residual cancer foci (arrows) are found in tumor bed; B: G3, residual cancer occupy > 50% of tumor bed. The scale bar indicates 2 mm.

response to pre-CTx was retrospectively evaluated by using only MLNs information according to the method reported by Schwartz *et al*[17]. In brief, a cine-mode display of contrast-enhanced MDCT images, which was performed two to four weeks after the completion of pre-CTx, was mainly used. The regional LNs were considered to show metastatic involvement if their longest diameter was ⩾ 15 mm, which is according to the RECIST guidelines. An experienced radiologist (Ichijo Y) who was blind to the patients' outcome reviewed the images and selected one or two of the most reproducible target MLNs per patient. Consequently, the MLNs were graded as "complete response" (CR), "partial response" (PR), "stable disease" (SD), and "progressive disease" (PD).

Histological evaluation

Immediately after resection, all the regional LNs were manually retrieved from the resected specimens. Following Japanese guidelines[15], the primary tumors were cut crosswise through the center of the tumor, and the retrieved LNs were cut longitudinally through the hilus. All slides were stained with hematoxylin and eosin in the routine fashion for use in histological evaluation.

The histological tumor regression grade (TRG) was evaluated using the grading system proposed by Becker *et al*[18,19]: G1a (complete response); G1b (< 10% residual tumor per tumor bed); G2 (10%-50% residual tumor per tumor bed); and G3 (> 50% residual tumor per tumor bed). We applied this grading system to the primary tumors and each individual's MLNs, comparing the patients' outcomes. Representative slides for TRG in the MLNs are shown in Figure 1. A pathologist specializing in gastrointestinal disorders (Kishimoto M and Yanagisawa A) who was blind to the patients' outcome reviewed the histology of all the slides.

Statistical analysis

All the analyses were implemented using the R statistical software program (The R Foundation for Statistical Computing, Vienna, Austria). The differences between the groups were analyzed using a χ^2 test. Differences were considered to be statistically significant at the $P < 0.05$ level.

RESULTS

Clinical evaluation and effects in primary tumors

A total of 28 patients were enrolled in the study. Based on the TNM classification from the Union for International Cancer Control, 15 patients (54%) were clinically diagnosed as Stage Ⅲ; 13 (46%) were diagnosed as Stage Ⅳ. Of the 28 patients, 27 received a postoperative chemotherapy regimen including S-1. As for TRGs in primary tumors, two cases (7%) were graded as G1b, six (21%) as G2, and 20 (71%) as G3. However, no cases were found with a complete tumor regression (G1a).

Evaluation for clinical response to pre-CTx was performed based on MLN findings in 11 patients (43%), whose pre- and post-therapeutic MDCT images were both available. Of these, two cases were graded as CR, four cases as PR, and two cases as SD, while no applicable target MLNs were found in three cases. The details of the other patient characteristics are listed in Table 1.

MLNs

A total of 1044 regional LNs (mean: 37.3 in each patient; range: 8-71) were retrieved from the 28 patients. Of those, 438 were diagnosed as positive for lymph node metastasis; 22 (5%), 48 (11%), 63 (14%), and 305 (70%) LNs were assigned to G1a, G1b, G2, and G3, respectively. As summarized in Table 2 and Figure 2, the TRGs of the primary tumors were significantly associated with those of the MLNs ($P < 0.0001$, χ^2 test). As for pathological complete response LN graded G1a ($n = 22$), 13 LNs belonged to the peri-gastric region (D1) and nine LNs belonged to regions along the named vessels of the celiac axis (D2). On the other hand, stratification of the residual MLNs based on the TRGs was as follows: 28 G1b MLNs (9%),

Table 1 Patients' characteristics (*n* = 28) *n* (%)

Variables	
Age (yr) [mean ± SD, (range)]	60 ± 10.7, (28-81)
Sex	
Male	16 (57)
Female	12 (43)
Tumor location	
Upper	9 (32)
Middle	15 (54)
Lower	4 (14)
Histological type	
Differentiated	10 (36)
Undifferentiated	18 (64)
Pre-therapeutic staging	
cStage Ⅲ	15 (54)
cStage Ⅳ	13 (46)
Clinical response	
CR	0 (0)
PR	10 (36)
SD	16 (57)
PD	2 (7)
Post-therapeutic T status	
ypT2	1 (4)
ypT3	11 (39)
ypT4	16 (57)
Post-therapeutic N status	
ypN0	1 (4)
ypN1	1 (4)
ypN2	4 (14)
ypN3	22 (79)
Post-therapeutic M status	
ypM0	9 (32)
ypM1	19 (68)
Post-therapeutic staging	
ypStage Ⅱ	2 (7)
ypStage Ⅲ	7 (25)
ypStage Ⅳ	19 (68)
TRG in primary tumors	
G1a	0 (0)
G1b	2 (7)
G2	6 (21)
G3	20 (71)
Preoperative chemotherapy	
S-1 alone	5 (18)
S-1 plus cisplatin	16 (57)
S-1 plus docetaxel	7 (25)
Operative procedure	
Distal gastrectomy	7 (25)
Total gastrectomy	21 (68)
Postoperative chemotherapy	
With	27 (96)
Without	1 (4)

CR: Complete response; PR: Partial response; SD: Stable disease; PD: progressive disease.

48 G2 MLNs (15%), and 253 G3 MLNs (76%) in the D1 region; 20 (23%), 15 (17%), and 52 (60%) in the D2 region, respectively.

In the subgroup of 11 cases for which MDCT images were available, a total of 436 regional LNs were retrieved. Of these, 226 MLNs were available for histological evaluation; 6 (3%), 23 (10%), 28 (12%), and 169 (76%) LNs were assigned to G1a, G1b, G2, and G3, respectively. Table 3 shows a breakdown of TRG in MLNs according to clinical response based on RECIST classification; however, there was no significant correlation between the clinical and histological response.

Table 2 Comparison of tumor regression grade in primary tumors *n* (%)

Variables		TRG in primary tumors			*P* value
		G1b (*n* = 2)	G2 (*n* = 6)	G3 (*n* = 20)	
TRG in MLNs	G1a	9 (2)	6 (1)	7 (2)	< 0.0001
	G1b	2 (0)	22 (5)	24 (5)	
	G2	2 (0)	17 (4)	44 (10)	
	G3	7 (2)	45 (10)	253 (60)	

TRG: Tumor regression grade; MLNs: Metastatic lymph nodes.

Table 3 Correlation between clinical response and histological evaluation *n* (%)

Variables		Clinical response			
		CR (*n* = 2)	PR (*n* = 4)	SD (*n* = 2)	No applicable target LNs (*n* = 3)
TRG in MLNs	G1a	1 (0)	4 (2)	1 (0)	0 (0)
	G1b	0 (0)	23 (10)	0 (0)	0 (0)
	G2	2 (1)	20 (9)	6 (3)	0 (0)
	G3	15 (7)	79 (35)	51 (23)	24 (11)

CR: Complete response; PR: Partial response; SD: Stable disease; TRG: Tumor regression grade; MLNs: Metastatic lymph nodes.

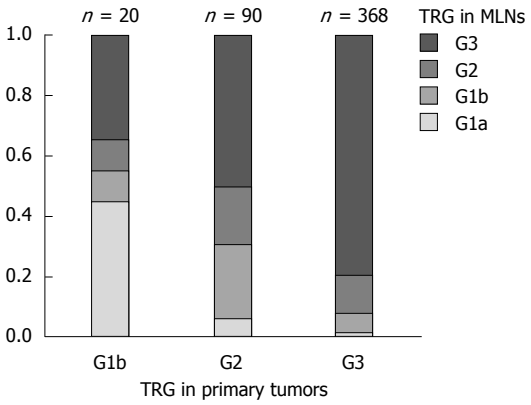

Figure 2 Breakdown of tumor regression grades in metastatic lymph nodes according to that in primary tumors. TRG: Tumor regression grade; MLNs: Metastatic lymph nodes.

DISCUSSION

In Asian countries, gastrectomy with D2 lymph node dissection has been generally regarded as the standard treatment for achieving a radical cure[15]. Recently, the D2 lymphadenectomy is increasingly recognized to be associated with lower locoregional recurrence and gastric cancer-related death rates than D1 lymphadenectomy in Western countries; therefore, it is the recommended surgical approach for patients with resectable gastric cancer[20]. On the other hand, pre-CTx has also been recognized as effective for latent

lymph node micrometastasis[21]. However, there is no detailed previous report concerning the effects of pre-CTx on MLNs in GC patients, and there is no consensus as to whether limited lymph node dissection is possible for GC patients treated by pre-CTx.

This study investigated histological effects in each individual's MLNs in 28 patients with advanced GC who were treated with pre-CTx, and to the best of our knowledge, this is the first report to address this issue. One of the most important findings in this study underlined that even MLNs clinically exhibiting favorable pre-CTx response showed an unsatisfactory histological response in practice. Kurokawa *et al*[22] also compared clinical and histological responses of GC to treatment with pre-CTx, focusing particularly on survival rates, and concluded that histological criteria showed higher response assessment validity than RECIST criteria and yielded the best surrogate endpoint for overall survival. Our results showed that histologically proven residual MLNs were located irrespective of D1 and D2 region. The residual tumor also existed in MLNs regardless of the degree of clinical response based on RECIST. Only 1 of 18 MLNs (5%) graded as clinically CR could achieve complete tumor regression (G1a,) and most MLNs had limited response. Taken together, appropriate D2 lymphadenectomy should be routinely performed in advanced GC patients who become candidates for curable surgical treatment by pre-CTx irrespective of the clinical response, as suggested by previous reports[23]. Some authors proposed that the clinical evaluation using MLNs information of GC patients treated with pre-CTx contributed to improving the complete resection rate by D2 lymphadenectomy[7]. However, Hayashi *et al*[24] called attention to the fact that D2 lymphadenectomy for GC patients, who had lower creatinine clearance treated with pre-CTx, caused greater surgical complications.

Another interesting finding of this study was that pre-CTx response in MLNs was, to some extent, correlated to the response in the primary tumors. We made an unwarranted assumption that the pre-CTx response in regional MLNs would parallel that of primary tumors; however, there is limited histological data supporting this correlation. The extent to which MLNs would histologically benefit from pre-CTx is unclear. Our present study revealed that 45% of the MLNs had a limited response (G2 or G3), even with G1b primary tumors, and that only 5% of the total MLNs achieved complete tumor regression (G1a). Similar findings were previously described by Mandard *et al*[25] for esophageal cancer treated with pre-CTx. Some previously published circumstantial evidence has also revealed that the interaction of the tumor cell with the organ environment creates differences between the primary tumors and their metastatic lesions in terms of their histology as well as their gene or protein expression[26,27]. Taken together, these findings suggest that pre-CTx may have no outstanding benefit for regional MLNs, even when the primary could achieve a considerable therapeutic effect. However, cumulative evidence[3-8] showed that some patients benefited from pre-CTx, in this context, with more effective pre-CTx regimens. Patient selection might be required for further effect.

Our study had several limitations, the first of which is its small sample size: the total number of patients was 28, and the final number of cases available for clinical evaluation was only 11. Further investigations using larger sample sizes would therefore be needed to confirm our findings. Second is that this study cohort included many advanced cases and limited information concerning patients in earlier stages of GC. Staging laparoscopy was not mandatory for patients suspected to have peritoneal dissemination during this period, and subsequently, this study cohort included many advanced cases of GC. The advanced stage of cancer progression and the large amount of tumor potentially influence the therapeutic effects of pre-CTx on MLNs in this study.

In summary, pre-CTx for advanced GC does not provide any outstanding histological regression for regional MLNs, and residual MLNs were located irrespective of D1 and D2 region. Further, little correlation was found between TRGs in MLNs and their clinical evaluation. Consequently, an appropriate D2 lymphadenectomy should always be performed in order to offer the chance of curative resection of advanced GC treated with pre-CTx. However, this study was based on a small number of patients with advanced GC, and limited data was given concerning patients in earlier stages of GC. Thus, a well-selected larger cohort study would be required to confirm our findings.

COMMENTS

Background

To reduce the mortality from gastric cancer (GC), improvement of perioperative intervention is essential and is still challenging. Since several large, randomized trials have demonstrated that preoperative chemotherapy (pre-CTx) markedly improves the survival rates of patients with GC, therapeutic strategies including pre-CTx have gradually been introduced into clinical settings around the world.

Research frontiers

One important concern for surgeons relates to interventions for patients with GC who become surgical candidates after pre-CTx. However, despite the cumulative evidence for pre-CTx in GC, the extent to which metastatic lymph nodes (MLNs) would histologically benefit from pre-CTx is unclear, and there is no detailed previous report concerning this issue.

Innovations and breakthroughs

These results showed a histological pre-CTx effect on regional MLNs using tumor regression grade (TRG). The TRGs of MLNs were closely correlated with those of the primary tumors. Furthermore, in this study, the clinical response to pre-CTx, which was retrospectively evaluated using only MLNs information, was compared with the histological pre-CTx effect. However, there was no significant correlation between the clinical and histological response in regional MLNs.

Applications

Pre-CTx for advanced GC does not provide promising histological regression for regional MLNs; consequently, an appropriate D2 lymphadenectomy should always be performed in order to offer the chance of curative resection. However, this study was based on a small number of patients with advanced GC, and limited data concerning patients in earlier stages of GC was available. A well-selected larger cohort study is necessary to confirm our findings.

Peer-review

The manuscript draws potentially interesting conclusions, although based on a limited number of patients.

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ORIGINAL ARTICLE

Retrospective Study

Use of a clinical pathway in laparoscopic gastrectomy for gastric cancer

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Abstract

AIM: To evaluate the implementation of a clinical pathway and identify clinical factors affecting the clinical pathway for laparoscopic gastrectomy.

METHODS: A standardized clinical pathway for gastric cancer (GC) patients was developed in 2001 by the GC surgery team at the Asan Medical Center. We reviewed the collected data of 4800 consecutive patients treated using the clinical pathway following laparoscopic gastrectomy with lymph node dissection for GC involving intracorporeal and extracorporeal anastomosis. The patients were treated between August 2004 and October 2013 in a single institution. To evaluate the rate of completion and risk factors affecting dropout from the clinical pathway, we used a multivariate logistic regression analysis.

RESULTS: The overall completion rate of the clinical pathway for laparoscopic gastrectomy was 84.1% (n = 4038). In the comparison between groups of intracorporeal anastomosis and extracorporeal anastomosis patients, the completion rates were 83.88% (n = 1740) and 84.36% (n = 2071), respectively, showing no statistically significant difference. The main reasons for dropping out were postoperative complications (n = 463, 9.7%) and the need for patient observation (n = 299, 6.2%). Among the discharged patients treated using the clinical pathway, the number of patients who were readmitted

within 30 d due to postoperative complications was 54 (1.1%). In a multivariate analysis, the intraoperative events (OR = 2.558) were the most predictable risk factors for dropping out of the clinical pathway. Additionally, being male (OR = 1.459), advanced age (OR = 1.727), total gastrectomy (OR = 2.444), combined operation (OR = 1.731), and ASA score (OR = 1.889) were significant risk factors affecting the dropout rate from the clinical pathway.

CONCLUSION: Laparoscopic gastrectomy appears to be a good indication for the application of a clinical pathway. For successful application, patients with risk factors should be managed carefully.

Key words: Clinical pathway; Laparoscopic gastrectomy; Gastric cancer; Extracorporeal anastomosis; Intracorporeal anastomosis

Core tip: Laparoscopic gastrectomy has been proven to enhance postoperative recovery compared to open gastrectomy for gastric cancer (GC) patients. Therefore, laparoscopic gastrectomy is thought to be a suitable procedure for a clinical pathway. In this study, we retrospectively analyzed the outcomes of a clinical pathway application for laparoscopic gastrectomy and tried to investigate the clinical factors that may influence a clinical pathway in a high-volume center. Laparoscopic gastrectomy for GC appears to be a good indicator for the application of a clinical pathway. For successful application, patients with risk factors (male, advanced age, total gastrectomy, combined operation, intraoperative events, American Society of Anesthesiologists score) should be managed carefully.

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INTRODUCTION

A clinical pathway (CP) is a comprehensive, systematized plan that details the essential steps in patient care in a given process, including any time-dependent clinical decisions[1,2]. The purpose of the CP is to minimize the hospital stay and to provide resources to achieve the best results and increase postoperative quality of life[3]. Therefore, CPs for multidisciplinary processes have been used to provide a coordinated program after various surgical procedures[4].

Gastric cancer (GC) is the most prevalent malignancy in South Korea and remains the second most common cause of cancer-related deaths in the world[5-7]. The proportion of early gastric cancers (EGCs) has increased to over 50% in South Korea and Japan as a result of early detection through mass screening[8,9]. GC detected at an early stage can be cured by surgical treatment, and the subsequent prognosis is excellent[10]. However, CP has rarely been suggested for conventional open gastrectomy (OG) in GC patients due to the complexity of the procedure, which involves postoperative hemodynamic changes, compared with the procedures for patients with benign diseases[11,12]. Recently, laparoscopic gastrectomy (LG) has been established as an alternative modality for the treatment of EGC patients, and it has better surgical outcomes. LG causes less postoperative pain, enhances postoperative recovery, reduces the length of hospital stay, and increases post-operative quality of life compared to open surgery[13-16]. It seems that LG improves outcomes because the less-invasive procedure decreases surgical trauma. It has been proposed that minimally invasive surgery might be a good candidate for a CP[1,12,17]. Therefore, LG may be suitable for the use of a CP that provides a time-based schedule for patients. Despite the usefulness of CPs in surgical settings, the use of a CP for LG for GC has not been adequately investigated. LG has become a primary minimally invasive operation for GC in the Asan Medical Center, especially for EGC patients. Since 2004, our institute has used a CP for large numbers of GC patients undergoing LG performed by experienced surgeons.

In this study, we retrospectively analyzed the outcomes of the CP for LG and investigated the clinical factors that influence the dropout rate from the CP.

MATERIALS AND METHODS

Patients and methods

A standardized CP for GC patients was developed in 2001 by a committee consisting of gastric surgeons, nurses, nutritionists, and members of the clinical support services in the Asan Medical Center. In 2004, we started LG in our gastric division, and a revised version of the CP was created for patients who underwent LG.

We reviewed the collected data of 4800 consecutive patients treated by the CP following LG for GC involving both extracorporeal and intracorporeal anastomoses at the Asan Medical Center between August 2004 and October 2013. Preoperative clinical staging was based on the depth of invasion using esophagogastroduodenoscopy and endoscopic ultrasound (EUS) and on nodal status by computed tomography (CT) scan. The absolute indications for LG were EGC, cT1N0-1, and serosa-negative cases without distant lymph node metastasis, while cT2-3N1-2 was a relative indication in our division according to the preoperative clinical staging. The contraindication was serosa-positive (cT4) advanced GC (AGC) or AGC with cN3 at preoperative evaluation for LG. All

patients underwent a standardized laparoscopic radical gastrectomy with D1 + β or D2 lymph node dissection according to the Japanese classification of gastric carcinoma[18]. Nine experienced gastric surgeons participated, all of whom had performed more than 150 conventional OGs and over 50 LGs for GC. Patients undergoing emergency surgery or palliative surgery or having concomitant malignancies and neo-adjuvant chemotherapy were excluded from application of the CP.

The contents of the CP for LG are composed of three components for preoperative, perioperative and postoperative care, which are listed in Table 1.

Summary of the clinical pathway

All preoperative examinations were performed on an outpatient basis, and the patients were admitted one day before the operation. Patients and their families were given preoperative information and education regarding the schedule of the CP by members of our stomach surgery team. Information consisted of the following categories: nursing care, activity, diet, treatment procedures, medication, laboratory tests, and education. In most cases, no nasogastric tube was used before surgery. Patients were permitted sips of water 24 h after surgery. Laboratory examinations were performed on postoperative days 1, 3 and 5. A liquid diet (LD) was given three days after surgery regardless of passing flatus, and a soft diet (SD) was given after passing flatus. If there was no issue with the SD, the patient's intra-abdominal drain was removed. Patients were well educated about diet, and nutrition information was provided by a nutritionist and a clinical nurse specialist. After consuming a SD three times and showing no postoperative complications, patients were discharged on the 5th or 6th postoperative day. Information about the postoperative follow-up schedule was given in the outpatient clinic before discharge.

Surgical procedure

Extracorporeal anastomosis: After dissection of all the lymph nodes, a 6-9 cm mini-laparotomy incision was made in the epigastric area in the form of midline incision, and a wound protector was applied. All anastomoses were performed in the same way as in conventional OG.

Intracorporeal anastomosis: After dissection of all of the lymph nodes, the stomach was resected into the abdominal cavity using endoscopic linear staplers and then removed through the umbilical port site by extending the incision by 2-3 cm. For reconstruction of the intracorporeal anastomosis, a double staple was inserted with a linear stapler. For a distal gastrectomy, a gastroduodenostomy was performed *via* a delta-shaped anastomosis, and a gastrojejunostomy was mainly performed *via* antecolic Roux-En-Y type anastomoses. For a total gastrectomy, functional-type esophagojejunostomies were mainly performed[19-22].

Criteria for completion of the CP

The CP was considered to be completed if the patient was discharged within 8 d after surgery without any complications, and the patients were divided into two categories. In the first category, the patient was discharged 5 to 6 d after surgery without any complications (planned), and in the second category, the patient voluntarily decided to stay longer for personal reasons and was discharged 7 to 8 d after surgery (wanted). A patient was considered to have dropped out of the CP if the surgeon decided to change the schedule because of a patient's postoperative condition or complication. Early post-operative complications occurred within 30 d after surgery and were classified according to the Clavien-Dindo classification system[23]. Readmission within 30 d after discharge was included in the category of complications because all readmissions were due to complications. The following clinical features were analyzed: patient characteristics and data from hospital records [sex, age, body mass index (BMI), American Society of Anesthesiologists (ASA) score, history of previous abdominal operations, and TNM stage]; operative methods (method of anastomosis, percent of the resection); and postoperative outcomes (early postoperative complications, postoperative hospital stay). The study was reviewed and approved by the Ethics Committee of the Asan Medical Center, Ulsan University, Seoul, South Korea.

Statistical analysis

Statistical analysis was performed by the Statistical Package for Social Sciences (SPSS) version 21.0 J for Windows (SPSS, Inc., Chicago, IL, United States). Patient characteristics were expressed as number and percent (%) for categorical variables, and mean ± SD for continuous variables. Chi-square tests were used to compare the anastomosis groups, and binary logistic regression was used to evaluate risk factors for dropping out of the CP. Multiple regressions were constructed by backward elimination, and the anastomosis groups were further adjusted in the final model. All tests were two sided, and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics are listed in Table 2. Of a total of 4800 patients, 2920 (60.8%) were men and 1880 (39.2%) were women. The mean age was 56.7 ± 11.7 years and the mean BMI was 23.71 ± 3.0. Intracorporeal and extracorporeal anastomoses were performed in 2345 patients (48.9%) and 2455 patients (51.1%), respectively. Distal gastrectomy was performed in 4218 (87.9%) patients, and total

Table 1 Structure of the clinical pathway for laparoscopic gastrectomy

Activities date	1 d before OP (Admission)	Pre-OP (the day of OP)	Operation (the day of OP)	Post-OP (the day of OP)	POD 1	POD 2	POD 3	POD 4	POD 5 and 6 (D/C)
Nursing care	Admission to room: (1-3 pm) Fluid balance Surveillance V/S Weight measurement	Room nurse: Fluid balance, Check V/S	Surgical nurse: OP preparation	Room nurse: Fluid balance Check V/S, drainage	Room nurse: Fluid balance Check V/S, drainage	Room nurse: Fluid balance Check V/S, drainage	Room nurse: Fluid balance, Check V/S, Drainage	Room nurse: Check V/S	Room nurse: Check V/S
	Check (Surgeon/ anesthesiologist): Preoperative study Written consent Protocol for OP preparation						Remove peripheral line after start of 2nd SD		
Activity Treatment procedure	Usual Skin preparation No Levin tube Breathing exercises use of IS Bowel preparation: Magcorol solution, 250 mL: Dulcolax supplement, 2 sup	Bed rest	Bed rest Surgical nurse: Foley catheter Insertion	Bed rest Breathing exercises : use of IS	Ambulation Breathing exercises : use of IS	Ambulation Breathing exercises : use of IS	Ambulation Breathing exercises : use of IS remove JP	Ambulation	Ambulation Surgeon: Wound S/O Evaluation on D/C criteria
			Surgeons: OP	Anesthesiologist: PCA (Fentanyl 3000 mg)					
Medication		Prophylaxis: TE	Prophylaxis: ATB	Prophylaxis: ATB Mucolytic agent	Prophylaxis: TE				
				Anesthesiologist: PCA	PCA	PCA			
Medication on demand				Pain killer: (IV) Demerol NSAIDs Antiemetics	Pain killer: (IV) Demerol NSAIDs Antiemetics	Pain killer: (IV) Demerol NSAIDs Antiemetics	Pain killer: (IV) Demerol NSAIDs Antiemetics	Pain killer: (Oral) NSAIDs	Pain killer: (Oral) NSAIDs
Laboratory test				Laboratory test	Laboratory test		Laboratory test		Laboratory test
Diet	Usual diet at breakfast NPO after breakfast	NPO	NPO	NPO	SOW: post 24 h OP		LD at breakfast SD after G/O	SD	SD
Education and information	Information on CP Permission		Information on leaving OP room				Education: diet for patient and/or relative	Education: diet for patient and/or relative	Information on D/C

OP: Operation; V/S: Vital signs; IS: Incentive spirometry; NPO: Nil per os; CP: Clinical pathway; TE: Thromboembolic; ATB: Antibiotic; PCA: Patients controlled analgesia; IV: Intravenous; NSAIDs: Nonsteroidal anti-inflammatory drugs; POD: Postoperative day; SOW: Sips of water; JP: Jackson-Pratt; LD: Liquid diet; SD: Soft diet; D/C: Discharge; S/O: Stitch out; G/O: Gas out.

Table 2 Demographic data for the enrolled patients *n* (%)

Variables		No. of patients (total *n* = 4800)
Sex	Male:Female	2920:1880 (60.8:39.2)
Age (yr)	mean ± SD	56.7 ± 11.7
BMI (kg/m^2)	mean ± SD	23.71 ± 3.0
Anastomosis method	Intra:Extra	2345:2455 (48.9:51.1)
Resection	Distal:Total	4218:582 (87.9:12.1)
Combined OP	None:Yes	4531:269 (94.4:5.6)
Event during OP	None:Yes	4723:77 (98.4:1.6)
Number of comorbidities	0:1:2 and more	3196:1137:467 (66.6:23.7:9.7)
ASA	1:2:3	3025:1549:226 (63.0:32.3:4.7)
Previous Abd. OP history	N:Y	4057:743 (84.5:15.5)
TNM stage	1:2:3	4380:314:106 (91.3:6.5:2.2)

SD: Standard deviation; BMI: Body mass index; Intra: Intracorporeal anastomosis; Extra: Extracorporeal anastomosis; OP: Operation; ASA: American Society of Anesthesiologists; Abd: Abdominal.

Table 3 Events during operation

	Intra (*n* = 25)	Extra (*n* = 52)
Anastomosis failure (*n* = 31)	8	23
Esophagojejunostomy failure	7	9
Gastroduodenostomy failure	1	14
Organ injury (*n* = 30)	10	20
Spleen injury	6	10
Duodenum	0	5
Small bowel injury	1	1
Colon injury	1	2
Pancreas injury	1	1
Liver injury	1	1
Vessel injury (*n* = 12)	4	8
Splenic artery injury	2	6
Splenic vein injury	0	1
Common hepatic artery injury	1	1
Proper hepatic artery injury	1	0
Emphysema (*n* = 4)	3	1

Intra: Intracorporeal anastomosis; Extra: Extracorporeal anastomosis.

gastrectomy was performed in 583 patients (12.1%). Additional operations on other organs were performed in 269 cases (5.6%). The most frequent additional operation was a laparoscopic cholecystectomy (208 cases); there were also 24 cases of gynecological surgery, such as laparoscopic salpingo-oophorectomy, laparoscopic ovarian cysterectomy, laparoscopic myomectomy, and laparoscopic total hysterectomy. Other additional operations included laparoscopic herniorrhaphy, video-assisted thoracoscopic surgery, laparoscopic distal pancreatectomy, laparoscopic nephrectomy, laparoscopic appendectomy, laparoscopic adrenalectomy, and laparoscopic colectomy. Intraoperative events occurred in a total of 77 cases (1.6%) (Table 3). Among them, there were 31 intraoperative events during anastomosis, and almost all of them developed during esophagojejunostomies after total gastrectomy. Organ injuries occurred in 30 cases during lymph node dissection, mostly due to spleen injury. Vessel injuries occurred in 12 cases, and spleen artery injuries mainly occurred during the dissection of lymph node number 11 or retraction of the stomach. Seven of the 30 instances of organ injury involved severe adhesions due to previous abdominal operations, and these patients had a history of upper gastrointestinal surgery. Four of the remaining intraoperative events were subcutaneous emphysemas. One or more comorbidities were identified in 1604 patients (34.4%), including diabetes mellitus, hypertension, coronary artery disease, and asthma. The majority of patients were classified as ASA grade Ⅰ (3025 patients) or Ⅱ (1549 patients). A total of 743 patients (15.5%) had histories of previous abdominal surgery. Most were gynecologic surgery (351 cases) followed by appendectomy (281 cases) and cholecystectomy (51 cases). The others were bowel surgery, pancreatectomy, and incisional herniorraphy. Most of the patients were at stage Ⅰ according to the American Joint Committee on Cancer - International Union for Cancer Control 7th edition[24]. There was no case converted to a laparotomy. However, six cases of intracorporeal anastomosis were converted to extracorporeal anastomosis due to intraoperative events, such as anastomosis failure and bleeding.

Comparisons between the intracorporeal and extracorporeal anastomosis groups are shown in Table 4. There were no statistically significant differences in terms of sex, age, combined operations, or history of previous abdominal operations. However, BMI, extent of resection, ASA classification system score, and TNM stage were significantly higher in the intracorporeal anastomosis group, and the number of intraoperative events was higher in the extracorporeal anastomosis group ($P < 0.005$).

The overall completion rate of the CP was 84.1%; it was 83.9% in the intracorporeal anastomosis group and 84.4% in the extracorporeal anastomosis group (Table 5). Of the 4038 patients who completed the CP, 3781 patients (78.8%) were planned, and 257 (5.3%) patients were wanted. The main reasons for dropping out were postoperative complications and need for additional patient observation. Early postoperative complications occurred within 30 d of surgery, and they were classified according to the Clavien-Dindo classification system[23]. Complications higher than grade Ⅰ were considered clinically significant, such as anastomosis stenosis, leakage, fluid collection, and bleeding (Table 6). The most common complications were fluid collection in the intracorporeal anastomosis group and wound infection in the extracorporeal anastomosis group. There were two cases of mortality.

Table 4 Clinicopathological characteristics of patients who underwent total laparoscopic gastrectomy (intracorporeal anastomosis) and laparoscopic assisted gastrectomy (extracorporeal anastomosis) *n* (%)

Variables	Intra (*n* = 2345)	Extra (*n* = 2455)	*P* value[1]
Sex			0.118
Male	1453 (61.96)	1467 (59.76)	
Female	892 (38.04)	988 (40.24)	
Age (yr)			0.110
≤ 65	1716 (73.18)	1846 (75.19)	
> 65	629 (26.82)	609 (24.81)	
BMI (kg/m^2)			< 0.001
≤ 25	1490 (63.54)	1835 (74.75)	
> 25	855 (36.46)	620 (25.25)	
Resection			< 0.001
Distal	2018 (86.06)	2200 (89.61)	
Total	327 (13.94)	255 (10.39)	
Combined OP			0.958
None	2214 (94.41)	2317 (94.38)	
Yes	131 (5.59)	138 (5.62)	
Event during OP			0.002
None	2321 (98.98)	2402 (97.84)	
Yes	24 (1.02)	53 (2.16)	
Number of comorbidities			< 0.001
0	1477 (62.99)	1719 (70.02)	
1	588 (25.07)	549 (22.36)	
2 and more	280 (11.94)	187 (7.62)	
ASA			< 0.001
1	1404 (59.87)	1621 (66.03)	
2	822 (35.05)	727 (29.61)	
3	119 (5.07)	107 (4.36)	
Abd OP history			0.008
None	1949 (83.11)	2108 (85.87)	
Yes	396 (16.89)	347 (14.13)	
TNM stage			< 0.001
Ⅰ	2066 (88.10)	2314 (94.26)	
Ⅱ	197 (8.40)	117 (4.77)	
Ⅲ	82 (3.50)	24 (0.98)	

[1]χ^2 test. Intra: Intracorporeal anastomosis; Extra: Extracorporeal anastomosis; BMI: Body mass index; OP: Operation; ASA: American Society of Anesthesiologists; Abd: Abdominal.

One was caused by esophagojejunal leakage in extracorporeal anastomosis and the other by duodenal stump leakage in intracorporeal anastomosis. Reasons for readmissions were intra-abdominal fluid collection (23 patients), ileus (19 patients), anastomosis stenosis (5 patients), internal herniation (3 patients), duodenal stump leakage (2 patients), and wound infection (2 patients). There were eight (14.9%) reoperations from the readmission cases. Patients who needed additional observation without definite postoperative complications were classified as observation cases (Table 7). The causes of observation were laboratory test abnormality, underlying disease, turbid drainage, and need for an upper gastrointestinal (UGI) series for cases of anastomotic site problems or ileus.

Table 8 shows the result of an analysis of the risk factors for dropping out of the CP. Male (OR = 1.459), advanced age (OR = 1.727), total gastrectomy (OR = 2.444), combined operation (OR = 1.731), intraoperative event (OR = 2.558), and ASA score (OR = 1.889) were all risk factors in the multivariate analysis.

Table 5 Results of the clinical pathway *n* (%)

Results	Total (*n* = 4800)	Intra (*n* = 2345)	Extra (*n* = 2455)	*P* value[1]
Complete	4038 (84.1)	1967 (83.9)	2071 (84.4)	0.651
Planned	3781 (78.8)	1740 (74.2)	2041 (83.2)	
Wanted	257 (5.3)	227 (9.7)	30 (1.2)	
Drop	762 (15.9)	378 (16.1)	384 (15.6)	
Complication	463 (9.7)	230 (9.8)	233 (9.5)	
(readmission)	(54) [(1.1)]	(29) [(1.2)]	(25) [(1.0)]	
Observation	299 (6.2)	148 (6.3)	151 (6.1)	

[1]χ^2 test comparing proportions of dropouts. Intra: Intracorporeal anastomosis; Extra: Extracorporeal anastomosis.

Table 6 Reasons for dropping out: Early postoperative complications *n* (%)

	Total (*n* = 4800)	Intra (*n* = 2345)	Extra (*n* = 2455)
Complications	463 (9.7)	230 (9.8)	233 (9.5)
Wound infection	127 (2.6)	41 (1.7)	86 (3.5)
Fluid collection	77 (1.6)	49 (2.1)	28 (1.1)
Anastomosis leakage	57 (1.2)	31 (1.3)	26 (1.1)
Anastomosis stenosis	16 (0.3)	11 (0.5)	5 (0.2)
Luminal bleeding	58 (1.2)	25 (1.1)	33 (1.3)
Extraluminal bleeding	19 (0.4)	10 (0.4)	9 (0.4)
Passage disturbance	20 (0.4)	8 (0.3)	12 (0.5)
Paralytic ileus	24 (0.5)	13 (0.6)	11(0.5)
Mechanical ileus	7 (0.2)	2 (0.1)	5 (0.2)
Medical problem	41 (0.9)	26 (1.1)	15 (0.6)
Internal herniation	5 (0.1)	5 (0.2)	0 (0.0)
Others	12 (0.3)	9 (0.4)	3 (0.1)

Intra: Intracorporeal anastomosis; Extra: Extracorporeal anastomosis.

DISCUSSION

The use of CP for patients undergoing surgical procedures can help with postoperative care and reduce the cost and the length of hospital stays[25,26]. Several researchers have reported that the CP was effective for gastrectomy for stomach cancer[4,27,28]. On the other hand, some studies have shown relatively low CP completion rates of 19%[11] and 40.6%[12]. These reports suggest that the CP is not suitable for patients undergoing gastrectomy for GC because it is frequently associated with postoperative hemodynamic changes that are risk factors for morbidity and mortality. Advanced age, combined disease, and poor nutrition are common in GC patients, and these factors may increase the risk of postoperative complications[4,29]. The long upper abdominal incisions needed for OG can cause significant postoperative pain and lead to lung problems, such as atelectasis, because patients do not care for their lungs appropriately because of the pain[30]. Recently, LG has been performed as the standard treatment for EGC, whereas OG is usually done for AGC. Severe AGC has a higher possibility of intraoperative

Table 7 Reasons for dropout: Observation cases *n* (%)

	Total (*n* = 4800)	Intra (*n* = 2345)	Extra (*n* = 2455)
Observation cases	299 (6.2)	148 (6.3)	151 (6.1)
Laboratory test abnormality	220 (4.6)	107 (4.6)	113 (4.6)
Underlying disease	20 (0.4)	14 (0.6)	6 (0.2)
JP turbid	5 (0.1)	4 (0.2)	2 (0.1)
Due to UGI series	29 (0.6)	10 (0.4)	19 (0.8)
Others	25 (0.5)	13 (0.5)	11 (0.4)

Intra: Intracorporeal anastomosis; Extra: Extracorporeal anastomosis; JP: Jackson-Pratt; UGI: Upper Gastrointestinal.

events and complications due to difficult node dissection and extended surgery. Some studies have suggested that the CP is more effective in patients with benign diseases or those receiving minimally invasive surgery than in those undergoing conventional OG[12,17]. The most common causes of dropout from the CP were postoperative complications that needed additional medical treatment. Postoperative complications, such as leakage, stricture, and bleeding, occurred more often in gastrointestinal cancer surgery than in benign or other types of cancer surgery. In this study, the CP completion rate was 84.13%, which was higher than in other reports[4,11,12,31]. One of the most important factors that can influence early surgical outcomes and CP dropout is the surgeon's experience. However, surgeons who participated in this study were highly experienced gastric surgeons working in a high-volume center, where more than 2000 gastrectomies for GC are performed each year.

Many surgeons still advocate the slow and careful introduction of oral intake after a gastrectomy because of concerns over the functioning of the remnant stomach and the risk of disrupting the anastomosis or of postoperative paralytic ileus. These factors may make surgeons hesitant to apply the planned dietary schedule to patients undergoing a gastrectomy.

Since LG was first described by Kitano *et al*[32] in 1994, it has become well established as a minimally invasive operation for GC. Its benefits include the need for a small incision, reduced postoperative pain, fewer postoperative adhesions, earlier recovery of bowel movement because of reduced trauma compared to open surgery, and a shorter postoperative hospital stay[13-15]. LG is thought to be suitable for the application of a CP because it reduces the incidence of postoperative complications and provides a more rapid recovery and earlier hospital discharge due to its low invasiveness[33-35]. Recently, LG's effectiveness in enhancing recovery after surgery has been reported[15,36,37]. Furthermore, LG has a predictable clinical course, which facilitates the use of a CP in GC surgery[17]. Choi *et al*[12] reported that the completion rate of a CP in LG for GC was 76.2%, and the expected completion rate in selected patients with no risk factors was 85.4%, which was similar to that of this study. These results support the belief that it is possible to develop and apply a CP for LG in GC patients.

In LG for GC, there are two methods of reconstruction after a gastrectomy. The intracorporeal anastomosis method is not yet performed as frequently as the extracorporeal anastomosis method because of its greater technical difficulty. Despite the complexity of intracorporeal anastomosis, our studies have shown that it is feasible and safe when performed by experienced gastric surgeons[14,19-22,38]. In the present study, the completion rates of the CP in both the intracorporeal and the extracorporeal anastomosis groups were considerably higher than those in other reports[39]. Intracorporeal anastomosis could improve early surgical outcomes because it can provide a wide operating view with direct sight, which can make anastomosis safer, and an endoscopic linear stapler provides greater tensile strength than a circular stapler[19,20,40]. The most common reason for complications in this study was wound infection in extracorporeal anastomosis due to an additional mini-laparotomy. The second most frequent cause of termination of the CP was the need for additional patient observation and further laboratory tests or a UGI series, which led the surgeon to change the schedule. The laboratory tests were required to identify abnormalities, such as leukocytosis or aberrant artery ligation, during the gastrectomy. For patients who experienced intraoperative events during anastomosis, dietary intake was postponed until after the UGI series confirmed that there was no leakage or stricture, and the CP was terminated. The other reason for dropout was postoperative aggravation of an underlying disease, independent of surgical complications.

There is a striking difference of the numbers of "wanted completion CPs" between the intracorporeal anastomosis and the extracorporeal anastomosis groups. We believe that the reason can be explained as follows: Starting in 2004, the LG was performed by extracorporeal anastomosis, and starting in approximately 2008, it gradually converted to intracorporeal anastomosis. Since 2010, intracorporeal anastomosis procedures outnumbered extracorporeal anastomosis procedures. Over the last ten years, there have been many changes in the healthcare and reimbursement system in South Korea, and the national health insurance allows cancer patients to stay in the hospital longer for treatment at reduced costs. These changes seem to influence the patient's desire to stay in the hospital.

In a multivariate analysis, being male (OR = 1.459), advanced age (OR = 1.727), total gastrectomy (OR = 2.444), combined operation (OR = 1.731), intraoperative event (OR = 2.558), and ASA score (OR = 1.889) were risk factors for dropout. A large amount of intra-abdominal fat deposition or intra-abdominal adhesions from previous operations can make the surgery more difficult, resulting in intraoperative

Table 8 Clinical factors that affect dropout *n* (%)

	Total	Drop	Univariate		Multivariable[1]	
			OR (95%CI)	*P* value	OR (95%CI)	*P* value
Sex				< 0.001		< 0.001
Male	2920	534 (18.29)	1.622 (1.372-1.917)		1.459 (1.228 -1.734)	
Female	1880	228 (12.13)	1		1	
Age (yr)						
≤ 65	3562	462 (12.97)	1		1	
> 65	1238	300 (24.23)	2.146 (1.824 -2.525)	< 0.001	1.727 (1.448-2.059)	< 0.001
BMI (kg/m^2)						
≤ 25	3325	496 (14.92)	1			
> 25	1475	266 (18.03)	1.255 (1.066-1.478)	0.006		
Anastomosis method						
Intra	2345	378 (16.12)	1		1	
Extra	2455	384 (15.64)	0.965 (0.826-1.126)	0.651	1.057 (0.900-1.242)	0.499
Resection						
Distal	4218	593 (14.06)	1		1	
Total	582	169 (29.04)	2.501 (2.050-3.052)	< 0.001	2.444 (1.988-3.005)	< 0.001
Resection group						
Ⅰ-distal	2018	285 (14.12)	1	< 0.001		
Ⅰ-total	327	93 (28.44)	2.417 (1.843 -3.169)	< 0.001		
E-distal	2200	308 (14.00)	0.990 (0.832 -1.178)	0.909		
E-total	255	76 (29.80)	2.582 (1.920-3.472)	< 0.001		
Combined OP						
None	4531	694 (15.32)	1		1	
Yes	269	68 (25.28)	1.870 (1.404-2.491)	< 0.001	1.731 (1.284-2.334)	< 0.001
OP event						
None	4723	732 (15.50)	1		1	
Yes	77	30 (38.96)	3.480 (2.187-5.539)	< 0.001	2.558 (1.554-4.212)	< 0.001
Number of comorbidities						
0	3196	411 (12.86)	1	< 0.001	1	0.031
1	1137	221 (19.44)	1.635 (1.366 -1.957)	< 0.001	1.179 (0.882 -1.576)	0.266
2 and more	467	130 (27.84)	2.614 (2.082-3.281)	< 0.001	1.564 (1.110-2.204)	0.011
ASA score						
1	3025	382 (12.63)	1	< 0.001	1	0.006
2	1549	305 (19.69)	1.696 (1.438-2.000)	< 0.001	1.182 (0.893-1.565)	0.242
3	226	75 (33.19)	3.437 (2.554-4.625)	< 0.001	1.889 (1.271-2.808)	0.002
Abdominal OP history						
None	4057	661 (16.29)	1			
Yes	743	101 (13.59)	0.808 (0.645-1.013)	0.065		
TNM stage						
Ⅰ	4380	688 (15.71)	1	0.274		
Ⅱ	314	51 (16.24)	1.041 (0.763-1.420)	0.802		
Ⅲ	106	23 (21.70)	1.487 (0.930-2.377)	0.097		

[1]Backward elimination. Logistic regression to predict dropout (= 762/4800). OR: Odd ratio; BMI: Body mass index; Intra/I: Intracorporeal anastomosis; Extra/E: Extracorporeal anastomosis; OP: Operation; ASA: American Society of Anesthesiologists.

events such as bleeding[41]. Intraoperative events (OR = 2.558) were the most significant risk factors for dropout because they are related to a longer operation time and more postoperative complications. Many researchers have reported that advanced age, type of reconstruction, combined operation, and ASA score contribute to postoperative morbidities in GC[42-44]. We found that being male was a risk factor for dropout even though there was no difference in the BMI between male and female patients. This suggests that men have a larger proportion of visceral adipose tissue than women[45-47], which might increase postoperative complications. Women generally have a higher amount of body fat in subcutaneous areas, whereas men have more body fat in the abdominal (visceral) region[48]. However, BMI does not accurately reflect the extent of a patient's visceral fat because the distribution of fat varies with gender, and the visceral fat area is a more accurate risk factor for postoperative complications than BMI in LG for GC[41,49].

It was difficult for patients to follow the CP if they did not understand the concept. Most of them were afraid of early food intake and discharge because their food intake had to be strictly limited. Post-gastrectomy symptoms (PSGs), including weight loss, early satiety, eating restriction, appetite loss, dysphagia, reflux, nausea and vomiting are inevitable consequences of gastrectomy, and patients need to receive information about the CP and PSGs in the outpatient clinic. Diet is more challenging after gastrectomy than after other operations. Therefore, it is important that all patients and their family members learn about the

diet. Most patients without major complications were able to tolerate early oral intake as specified in the CP. All patients were permitted sips of water 24 h after surgery, as well as early oral intake of an LD. To maintain the high quality of the CP, all members of the gastric surgery team, including gastric surgeons, nurses, nutritionists and members of the clinical support services, need to actively participate and cooperate. In our stomach division, a clinical nurse specialist provides patients with detailed information about the CP, including postoperative course, dietary schedule, expected hospital stay, and anticipated return to normal activities. In addition, the surgical team should attempt to reduce the incidence of intraoperative events, which are the most significant risk factor for dropout from the CP. Intraoperative events mainly occurred during the reconstruction of anastomoses, and most involved esophagojejunostomy after total gastrectomy or organ injury during dissection of the lymph nodes. Therefore, LG should be performed by expert surgeons who are experienced in OG and various laparoscopic procedures. Moreover, the surgeons should pay special attention to complex cases with risk factors such as total gastrectomies and combined operations, as well as intraoperative events.

Our findings suggest that LG for GC is a suitable indication for the use of a CP because it can provide better early surgical outcomes due to low invasiveness, and make an early hospital discharge possible. The use of a CP for LG could be helpful in East Asian countries, where the incidence of GC is high, because of the cost benefits and short hospital stays. For successful application of a CP, patients with risk factors should be managed carefully. However, this study was analyzed retrospectively and was not a randomized controlled study, and the groups were not homogeneous, so the results could not be significant. Therefore, a prospective randomized controlled study should be conducted in the near future.

COMMENTS

Background

Gastric cancer (GC) is the most prevalent malignancy in South Korea and Japan. The proportion of early gastric cancers (EGC) has increased to over 50% as a result of early detection through mass screening. Recently, laparoscopic gastrectomy (LG) has been established as a standard treatment for the EGC patients, and it has better surgical outcomes than open gastrectomy (OG). LG provides enhanced postoperative recovery compared to OG for GC patients. Therefore, LG is thought to be the one of suitable procedures for a clinical pathway (CP) that provides a time-based schedule for patients. In this study, we developed a standardized CP for GC patients in 2001 by a committee consisting of gastric surgeons, nurses, nutritionists, and members of the clinical support services in the Asan Medical Center. In 2004, our institute implemented a CP for large numbers of GC patients undergoing LG performed by experienced surgeons. In this study, the authors showed the rate of completion of a CP and identified clinical factors affecting the CP for LG.

Research frontiers

Despite the usefulness of a CP in various surgical settings, its use for LG for GC has not been adequately investigated. LG is popular as a minimally invasive operation for GC in the Asan Medical Center, especially for EGC. Since 2004, the authors' institute has used a CP for large numbers of GC patients undergoing LG performed by experienced surgeons. Their CP will contribute a standard structure for GC patients and provide guidelines for LG.

Innovations and breakthroughs

This study evaluated the rate of completion of a CP and the clinical factors affecting the CP after laparoscopic gastrectomy for GC patients. The overall completion rate of the CP was 84.1%; it was 83.9% in the intracorporeal anastomosis group and 84.4% in the extracorporeal anastomosis group. In the current study, the completion rates of the CP in both the intracorporeal and the extracorporeal anastomosis groups were considerably higher than in other reports because there was no bias caused by the different technique of each surgeon. Surgeons who participated in this study were all trained in the same surgical technique and had each performed more than 150 conventional OGs as well as more than fifty LGs for GC at a single, high-volume center. A multivariate analysis revealed that being male (OR = 1.459), advanced age (OR = 1.727), total gastrectomy (OR = 2.444), combined operation (OR = 1.731), intraoperative events (OR = 2.558), and ASA score (OR = 1.889) were risk factors for dropping out of the CP. These results can give useful information to the surgeon who is a novice at LG and works in small-volume center. For successful application, patients with risk factors (male, advanced age, total gastrectomy, combined operation, intraoperative events, American Society of Anesthesiologists score) should be managed carefully.

Applications

LG appears to be a good indication for the application of a CP. The authors' CP can be applied to LG for GC patients.

Terminology

CP, clinical pathway, is a comprehensive systematized plan that details the essential steps in patient care in a given process, including any time-dependent clinical decisions.

Peer-review

The authors of this paper evaluated the rate of completion for the CP, identified risk factors for dropping out of the CP, and conclude that those patients who had a risk factor should be managed more carefully. This is an interesting analysis that addresses a clinically relevant question and provides somewhat valuable results due to the large number of patients enrolled in the study.

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ORIGINAL ARTICLE

Retrospective Study

Second-look endoscopy with prophylactic hemostasis is still effective after endoscopic submucosal dissection for gastric neoplasm

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Author contributions: Jung JH analysed the data and wrote the paper; Kim BJ designed the research; Choi CH and Kim JG collected the data.

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Abstract

AIM: The clinical value of second-look endoscopy (SLE) after endoscopic submucosal dissection (ESD) has been doubted continuously. The aim of this study was to assess the effectiveness of SLE based on the risk of delayed bleeding after ESD.

METHODS: A total of 310 lesions of gastric epithelial neoplasms treated by ESD were reviewed. The lesions were divided into two groups based on the risk of post-procedural bleeding estimated by Forrest classification. The high risk of rebleeding group (Forrest Ⅰa, Ⅰb and Ⅱa) required endoscopic treatment, while the low risk of rebleeding group (Forrest Ⅱb, Ⅱc and Ⅲ) did not. Delayed bleeding after ESD was investigated.

RESULTS: Sixty-six lesions were included in the high risk of rebleeding group and 244 lesions in the low risk of rebleeding group. There were no significant differences in delayed bleeding between the high risk group (1/66) and the low risk group (1/244) (P = 0.38). The high risk of rebleeding group tended to be located more often in the mid-third and had higher appearance of flat or depressed shape than the low risk group (P = 0.004 and P = 0.006, respectively).

CONCLUSION: SLE with pre-emptive prophylactic endoscopic treatment is still effective in preventing delayed bleeding after ESD.

Key words: Second-look endoscopy; Forrest classification; Endoscopic submucosal dissection; Delayed bleeding

Core tip: This is a retrospective study to assess the effectiveness of second-look endoscopy (SLE) based on the risk of delayed bleeding after endoscopic submucosal dissection (ESD). A total of 310 lesions of gastric epithelial neoplasms treated by ESD were reviewed. The lesions were divided into two groups based on the risk of post-procedural bleeding estimated by Forrest classification. The high risk of rebleeding group (Forrest Ⅰa, Ⅰb and Ⅱa) required endoscopic treatment, while the low risk of rebleeding group (Forrest Ⅱb, Ⅱc and Ⅲ) did not. Delayed bleeding after ESD was investigated. As a result, there were no significant differences in delayed bleeding between the high risk group and the low risk group. However, the high risk of rebleeding group tended to be located more often in the mid-third and had higher appearance of flat or depressed shape than the low risk group. In conclusion, SLE with pre-emptive prophylactic endoscopic treatment is still effective in preventing delayed bleeding after ESD.

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INTRODUCTION

Gastric cancer is the most common malignant neoplasm and the third most common cause of cancer related death in South Korea[1]. Endoscopy for screening of gastric cancer is widely performed in high incidence areas such as Korea and Japan. Recently, advances in endoscopic equipment have increased the early detection rate. There is a good chance of being cured when gastric cancer is diagnosed at an early state, for which surgery is the treatment of choice. Endoscopic submucosal dissection (ESD) for node-negative early gastric cancer (EGC) has been recognized as an outstanding endoscopic treatment instead of open surgery[2,3]. However, bleeding from artificial ulcers after gastric ESD has remained a major complication. Although the bleeding rate is relatively low after gastric ESD, uncontrolled bleeding is related to mortality[3]. Therefore, second-look endoscopy (SLE) continues to be performed at most institutions after gastric ESD to check for post-procedural bleeding. Nevertheless, there has been no consensus nor significant evidence of the clinical utility of SLE in patients without any sign of bleeding. In most cases of bleeding, endoscopic hemostasis effectively stops bleeding when properly performed during emergency endoscopy. For this reason, the clinical value of SLE is continuously under debate. Two retrospective analysis reported that SLE after gastric ESD may contribute little to the prevention of delayed bleeding[4,5]. In that study, gross type (Ⅱb/Ⅱc) was the only considerable predictor for post-ESD bleeding with a significant difference[4]. In consideration of that point, gastric ESD cases were evaluated herein to verify the clinical and pathological conditions depending on the state of the iatrogenic ulcer base of post-ESD. The aim of this study was to assess the clinical role of routine SLE after ESD based on the delayed bleeding estimated by Forrest classification, and to confirm whether SLE with prophylactic hemostasis is useful for the prevention of delayed bleeding after gastric ESD.

MATERIALS AND METHODS

Patients and materials

A total of 319 patients with gastric epithelial neoplasm who were consecutively treated with ESD in Chung-Ang University Hospital from January 2009 to December 2013 were retrospectively reviewed. ESD was principally indicated for node-negative EGC or gastric adenoma. A total of four cases were excluded due to deep invasion (n = 3) or perforation (n = 1). As a result, a total of 310 lesions (74 cases of EGCs and 236 cases of gastric adenoma) were explored by SLE, defined as performing elective endoscopy without any sign of bleeding within 72 h after ESD.

The 310 lesions were divided into two groups based on the risk of post-procedural bleeding (high risk group *vs* low risk group) estimated by Forrest classification. The high risk of rebleeding group (Forrest Ⅰa, Ⅰb and Ⅱa) required endoscopic treatment, while the low risk of rebleeding group (Forrest Ⅱb, Ⅱc and Ⅲ) did not. More specifically, the high risk of rebleeding group included cases with blood oozing and visible vessels without any sign of hemorrhage. A flow chart for inclusion in this study is shown in Figure 1. The study protocol was approved by the institutional review board of Chung-Ang University College of Medicine [IRB No. C2014088(1284)].

ESD procedures

The ESD was performed under conscious sedation. All patients provided written informed consent before treatment. Patients fasted the morning of the treatment. After circumferential marking with an argon plasma coagulator (APC) from the tumor edge, a mixture of 0.9% saline with epinephrine, glycerol, or hyaluronic acid containing indigo carmine was injected into the submucosal layer. The muscularis mucosa was then cut and submucosal dissection was performed. An insulation-tipped knife (Olympus, Tokyo, Japan), Hook knife (Olympus, Tokyo, Japan), or Flex knife (Olympus, Tokyo, Japan) was selected as the electrosurgical knife according to the preference of the endoscopist and/or

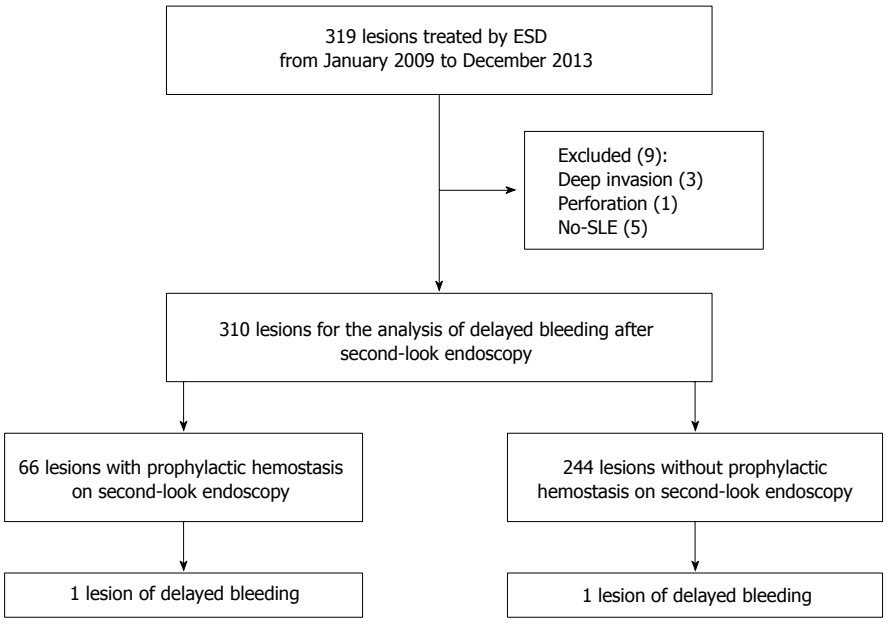

Figure 1 Flowchart for the efficacy of second-look endoscopy. Among 310 lesions treated by ESD, delayed bleeding occurred in 2 (0.64%), one per group; high risk group (1/66) and low risk group (1/244). Delayed bleeding was defined bleeding event after SLE within 30 d. ESD: Endoscopic submucosal dissection; SLE: Second-look endoscopy.

tumor characteristics. Oozing lesions or non-bleeding visible vessels in the ulcer bed were treated by hemostatic forceps (FD-410LR; Olympus) or hemoclips immediately after ESD.

In principle, treatment with antiplatelet or anti-coagulant agents was stopped 1 wk before ESD. 40mg pantoprazole was administered intravenously once daily from the day of ESD to the first day of feeding. Complete blood count was checked immediately after ESD, the day after, at discharge, and after 1 and 4 wk.

Second-look endoscopies

The purpose of SLE was to check and prevent post-ESD bleeding from the artificial ulcer. SLE was performed within 72 h after ESD. SLE was mainly performed the day after ESD; however, emergency endoscopy was performed anytime within 24 h in patients with suspected bleeding or perforation. During SLE, prophylactic hemostasis with thermocoagulation or hemostatic clipping were performed on actively bleeding (Forrest classification Ⅰa) or blood-oozing ulcers (Forrest classification Ⅰb) or non-bleeding visible vessels (Forrest classification Ⅱa). After SLE, patients without evidence of bleeding were usually allowed to have a liquid diet with an oral proton pump inhibitor. Most patients were discharged within 3 d after ESD if a bleeding event did not occur. The patients were scheduled to visit the hospital at 1 wk, 1 mo, and 3 mo after discharge. All patients were instructed to visit the hospital in case of hematemesis, hematochezia, melena or dizziness.

Data analysis

Post-ESD bleeding was defined as the appearance of hematemesis, hematochezia, melena, hypotension or decrease in hemoglobin of more than 2g/dL after ESD. Diagnosis was made by endoscopy. Delayed bleeding was defined as bleeding events after SLE which occurred within 30 d. The following variables were analyzed: age, sex, comorbidities with Charlson comorbidity scale, use of antiplatelet or anticoagulant agents, use of steroid or nonsteroidal anti-inflammatory drugs, procedure time, resection type (*en bloc* or piecemeal), lesion location (upper third-high body or cardia, middle third-mid body or lower body, lower third-antrum or angle), lesion characteristics (elevated or flat, depressed), lesion size (maximum diameter), resected specimen size (maximum diameter), histologic type, presence of *Helicobacter pylori (H. pylori)*, Forrest classification at SLE (high risk-Ⅰa, Ⅰb, Ⅱa or low risk-Ⅱa, Ⅱb, Ⅲ), whether or not prophylactic hemostasis occurred, and presence of delayed bleeding (≤ 30 d after ESD).

Statistical analysis

Data were analyzed with comparison between the two bleeding groups. Statistical analysis was performed using the SPSS software version 18.0 (SPSS Inc., Chicago, IL, United States). Univariate analysis by the Student's *t*-test was performed for age, lesion size, resected specimen size, and procedure time. χ^2 test was performed for sex, cormorbidities, the use of antiplatelet or anticoagulant agents or of steroid or non-steroidal anti-inflammatory drugs, technique of resection, resection type, lesion location, lesion characteristics, histologic type, presence of *H. pylori*, whether or not prophylactic hemostasis occurred, and presence of delayed bleeding. The statistical

Table 1 Clinical characteristics before second look endoscopy according to the rebleeding risk after endoscopic submucosal dissection *n* (%)

	High risk group (*n* = 66)	Low risk group (*n* = 244)	*P* value
Forrest classfication	Ⅰa, Ⅰb, Ⅱa (21.2) Ⅰa (*n* = 0) Ⅰb (*n* = 48) Ⅱa (*n* = 18)	Ⅱb, Ⅱc, Ⅲ (78.8) Ⅱb (*n* = 55) Ⅱc (*n* = 14) Ⅲ (*n* = 175)	
Age, mean (mean ± SD, yr)	65.2 ± 8.6	64.7 ± 9.4	0.70
Sex, male/female	43/23	150/94	0.58
Comorbidities (Charlson scale)			0.96
0	49 (74.2)	178 (73.0)	
1	13 (19.7)	52 (21.3)	
2	3 (4.5)	9 (3.7)	
3	1 (1.5)	3 (1.2)	
4	0 (0)	2 (0.8)	
Use of antiplatelet or anticoagulant agents or steroid or nonsteroidal anti-inflammatory drugs			0.03
Yes	10 (15.2)	17 (7.0)	
None or unknown	56 (84.8)	227 (93.0)	

significance was set at a *P* value < 0.05.

RESULTS

When the 310 lesions were classified into two groups according to the Forrest classification, 66 lesions (21.2%) were included in the high risk of rebleeding group (Forrest classification: Ⅰa, Ⅰb, Ⅱa) and 244 lesions (78.8%) were included in the low risk of rebleeding group (Forrest classification: Ⅱb, Ⅱc, Ⅲ). No bleeding occurred within 3 d after SLE in both groups. Delayed bleeding occurred in only two of the 310 lesions (0.64%). All cases of bleeding were controlled with endoscopic management (e.g. hemostatic forceps and hemoclips) without surgical intervention. Blood transfusion was not performed in delayed bleeding cases because of stable vital signs and hemoglobin levels above 9 g/dL. No rebleeding occurred after post-ESD hemostasis. There were no statistically significant differences between the two groups according to age, sex, comorbidities using the Charlson comorbidity scale (Table 1). In addition, there were no significant differences in procedure time, lesion size, resected specimen size, histologic type, presence of *H. pylori*, whether or not prophylactic hemostasis occurred, and presence of delayed bleeding (Table 2). The lesions of the high risk of rebleeding group tended to be located more often in the mid-third, whereas those of the low risk of rebleeding group were located more in the upper or lower third (*P* = 0.004). As for lesion characteristics, the high risk of rebleeding group had higher appearance of flat or depressed shape than the low risk group (*P* = 0.006). Delayed bleeding after SLE occurred on POD 5 in the low risk of rebleeding group and on POD 23 in the high risk of rebleeding group. There were no significant differences between the groups (*P* = 0.38). The three patients with double lesions had low grade dysplasia of elevated shape, and were included in the low risk of rebleeding group without delayed bleeding.

DISCUSSION

Performance of a routine SLE after gastric ESD was supported by several prospective, randomized trials owing to its efficacy after endoscopic hemostasis for peptic ulcer bleeding[6-8]. However, there are fundamental pathophysiological differences in ulcer formation between peptic and post-ESD ulcers. Peptic ulcers may occur due to breakdown of the mucosal defense mechanism and hyperacidic environment. On the other hand, post-ESD ulcers occur in a relatively less acidic environment. Furthermore, post-ESD ulcers are relatively shallow compared to peptic ulcers due to electively performed submucosal dissection[4,9].

Several current randomized trials suggested that routine SLE had little or no influence on the prevention of delayed bleeding[4,10]. However, most of them were limited to making generalizations because of the small scale, single center-based studies. In addition, recurrent bleeding is one of the most important risk factors for mortality, even though there are fundamental difference in the pathophysiology of peptic ulcers and post-ESD ulcers[8]. Therefore, the study of multiple aspects for predicting the likelihood of post-ESD ulcer rebleeding is in progress. According to several studies, tumor location, tumor size, ulcerative findings, and long procedure time are suggested as risk factor for delayed bleeding[4,11]. However, these factors proved not to be significant in this study. In this study, the use of antiplatelet or anticoagulant agents, steroids, and non-steroidal anti-inflammatory drugs (NSAIDs) was related with high risk of rebleeding after ESD. This result is consistent with previous studies on peptic ulcer bleeding[12].

The presence of *H. pylori* infection was confirmed using Giemsa staining or urea breath test, because this bacterium disturbs the healing process of gastric ulcers.

As a result, no significant differences were observed between the two groups (*P* = 0.88).

In this study, the post-ESD lesions were classified according to Forrest classification. Forrest classification is known to be the most important factor among various predictive variables for gastroduodenal ulcer rebleeding[13,14]. There is no doubt that higher Forrest classification has more bleeding risk than lower Forrest classification. In addition, Takizawa et al. suggested that preventive coagulation of visible vessels in the resection area after ESD may lead to a lower bleeding rate[15]. In this study, pre-emptive prophylactic endoscopic treatment for the high risk of rebleeding group during SLE was found to reduce

Table 2 Outcomes of endoscopic submucosal dissection *n* (%)

	High risk group (*n* = 66)	Low risk group (*n* = 244)	*P* value
Prodecure time (mean ± SD, min)	50.5 ± 27.6	44.2 ± 29.7	0.12
Resection type (*en bloc*)	66 (100.0)	244 (100.0)	
Lesion location			0.004
High body, cardia	0 (0.0)	10 (4.1)	
Mid body, lower body	9 (13.6)	9 (3.7)	
Antrum, angle	57 (86.4)	225 (92.2)	
Lesion characteristics			0.006
Elevated	54 (81.8)	227 (93.0)	
Flat or depressed	12 (18.2)	17 (7.0)	
Lesion size (maximum diameter)	12.0 ± 7.3	12.5 ± 8.4	0.69
Resected specimen size (maximum diameter)	32.3 ± 9.4	30.7 ± 10.8	0.25
Histologic type			0.36
Cancer	20 (30.3)	54 (22.1)	
High grade dysplasia	6 (9.1)	29 (11.9)	
Lower grade dysplasia	40 (60.6)	161 (66.0)	
Presence of *Helicobacter pylori*			0.87
Yes	11 (16.7)	36 (14.8)	
No	54 (81.8)	204 (83.6)	
Unknown	1 (1.5)	4 (1.6)	
Endoscopic hemostasis			1.00
Yes	66 (100.0)	0 (0.0)	
No	0 (0.0)	244 (100.0)	
Occurrence of delayed bleeding			0.38
Yes	1 (1.5)	1 (0.4)	
No	65 (98.5)	243 (99.6)	

delayed bleeding, as much as the low risk group. Although SLE did not prevent all the delayed bleeding, early detection of delayed bleeding may prevent cardiovascular complications.

From the viewpoint of probability, the high risk of rebleeding group can be recognized as a group with potential for bleeding. In fact, cases with active arterial bleeding (Forrest class Ⅰa and Ⅰb) have a 90% risk of rebleeding. Furthermore, non-bleeding visible vessels (Forrest class Ⅱa) have a 50% risk of rebleeding in spite of proper initial medical management. As for the potential bleeding, theoretically, at least 50% of the high risk of rebleeding group in this study had the chance of experiencing bleeding. This estimation is by no means a negligible level in post-ESD care. In this study, the incidence of complications after ESD, such as perforation or delayed bleeding, was much lower than reported in previous studies[4,9,16]. *En bloc* resection rate was 100% (314/314), and curative resection rate was 99.0% (311/314) in the present study. This result may be derived from excellent outcomes owing to improvement in the techniques and therapeutic modalities. In a previous study, positive/indeterminate lateral margin was suggested as a significant risk factor for delayed bleeding from ESD[17]. Interestingly, the low risk of rebleeding group was taking more medication with bleeding tendency compared with the high risk of rebleeding group. Exposure to such drugs is well known to substantially increase the bleeding risk of peptic ulcers[13,18,19]. In particular, the low risk of bleeding group in this study included a significant number of patients with relatively higher potential bleeding risk. Thus, the imperfect organization of the low risk of rebleeding group might have led to misinterpretation. Even though the possibility of bleeding after gastric ESD is low, SLE should never be ignored.

In conclusion, based on our retrospective analysis, SLE with pre-emptive prophylactic endoscopic treatment is still effective in preventing delayed bleeding after ESD, especially when the lesion is located in upper locations and is flat or depressed in shape. In the future, large randomized controlled trials will be warranted to elucidate the effectiveness of SLE after ESD.

COMMENTS

Background

Owing to recent advances in endoscopic equipment enables, endoscopic submucosal dissection (ESD) for node-negative early gastric cancer has been recognized as an alternative modality of surgery. However, bleeding from artificial ulcers after gastric ESD has remained a major complication. Although the bleeding rate is relatively low after gastric ESD, uncontrolled bleeding is related to mortality. In most cases of bleeding, endoscopic hemostasis effectively stops bleeding when properly performed during emergency endoscopy. For this reason, the clinical value of second-look endoscopy (SLE) is continuously under debate. Therefore, this study evaluated the clinical efficacy of routine SLE after ESD based on the delayed bleeding estimated by Forrest classification, and to confirm whether SLE with prophylactic hemostasis is useful for the prevention of delayed bleeding after gastric ESD.

Research frontiers

Second-look endoscopy is important for preventing delayed bleeding after gastric ESD. The results of this study contribute to clarifying the clinical efficacy of second-look endoscopy with prophylactic hemostasis.

Innovations and breakthroughs

In this study, second-look endoscopy with pre-emptive endoscopic treatment was still useful tool for preventing delayed bleeding after ESD. Especially, estimation of rebleeding risk based on the Forrest classification is also applicable to post-procedural bleeding risk assessment as well as peptic ulcer bleeding.

Applications

This study suggests that second-look endoscopy with prophylactic hemostasis is still effective for preventing delayed bleeding after gastric ESD. Especially, high risk of rebleeding represented by high grade of Forreset classification (Ⅰa, Ⅰb, and Ⅱa) should be managed on the second-look endoscopy.

Terminology

ESD: An endoscopic procedure that resect gastrointestinal neoplasm in en bloc fashion with circumferential mucosal incision and submucosal dissection. SLE: An endoscopic technique that confirm recurred or residual bleeding from the gastrointestinal tract after endoscopic procedure.

Peer-review

The author of this paper evaluated the efficacy of second-look endoscopy with prophylactic hemostasis based on the Forrest classification and compared the results of the high risk of rebleeding group with the low risk of rebleeding group. As a result, the high risk of rebleeding group tended to be located more often in the mid-third and had higher appearance of flat or depressed shape than the low risk group. In conclusion, SLE with pre-emptive prophylactic endoscopic treatment is still effective in preventing delayed bleeding after ESD.

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ORIGINAL ARTICLE

Retrospective Study

Liver metastasis from hepatoid adenocarcinoma of the stomach mimicking hepatocellular carcinoma: Dynamic computed tomography findings

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Author contributions: Lin YY and Chen CM collected and analyzed the data, and drafted the manuscript; Huang YH reviewed the pathological findings; Lin CY provided the clinical data and followed up the patients; Chu SY and Hsu MY revised the manuscript for important intellectual content; Pan KT and Tseng JH designed and supervised the study.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Chang Gung Memorial Hospital.

Informed consent statement: The need for patient informed consent was waived because of the retrospective and anonymous nature of this study.

Conflict-of-interest statement: There is not any conflict-of-interest problem in this study.

Data sharing statement: No additional data are available.

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Abstract

AIM: To evaluate the dynamic computed tomography (CT) findings of liver metastasis from hepatoid adenocarcinoma of the stomach (HAS) and compared them with hepatocellular carcinoma (HCC).

METHODS: Between January 2000 and January 2015, 8 patients with pathologically proven HAS and liver metastases were enrolled. Basic tumor status was evaluated for the primary tumor location and metastatic sites. The CT findings of the liver metastases were analyzed for tumor number and size, presence of tumor necrosis, hemorrhage, venous tumor thrombosis, and dynamic enhancing pattern.

RESULTS: The body and antrum were the most common site for primary HAS (n = 7), and observed metastatic sites included the liver (n = 8), lymph nodes (n = 7), peritoneum (n = 4), and lung (n = 2). Most of the liver metastases exhibited tumor

necrosis regardless of tumor size. By contrast, tumor hemorrhage was observed only in liver lesions larger than 5 cm (n = 4). Three patterns of venous tumor thrombosis were identified: direct venous invasion by the primary HAS (n = 1), direct venous invasion by the liver metastases (n = 7), and isolated portal vein tumor thrombosis (n = 2). Dynamic CT revealed arterial hyperattenuation and late phase washout in all the liver metastases.

CONCLUSION: On dynamic CT, liver metastasis from HAS shared many imaging similarities with HCC. For liver nodules, the presence of isolated portal vein tumor thrombosis and a tendency for tumor necrosis are imaging clues that suggest the diagnosis of HAS.

Key words: Computed tomography; Liver; Hepatoid adenocarcinoma; Hepatocellular carcinoma; Stomach

Core tip: Hepatoid adenocarcinoma of the stomach (HAS) is a rare form of gastric cancer with clinicopathological presentation mimicking hepatocellular carcinoma (HCC). The high similarity between the two diseases makes the differential diagnosis challenging, especially when the primary tumor is unknown, and the liver nodules are the only initial finding. In the present study, identical dynamic enhancing pattern (arterial hyperattenuation and late phase washout) between liver metastasis from HAS and HCC was confirmed. Moreover, the presence of isolated portal vein tumor thrombosis and a tendency of tumor necrosis are the imaging clues that suggest the diagnosis of HAS rather than HCC.

Lin YY, Chen CM, Huang YH, Lin CY, Chu SY, Hsu MY, Pan KT, Tseng JH. Liver metastasis from hepatoid adenocarcinoma of the stomach mimicking hepatocellular carcinoma: Dynamic computed tomography findings. *World J Gastroenterol* 2015; 21(48): 13524-13531 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13524.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13524

INTRODUCTION

Hepatoid adenocarcinoma of the stomach (HAS) is a rare form of gastric cancer with clinicopathological presentation mimicking hepatocellular carcinoma (HCC). Clinically, the neoplasm is characterized by a predilection for older age, high serum alpha-fetoprotein (AFP) levels, an aggressive clinical course, and poor prognosis[1]. The aggressive tumor behavior makes the liver metastasis being as the first clinical manifestation in more than 75% of HAS patients[2]. Pathologically, hepatoid morphology, immunoreactivity with AFP, and a tendency for vascular permeation are the shared features of HAS and HCC[3]. The high similarity between the two diseases makes differential diagnosis challenging, especially when the primary tumor is unknown, and the liver nodules are the only initial finding. Moreover, the role of dynamic computed tomography (CT) in liver metastasis from HAS is not well established. To our knowledge, only one case report mentioned a similar dynamic enhancing pattern in liver metastasis from HAS and HCC[4]. The aim of our study was to evaluate the dynamic CT findings of liver metastasis from HAS and compare them with the typical imaging findings of HCC. The clinical presentation and treatment results of HAS are also evaluated.

MATERIALS AND METHODS

Patient population

Institutional Review Board approval was obtained, and the need for patient informed consent was waived because of the retrospective and anonymous nature of this study. A retrospective search of January 2000 to January 2015 revealed 11 patients with pathologically proven HAS and liver metastases in our institution. Three patients were excluded because their CT studies were not available for review. Finally, 8 patients (6 men and 2 women; mean age 68.5 ± 6.1 years; range 60-78 years) constituted our study cohort. Clinical data including patient demographics, initial presentation, personal history (alcohol use and hepatitis infection), laboratory data (hemoglobin level and serum AFP level), treatments received, and therapeutic result were reviewed.

CT acquisition

CT examinations were requested in regard to clinical symptoms, abnormal liver ultrasound results, or abnormal endoscopic findings. Four patients underwent three-phase dynamic CT of the liver, 2 patients underwent two-phase dynamic CT for gastric tumor staging, and routine abdominal CT was arranged for the remaining 2 patients. Each patient received 100 mL of a nonionic, low-osmolar contrast material (Iohexol, Omnipaque, GE Healthcare, Milwaukee, WI, United States) administered intravenously at a rate of 2.5-3.0 mL/s. Dynamic CT scanning was conducted at 30 s (arterial phase) and 60 s (portal venous phase) after the beginning of intravenous injection. Additional scanning at 3 min (equilibrium phase) was included in the protocol of dynamic CT of the liver. For patients that underwent CT for gastric tumor staging, a total of 800-1000 mL of tap water was administered orally to obtain gastric distension just prior to scanning. Images were routinely reconstructed into coronal and sagittal planes and available for viewing on a picture-archiving and communication system (PACS, GE Healthcare, Milwaukee, WI, United States).

Statistical analysis

CT images were retrospectively reviewed by two

Table 1 Clinical features of hepatoid adenocarcinoma of the stomach

Case No.	Sex/age (yr)	Initial presentation	Location (largest size, cm)	Metastasis	Serum AFP (ng/mL)	Treatment	Follow-up status
1	M/64	Epigastric discomfort	Body, antrum (7.5)	Liver Peritoneum Perigastric lymph nodes	1133.7	Gastrectomy Chemotherapy TACE for liver metastases	Died at 19 mo
2	M/69	Body weight loss	Antrum (7.3)	Liver Lung	281.1	Gastrectomy Chemotherapy	Died at 3 mo
3	M/78	Epigastric discomfort	Antrum (4.5)	Liver Perigastric lymph nodes	3124.9	Gastrectomy Chemotherapy	Died at 5 mo
4	M/63	Epigastric discomfort	Cardia (5.2)	Liver Peritoneum Perigastric lymph nodes	2170.3	Chemotherapy TACE for liver metastases	Died at 6 mo
5	F/70	Palpable mass	Body, antrum (3.8)	Liver Perigastric lymph nodes	890.3	Chemotherapy TACE for liver metastases	Died at 23 mo
6	F/69	Epigastric discomfort	Body, antrum (7.5)	Liver Perigastric lymph nodes	6442.6	Chemotherapy	Died at 9 mo
7	M/60	Epigastric discomfort	Antrum (4.5)	Liver Peritoneum Paraaortic lymph nodes Perigastric lymph nodes	1419.7	Chemotherapy	Died at 3 mo
8	M/75	Body weight loss	Body (4.0)	Liver Lung Peritoneum Perigastric lymph nodes	2904.5	Supportive care	Died at 3 mo

M: Male; F: Female; AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization.

board-certified radiologists in consensus. Basic tumor status was evaluated for the primary tumor location, tumor size, and metastatic sites (for both nodal metastasis and distant metastasis). Image findings of liver metastases were analyzed for the following factors: tumor number and size, presence of tumor necrosis, hemorrhage, venous tumor thrombosis, and dynamic enhancing pattern. The presence of a gastric tumor was suggested when the gastric wall was 6 mm or greater in thickness or with abnormal contrast enhancement[5]. Lymph node involvement was considered as present when the short-axis diameter is larger than 8 mm for perigastric lymph nodes and larger than 10 mm for distant lymph nodes[6,7]. The attenuation of tumor content was analyzed as Hounsfield units[4] when a region of interest was placed in the center of the lesion. The presence of a low-attenuation (10-30 HU), nonenhancing area within the tumor was defined as tumor necrosis[8]. By contrast, tumor hemorrhage was considered as a high-attenuation (50-100 HU), nonenhancing area within the tumor[9]. Venous tumor thromboses, including those of the portal veins, hepatic veins, inferior vena cava, and perigastric veins, were judged by identifying intravenous filling defects with enlargement of the involved venous segment and with minimal contrast enhancement on portal venous phase of CT[10-12]. Isolated portal vein tumor thrombosis was defined as the presence of tumor thrombosis of the portal vein without evidence of liver metastasis of the ipsilateral liver lobe[13]. The degree of tumor attenuation (hypoattenuation, isoattenuation, and hyperattenuation) on CT studies was determined by the largest lesion against the background of the adjacent normal liver parenchyma on arterial phase and late phase (portal venous and equilibrium phases) images[14]. Heterogeneously enhancing lesions were classified as hyperattenuating when most of the solid component was well-enhanced[15]. Washout was defined as occurring when any part of the lesion that was hyperattenuating on arterial phase images exhibited a corresponding hypoattenuating area on late phase images[14].

RESULTS

Clinical findings

Table 1 summarizes the relevant clinical information and basic tumor status of the enrolled patients. Among the 8 patients with pathologically proven HAS and liver metastasis, 6 were male and the mean age at diagnosis was 68.5 ± 6.1 years. All patients were serologically negative for hepatitis B and C, had no history of alcohol abuse, and did not exhibit any clinical or imaging signs of liver cirrhosis. Markedly elevated serum AFP levels (mean 2295.9 ± 1942.8 ng/mL; range 281.1-6442.6 ng/mL) and various degrees of anemia (mean 8.3 ± 0.9 g/dL; range 6.8-9.4 g/dL) were the main laboratory abnormalities. In 4 patients, the symptoms (epigastric discomfort and palpable

Table 2 Computed tomography features of liver metastases from hepatoid adenocarcinoma of the stomach

Case No.	Tumor location	Tumor number (largest size, cm)	Necrosis	Hemorrhage	Venous tumor thrombus	Dynamic enhancing pattern
1	Lateral segment	Single (9.7)	Yes	No	Left portal vein Left hepatic vein Inferior vena cava	Arterial hyperattenuation Late phase washout
2	Bilateral lobes	Multiple (8.7)	Yes	Yes	Right portal vein Left hepatic vein Inferior vena cava	Arterial hyperattenuation Late phase washout
3	Segment 7	Single (2.1)	Yes	No	Right portal vein	NA
4	Right lobe	Multiple (2.8)	Yes	No	Right portal vein Left portal vein1	Arterial hyperattenuation Late phase washout
5	Segment 4	Single (7.2)	Yes	No	nil	Arterial hyperattenuation Late phase washout
6	Right lobe	Multiple (9.8)	Yes	Yes	Right portal vein Left portal vein[1] Gastroepiploitc vein[2]	Arterial hyperattenuation Late phase washout
7	Bilateral lobes	Multiple (7.5)	Yes	Yes	Right portal vein Left hepatic vein	Arterial hyperattenuation Late phase washout
8	Bilateral lobes	Multiple (8.1)	Yes	Yes	Left portal vein	NA

[1]Isolated portal vein tumor thrombosis; [2]By direct invasion of primary hepatoid adenocarcinoma of the stomach. NA: Not available.

mass) led to the ultrasonographic detection of hepatic nodules as the initial clinical finding. Only 25% of the patients (*n* = 2) underwent endoscopy prior to CT examinations. The metastatic tumor status made Fluorouracil (5-FU)-based chemotherapy being as the main treatment for most of the patients (*n* = 7). Three patients complicated with gastric outlet obstruction were treated with palliative distal gastrectomy, and transarterial chemoembolization (TACE) with Lipiodol and Doxorubicin infusion was performed for liver metastases in 3 patients. Supportive care was suggested for one patient because of poor performance status. The mean survival time from the first diagnosis was 8.9 ± 7.8 mo (range 3-23 mo).

CT findings

The body and antrum were the most common site for primary HAS (*n* = 7; mean size 5.5 ± 1.6 cm), and the observed metastatic sites included the liver (*n* = 8), lymph nodes (*n* = 7), peritoneum (*n* = 4), and lung (*n* = 2). Table 2 summarizes the CT characteristics of the liver metastases from HAS. Liver metastases usually presented as multiple nodules with variable sizes (*n* = 5), but single presentation was also noted (*n* = 3). Most of the liver metastases exhibited tumor necrosis regardless of tumor size. By contrast, tumor hemorrhage was observed only in liver lesions larger than 5 cm (*n* = 4). Venous tumor thrombosis was identified in 7 patients, and the locations included the portal veins (*n* = 7), hepatic veins (*n* = 3), inferior vena cava (*n* = 2), and gastroepiploic vein (*n* = 1). Three patterns of venous tumor thrombosis were found: direct venous invasion by the primary HAS (*n* = 1), direct venous invasion by the liver metastases (*n* = 7), and isolated portal vein tumor thrombosis (*n* = 2). Dynamic CT revealed arterial hyperattenuation and late phase washout in all the liver metastases.

DISCUSSION

The typical dynamic enhancing pattern of HCC is arterial hyperattenuation followed by washout on late phase images[16]. This pattern is consistent with a multistep process of hepatocarcinogenesis, which results in tumor vascular changes toward a predominant hepatic arterial supply with a lack of portal venous inflow[17,18]. According to the diagnostic guidelines of the American Association for the Study of Liver Diseases in 2010[19], in patients with cirrhosis or chronic hepatitis B, liver nodules larger than 1 cm with typical enhancing pattern on contrast-enhanced CT can be considered HCC and the need for biopsy is obviated. In our study, all the liver metastases from HAS presented with arterial hyperattenuation and washout on dynamic CT studies (Figures 1 and 2). The identical dynamic enhancing pattern, accompanied with high serum AFP levels, makes liver metastasis from HAS a great mimic of HCC. Clinically, HAS is characterized by the absence of risk factors for HCC[20]. However, in endemic areas with a high incidence of chronic hepatitis B, chronic hepatitis C, and cirrhosis, a HAS patient being a hepatitis carrier or cirrhotic patient simultaneously may not be rare[21].

In our study, most of the liver metastases from HAS exhibited tumor necrosis regardless of tumor size. Central necrosis can be detected even in liver nodules with a diameter of less than 1 cm (Figure 1). By contrast, spontaneous central necrosis occurs only in HCCs larger than 3 cm[22], and the reported necrosis rate for HCC is relatively low (10%-40%)[23]. For liver metastases from HAS, the high incidence of tumor necrosis is believed to be due to outgrowth of the blood supply by the tumor causing hypoxia and subsequent necrosis at the center of the tumor[24].

The tendency for tumor hemorrhage was a shared

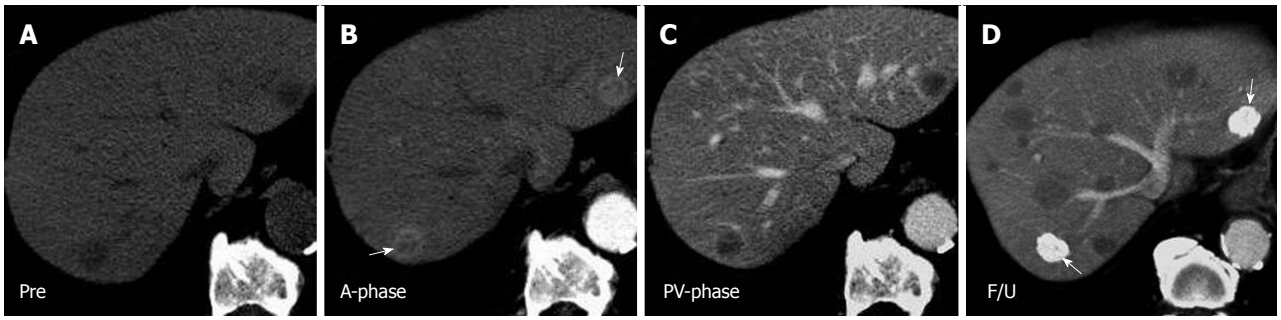

Figure 1 Dynamic enhancing pattern of the liver metastasis from hepatoid adenocarcinoma of the stomach in a 63-yr-old male. A: Liver metastases from hepatoid adenocarcinoma of the stomach presented with hypoattenuation on precontrast computed tomography (CT); B and C: The nodules revealed arterial hyperattenuation (B) and late phase washout (C) on dynamic CT study. Central necrosis (arrows) was found in the small liver nodules; D: The patient received transarterial chemoembolization for liver metastases. The treated liver nodules were well embolized by densely packed Lipiodol (arrows). However, new liver metastases were observed on follow-up CT. Pre: Precontrast; A-phase: Arterial phase; PV-phase: Portal venous phase; F/U: Follow-up image.

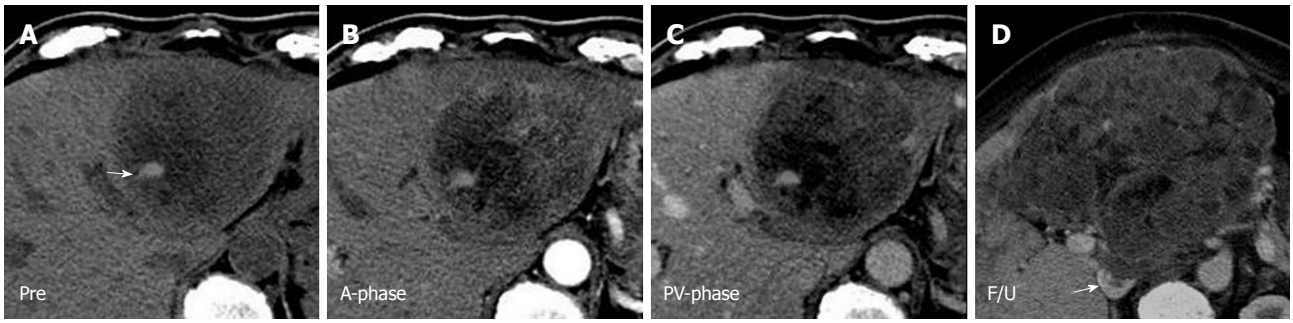

Figure 2 Dynamic enhancing pattern of the liver metastasis from hepatoid adenocarcinoma of the stomach in a 69-yr-old male. A: Liver metastasis from hepatoid adenocarcinoma of the stomach presented with hypoattenuation on precontrast computed tomography (CT); B and C: The liver mass revealed arterial hyperattenuation (B) and late phase washout (C) on dynamic CT study. Central necrosis and tumor hemorrhage (arrow) were noted; D: The patient received total gastrectomy combined with systemic chemotherapy. Progression of the liver metastasis with direct IVC invasion (arrow) was observed on follow-up CT. Pre: Precontrast; A-phase: Arterial phase; PV-phase: Portal venous phase; F/U: Follow-up image.

feature between liver metastasis from HAS and HCC. In our study, tumor hemorrhage was observed only in liver lesions larger than 5 cm (Figure 2). Similarly, HCC with a larger diameter carried a higher risk of tumor hemorrhage[25]. Intratumoral bleeding is usually a result of local ischemia caused by rapid growth of the lesion[26]. In 3%-26% of patients with HCC, hemoperitoneum occurs after tumor bleeding[27,28]. By contrast, neither hemoperitoneum nor tumor rupture has been reported in patients with HAS.

Lee *et al*[7] proposed two patterns of liver metastasis from HAS: a dominant bulky mass with adjacent portal vein tumor thrombosis and multiple nodules of a similar size without tumor thrombosis. The dominant bulky mass pattern was reported to be more common. In our study, a predilection existed for a dominant bulky mass (Figures 2 and 3). However, the tumor number and size were unrelated to the presence of portal vein tumor thrombosis. On CT images, most of the tumor thromboses were caused by direct venous invasion of the metastatic liver tumors (Figure 3C). Similarly, most of the reported venous tumor thromboses in HCC were caused by direct tumor invasion[29]. In one patient with gastroepiploic vein tumor thrombosis, the tendency for vascular permeation by the primary HAS was demonstrated. This finding supports those of two previous studies[4,7]. Moreover, our study demonstrated a unique type of venous tumor thrombosis: isolated portal vein tumor thrombosis (Figure 3D). This finding implies a possible route of tumor spread for the primary HAS and could be useful in differential diagnosis.

A percutaneous liver biopsy is usually performed as a problem-solving tool on liver nodules of uncertain nature. However, the procedure may play a limited role in differentiation between liver metastasis from HAS and HCC. HAS shares strikingly morphologic similarity with HCC in histology[20]. Routine immunohistochemical stains, like AFP, HepPar1, and GPC-3, are useful but not specific for differential diagnosis[21]. Although some novel immunohistochemical markers, such as PIVKA-Ⅱ[30], CEA, PLUNC, and CK19[3], have exhibited varying degrees of ability in differentiation, they are performed only upon adequate clinical and imaging suspicions of HAS. By contrast, for patients with suspected HAS and liver metastasis, liver biopsy along with endoscopic biopsy can be performed to confirm the identical tumor origin in both the liver and stomach, and exclude the diagnosis of synchronous HCC and gastric cancer, which is the most crucial differential diagnosis.

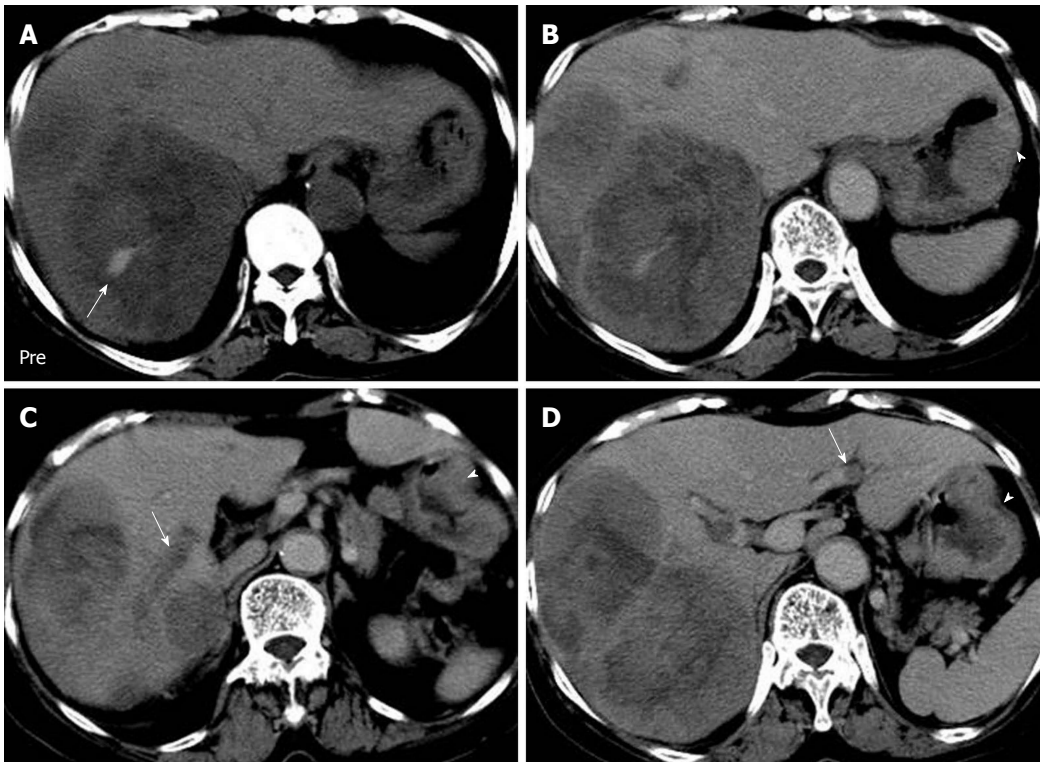

Figure 3 Venous tumor thrombosis in a 69-yr-old female with hepatoid adenocarcinoma of the stomach and liver metastases. A: Bulky liver metastases presented with tumor hemorrhage (arrow) on precontrast computed tomography; B and C: Eccentric wall thickening and heterogenous enhancement of the gastric body (arrowheads) implied gastric malignancy. On portal venous phase, the liver mass presented irregular central necrosis (B) and right portal vein tumor thrombosis (arrow; C); D: Isolated left portal vein tumor thrombosis (arrow) was observed. No visible liver nodule was found at the left liver lobe. Pre: Precontrast.

The prognosis of HAS is extremely poor, mainly resulting from the strong tendency for vascular permeation and early distant metastases[4]. Aggressive treatments, such as radical surgery combined with chemotherapy, have been proven to positively affect the clinical outcome of HAS patients[31,32]. Moreover, because striking pathological similarities are shared by HAS and HCC, the treatments recommended for HCC may also be effective for HAS. Petrelli *et al*[33] suggested Sorafenib (Nexavar, Bayer HealthCare, Montville, NJ, United States) as a possible treatment for hepatoid adenocarcinoma. In our study, TACE was performed along with 5-FU-based systemic chemotherapy in 3 patients. Most of the treated liver nodules were well embolized by densely packed Lipiodol. However, new liver metastases were observed on follow-up CT (Figure 1D), which may be contributed by the continuous tumor spread from the poorly controlled primary HAS through the portal venous system. Our results suggest that TACE, similarly to its role in HCC treatment, may serve as a local therapy for liver nodules of HAS but must be accompanied with systemic chemotherapy or radical surgery for complete tumor control.

The presence of gastric malignancy was not always easily identified on CT images. The detectability of primary HAS was influenced by morphologic features, the thickness of the gastric wall, and the degree of tumor enhancement. Moreover, abdominal CT studies performed as a result of nonspecific initial symptoms or abnormal liver ultrasonographic findings would not follow the standard protocol for gastric tumor staging and would thus impair the detectability of gastric malignancy on CT. D'Elia *et al*[34] reported conventional CT studies possess a low range of detectability (40%-90%) in preoperative gastric cancer staging. When the primary tumor is unknown, and the liver nodules are the only initial finding, correct diagnosis of HAS can be challenging.

This study has some limitations. First, it is a single-center, retrospective, and nonrandomized study. Because of the rarity of HAS, only 8 patients were enrolled in this study. The small sample size may limit accurate analysis and lead to bias of the results. Second, the presence of venous tumor thrombosis, central necrosis, and intratumoral bleeding was determined according to the imaging criteria, not by histologic proof. However, all the enrolled patients had their HAS confirmed histologically through an operation or biopsy and had no other conditions predisposing them to portal venous thrombosis. Third, this study covered patients over a span of 15 years. Images from different CT scanner with various parameters were included for review. However, this was not a major limitation since the intra-abdominal lesions were usually bulky.

In conclusion, liver metastasis from HAS shared many CT imaging similarities with HCC. For the liver

nodules, the presence of isolated portal vein tumor thrombosis and a tendency for tumor necrosis are imaging clues that suggest the diagnosis of HAS. Liver biopsy along with endoscopic biopsy can be performed to confirm the identical tumor origin in both the liver and stomach for the patients with suspicious HAS.

COMMENTS

Background

Hepatoid adenocarcinoma of the stomach (HAS) is a rare gastric cancer with clinicopathological presentation mimicking hepatocellular carcinoma (HCC). The high similarity between the two diseases makes differential diagnosis challenging, especially when the primary tumor is unknown, and the liver nodules are the only initial finding. Moreover, the role of dynamic computed tomography (CT) in liver metastasis from HAS is not well established.

Research frontiers

To date, only one case report mentioned the dynamic enhancing pattern in liver metastasis from HAS and HCC. This study was designed to evaluate the dynamic CT findings of liver metastasis from HAS and compare them with the typical imaging findings of HCC.

Innovations and breakthroughs

Identical dynamic enhancing pattern (arterial hyperattenuation and late phase washout) between liver metastasis from HAS and HCC was confirmed in the present study. Moreover, most of the liver metastases from HAS exhibited tumor necrosis regardless of tumor size. Isolated portal vein tumor thrombosis, a unique type of venous tumor thrombosis, implies a possible route of tumor spread for the primary HAS and could be useful in differential diagnosis.

Applications

The presence of isolated portal vein tumor thrombosis and a tendency of tumor necrosis are the imaging clues that suggest the diagnosis of HAS rather than HCC.

Terminology

HAS is a rare gastric cancer, which usually presents high serum alpha-fetoprotein (AFP) levels, an aggressive clinical course, and poor prognosis. Pathologically, hepatoid morphology, immunoreactivity with AFP, and a tendency for vascular permeation are the shared features of HAS and HCC.

Peer-review

This is a very good paper.

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ORIGINAL ARTICLE

Retrospective Study

Clinical comparison of laparoscopy *vs* open surgery in a radical operation for rectal cancer: A retrospective case-control study

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Institutional review board statement: Our study was a retrospective case-control study between laparoscopic surgery and open surgery in rectal cancer. And the study was and approved by the Ethics Committee of Shanghai First People's Hospital Affiliated Shanghai Jiao Tong University.

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Abstract

AIM: To assess the diverse immediate and long-term clinical outcomes, a retrospective comparison between laparoscopic and conventional operation was performed.

METHODS: A total number of 916 clinical cases, from January 2006 to December 2013 in our hospital, were analyzed which covered 492 patients underwent the laparoscopy in radical resection (LRR) and 424 cases in open radical resection (ORR). A retrospective analysis was proceeded by comparing the general information, surgery performance, pathologic data, postoperative recovery and complications as well as long-term survival to investigate the diversity of immediate and long-term clinical outcomes of laparoscopic radical operation.

RESULTS: There were no statistically significance differences between gender, age, height, weight, body mass index (BMI), tumor loci, tumor node metastasis stages, cell differentiation degree or American Society of Anesthesiologists scores of the patients (P > 0.05). In contrast to the ORR group, the LRR group experienced less operating time (P < 0.001), a lower blood loss (P < 0.001), and had a 2.44% probability of conversion to open surgery. Postoperative bowel function recovered more quickly, analgesic usage and the average hospital stay (P < 0.001) were reduced after LRR. Lymph node dissection during LRR appeared to be slightly more than in ORR (P = 0.338). There were no obvious differences in the lengths and margins (P = 0.182). And the occurrence rate in the two groups was similar (P = 0.081). Overall survival rate of ORR and LRR for 1, 3 and 5 years were 94.0% and 93.6% (P = 0.534), 78.1% and 80.9% (P = 0.284) and 75.2% and 77.0% (P = 0.416), respectively.

CONCLUSION: Laparoscopy as a radical operation for rectal cancer was safe, produced better immediate outcomes. Long-term survival of laparoscopy revealed that it was similar to the open operation.

Key words: Laparoscopic; Open surgery; Short-term outcomes; Long-term outcomes; Rectal cancer

Core tip: This is a retrospective case-control study between laparoscopic surgery and open surgery in rectal cancer. There are 916 clinical cases, collecting from January 2006 to December 2013 in our hospital, which covered 492 cases in laparascopic group and 424 cases in open group. We compared the general information, surgery performance, pathologic data, postoperative recovery and complication as well as the long-term survival of the patients. And than we concluded that laparoscopy can produce better immediate outcomes in rectal cancers. And the long-term survival of laparoscopy was similar to the open operation.

Huang C, Shen JC, Zhang J, Jiang T, Wu WD, Cao J, Huang KJ, Qiu ZJ. Clinical comparison of laparoscopy *vs* open surgery in a radical operation for rectal cancer: A retrospective case-control study. *World J Gastroenterol* 2015; 21(48): 13532-13541 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13532.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13532

INTRODUCTION

It has been estimated that about 1.4 million new cases of colorectal cancer are diagnosed worldwide and nearly 0.7 million colorectal cancer-related deaths occur, of which about 33% are due to rectal cancer[1]. Owing to the restriction of surgery and medicine the prognoses of rectal cancer patients are critically poor, with up to 40% locoregional recurrence and less than a 50% 5-year survival rate before the 1980s[2]. With improvement of surgery and treatment with neoadjuvant chemoradiotherapy, radiotherapy and immune therapy, the recurrence rates have been reduced with survival rates now greater than 70%[3].

It is more than 20 years since laparoscopy was applied to the treatment of malignant colorectal cancer by Jacobs and others[4]. With improved techniques and updated surgical instruments, laparoscopy is more widely used to treat rectal cancer. Its principal advantages are its clear view and reduced wounding, less interference to the immune system, quick post-operative recovery and a lower overall operating expense[5-7]. Yet many controversies remain whether laparoscopy, although minimally invasive, is inferior to conventional open surgery for the long-term treatment of this serious cancer. For example, it has been suggested that laparoscopy may stimulate tumor invasion and metastasis. Most large scale randomized controlled trials (RCT) worldwide have conducted research [conventional *vs* laparoscopic-assisted surgery in colorectal cancer (CLASICC), colorectal cancer laparoscopic or open resection (COLOR), *etc*.] on colon rather than rectal cancer. In 2013, National Comprehensive Cancer Network tended to recommend laparoscopy for rectal cancer patients with clinical research trials encouraged to compare laparoscopic and conventional operations.

A large body of research exists, which involved a comparison between laparoscopic and open radical resection for rectal cancer[8]. The research included resection margin length of both sides, mesentery length and the number of lymph nodes retrieved, which suggested the same effectiveness of radical laparoscopy to an open operation[9,10]. However, laparoscopic surgery also has the advantage of less trauma, less pain postoperatively and a reduced hospital stay for patients. In previous years, laparoscopy took longer than open section surgery but now laparoscopy can be completed in about the same time as open section, mainly due to the accumulation of practical surgical experience using this technique; one additional benefit is the reduced occurrence of complications[11]. Research on the long-term curative effect has revealed closely similar 1-, 3- and 5-year survival rates of laparoscopic rectal cancer surgery compared to open resection[6,12-14]. Earlier research found a high incidence of postoperative implantation metastasis and peritoneal implantation metastasis after laparoscopic radical surgery for rectal cancer[15]. But recent research has reported a similar implantation metastasis incidence for laparoscopic and open section[16]. It was assumed that earlier outcomes could have been related to the neglect of tumor-free principles during the operations.

RCT research includes that of Milson, Schwandner,

CLASICC[9,10,17] and others, who reached the conclusion of the similar long-term benefits of laparoscopic and open resection for rectal cancer. Leung also concluded that there were similar long-term effects of laparoscopic and conventional surgery for recto sigmoid colon cancer[16]. The RCT research focused on laparoscopic and open section for rectal tumors, which was conducted by Gong, Park and the COLOR Ⅱ stage experiments are still in progress[13,18,19]. From the perspective of evidence-based medicine, a RCT outcome based on large number of cases should prove the long-term efficacy or otherwise of a laparoscopic radical operation for rectal cancer.

MATERIALS AND METHODS

Patients

Between January 2006 and December 2013, 492 patients in group LRR and 424 patients in group ORR in the Shanghai First People's Hospital Affiliated Shanghai Jiao Tong University were enrolled in our study. Open surgeries (OP) and laparoscopic-assisted surgeries (LAP) were performed by the same surgical teams, respectively. Two groups of patients underwent preoperative colonoscopy and had biopsy-proven adenocarcinoma. The trial received approval from the Shanghai First Peoples' Hospital Affiliated Shanghai Jiao Tong University research ethics committee and prior written informed consent was obtained from all patients.

Patients diagnosed with rectal cancer without other serious diseases were included in the study. Patients with distant metastasis of the tumor or associated malignant tumor; intestinal obstruction; recurrence of the tumor or other digestive system malignancy; those who had a palliative operation for the tumor which could not be resected or had widespread metastasis in the abdominal cavity, were excluded from the study.

Preoperative preparation and operation procedures

Both groups had preoperative fiber colonoscopy and biopsy for pathology confirmation of a clear diagnosis of rectal cancer. Preoperative staging was evaluated by enhanced CT or MRI scanning of the abdomen and pelvis, as well as chest X-ray films, ultrasonography or other assisted auxiliary examination. Both teams employed preoperative intestinal preparations: twice oral gentamicin 80000 U/d, 3 times 0.4 g oral metronidazole for 3 d, and 1 d fluid food before surgery. Polyethylene glycol-electrolyte powder was given to prepare the bowel.

Both groups were operated on by a fixed team of experienced physicians. Under the tumor-free technique principle, the operation should be performed based on colorectal cancer radical excision and rectal tumor patients should be operated according to TME principles. The preoperative preparation of the laparoscopy group closely mimicked the open resection group. Patients were placed in the bladder lithotomy position, with tracheal intubation anesthesia and a pneumoperitoneum established under a maintained pressure of 10-14 mmHg. The 5-trocar technique was adopted by using an ultrasound knife to incise the sigmoid right-sided mesentery and clear the peritoneum to free up presacral space to the right side of the rectum. A medical grasper was used to retract the superior rectum vessels and was directed upwards in a retrograde direction to facilitate separation of the left-sided Toldt anadesma, reveal the ureter and bare the root of the upper mesentery artery. A part of the upper mesentery artery and the inferior mesenteric aorta in the horizontal position was separated using a titanium clamp. Downward pressure was used to separate the presacral space and remove the peritoneum from the right side of the rectum. The sigmoid was isolated and the peritoneum removed to the left side of the rectum and also the anterior sacral fascia to protect the hypogastric nerve and pots plexus. Next, the antetheca denonvillier fascia was separated as well as the right and left ligament down to the pelvic floor, and a further incision made 3-5 cm below the umbilicus in the center. Through this incision, gradually the rectal tumor was removed in the same way as during an open operation. Subsequently, an intestinal anastomosis was completed or an enterostomy, and the pelvic region rinsed with copious quantities of distilled water to decrease the growth of cancer cells. A regular drainage tube was indwelled close to the anastomotic area and was routed to the lower abdomen or ischiorectal fossa. Open section mainly used a high frequency electric knife and the operative principle and methods were identical to LRR operated group.

Perioperative surveillance, postoperative management and follow-up evaluation

Common data included gender, age, height, weight, body mass index (BMI), tumor location, tumor node metastasis (TNM) staging, cell differentiation grade, American Society of Anesthesiologists (ASA) scores and so on. The operative index included the time to complete the operation, blood loss, sample lengths, average retrieved lymph node numbers and the conversation rates. The postoperative pain score on day 1 was adopted as the basis of a numerical rating scale. The degree of pain was assessed by a number from 0 to 10 in which 0 was pain-free and 10 was the highest pain intensity experienced by patients. Individual patients specified their degree of pain: 0-4 was mild pain; 5-6 medium pain, and 7-10 severe pain. Postoperative data was recorded which included comprised peristalsis recovery time, exsufflation time, time until off-bed, time until the first liquid and semi-liquid intake, duration of hospital stay and the overall hospitalization duration.

FOLFOX plan, XELOX plan and the Capecitabine

Table 1 Clinical comparison of radical operation data for rectal cancer

	LRR (n = 492)	ORR (n = 424)	P value
Gender			
Male	301	243	0.235
Female	191	181	
Age (yr, mean ± SD)	64.5 ± 11.9	63.3 ± 12.3	0.290
BMI (kg/m^2)	23.274 ± 3.463	23.438 ± 3.533	0.999
Preoperative comorbid diseases	52.8% (260/492)	58.7% (249/424)	0.074
Hypertension	60	57	
Coronary heart disease	37	34	
Arrhythmia	21	20	
COPD	10	13	
Pulmonary infection	3	5	
Asthma	2	3	
Diabetes	42	70	
Hepatic cirrhosis	4	3	
Cerebral infarction	20	6	
Renal failure	17	7	
Autoimmune	5	0	
Others	39	31	
ASA score			0.498
Ⅰ	109	108	
Ⅱ	321	264	
Ⅲ	62	52	
Previous abdominal surgery	75 (15.2%)	65 (15.3%)	0.971

LRR: Laparoscopy in radical resection; ORR: Open radical resection; BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; ASA: American Society of Anesthesiologists.

plan were regularly deployed in postoperative chemotherapy for 6 mo, apart from cancer stage 0 and Ⅰ. The postoperative follow-up commonly involved a clinical visit or telephone follow-up. As of 28th February 2014, the shortest follow-up duration was 2 mo, the longest 109 mo and the average 55 mo, with a 95.1% follow up success rate.

Statistical analysis

SPSS Statistics for Windows, Version 17.0 (SPSS, Inc., Chicago, IL, United States) was used for analyses. A t-test was used to analyze normally distributed data and a Mann-Whitney U-test for other types of distributions. A χ^2 test was used to analyze count data. $P < 0.05$ was considered to be statistically significant. The survival cures for two group were used Kaplan-Meier method, and the log-rank test was applied to analyze the differences between the results.

RESULTS

Clinical data of patients

Of the 916-recorded cases, 492 cases were LRR. There were 301 males and 191 females, aged 64.5 ± 11.9, BMI index 23.274 ± 3.463, Ⅰ stage 93 cases, Ⅱ stage 218 cases and Ⅲ stage 181 cases in TNM staging, with one-score for 109 cases, two-scores for 321 cases and three-scores for 62 cases in ASA assessments. In 424 cases in the ORR group, there were 243 males and 181 females, aged 63.3 ± 12.3, BMI index 23.438 ± 3.533, Ⅰ stage 83 cases, Ⅱ stage 211 and Ⅲ stage 130 cases in TNM staging, with one-score 108 cases, two-score 264 and three-score 52 cases in ASA scores. The cases of previous abdominal surgery in ORR and LRR group were 75 and 65. There was comparability between the two groups in terms of age, gender, tumor staging, ASA scores and so on ($P > 0.05$; see Table 1).

Table 2 Surgery index

	LRR (n = 492)	ORR (n = 424)	P value
Surgery duration (min)	143.89 ± 50.865	164.86 ± 67.993	< 0.001
Surgery bleeding (mL)	111.54 ± 97.148	154.03 ± 154.545	< 0.001
Maximum incision (cm)	4.14 ± 0.738	13.8 ± 2.603	< 0.001
Conversions to open	12 (2.4%)		
Tumor diameter (cm)	4.338 ± 1.6387	4.325 ± 1.8274	0.914
Sample length(cm)	18.050 ± 5.1748	17.553 ± 5.7995	0.182
Proximal margin (cm)	10.487 ± 4.2906	10.673 ± 5.2175	0.567
Distal margin (cm)	3.214 ± 1.8727	2.873 ± 2.4913	0.022
Lymph nodes retrieved (unit)	11.09 ± 6.503	10.68 ± 6.321	0.338

LRR: Laparoscopy in radical resection; ORR: Open radical resection.

Comparison of LRR and ORR operations

The operation time of LRR was shorter than ORR (143.89 ± 50.865 min *vs* 164.86 ± 67.993 min), with a difference that reached statistical significance ($P < 0.001$). ORR produced more blood loss than LRR (111.54 ± 97.148 mL *vs* 154.03 ± 154.545 mL) and the difference was statistically significant ($P < 0.001$). Proximal and distal margins for both samples were negative. The sample length obtained from LRR and ORR were not significantly different ($P = 0.182$) but the distal margin of LRR was longer compared to ORR (3.214 ± 1.8727 cm *vs* 2.873 ± 2.4913 cm) the difference being statistically significant ($P = 0.022$). The average retrieved lymph node numbers during LRR appeared to be slightly above ORR but it was not statistically different ($P = 0.338$). In LRR there were 12 cases of conversion (2.4% conversion rate) to open resection comprising 1 case of surgical bleeding, 6 cases of tight adhesion between the tumor and surrounding tissues and 5 cases of serious abdominal and pelvic adhesion. Table 2 summarizes these operative results.

Pathology findings

After postoperative pathology, 88 cases were well-differentiated, 285 cases moderately-differentiated, 86 cases poorly-differentiation and 33 cases of mucinous cancer in LRR, while 92 cases were well-differentiated, 237 moderately-differentiated, 72 cases poorly-differentiated and 23 cases of mucinous cancer in ORR. The differences were not statistically significantly different ($P = 0.476$). For TNM staging, LRR contained 93 cases of Ⅰ stage, 218 cases of Ⅱ stage and 181 cases of Ⅲ stage while ORR were 83 cases of Ⅰ stage, 211 cases of Ⅱ stage and 130 cases

Table 3 Pathology findings

	LRR	ORR	χ^2	*P* value
Differentiation			2.495	0.476
Well-differentiated	88	92		
Moderate-differentiation	285	237		
Poorly-differentiated	86	72		
Mucinous cancer	33	23		
pT			7.468	0.058
T1	33	29		
T2	164	108		
T3	207	193		
T4	88	94		
pN			6.865	0.076
N0	252	233		
N1	147	96		
N2	75	73		
Nx	18	22		
Lymph node metastasis				0.259
Yes	240	191		
No	252	233		
TNM stage				0.134
Ⅰ	93	83		
Ⅱ	218	211		
Ⅲ	181	130		

pT: Pathology Tumor; pN: Pathology Node; pM: Pathology metastasis; TNM: Tumor node metastasis.

of Ⅲ stage cancer, but the apparent differences were not statistically significant (P = 0.134). Table 3 shows the results in detail.

Perioperative complications

There were 95 cases of perioperative complications (19.3% incidence rate) for LRR which included: 3 cases of ureter injury, 2 cases of massive hemorrhage, 7 cases of postoperative bleeding, 3 cases of anastomotic hemorrhage, 24 cases of anastomotic leakage, 7 cases of incisional infection, 8 cases of ileus, 1 case of pelvic abscess, 8 cases of pulmonary infection, 2 cases of acute cardiac failure, 1 case of deep vein thrombosis, and 1 case of lymphatic fistula. For ORR, with (a 24.1% occurrence rate) there were 102 cases of perioperative complications in the ORR group, which were: 1 case of ureter injury, 15 cases of anastomotic leakage, 27 cases of incisional infection, 1 case of postoperative pelvic abscess, 9 cases of inflammatory ileus, 10 cases of acute cardiac failure and 2 cases of sudden death. The incidence rate of perioperative complications in the LRR and ORR groups were not significantly different (P = 0.081). Table 4 shows the results in detail.

Postoperative recovery

The duration of LRR was distinctly shorter than ORR in terms of peristalsis recovery (P < 0.001), off-bed time (P = 0.017), gastrointestinal decompression (P < 0.001), retention catheterization (P < 0.001), oral food time (P < 0.001), abdominal drainage (P = 0.008), hospital stay (P < 0.001) and various other aspects. The difference in duration for the two groups was statistically different. Table 5 shows the detailed results.

Postoperative analgesics and pain scores

LRR was significantly less than ORR in terms of the requirement for postoperative analgesic administration (P < 0.001). The difference in the NRS postoperative pain score between the 2 groups was statistically significant showing that the degree of postoperative pain for LRR was evidently less than that after ORR (Table 6).

Economic cost

The overall hospitalization cost for LRR and ORR was little different because although the operation costs of LRR were more than ORR, the cost of medication for LRR was lower than ORR. Thus, overall there was no statistically significantly difference between the costs of the operation as detailed in Table 7.

Post-operation survival

A 95.1% patient follow-up was achieved with an average duration time of 55 mo. For the total survival rate, the 1-year overall survival rate for ORR and LRR was 94.0% and 93.6% (P = 0.534), 3-year overall survival rate 78.1% and 80.9% (P = 0.284) and the 5-year overall survival rate 75.2% and 77.0% (P = 0.416), respectively. In stage Ⅰ, the 1-year overall survival rate for ORR and LRR was 93.8% and 99.2% (P = 0.402), 3-year survival rate 91.6% and 90.3% (P = 0.774) and the 5-year survival rate 89.2% and 88.2% (P = 0.837), respectively. In stage Ⅱ, the 1-year overall survival rate for ORR and LRR was 92.8% and 94.7% (P = 0.489), 3-year survival rate 79.1% and 86.2% (P = 0.052) and the 5-year survival rate 76.8% and 81.7% (P = 0.140), respectively. For Stage Ⅲ, the 1 year overall survival rate for ORR and LRR was 87.8% and 91.9% (P = 0.178), the 3-year survival rate 69.2% and 70.7% (P = 0.777) and the 5-year survival rate 65.4% and 66.9% (P = 0.787), respectively. Refer to Table 8 and the survival curves in Figures 1A-D for the relevant data.

DISCUSSION

Due to advances in surgical techniques and medication, the outcomes of rectal cancer treatment have considerably improved[20]. In spite of various published studies[3,13] concerning the similar long-term outcomes of rectal cancer produced by laparoscopic surgery, the argument for choosing laparoscopy or open surgery is still heated. In the latest randomized trial for rectal cancer, it was reported that the disease-free survival (DFS) and overall survival rates for LRR and ORR group were similar[20]. In our current trial, we studied the advantages and disadvantages of laparoscopic *vs* an open operation, and have retrospectively compared perioperative complications, postoperative recovery,

Table 4 Perioperative complications for the 2 groups *n* (%)

	LRR (*n* = 492)	ORR (*n* = 424)	χ^2	*P* value
Occurrence rate of perioperative complications	19.3% (95/492)	24.1% (102/424)	3.041	0.081
Intraoperative complications	10 (2)	4 (0.9)	1.795	0.180
Massive hemorrhage (> 1000 mL)	2 (0.41)	1 (0.24)		
Organ injury	3 (0.61)	2 (0.47)		
Equipment disorders	4 (0.81)	0		
Other	3 (0.61)	1 (0.24)		
Post-operative complications	85 (17.3)	98 (23.31)	4.853	0.028
Anastomotic leakage	24 (4.88)	15 (3.54)		
Wound infection	7 (1.42)	27 (6.37)		
Ileus	8 (1.63)	9 (2.12)		
Anastomotic hemorrhage	3 (0.61)	2 (0.47)		
Pelvic abscess	1 (0.20)	1 (0.24)		
Abdominal hemorrhage	7 (1.42)	4 (0.94)		
Peritonitis/septic shock	9 (1.83)	7 (1.65)		
Incisional/port herniation	4 (0.81)	10 (2.36)		
Deep vein thrombosis	1 (0.20)	0		
Lymphatic fistula	1 (0.20)	0		
Pulmonary infection	8 (1.63)	2 (0.47)		
Acute cardiac failure	2 (0.41)	10 (2.36)		
Urinary infection	4 (0.81)	4 (0.94)		
Incision split	4 (0.81)	5 (1.18)		
Sudden death	2 (0.41)	2 (0.47)		

LRR: Laparoscopy in radical resection; ORR: Open radical resection.

Table 5 Postoperative recovery

	LRR (*n* = 407)	ORR (*n* = 326)	*P* value
Peristalsis recovery (d)	1.91 ± 0.89	2.41 ± 1.13	< 0.001
Exsufflation recovery (d)	3.03 ± 1.25	3.96 ± 1.53	< 0.001
Off-bed (d)	2.80 ± 1.26	3.38 ± 1.07	< 0.017
Liquid intake (d)	3.90 ± 1.446	5.08 ± 1.763	< 0.001
Semi-liquid intake (d)	6.55 ± 1.910	7.59 ± 2.065	< 0.001
Abdominal drainage (d)	9.48 ± 7.386	11.07 ± 10.484	< 0.008
Retention catheterization (d)	5.63 ± 3.613	6.67 ± 4.043	< 0.001
Post-op hospital stay (d)	12.27 ± 3.156	18.32 ± 5.406	< 0.001
Total hospital stay (d)	21.50 ± 4.991	25.81 ± 7.868	< 0.001

LRR: Laparoscopy in radical resection; ORR: Open radical resection.

Table 6 Postoperative analgesics and pain scores

	LRR (*n* = 492)	ORR (*n* = 424)	χ^2	*P* value
Analgesic usage			95.31	< 0.001
No	299	121		
PCIA	152	273		
Short-acting drug	41	30		
Post-op pain degree			21.43	< 0.001
Mild	263	193		
Medium	213	186		
Severe	16	45		

LRR: Laparoscopy in radical resection; ORR: Open radical resection; PCIA: Patient controlled intravenous analgesia.

economic costs, survival and so on, of rectal cancer treated in our hospital from January 2006 to December 2013.

Based on published literature[5,7,21], we understand that the short-term outcomes of the LRR group are superior to the ORR group. In early stage laparoscopic radical rectal tumor surgery, much more time is required than for an open operation[9]. With the improvement of medical devices, sufficient training and accumulated experience, the operating time between the two groups was shown to be similar with no statistical significance[22]. In the present study, we found that the operating time of the LRR group was shorter than that of the ORR group, with statistical significance being achieved. It was reported in COLOR Ⅱ[18] that the blood loss volume was much less than that of open surgery proving definitively that laparoscopic surgery is capable of decreasing the hemorrhage volume. In the present study, the volume of intraoperative bleeding in the LRR group was clearly less than that in the ORR group. This finding can probably be attributed to the fact that operators benefit from the magnified view of laparoscopy, and also improved equipment that can effectively stop bleeding. The clearance and sufficient length of the resected sample was markedly key for the success of each operation. CLASICC showed that the resected intestinal segment in laparoscopic and in open surgery were similar in length[9], and our research verified further that the lengths of the resected sample in the LRR and ORR groups were similar, with no statistical difference being found. However, we established that the distal margin of tumor in the LRR group was longer than in the ORR group and we hypothesized that the difference between the two groups may be caused by preponderant visualization of laparoscopy in the lower

Table 7 Economic costs of the operations

	LRR (n = 492)	ORR (n = 424)	P value
Operation fee (RMB)	13628 ± 6771	10761 ± 5056	< 0.001
Medication (RMB)	15859 ± 10203	17562 ± 7006	0.06
Total hospitalization (RMB)	40889 ± 19356	42381 ± 17915	0.234

LRR: Laparoscopy in radical resection; ORR: Open radical resection.

Table 8 Postoperative survival rates

	LRR	ORR	P value
Post-op 1-yr			
Ⅰ	99.2%	93.8%	0.402
Ⅱ	94.7%	92.8%	0.489
Ⅲ	91.9%	87.8%	0.178
Total survival rate	93.6%	94.0%	0.534
Post-op 3-yr			
Ⅰ	90.3%	91.6%	0.774
Ⅱ	86.2%	79.1%	0.052
Ⅲ	70.7%	69.2%	0.777
Total survival rate	80.9%	78.1%	0.284
Post-op 5-yr			
Ⅰ	88.2%	89.2%	0.837
Ⅱ	81.7%	76.8%	0.140
Ⅲ	66.9%	65.4%	0.787
Total survival rate	77.0%	75.2%	0.416

LRR: Laparoscopy in radical resection; ORR: Open radical resection.

pelvis. It was illustrated in detailed by Schwandner *et al*[10] that there was no difference in the lymph nodes harvested in the two groups, findings consistent with our data. The average lymph nodes retrieved from LRR and ORR were 11.09 ± 6.503 and 10.68 ± 6.321 (P = 0.338) in our study, which suggested a small disparity in the number of lymph nodes retrieved in LRR compared to ORR.

The NRS pain score of the ORR group was much higher than the scores of the LRR group (P < 0.001), and the average postoperative analgesics usage for LRR patients was much less than ORR patients (P < 0.001). We conclude that the postoperative pain degree of the LRR group was obviously milder than in the ORR group, findings consistent with previous reports[6,12,23]. Additionally, the recovery time of intestinal function, off-bed time, regular food intake time, hospital stay and other aspects were evidently less than after ORR, which is in accordance with Schwandner[10], COLOR Ⅱ[18] and other reports.

It is well documented that the leading reasons for conversion to open surgery included extensive abdominal metastasis, over-sized tumors and a confused anatomic relationship due to serious pelvic adhesion, *etc.*[6,12,24-26]. The conversion rate was 2.44% in the current research, which is lower than the well-documented 5% of other studies. The potential cause of this discrepancy could be due to the full assessment of partial status for rectal tumor, the high admittance standard for studied cases and the elimination of advanced tumor patients from the study. In addition, a large amount of laparoscopic radical surgery for rectal cancer had been undertaken in our institute prior to this study. Thus, we had had completed the study curve and obtained extensive experience for a lower conversion rate between the two surgical techniques. Moreover, the incidence of perioperative complication rates in the LRR group was similar to that in the ORR group as previously reported[27], findings parallel to our results, with no statistical significance between the LRR (19.3%) and ORR (24.1%) groups.

Economic efficiency is also a vital appraisal index for technology. It has been well documented that the surgical expenses for laparoscopic are well above those for open surgery due to its use of disposable surgical instruments and higher anesthesia costs, and technology equipment standards[28,29]. However, Choi assumed that the surgical expenses only consisted of partial expenses which should have included anesthesia, inspection, medication, medical care agents and other consumption goods[28]. In our research, LRR surgical expenses were a slightly more costly than conventional procedures (P < 0.001) while the medication required for LRR was less than that of ORR, which possessed no difference of statistical importance (P = 0.06). In general, there was no obvious difference between LRR and ORR in terms of total hospitalization costs (P = 0.234). Considering a briefer hospital stay and quick recovery after LRR, the nursing labor costs will be significantly lower, which suggests that the laparoscopic approach is the most cost effective.

Concerning the survival outcomes, various studies have shown that the short-term survival and long-term survival between the LRR and ORR groups is similar. The survival outcomes of the COREAN trial revealed that the 3-year DFS rate was 72.5% in the ORR group and therefore similar to that in the LRR group (79.2%)[30]. Based on published comparative retrospective studies, we have found that the 5-year DFS between the LRR and ORR groups was separately 82% and 79% with no statistical difference[31]. Importantly, a randomized multicenter study revealed that the DFS rates for LRR and ORR group were 74.8% and 70.8% respectively, and that the overall survival rates for the LRR and ORR group were 86.7% and 83.6%, with no statistical difference being detected[20]. In the present study, the follow-up duration of the 2 groups was 55 mo in total, with a 95.1% follow-up rate being achieved. We have illustrated that the 1-, 3- and 5-year survival rate between the ORR and LRR groups were not statistically significant (94.0% *vs* 93.6%, P = 0.534; 78.1% *vs* 80.9%, P = 0.284; 75.2% *vs* 77.0%, P = 0.416), findings similar to the published literature. The results here suggest that the laparoscopic radical operation is analogous to open resection and has prior immediate and long-term clinical effects. It may be related to the proficiency of laparoscopic techniques as well as the relatively protective functions of

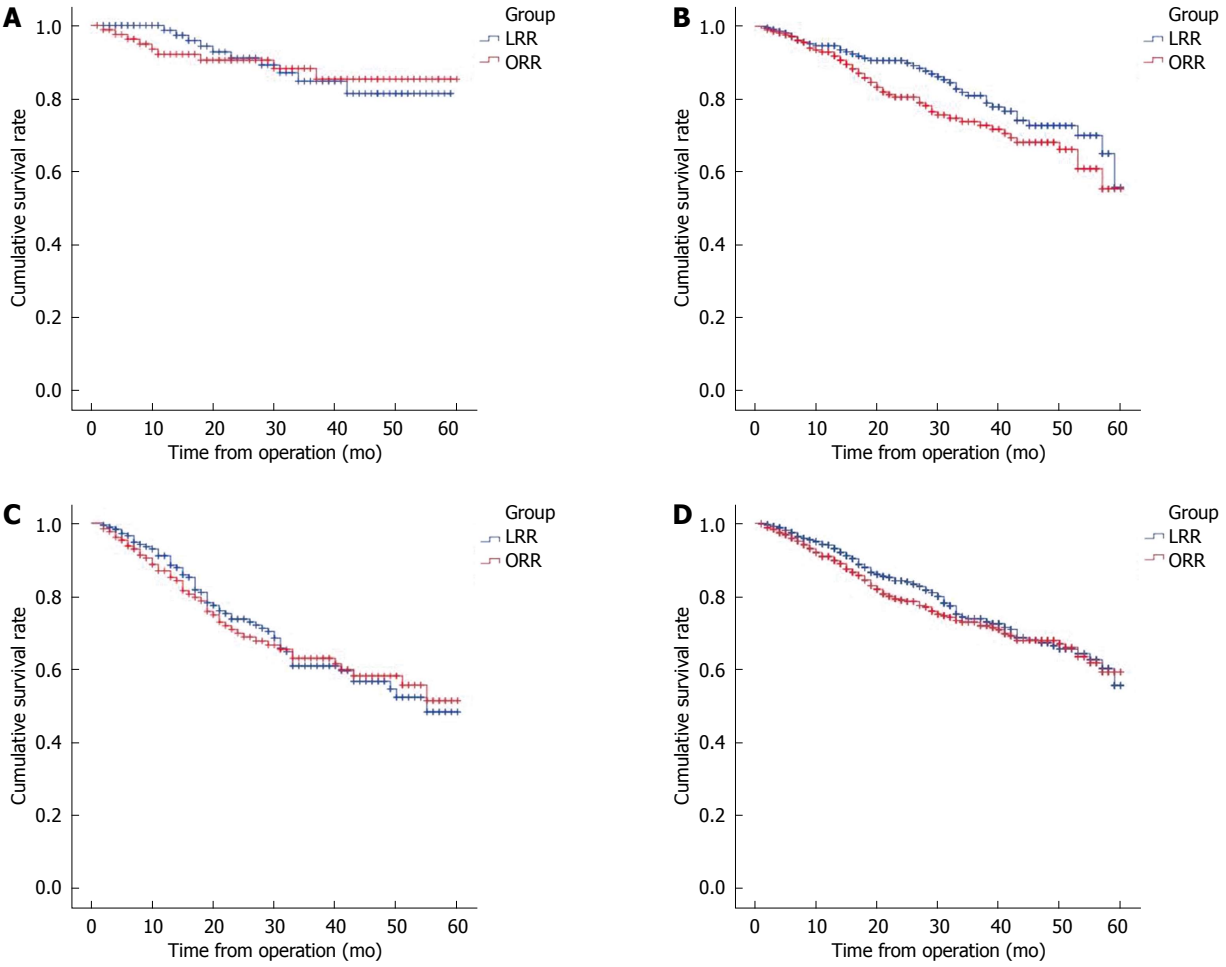

Figure 1 Different stages of postoperative survival status. A: Stage Ⅰ postoperative survival status; B: Stage Ⅱ postoperative survival status; C: Stage Ⅲ postoperative survival status; D: Overall postoperative survival status for both groups. LRR: Laparoscopy in radical resection; ORR: Open radical resection.

laparoscopy for its less interference in cell immunity functions. Thus, the long-term survival of laparoscopic patients for rectal cancer proved equal to those given open operations.

In this original clinical research, we conclude that a laparoscopic-assisted operation has more benefits due to reduced trauma, less postoperative pain and a reduced stay in hospital, as well as faster recovery from radical rectal malignancy therapy. It is clear that the surgical scope and radical effects of tumors were similar in the laparoscopic and open resection techniques. In addition, overall hospitalization expenses, immediate perioperative complications and the long-term survival rates of patients who experienced laparoscopic surgery closely resembled the conventional operation. Thus, laparoscopic surgery may become the most effective therapy for rectal malignancy in the future.

COMMENTS

Background

As we all know, the argument for choosing laparoscopy or open surgery in rectal cancer is heated due to the inconsistent outcomes reported by different research centers. In the latest randomized trial for rectal cancer, it was reported that the disease-free survival (DFS) and overall survival rates for laparoscopy in radical resection (LRR) and open radical resection (ORR) group were similar. In this study, they compared the short-term outcomes and long-term outcomes between the LRR group and ORR group.

Research frontiers

The outcomes between LRR group and ORR group in rectal cancer was inconsistent. Their study compared the short-term and long-term outcomes between LRR group and ORR group, and concluded the similar results reported in the lasted randomized trial for rectal cancer.

Innovations and breakthroughs

It is a retrospective case-control study performed in rectal cancer with different operations, and the authors compared the short-term and long-term outcomes between the LRR group and ORR group.

Applications

The outcomes between LRR group and ORR group in rectal cancer verified that the laparascopic surgery could have a better result than open surgery. It may affect the choice of surgery in rectal cancer.

Terminology

Conventional *vs* laparoscopic-assisted surgery in colorectal cancer and colorectal cancer laparoscopic or open resection: Two trials mainly compared the survival of overall survival, disease-free survival, progression-free survival between the LRR and ORR groups in colorectal cancer.

Peer-review

The authors conducted a clinical retrospective study to compare the short and long-term outcomes between laparoscopic and open surgery for rectal cancer in the Chinese population. They concluded that the laparoscopic resection as a radical operation was safe and effective, while the long-term survival of patients treated with laparoscopic surgery was similar to those with open surgery.

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ORIGINAL ARTICLE

Retrospective Study

Endoscopic resection of colorectal granular cell tumors

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Author contributions: Take I, Shi Q and Qi ZP contributed equally to this work; Take I and Qi ZP collected the data; Take I analyzed and drafted the manuscript; Shi Q designed and supervised the study; Cai SL, Zhou PH and Yao LQ revised the manuscript for important intellectual content; Yao LQ, Zhou PH and Zhong YS provided administrative support; all authors have read and approved the final version to be published.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Zhongshan Hospital of Fudan University, Shanghai, China.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: No additional data are available.

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Abstract

AIM: To determine the feasibility and effectiveness of endoscopic resection for the treatment of colorectal granular cell tumors (GCTs).

METHODS: This was a retrospective study performed at a single institution. From January 2008 to April 2015, we examined a total of 11 lesions in 11 patients who were treated by an endoscopic procedure for colorectal GCTs in the Endoscopy Center, Zhongshan Hospital of Fudan University, Shanghai, China. Either endoscopic mucosal resection or endoscopic submucosal dissection (ESD) was performed by three surgeons with expertise in endoscopic treatment. The pre- and post-operative condition and follow-up of these patients were evaluated by colonoscopy and endoscopic ultrasonography (EUS).

RESULTS: Of these 11 lesions, 2 were located in the cecum, 3 were in the ileocecal junction, 5 were in the ascending colon, and 1 was in the rectum. The median maximum diameter of the tumors was 0.81 cm (range 0.4-1.2 cm). The *en bloc* rate was 100%, and the complete resection rate was 90.9% (10/11). Post-operative pathology in one patient showed a tumor

at the cauterization margin. However, during ESD, this lesion was removed *en bloc*, and no tumor tissue was seen in the wound. No perforations or delayed perforations were observed and emergency surgery was not required for complications. All patients were followed up to May 2015, and none had recurrence, metastasis, or complaints of discomfort.

CONCLUSION: Endoscopic treatment performed by endoscopists with sufficient experience appears to be feasible and effective for colorectal GCTs.

Key words: Endoscopic submucosal dissection; Granular cell tumors; Endoscopic mucosal resection; Colorectal

Core tip: Granular cell tumors (GCTs) are asymptomatic and are potentially malignant, which can pose a significant diagnostic and therapeutic challenge for endoscopists. The development of endoscopic techniques has had a marked influence on the diagnosis and treatment of colorectal submucosal tumors. We determined the feasibility and effectiveness of endoscopic resection for the treatment of colorectal GCTs. We conclude that endoscopic resection is safe and effective for treating colorectal GCTs, which allows *en bloc* resection in one visit, with a clear histological diagnosis, provides patients with a greater degree of comfort and leads to a better compliance.

Take I, Shi Q, Qi ZP, Cai SL, Yao LQ, Zhou PH, Zhong YS. Endoscopic resection of colorectal granular cell tumors. *World J Gastroenterol* 2015; 21(48): 13542-13547 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13542.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13542

INTRODUCTION

Granular cell tumor (GCT) is an uncommon, potentially malignant, asymptomatic mesenchymal tumor arising from Schwann cells[1,2]. In the gastrointestinal tract, GCTs often appear as a round, yellowish submucosal nodule covered by normal mucosa during endoscopy[3-5]. The development of endoscopic techniques and immunohistochemical analysis has had a marked influence on the diagnosis and treatment of gastrointestinal tract submucosal tumors (SMT)[6-9]. Recently, there have been many reports on upper gastrointestinal GCTs[3,9-11]; however, there are few studies on colorectal GCTs. The present study was conducted to evaluate the feasibility and effectiveness of endoscopic resection for the treatment of colorectal GCTs.

MATERIALS AND METHODS

Patient information

During a 7-year period between January 1, 2008 and April 30, 2015, a total of 531 colorectal SMTs were treated at the Endoscopy Center of Zhongshan Hospital of Fudan University, Shanghai, China. A computer search of the SMT pathology files was carried out to determine GCTs arising in the colon, and 11 cases were identified. Patient demographics, pathological data and surgery reports were obtained for all cases (Table 1). All the GCTs were discovered incidentally during endoscopy performed as a screening examination or for unrelated indications. All patients were informed about the option of endoscopic surgery and provided written informed consent.

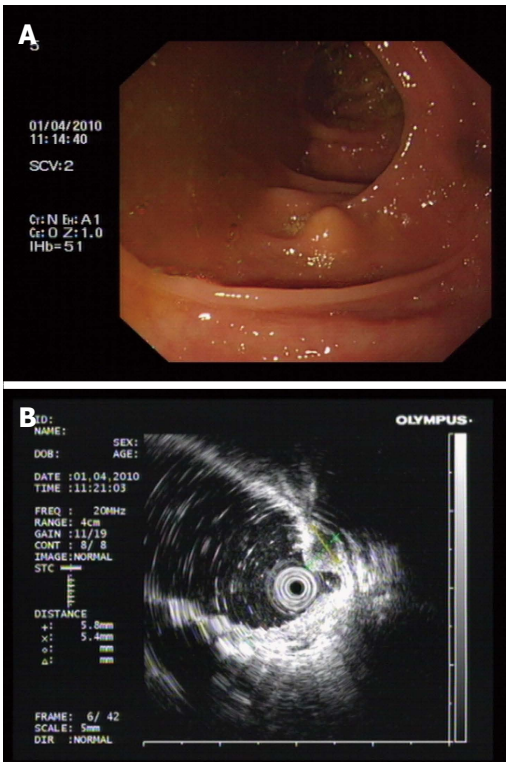

Figure 1 Preoperative examination. A: A 0.8 cm submucosal lesion was observed 60 cm from the anal verge (ascending colon). The mass was sessile, the mucosal surface was smooth, with non-necrotic erosion, and was non-circumferential. B: Ultrasonographic examination revealed a 0.5 cm hypoechoic submucosal lesion without muscularis propria involvement.

Preoperative management

Lesion size, location, color, and surface conditions, with or without ulceration were recorded. Endoscopic ultrasonography (EUS) (Figure 1) was performed to evaluate the depth and echo of the lesion. All patients were asked to finish bowel preparation according to established principles for colorectal surgery.

Endoscopic equipment and accessories

Standard single-accessory-channel endoscopy (GIT-H260; Olympus, Tokyo, Japan), and AQ100 (Aohua, Shanghai, China) were used during the procedures. A short, transparent cap (ND-201–11802; Olympus) was attached to the front of the endoscope to provide a constant endoscopic view and to apply

Table 1 Clinicopathologic features of 11 patients with colorectal granular cell tumors

Patient number	Gender	Age (yr)	Location	Maximum tumor size (cm)	Treatment methods	Length of stay (d)	*En bloc* resection	Margin	S-100
1	F	27	Cecum	1.0	EMR	0	Yes	Negative	+
2	F	33	Ascending colon	0.4	EMR	1	Yes	Negative	++
3	M	42	Ileocecal junction	0.6	EMR	0	Yes	Negative	+
4	M	47	Cecum	0.6	ESD	1	Yes	Negative	++
5	F	51	Ascending colon	0.6	EMR	0	Yes	Negative	+
6	M	49	Rectum	0.8	ESD	1	Yes	Negative	+
7	M	48	Ascending colon	0.7	ESD	2	Yes	Positive	+
8	F	37	Ascending colon	0.8	ESD	1	Yes	Negative	++
9	M	49	Ileocecal junction	1.0	ESD	0	Yes	Negative	++
10	F	45	Ileocecal junction	1.2	ESD	1	Yes	Negative	++
11	M	60	Ascending colon	1.2	ESD	1	Yes	Negative	+++

M: Male; F: Female; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

tension to the connective tissues during dissection. An IT-knife and/or a hook-knife (KD-611 and KD-620LR; Olympus) was used to dissect the submucosal layer and to peel the tumor. The high-frequency generator used was the HybridKnife system (ERBE, Tuebingen, Germany). Other equipment included injection needles (NM-4L-1), snares (SD-230U-20), hot biopsy forceps (FD-410LR), and clips (HX-610–135) (all from Olympus).

Endoscopic resection

All procedures were performed under general anesthesia by three surgeons with expertise in endoscopic treatment (Zhou PH, Yao LQ and Zhong YS).

Endoscopic mucosal resection procedure

Endoscopic mucosal resection (EMR) procedures were performed in a standardized manner as follows: (1) Marking; marking dots were made approximately 5-10 mm from the lesion by argon plasma coagulation (APC); (2) Submucosal injection; several milliliters of solution (100 mL saline, 5 mL 0.8% indigo carmine, and 1 mL of epinephrine) were injected around the lesion using a 23-gauge disposable needle; (3) Snare resection; an endoloop was used to snare and ligate the lesion with the aid of suction, and then the lesion was completely resected; and (4) Closure; exposed vessels on the artificial ulcer were coagulated with APC or hot biopsy forceps to prevent delayed bleeding, and metallic clips were always used to close the deeply dissected areas.

Endoscopic submucosal dissection procedure

The following steps were used in this procedure (Figure 2): (1) Marking; (2) Submucosal injections were the same as for EMR; (3) Circumferential incision; we used an IT knife or a hook knife to cut the mucosa initially along the marked points; (4) Strip lesions; the submucosal connective tissue beneath the lesion was gradually dissected with the aid of the transparent cap; the above-mentioned solution was injected repeatedly during the dissection when necessary; direct dissection of the submucosal layer was carried out until complete removal was achieved; and (5) Closure was the same as for EMR.

Postoperative management and follow-up

Patients were allowed oral intake from the second day unless serious complications occurred. Antibiotics (second-generation cephalosporins, such as celaclor or cefuroxime) and hemocoagulase injections (ethamsylate or P-aminomethybenzoic acid) were routinely administered after the procedure.

Pathologic evaluation

Tissue specimens were fixed to a plastic foam plate using thin needles along their edges and were then fixed in formalin solution. Processing of the resected specimens and histopathological evaluations were performed after endoscopic resection by highly experienced pathologists. A resection with a tumor-free margin in which both the lateral and basal margins were free of tumor cells was considered a complete resection. A resection in which the tumor extended into the lateral or basal margin or in which the margins were indeterminate due to artificial burn effects was considered an incomplete resection and recommended for surgical intervention.

RESULTS

Of the 11 colorectal GCTs in 11 patients, 2 (18.2%) were located in the cecum, 3 (27.3%) were in the ileocecal junction, 5 (45.4%) were in the ascending colon, and 1 (9.1%) was in the rectum. The median maximum diameter of the tumors was 0.81 cm (range 0.4-1.2 cm).

All patients underwent endoscopic resection, including 4 cases of EMR and 7 cases of endoscopic submucosal dissection (ESD). Each lesion was removed by *en bloc* resection, which was defined as no tumor identified at the resection site by endoscopy. All lesions were submucosal without muscularis layer

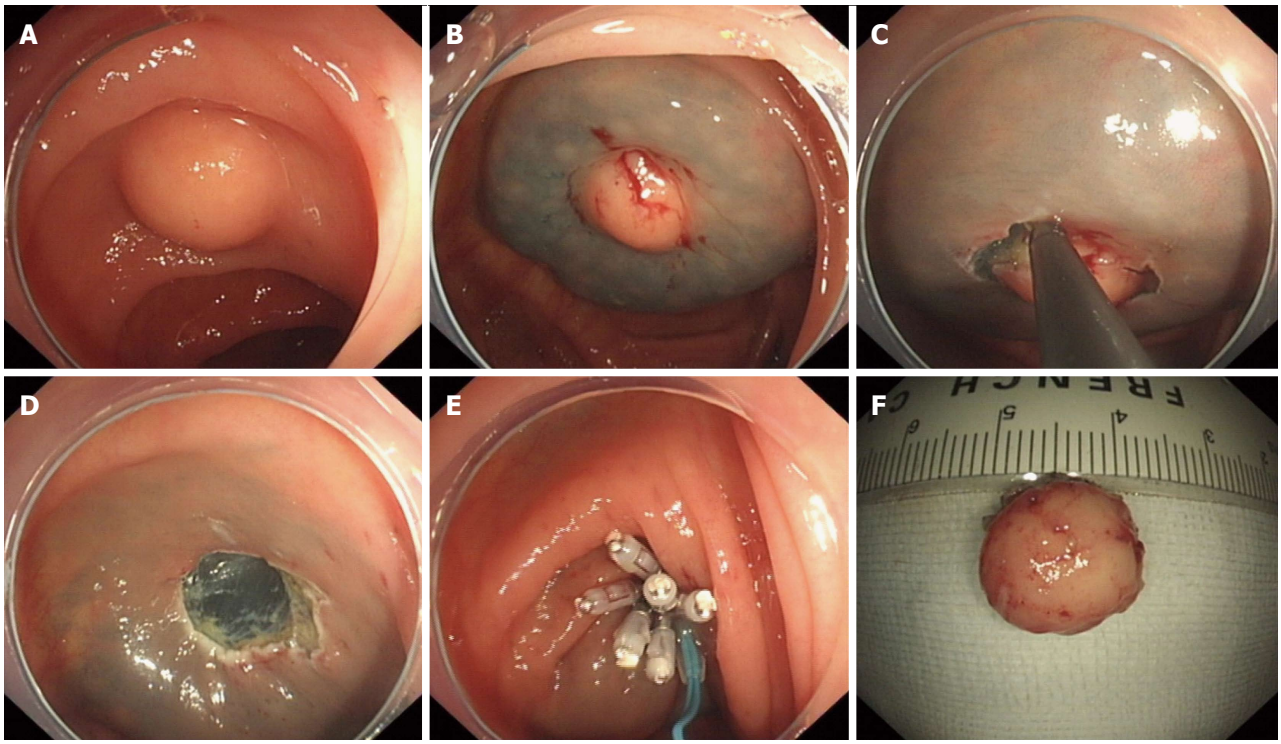

Figure 2 Endoscopic submucosal dissection procedure. A: A 1.0 cm × 1.2 cm hemispherical submucosal lesion which had a hard texture, fixed, smooth surface, was observed in the ileocecal junction; B: Saline solution with a small amount of epinephrine and indigo carmine was injected beneath the lesion, and resulted in good elevation; C: Complete dissection of the lesion after circumferential incision; D: The lesion was completely removed without any bleeding or perforation; E: Purse-string suture with metallic clips and endoloop; F: Dissected specimen was sent for pathologic examination.

invasion. There were 10 (90.9%) complete resections, which were defined as no granular cell tissue seen microscopically at the resection margin. Post-operative pathology of one lesion showed that the tumor was seen at the cauterization margin, indicating incomplete resection. However, during ESD, this lesion was removed *en bloc*, and no tumor tissue was seen in the wound. The patient refused surgery or any other additional treatment after a full discussion with his physician. There was no recurrence in this patient during close follow-up.

The procedures were successful, with no perforations, endoscopically uncontrolled bleeding, or any other severe adverse events requiring emergency surgery. The patients did not experience abdominal pain, abdominal distension, or any sign of peritonitis after endoscopic surgery. All samples were diagnosed as GCTs by hematoxylin and eosin (HE) staining and immunohistochemical (IHC) analysis (Figure 3).

One rectal GCT patient received a follow-up endoscopy one week after ESD. In the examination up to the rectum, wound healing was satisfactory, dry, and no bleeding was seen. In May 2015, all patients were followed up by telephone, and none complained of discomfort, including hematochezia or changed bowel habits. All 11 patients underwent complete colonoscopy up to the ileocecal junction annually after endoscopic treatment and no abnormalities were observed.

DISCUSSION

GCTs often occur in the tongue and the skin, and approximately 1%-8% of GCTs occur in the gastrointestinal tract[2,12], one-third of which occur in the esophagus[3,9,11]. Colorectal GCTs are even rarer. With improvements in endoscopy and IHC, there is the potential for an increase in the identification of GCTs in the gastrointestinal tract. Based on our experience, colorectal GCTs are found more frequently in the ileocecal junction and ascending colon. Of the 11 colorectal GCTs in the present study, 2 (18.2%) were located in the cecum, 3 (27.3%) were in the ileocecal junction, 5 (45.4%) were in the ascending colon, and 1 (9.1%) was in the rectum.

Most GCTs are asymptomatic and are clinically insignificant. However, 1%-3% of all GCTs have malignant potential[13,14], thus it is essential to distinguish them from other benign or malignant lesions. Malignant GCTs are often larger than 5 cm, have an unclear margin with the surrounding tissue, invade fat and/or muscle tissue, and are strongly positive for S-100 and neuron-specific enolase on IHC. Neither biopsies nor EUS can reliably distinguish benign GCTs from malignant GCTs; therefore, GCTs can pose a significant diagnostic and therapeutic challenge for endoscopists.

For asymptomatic and relatively small gastrointestinal SMTs, clinical follow-up is standard care.

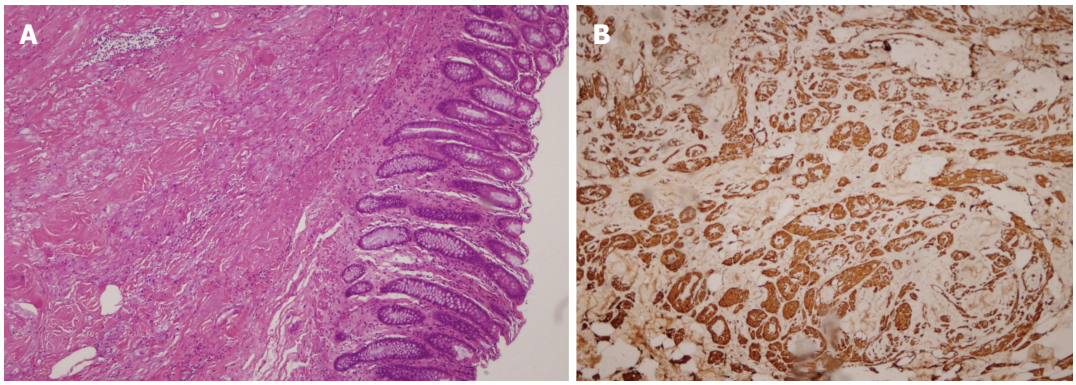

Figure 3 Pathologic findings. A: Infiltrative growth pattern of colorectal granular cell tumor (× 400); B: Tumor cells were strongly positive for cytoplasmic S-100 protein (+++) (× 400).

This usually requires patients to undergo multiple endoscopies, which often leads to noncompliance. Furthermore, histopathological diagnoses of SMTs are unobtainable unless the lesion is removed. Endoscopic resection has the advantage of being a minimally invasive technique, which typically results in a shorter operation time, minor post-operative pain, shorter hospital stay, and lower cost than traditional surgery[15,16]. EMR and ESD are both safe and effective for treating superficial gastrointestinal SMTs[17-19].

All our 11 colorectal GCTs evaluated were submucosal without muscularis layer involvement. In view of our results[17,18,20] and those in other studies[21,22], ESD and EMR are both suitable for GCTs less than 2 cm, as their complication rates and long-term outcomes are equivalent. In the present study, the lesions were approximately 1 cm; and the median maximum diameter of the tumors was 0.81 cm (range 0.4-1.2 cm). Eleven lesions were removed *en bloc* by both ESD and EMR, and the *en bloc* resection rate was 100%. Post-operative pathology showed a complete resection rate of 100% (4/4) for EMR, and 85.7% (6/7) for ESD. None of the patients in the two groups experienced bleeding, perforation, or disease progression. For lesions larger than 1 cm and/or closely related with the muscularis propria, we suggest performing ESD. During excavation, using the knife to dissect the submucosal connective tissue, the complete tumor capsule must be preserved and perforation avoided. Post-operative pathology showed a positive margin in only one patient; however, ESD achieved *en bloc* resection, and no tumor tissue was seen in the wound. The patient refused surgery after a full discussion with his physician. No recurrence was observed in this patient during close follow-up.

The main complication of endoscopic treatment for colorectal SMTs is perforation; this is also the bottleneck which limits endoscopic treatment of colorectal SMTs. With the widespread use of endoscopic full-thickness resection for gastric SMTs and improvements in suturing techniques[7,8,17], colorectal defect repair skills have also significantly improved. Common endoscopic suture methods suitable for colorectal defect repair include: (1) clipping; (2) clipping then strengthening with the endoloop; (3) purse-string suture with metallic clips and endoloop; and (4) interrupted suture with endoloop and metallic clips[23]. Furthermore, we have reported the submucosal tunneling endoscopic resection for the treatment of rectal SMTs[7], which solves the problem of difficulties in complete closure using the endoscopic suture technique. By mastering such techniques, breaking through the bottleneck can lead to expanded indications for endoscopic treatment, and the successful treatment of colorectal SMTs using minimally invasive endoscopic techniques.

Endoscopic resection is a safe, feasible and effective treatment for colorectal GCTs, allows *en bloc* resection in one visit, and a clear histological diagnosis.

In conclusion, endoscopic treatment performed by endoscopists with sufficient experience appears to be feasible and effective for colorectal GCTs.

COMMENTS

Background

Colorectal granular cell tumors (GCTs) are often asymptomatic and potentially malignant. As the tumor grows, patients present with different symptoms depending on the tumor location and size. Diagnosis and management of colorectal GCTs remain challenging.

Research frontiers

Due to the rarity of these tumors, few studies have focused on colorectal GCTs. Therefore, this study described the authors' experience in the diagnosis and surgical treatment of patients with colorectal GCTs.

Innovations and breakthroughs

Based on this study, the use of endoscopy, endoscopic ultrasonography and immunohistochemistry are effective means of diagnosing colorectal GCTs. The authors focused on the treatment of colorectal GCTs by endoscopic resection, which proved to be a feasible and effective treatment.

Applications

Endoscopic resection is a feasible and effective treatment for colorectal GCTs. Future randomized controlled trials comparing endoscopic resection and surgery are needed to address quality of life issues.

Terminology

Endoscopic submucosal dissection and endoscopic mucosal resection are two treatment modalities used for gastrointestinal submucosal tumors.

Peer-review

Early detection and simultaneous removal of colorectal granular cell tumors with endoscopy are safe and effective which provide patients with a greater degree of comfort and lead to a better compliance.

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ORIGINAL ARTICLE

Clinical Trials Study

First-line eradication for *Helicobacter pylori*-positive gastritis by esomeprazole-based triple therapy is influenced by *CYP2C19* genotype

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Author contributions: Saito Y and Serizawa H designed the research; Saito Y, Serizawa H, Kato Y and Nakano M performed the clinical research; Nakamura M, Saito H, Suzuki H and Kanai T supervised the research; Saito Y and Serizawa H analyzed the data and wrote the paper.

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Clinical trial registration statement: This study was registered with the UMIN Clinical Trials Registry, No. UMIN000009642.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: None of the authors have any conflicts of interest to declare.

Data sharing statement: No additional data are available.

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Abstract

AIM: To evaluate the effect of first line esomeprazole (EPZ)-based triple therapy on *Helicobacter pylori* (*H. pylori*) eradication.

METHODS: A total of 80 Japanese patients with gastritis who were diagnosed as positive for *H. pylori* infection by endoscopic biopsy-based or ^{13}C-urea breath tests were included in this study. The average age of the patients was 57.2 years (male/female, 42/38). These patients were treated by first-line eradication therapy with EPZ 40 mg/d, amoxicillin 1500 mg/d, and clarithromycin 400 mg/d for 7 d. All drugs were given twice per day. Correlations between *H. pylori* eradication, *CYP2C19* genotype, and serum pepsinogen

(PG) level were analyzed. This study was registered with the UMIN Clinical Trials Registry (UMIN000009642).

RESULTS: The *H. pylori* eradication rates by EPZ-based triple therapy evaluated by intention-to-treat and per protocol were 67.5% and 68.4%, respectively, which were similar to triple therapies with other first-generation proton pump inhibitors (PPIs). The eradication rates in three different *CYP2C19* genotypes, described as extensive metabolizer (EM), intermediate metabolizer, and poor metabolizer, were 52.2%, 72.1%, and 84.6%, respectively. The *H. pylori* eradication rate was significantly lower in EM than non-EM ($P < 0.05$). The serum PG Ⅰ level and PG Ⅰ/Ⅱ ratio were significantly increased after eradication of *H. pylori* ($P < 0.01$), suggesting that gastric atrophy was improved by *H. pylori* eradication. Thus, first-line eradication by EPZ-based triple therapy for patients with *H. pylori*-positive gastritis was influenced by *CYP2C19* genotype, and the eradication rate was on the same level with other first-generation PPIs in the Japanese population.

CONCLUSION: The results from this study suggest that there is no advantage to EPZ-based triple therapy on *H. pylori* eradication compared to other first-generation PPIs.

Key words: *CYP2C19*; Esomeprazole; *Helicobacter pylori*; Pepsinogen; Proton pump inhibitor

Core tip: Esomeprazole (EPZ) is considered to be more effective for inhibition of gastric acid secretion than other first-generation proton pump inhibitors (PPIs) because its metabolism is not influenced by *CYP2C19* genotype. In the present study, however, first-line eradication by EPZ-based triple therapy for patients with *Helicobacter pylori* (*H. pylori*)-positive gastritis was influenced by *CYP2C19* genotype, and the eradication rate was on the same level with triple therapies with other first-generation PPIs in the Japanese population. Unlike previous studies, our results suggest that there is no advantage for EPZ-based triple therapy on *H. pylori* eradication in comparison with other first-generation PPIs.

Saito Y, Serizawa H, Kato Y, Nakano M, Nakamura M, Saito H, Suzuki H, Kanai T. First-line eradication for *Helicobacter pylori*-positive gastritis by esomeprazole-based triple therapy is influenced by *CYP2C19* genotype. *World J Gastroenterol* 2015; 21(48): 13548-13554 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13548.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13548

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most prevalent bacterial pathogens and is associated with upper gastrointestinal disorders, such as gastritis, peptic ulcers, functional dyspepsia, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer[1-3]. Eradication of *H. pylori* infection is reported to be an effective approach to curing or preventing these *H. pylori*-associated diseases[4,5]. One-week of triple therapy with a proton pump inhibitor (PPI), amoxicillin (AMPC), and clarithromycin (CAM) is recommended as first-line *H. pylori* eradication therapy and covered under the national health insurance system in Japan. However, the eradication rates for *H. pylori* have declined to approximately 70%[6].

The use of PPIs combined with antibiotics in *H. pylori* eradication therapy has been demonstrated to not only protect the stomach, but also increase the eradication rate. As antibiotics are more stable in higher pH gastric environments, strong gastric acid inhibition increases the efficacy of *H. pylori* eradication. The metabolism of first-generation PPIs such as omeprazole (OPZ) is influenced by genetic polymorphism of *CYP2C19*[7]. Based on the wild-type allele (*1) and the two mutated alleles (*2 and *3) of the *CYP2C19* gene, patients can be categorized into three groups: extensive metabolizer (EM; *1/*1), intermediate metabolizer (IM; *1/*2 or *1/*3), and poor metabolizer (PM; *2/*2, *2/*3, or *3/*3). As EM metabolizes OPZ rapidly, the success rate of *H. pylori* eradication by OPZ-based therapy in EM is lower than that of PM[7-9].

Esomeprazole (EPZ), the S-isomer of OPZ, is the most recent member of the PPI family and is a more potent acid inhibitor than other first-generation PPIs[10,11]. The metabolism of EPZ is considered to be unaffected by *CYP2C19* genotype. Indeed, recent studies have reported that there were no significant differences in *H. pylori* eradication by EPZ-based therapy among EM, IM, and PM of the *CYP2C19* genotype, and that EPZ showed better overall *H. pylori* eradication rates than first-generation PPIs[9,12-14]. However, Nishida *et al*[15] demonstrated that the *H. pylori* eradication rate of EPZ-based triple therapy was lower than lansoprazole (LPZ) in the Japanese population. Thus, the effect of *H. pylori* eradication by EPZ-based therapy is controversial. To evaluate the effect of first line EPZ-based triple therapy on *H. pylori* eradication, we investigated eradication rate, *CYP2C19* genotype, and serum pepsinogen (PG) level in Japanese patients with *H. pylori*-positive gastritis.

MATERIALS AND METHODS

Patients and study design

A total of 80 Japanese patients with gastritis who were diagnosed as positive for *H. pylori* infection by a ^{13}C-urea breath test (UBT) or endoscopic biopsy-based test (*i.e.,* histologic examination and *H. pylori* culture) were included in this study. Patients were recruited between January and September 2013 at the Kitasato

Table 1 Serum pepsinogen level and *Helicobacter pylori* eradication in the extensive metabolizer group (*CYP2C19* genotype: *1/*1)

Case	Age	Sex	PG Ⅰ/Ⅱ				UBT	Eradication
			Before eradication		After eradication			
E1	45	F	3.6	-	5.0	-	0.1	○
E2	39	M	3.3	-	6.7	-	0.4	○
E3	59	M	3.1	-	5.2	-	0.5	○
E4	58	M	2.9	-	7.1	-	0.4	○
E5	50	F	2.2	1+	5.1	-	0.3	○
E6	43	F	2.2	1+	4.9	-	0.3	○
E7	63	F	1.6	1+	4.1	-	0.0	○
E8	56	F	2.8	2+	4.1	-	0.3	○
E9	36	F	2.2	2+	4.6	-	0.0	○
E10	75	F	2.5	2+	4.8	-	0.9	○
E11	67	M	3.7	-	2.2	2+	0.0	○
E12	64	M	0.8	3+	2.2	2+	1.3	○
E13	48	M	3.4	-	3.1	-	12.7	×
E14	50	F	4.3	-	6.4	-	12.7	×
E15	67	M	3.5	-	3.7	-	41.6	×
E16	34	F	3.0	-	3.4	-	19.5	×
E17	63	M	3.1	-	3.0	-	26.8	×
E18	71	F	1.2	3+	3.1	-	11.0	×
E19	55	M	2.4	1+	2.6	1+	24.6	×
E20	56	M	2.0	2+	2.2	1+	24.9	×
E21	80	M	1.7	2+	1.8	2+	20.9	×
E22	53	M	2.2	2+	2.2	2+	26.6	×
E23	43	F	2.2	2+	2.3	2+	11.5	×

(-), PG Ⅰ ≥ 70 ng/mL and PG Ⅰ/PG Ⅱ ratio ≥ 3.0; (1+), PG Ⅰ < 70 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (2+), PG Ⅰ < 50 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (3+), PG Ⅰ < 30 ng/mL and PG Ⅰ/PG Ⅱ ratio < 2.0. Eradication (○) rate = 12/23 (52.2%). M: Male; F: Female; PG: Pepsinogen; UBT: ^{13}C-urea breath test.

Institute Hospital (Tokyo, Japan). The average age of the patients was 57.2 years (male/female, 42/38).

These patients were treated by first-line eradication therapy with EPZ 40 mg/d, AMPC 1500 mg/d, and CAM 400 mg/d for 7 d. All drugs were given twice per day. Three months after eradication, *H. pylori* infection was validated by UBT. Correlations between *H. pylori* eradication, *CYP2C19* genotype, and serum PG level were analyzed. The study was approved by the ethics committee of the Kitasato Institute Hospital, and written informed consent was obtained from all patients prior to examinations. This study was registered with the UMIN Clinical Trials Registry, number UMIN000009642.

CYP2C19 genotyping

Blood samples were collected from the patients before eradication therapy. The *CYP2C19* genotyping for wild-type allele (*1) and two mutated alleles (*2 and *3) was conducted by SRL (Tokyo, Japan). The patients were categorized into three groups based on the *CYP2C19* genotype, EM (*1/*1), IM (*1/*2 or *1/*3), and PM (*2/*2, *2/*3, or *3/*3).

Serum PG level

Serum PG Ⅰ and Ⅱ levels were measured before and after eradication therapy. Gastric atrophy was evaluated as described previously[16,17]: (-), PG Ⅰ ≥ 70 ng/mL and PG Ⅰ/PG Ⅱ ratio ≥ 3.0; (1+), PGI < 70 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (2+), PG Ⅰ < 50 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (3+), PG Ⅰ < 30 ng/mL and PG Ⅰ/PG Ⅱ ratio < 2.0.

Statistical analysis

Data were analyzed using the SPSS statistics version 22 software package (IBM Corp., Armonk, NY, United States). The data were also analyzed using χ^2 test and multiple logistic regression analysis. *H. pylori* eradication rate was evaluated by intention-to-treat (ITT) and per protocol (PP). Differences at $P < 0.05$ were considered significant.

RESULTS

Influence of CYP2C19 genotype on H. pylori eradication by EPZ-based triple therapy

Among 80 patients who were treated by first-line eradication therapy with EPZ, AMPC, and CAM, one patient did not return for a UBT after the therapy. The *H. pylori* eradication rates of this first-line therapy evaluated by ITT and PP were 67.5% and 68.4%, respectively, which were similar to first-line therapy with LPZ, AMPC, and CAM (67.5%) in the Kitasato Institute Hospital. The results of *CYP2C19* genotype and serum PG level in association with *H. pylori* eradication are shown in Tables 1-3. The eradication rates of first-line therapy with EPZ in the three *CYP2C19* genotypes, EM, IM, and PM, were 52.2% (12/23), 72.1% (31/43), and 84.6% (11/13), respectively. The *H. pylori* eradication rate of EM was significantly lower than that of non-EM ($P = 0.048$).

Table 2 Serum pepsinogen level and *Helicobacter pylori* eradication in the intermediate metabolizer group (*CYP2C19* genotype: *1/*2, *1/*3)

Case	Age	Sex	*CYP2C19* genotype	PG Ⅰ/Ⅱ				UBT	Eradication
				Before eradication		After eradication			
I1	61	F	*1/*2	2.6	-	4.5	-	1.1	○
I2	66	M	*1/*2	3.6	-	6.0	-	0.0	○
I3	65	F	*1/*2	2.5	-	4.1	-	0.2	○
I4	71	F	*1/*2	3.5	-	7.4	-	0.9	○
I5	59	M	*1/*3	2.2	-	5.3	-	1.1	○
I6	62	M	*1/*3	3.3	-	8.3	-	0.0	○
I7	67	M	*1/*2	4.9	-	5.0	-	0.3	○
I8	64	F	*1/*2	2.8	-	5.2	-	0.5	○
I9	64	F	*1/*2	3.2	-	5.4	-	0.1	○
I10	34	M	*1/*2	3.1	-	6.9	-	0.3	○
I11	52	M	*1/*2	3.7	-	4.9	-	0.8	○
I12	64	M	*1/*2	3.6	-	4.5	-	0.2	○
I13	55	M	*1/*3	2.0	-	4.7	-	1.1	○
I14	58	M	*1/*2	3.8	-	9.4	-	0.6	○
I15	66	F	*1/*3	1.4	-	3.7	-	1.0	○
I16	47	M	*1/*2	4.0	-	5.4	-	0.0	○
I17	42	M	*1/*3	2.8	1+	5.3	-	0.4	○
I18	53	M	*1/*2	2.3	1+	3.7	-	0.8	○
I19	56	M	*1/*3	2.0	1+	4.0	-	2.4	○
I20	51	F	*1/*2	1.1	1+	3.3	-	0.0	○
I21	62	F	*1/*2	2.3	1+	4.1	-	0.3	○
I22	39	F	*1/*2	2.7	2+	6.0	-	0.4	○
I23	63	F	*1/*3	2.1	2+	4.0	-	0.1	○
I24	53	F	*1/*2	2.3	2+	4.1	-	0.3	○
I25	45	M	*1/*2	2.4	2+	4.9	-	0.6	○
I26	55	M	*1/*2	2.6	2+	4.2	-	0.5	○
I27	50	M	*1/*2	1.8	2+	3.9	-	0.6	○
I28	55	M	*1/*2	1.3	3+	3.6	-	0.0	○
I29	53	F	*1/*2	1.7	2+	2.9	2+	0.4	○
I30	64	M	*1/*2	1.2	3+	2.5	2+	1.5	○
I31	66	F	*1/*2	0.8	3+	0.8	3+	1.4	○
I32	53	M	*1/*3	4.1	-	4.5	-	49.8	×
I33	61	M	*1/*2	4.2	-	3.2	-	24.8	×
I34	44	F	*1/*2	3.2	-	3.2	-	44.5	×
I35	51	F	*1/*2	2.4	2+	3.1	-	21.7	×
I36	28	F	*1/*3	2.2	-	2.2	1+	43.6	×
I37	70	M	*1/*3	2.7	1+	2.7	1+	9.3	×
I38	70	F	*1/*2	2.5	1+	2.0	1+	39.2	×
I39	65	M	*1/*3	3.1	-	3.0	2+	28.0	×
I40	45	F	*1/*3	2.8	-	2.5	2+	13.0	×
I41	78	F	*1/*2	0.8	3+	1.4	3+	4.1	×
I42	45	F	*1/*2	1.5	3+	1.6	3+	17.0	×
I43	70	M	*1/*3	1.1	3+	1.1	3+	37.4	×

(-), PG Ⅰ ≥ 70 ng/mL and PG Ⅰ/PG Ⅱ ratio ≥ 3.0; (1+), PG Ⅰ < 70 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (2+), PG Ⅰ < 50 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (3+), PG Ⅰ < 30 ng/mL and PG Ⅰ/PG Ⅱ ratio < 2.0. Eradication (○) rate = 31/43 (72.1%). M: Male; F: Female; PG: Pepsinogen; UBT: ^{13}C-urea breath test.

Influence of PG level on H. pylori eradication by EPZ-based triple therapy

In addition to *H. pylori* infection, serum PG level is associated with gastric mucosal atrophy and gastric cancer risk, which is used for gastric cancer screening[17-19]. Serum PG Ⅰ level and PG Ⅰ/Ⅱ ratio in association with *H. pylori* eradication are shown in Tables 1, 2, and 3. Table 4 is a summary of PG Ⅰ/Ⅱ ratio and *H. pylori* eradication. Serum PG Ⅰ level and PG Ⅰ/Ⅱ ratio were significantly increased after eradication of *H. pylori* (P = 0.007), suggesting that gastric atrophy was improved by *H. pylori* eradication therapy. We performed a multiple logistic regression analysis to identify independent predictors associated with *H. pylori* eradication. As shown in Table 5, only *CYP2C19* genotype was statistically significant as an independent predictor associated with *H. pylori* eradication.

DISCUSSION

EPZ is a second-generation PPI that is broadly used for the treatment of acid-peptic diseases. It is believed that EPZ is more effective for inhibition of gastric acid secretion than other first-generation PPIs, because it is the S-isomer of OPZ and its metabolism is not affected by *CYP2C19* genotype. Recent studies have also shown that *H. pylori* eradication by EPZ-based

Table 3 Serum pepsinogen level and *Helicobacter pylori* eradication in the poor metabolizer group (*CYP2C19* genotype: *2/*2, *2/*3, *3/*3)

Case	Age	Sex	*CYP2C19* genotype	PG Ⅰ/Ⅱ				UBT	Eradication
				Before eradication		After eradication			
P1	64	F	*2/*2	3.6	-	5.8	-	0.2	○
P2	46	M	*2/*2	4.0	-	5.5	-	1.2	○
P3	64	F	*2/*2	2.2	1+	3.8	-	1.1	○
P4	63	F	*2/*2	2.1	2+	3.9	-	0.0	○
P5	62	F	*2/*3	2.3	2+	4.2	-	0.2	○
P6	64	M	*2/*3	2.2	2+	5.0	-	0.0	○
P7	63	M	*2/*2	2.7	2+	4.9	-	1.2	○
P8	77	F	*3/*3	1.7	3+	2.9	2+	0.1	○
P9	68	M	*2/*2	1.3	2+	1.9	3+	1.2	○
P10	60	M	*2/*2	0.6	3+	1.4	3+	0.8	○
P11	57	M	*2/*2	4.1	-	ND	ND	0.2	○
P12	65	F	*2/*2	2.0	1+	2.0	1+	30.2	×
P13	38	F	*2/*2	2.3	2+	2.5	1+	63.8	×

(-), PG Ⅰ ⩾ 70 ng/mL and PG Ⅰ/PG Ⅱ ratio ⩾ 3.0; (1+), PG Ⅰ < 70 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (2+), PG Ⅰ < 50 ng/mL and PG Ⅰ/PG Ⅱ ratio < 3.0; (3+), PG Ⅰ < 30 ng/mL and PG Ⅰ/PG Ⅱ ratio < 2.0. Eradication (○) rate = 11/13 (84.6%). M: Male; F: Female; ND: Not detected; PG: Pepsinogen; UBT: ^{13}C-urea breath test.

Table 4 Correlation between pepsinogen Ⅰ/Ⅱ ratio and *Helicobacter pylori* eradication

Eradication	PG Ⅰ/Ⅱ ratio			Total
	Increase	No change	Decrease	
Success	26	26	1	53
Failure	4	18	3	25
Total	30	44	4	78

Serum PG Ⅰ level and PG Ⅰ/Ⅱ ratio were significantly increased after eradication of *Helicobacter pylori* ($P < 0.01$). PG: Pepsinogen.

Table 5 Multiple logistic regression analysis to identify independent predictors associated with *Helicobacter pylori* eradication

Variable	*P* value
CYP2C19 genotype (EM *vs* non-EM)	0.048
Age	0.603
Sex	0.637
Pepsinogen Ⅰ/Ⅱ before eradication	0.809

EM: Extensive metabolizer.

therapy is not influenced by *CYP2C19* genotype, and that overall *H. pylori* eradication rates of EPZ-based therapy was better than first-generation PPIs[9,12-14]. On the other hand, Hunfeld *et al*[11] revealed that the acid-inhibitory effect of EPZ was influenced by *CYP2C19* genotype. Nishida *et al*[15] demonstrated that the *H. pylori* eradication rate of EPZ-based triple therapy was lower than LPZ in the Japanese population. Thus, the effect of EPZ-based therapy on *H. pylori* eradication is controversial.

In this study we evaluated the influence of *CYP2C19* genotype in patients with *H. pylori*-positive gastritis treated by EPZ-based triple therapy. Our results demonstrated that the *H. pylori* eradication rate was significantly lower in EM phenotype patients having the wild-type *CYP2C19* genotype, compared to the non-EM patients with at least one mutant allele (*2 and *3). The result of multiple logistic regression analysis also showed that *CYP2C19* genotype is an independent predictor associated with *H. pylori* eradication. These findings suggest that EM metabolizes EPZ more rapidly, and therefore plasma concentrations of EPZ become lower, resulting in a lower *H. pylori* eradication rate than that of non-EM. Nishida *et al*[15] conducted a multicenter, randomized, open-label, non-inferiority trial comparing EPZ and LPZ in triple therapy for *H. pylori* eradication in Japan. They reported that the *H. pylori* eradication rates of EPZ-based triple therapy (69.4%/76.9%, ITT/PP) were lower than LPZ-based triple therapy (73.9%/79.8%, ITT/PP). In this study, the overall *H. pylori* eradication rates of EPZ-based triple therapy were 67.5%/68.4% (ITT/PP), which were similar to the previous report. A recent study with a Japanese population has also shown that the *H. pylori* eradication rates by the regimen with rabeprazole, AMPC, and CAM were 73.3%/77.2% (ITT/PP)[6]. Thus, these findings indicate that the *H. pylori* eradication rate by EPZ-based triple therapy is at the same level with triple therapies with other first-generation PPIs.

Serum PG level is associated with gastric mucosal atrophy and gastric cancer risk[17-19]. In the present study, the serum PG Ⅰ level and PG Ⅰ/Ⅱ ratio were significantly increased after eradication of *H. pylori*, suggesting that gastric atrophy was improved by *H. pylori* eradication. Serum PG level and PG Ⅰ/Ⅱ ratio can be noninvasive biomarkers for screening of gastric atrophy and gastric cancer. *H. pylori* eradication has clinical benefit for improvement of gastric mucosal atrophy and prevention against gastric cancer.

In conclusion, first-line *H. pylori* eradication by EPZ-based triple therapy was influenced by *CYP2C19*

genotype, and the overall eradication rate was on the same level with triple therapies with other first-generation PPIs in Japanese patients with *H. pylori*-positive gastritis. Unlike previous reports, the results in this study suggest that there is no advantage to EPZ-based triple therapy on *H. pylori* eradication in comparison to other first-generation PPIs. Further studies are needed in a large population of patients in different countries before an accurate correlation between the EPZ-based therapy and *CYP2C19* genotype is completed. Evaluation of *CYP2C19* genotype and serum PG level is important to develop more effective personalized *H. pylori* eradication therapy with EPZ.

COMMENTS

Background

Esomeprazole (EPZ) is a second-generation proton pump inhibitor (PPI) that is broadly used for the treatment of acid-peptic diseases. Recent studies have shown that EPZ is more effective for inhibition of gastric acid secretion than other first-generation PPIs because its metabolism is not influenced by *CYP2C19* genotype. However, the effect of *Helicobacter pylori* (*H. pylori*) eradication by EPZ-based therapy is controversial.

Research frontiers

First-line eradication by EPZ-based triple therapy for patients with *H. pylori*-positive gastritis was influenced by *CYP2C19* genotype, and the eradication rate was at the same level found with other first-generation PPIs in the Japanese population.

Innovations and breakthroughs

Unlike previous reports, the results in this study suggest that there is no advantage to EPZ-based triple therapy on *H. pylori* eradication in comparison to other first-generation PPIs.

Applications

Evaluation of *CYP2C19* genotype and serum pepsinogen level is important to develop more effective personalized *H. pylori* eradication therapy with EPZ.

Terminology

EPZ is the most recent member of the PPI family and is a more potent acid inhibitor than other first-generation PPIs. The metabolism of first-generation PPIs is influenced by genetic polymorphism of *CYP2C19*. Based on *CYP2C19* genotype, patients can be categorized into three groups: extensive metabolizer, intermediate metabolizer, and poor metabolizer.

Peer-review

This study reports that the second-generation PPI inhibitor, EPZ, has no apparent advantage over triple therapies utilizing other first-generation PPIs for overall eradication of *H. pylori* in patients with gastritis. The EPZ-based therapy was influenced by the *CYP2C19* genotype of the studied Japanese patients.

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ORIGINAL ARTICLE

Prospective Study

Fatty liver disease: Disparate predictive ability for cardiometabolic risk and all-cause mortality

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Abstract

AIM: To assess the association of a surrogate of fatty liver disease (FLD) with incident type-2 diabetes, coronary heart disease, and all-cause mortality.

METHODS: In a prospective population-based study on 1822 middle-aged adults, stratified to gender, we used an algorithm of fatty liver index (FLI) to identify associations with outcomes. An index $\geqslant$ 60 indicated the presence of FLD. In Cox regression models, adjusted for age, smoking status, high-density lipoprotein cholesterol, and systolic blood pressure, we assessed the predictive value of FLI for incident

diabetes, coronary heart disease (CHD), and all-cause mortality.

RESULTS: At a mean 8 year follow-up, 218 and 285 incident cases of diabetes and CHD, respectively, and 193 deaths were recorded. FLD was significantly associated in each gender with blood pressure, total cholesterol, apolipoprotein B, uric acid, and C-reactive protein; weakly with fasting glucose; and inversely with high-density lipoprotein-cholesterol and sex hormone-binding globulin. In adjusted Cox models, FLD was (with a 5-fold HR) the major determinant of diabetes development. Analyses further disclosed significant independent prediction of CHD by FLD in combined gender [hazard ratio (HR) = 1.72, 95% confidence interval (CI): 1.17-2.53] and men (HR = 2.35, 95%CI: 1.25-4.43). Similarly-adjusted models for all-cause mortality proved, however, not to confer risk, except for a tendency in prediabetics and diabetic women.

CONCLUSION: A surrogate of FLD conferred significant high risk of diabetes and coronary heart disease, independent of some metabolic syndrome traits. All-cause mortality was not associated with FLD, except likely in the prediabetic state. Such a FLI may reliably be used in epidemiologic studies.

Key words: All-cause death; Coronary heart disease; Hepatic steatosis; Metabolic syndrome; Turkish adult risk factor study

Core tip: We prospectively assessed in 1822 adults the association between a validated surrogate of fatty liver disease (FLD) and the incidence of type-2 diabetes, coronary heart disease (CHD), and all-cause mortality by stratifying to gender and using adjusted Cox regression models. At a mean 8 year follow-up, FLD was the major determinant of developing diabetes and was a significant predictor of CHD. Similarly-adjusted models for all-cause mortality did not confer risk, except for slightly in prediabetics and diabetic women. Involvement of circulating lipoprotein(a) in autoimmune activation may be an underlying mechanism. Such a FLD surrogate may be used in epidemiologic studies.

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INTRODUCTION

Steatohepatitis designates fatty infiltration and inflammation of the liver and has features closely associated with the metabolic syndrome (MetS)[1]. Liver biopsy, ultrasonography, serum liver enzymes and, more recently, an algorithm-based surrogate have been commonly used in identifying the presence of non-alcoholic fatty liver disease (NAFLD) and its relationship to adverse outcomes. As a growing public health issue, NAFLD has been demonstrated in the past decade to be associated with MetS[2], type-2 diabetes[3-5], cardiovascular events[6-9], and chronic kidney disease[10-12]. A complex bidirectional relationship between the development of diabetes and progression to non-alcoholic steatohepatitis promoting hepatic fibrogenesis and insulin resistance has been identified[1]. Risk of all-cause death is also predicted by NAFLD[13-15], but conflicting results have been reported[9,16] regarding overall and cause-specific mortality. Despite an increased association with independent cardiovascular disease (CVD) prevalence and NAFLD in United States adults (The National Health and Nutrition Examination Survey (NHANES)-Ⅲ), NAFLD did not predict mortality over a 14 year period[16].

The complex inter-relationships between NAFLD, visceral obesity, and insulin resistance[17] require further elucidation. Since most of the prospective studies on NAFLD have been performed in population samples of Western Europe, United States, and East Asia; investigation of different ethnicities is necessary to clarify better variation in the relationship. The controversial relationship between NAFLD and risk of overall mortality, as compared to that of diabetes and CVD, may be highly relevant for the pathophysiology of the associations and possibly related to ethnic differences.

Turkish adults are prone to MetS[18], diabetes mellitus[19], and chronic hepatitis. Cardiovascular risk profiles are characterized by a high prevalence of abdominal obesity in males, overall obesity in females, low high-density lipoprotein (HDL)-cholesterol, high triglyceride, and intermediate total cholesterol levels. Current smoking protects against abdominal obesity[18]. One-fifth of non-diabetic Turks exhibit impaired fasting glucose[20]. On one hand, evidence is growing that microbiota contribute to the pathogenesis of insulin resistance, abdominal obesity, and progression of NAFLD[1,21]. On the other hand, autoimmune activation based on enhanced proinflammatory state may be a common mechanism underlying these diseases in middle-aged and elderly Turkish adults[22]. Prospective evaluation of the same sample regarding the relationships among fatty liver disease (FLD), cardiometabolic disease risk, and all-cause death might reveal novel information.

Clinical and epidemiological studies using an algorithm-based surrogate of FLD to investigate related outcomes have been published[13,23]. We, therefore, aimed in this study to assess prospectively and simultaneously the impact of an algorithm-derived surrogate of FLD on diabetes, CHD, and overall

mortality in a population-based sample representative of middle-aged Turkish adults at a lengthy follow-up period.

MATERIALS AND METHODS

Population sample

The Turkish Adult Risk Factor Study is a prospective survey on the prevalence of cardiac disease and risk factors in adults in Turkey that has been carried out periodically, almost biennially, since 1990 in 59 communities scattered throughout all geographical regions of the country[24]. It comprises a representative sample of the Turkish adult population. Serum γ-glutamyltransferase (GGT) determinations were made in the 2003/04 survey, during which GGT was measured in all 1822 participants who attended the survey (examination in 60%) out of an eligible 3037 participants. Follow-up extended to the 2012/13 survey.

The survey conformed to the principles embodied in the Declaration of Helsinki and was approved by the Istanbul University Ethics Committee. All individuals gave written consent to participation. Data were obtained by history questionnaire, physical examination of the cardiovascular system, sampling of blood, and recording of a resting electrocardiogram.

Measurements of risk variables

Blood pressure (BP) was measured in the sitting position on the right arm, and the mean of two recordings at least 5 min apart was recorded. Waist circumference was measured with a tape (Roche LI95 63B 00, Basel, Switzerland) with the subject standing and wearing only underwear at the level midway between the lower rib margin and the iliac crest. Self-reported cigarette smoking was categorized into never smokers, former smokers (discontinuance for 3 mo or longer), and current smokers (regularly 1 or more cigarettes daily). Anyone who consumed alcohol at least once a week was considered a user of alcoholic drinks.

Biochemical parameters were assayed in a central laboratory. Blood samples were shipped to Istanbul and stored in deep-freeze at -75 ℃ until analyzed. Serum concentrations of total and HDL-cholesterol (directly without precipitation) and triglycerides were determined using enzymatic kits from Roche Diagnostics with a Hitachi 902 analyzer (Tokyo, Japan). Concentrations of sex hormone-binding globulin (SHBG) and total testosterone were determined by the electrochemiluminescence immunoassay ECLIA on Roche Elecsys 2010 (Roche Diagnostics). Serum concentrations of apolipoprotein (apo) A-I, apo B, lipoprotein (Lp)(a) and high-sensitivity C-reactive protein (CRP) was measured with nephelometry by BN ProSpec analyzer (Siemens Healthcare Diagnostics, Munich, Germany). Serum GGT activity was assayed by Cobas c 501 analyzer (Roche Diagnostics GmbH). Plasma fibrinogen was assayed by the modified Clauss method using Fibrintimer Ⅱ coagulometer and Multifibren U kit (Siemens Healthcare Diagnostics).

Definitions

Individuals with diabetes were diagnosed with criteria of the American Diabetes Association[25], namely plasma fasting glucose ≥ 126 mg/dL (or 2 h postprandial glucose > 200 mg/dL) and/or the current use of diabetes medication. Prediabetes was identified by fasting glucose of 100-125 mg/dL. Individuals with MetS were identified when three out of the five criteria of the National Cholesterol Education Program (ATP Ⅲ) were met, modified for prediabetes and for abdominal obesity using ≥ 95 cm as cutoff point in men, as assessed in the Turkish Adult Risk Factor study[18]. For women, the cutoff point ≥ 88 cm was retained based on our own prospective analyses. Homeostatic model assessment (HOMA) was estimated by the standard equation using fasting glucose and insulin levels.

Identification of CHD was based on the presence of angina pectoris, a history of myocardial infarction with or without accompanying Minnesota codes of the electrocardiogram (ECG)[26], or a history of myocardial revascularization. Typical angina and, in women, age > 45 years were prerequisite for a diagnosis when angina was isolated. ECG changes of "ischemic type" greater than minor degree (Codes 1.1-2, 4.1-2, 5.1-2, 7.1) were considered as myocardial infarct sequelae or myocardial ischemia, respectively. Death was identified *via* the information from first-degree relatives, records of local health personnel, and/or the nation-wide Identity Participation System.

Estimation of hepatic steatosis by an algorithm

We used a previously reported algorithm to detect fatty liver based on body mass index (BMI), waist circumference, triglycerides, and GGT[27] using the following equation.

$$= (e^{0.953 \times \log_e(\text{triglycerides}) + 0.139 \times \text{BMI} + 0.718 \times \log_e(\text{GGT}) + 0.053 \times \text{waist circumference} - 15.745})/(1 + e^{0.953 \times \log_e(\text{triglycerides}) + 0.139 \times \text{BMI} + 0.718 \times \log_e(\text{GGT}) + 0.053 \times \text{waist circumference} - 15.745}) \times 100$$

In agreement with the authors, we used an index < 30 to indicate absence of FLD, ≥ 60 for presence of FLD, and 30-59 for probable presence of FLD.

Data analysis

Descriptive parameters are shown as mean ± standard deviation or in percentages. Two-sided *t*-tests and Pearson's χ^2 tests were used to analyze the differences in means and proportions between groups. Due to the skewed distribution, log-transformed values were used for triglycerides, CRP, GGT, and Lp(a) for analyses. Analysis of variance (ANOVA) was used to detect difference across multiple groups, whereby a difference between two groups was determined using Bonferroni corrections. Estimates [and 95% confidence

Table 1 Baseline characteristics of the study sample, by gender and fatty liver disease categories (n = 1822)

		Men (n = 877)				Women (n = 945)			
	n	No NAFLD mean ± SD n = 198	Probable NAFLD mean ± SD n = 263	NAFLD mean ± SD n = 416	ANOVA P value	No NAFLD mean ± SD n = 252	Probable NAFLD mean ± SD n = 241	NAFLD mean ± SD n = 452	ANOVA P value
Age, yr	1822	52.8 ± 12.7	53.4 ± 12	51.7 ± 9.3	0.16	47.5 ± 10.3	51.9[2] ± 10.5	54.8 ± 9.9	< 0.001
Waist circumference, cm	1811	83.3 ± 8.1	92.4[2] ± 7.4	100.6 ± 9	< 0.001	79 ± 9	89[2] ± 7.5	99.4 ± 9.7	< 0.001
Body mass index, kg/m^2	1808	24.1 ± 3.3	27.2[2] ± 2.7	30.9 ± 4	< 0.001	26.2 ± 4.4	30.2[2] ± 3.8	34.5 ± 5.5	< 0.001
Systolic BP, mmHg	1822	120 ± 18	126[2] ± 21	132.5 ± 23	< 0.001	121 ± 19	132[2] ± 24	142 ± 27	< 0.001
Diastolic BP, mmHg	1822	75 ± 11	80[2] ± 11	85.6 ± 13.5	< 0.001	77 ± 12	83[2] ± 13	88 ± 14	< 0.001
Total cholesterd, mg/dL	1822	168 ± 34	178[2] ± 34	192 ± 39	< 0.001	174 ± 34	190[2] ± 37	201 ± 41	< 0.001
LDL cholesterd, mg/dL	1424	108 ± 32	112 ± 29	116[2] ± 33	0.037	105[2] ± 28	120 ± 33	126 ± 36	< 0.001
HDL cholesterd mg/dL	1820	42[2] ± 12	37 ± 11	35.3 ± 11	< 0.001	47.7 ± 13	46 ± 13	43[2] ± 12	< 0.001
Lipoprotein(a)[1], mg/dL	1186	8.3 × 2.74	7.76 × 2.77	9.12 × 3.14	0.34	11.8 × 2.76	12.7 × 3.13	10.47 × 2.9	0.11
Fasted glucose, mg/dL	1822	99 ± 27	100 ± 30	104 ± 37	0.099	97 ± 19	100 ± 31	105[2] ± 35	0.001
F. triglyceride[1], mg/dL	1413	95 × 1.48	126[2] × 1.55	174 × 1.72	< 0.001	87 × 1.47	110[2] × 1.53	148 × 1.69	< 0.001
γ-glutamyl transferase[1], U/L	1822	17 × 1.48	21.9[2] × 1.59	37.2 × 1.87	< 0.001	12.9 × 1.62	15.8[2] × 1.65	24.5 × 1.87	< 0.001
Fasted insulin, mIU/L	1636	6.94 × 2.36	8.00 × 2.00	11.0[2] × 2.02	< 0.001	6.98 × 1.87	8.75[2] × 1.97	11.0 × 1.87	< 0.001
Apolipoprotein A-I, g/L	1740	1.40 ± 0.24	1.35 ± 0.24	1.346[2] ± 0.24	0.042	1.55 ± 0.30	1.51 ± 0.26	1.50 ± 0.28	0.082
Apolipoprotein B, g/L	1759	0.94[2] ± 0.26	1.05 ± 0.19	1.09 ± 0.29	< 0.001	1.03 ± 0.27	1.04 ± 0.28	1.13[2] ± 0.31	< 0.001
Creatinine, mg/dL	1504	0.937± 0.17	0.976 ± 0.26	1.01 ± 0.37	0.023	0.80 ± 0.42	0.80 ± 0.44	0.805 ± 0.20	0.98
SHBG[1], nmol/L	1304	43.8 × 1.7	39 × 1.6	33.4[2] × 1.63	< 0.001	55.4 × 1.7	46.2[2] × 1.7	48.2 × 1.73	< 0.001
Testosterone[1], nmol/L	1412	19.2 × 4.2	15.8 × 3.3	15.1 × 3.3	0.16	0.79 × 3.66	0.70 × 3.1	0.79 × 3.8	0.50
Uric acid, mg/dL	1821	5.51 ± 1.3	5.82 ± 1.3	6.36[2] ± 1.6	< 0.001	4.23 ± 1.2	4.6[2] ± 1.1	5.15 ± 1.4	< 0.001
C-reactive protein[1], mg/L	1788	1.64[2] × 3.13	1.93 × 3.0	2.39 × 2.75	< 0.001	1.41 × 3.15	2.53[2] × 2.8	3.75 × 2.65	< 0.001
Current; past smokers, %	1817	59.4[2]; 18.8	47.5; 23.4	46.1; 25.4	0.037	27.4[2] ; 3.2	14.1; 3.3	10.2; 4.4	< 0.001
CHD prevalence, n (%)	1820	5 (2.5)	11 (4.2)	31 (7.5[2])	0.02	6 (2.4)	11 (4.6)	29 (6.4)	0.056

[1]Log-transformed values, SD range is obtained by dividing or multiplying with the given SD; [2]Denote significant difference, from the remaining two groups, those in italics borderline significant difference. NAFLD: Non-alcoholic fatty liver disease; CHD: Coronary heart disease; LDL: Low density lipoprotein; BP: Blood pressure; HDL: High-density lipoprotein; SHBG: Sex hormone-binding globulin.

intervals (CI)] for relative risk (RR) of the dependent variable were obtained by use of Cox proportional hazard regression analyses in models that controlled for potential confounders, including cardiovascular risk factors and HOMA. A value of $P < 0.05$ on the two-sided test was considered statistically significant. Statistical analyses were performed using SPSS-10 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

The study sample consisted of 1822 middle-aged adults (877 men and 945 women). CHD in 93 and diabetes in 103 subjects were identified at baseline. FLD was detected in 48% and there was no FLD in one-quarter of the sample at baseline. Follow-up averaged 8.0 ± 2.7 and 7.8 ± 2.8 years for mortality and incident CHD, respectively, with similar gender distribution (P = 0.52), yielding 14540 person-years for mortality. Fourteen percent of men and 1% of women were categorized as alcohol users. Six percent of males used alcohol at a daily mean equivalent to 19 mL ethanol and the remainder much less. Liver diseases of specific causes were not reported.

Table 1 shows the characteristics of the study sample at baseline, stratified to gender and FLD status. Significant differences in values across the sex-specific categories are noted in virtually all variables, except creatinine and testosterone, as well as age, fasting glucose, and Lp(a) in men. Notably, MetS traits were increased in subjects with FLD (blood pressure, HDL-cholesterol and glucose [borderline in males]), and insulin, apoB, SHBG, uric acid, and CRP levels were elevated as well. A significantly higher proportion of participants with no FLD were current smokers.

Pearson correlations of the fatty liver index (FLI) with relevant variables in males and females are provided in Table 2. BP, fasting insulin, total cholesterol, apoB, uric acid, and CRP were positively correlated, and SHBG and HDL-cholesterol were inversely correlated, in each gender. Age and fasting glucose were positive correlates in women alone, and alcohol usage was a positive correlate in men, while apoA-I, Lp(a), creatinine, and testosterone were not correlated with the FLI.

Kaplan-Meier plots were constructed for survival and survival free of diabetes/CHD, as seen in Figure 1. These demonstrated significantly lower survival free of diabetes and of incident CHD for participants with FLD at baseline. Subjects categorized as probable FLD also separated from those with no FLD in regard to CHD. However, overall survival curves were similar in the three groups.

Table 3 displays findings of Cox regression analyses for the prediction by FLD of diabetes mellitus and CHD, adjusted for five other conventional cardiovascular risk factors and stratified to gender. It is evident that FLD was (with a 5-fold relative risk) the major determinant of the development of diabetes, besides (inversely) serum uric acid. In regard to incident CHD, FLD proved

Table 2 Pearson correlations of fatty liver index (n = 1822) with relevant variables

	Men		Women	
	n	*r*	*n*	*r*
Age	877	-0.04	945	0.28
Body mass index	869	0.71	939	0.68
Systolic BP	877	0.27	945	0.38
Diastolic BP	877	0.34	945	0.36
Total cholesterol	877	0.27	945	0.28
HDL cholesterol	877	-0.22	945	-0.19
C-reactive protein[1]	864	0.16	924	0.41
Apo A-I	841	-0.08	899	-0.07
Apo B	844	0.18	915	0.19
Lipoprotein(a)[1]	549	0.02	637	-0.05
Fasting glucose	877	0.09	945	0.12
Sex h-b globulin[1]	623	-0.23	681	-0.26
Testosterone[1]	642	-0.08	708	0.01
Uric acid	869	0.23	938	0.20
Creatinine	735	0.04	785	0.00
Fasting insulin	775	0.33	861	0.33
Alcohol usage	873	0.11	941	-0.06

[1]Coefficients in bold denote $P \leq 0.002$, in italics $P < 0.05$. BP: Blood pressure; HDL: High-density lipoprotein.

to be a significant predictor [hazard ratio (HR) = 1.72, 95%CI: 1.17-2.53] independent of age, presence of diabetes, systolic BP, and current smoking.

With respect to overall mortality, however, age, diabetes, and -in non-diabetic men- current smoking were determinants, whereas FLD and HOMA index did not emerge as independent predictors (Table 4). We further analyzed similar regression models for mortality stratifying to glucose categories (normoglycemia, impaired fasting glucose and diabetes). Tendency to excess independent mortality risk for FLD (HR = 2.69, 95%CI: 0.42-17) and HOMA index was restricted to prediabetic men in whom age had an exceptionally high HR. In women - albeit non-significant - increasing HRs were noted for both FLD and HOMA index from normoglycemia to diabetes categories.

In order to assess whether some of the components of the FLI, rather than the overall algorithm, were determinants of outcomes, we analyzed the Cox models separately with the four components (Table 5). These demonstrated a greater impact of triglycerides and GGT levels and - to some extent - of abdominal obesity but not of overall obesity, which interestingly and significantly protected against risk of death.

Risk of death related to the three glucose categories is schematized in Figure 2.

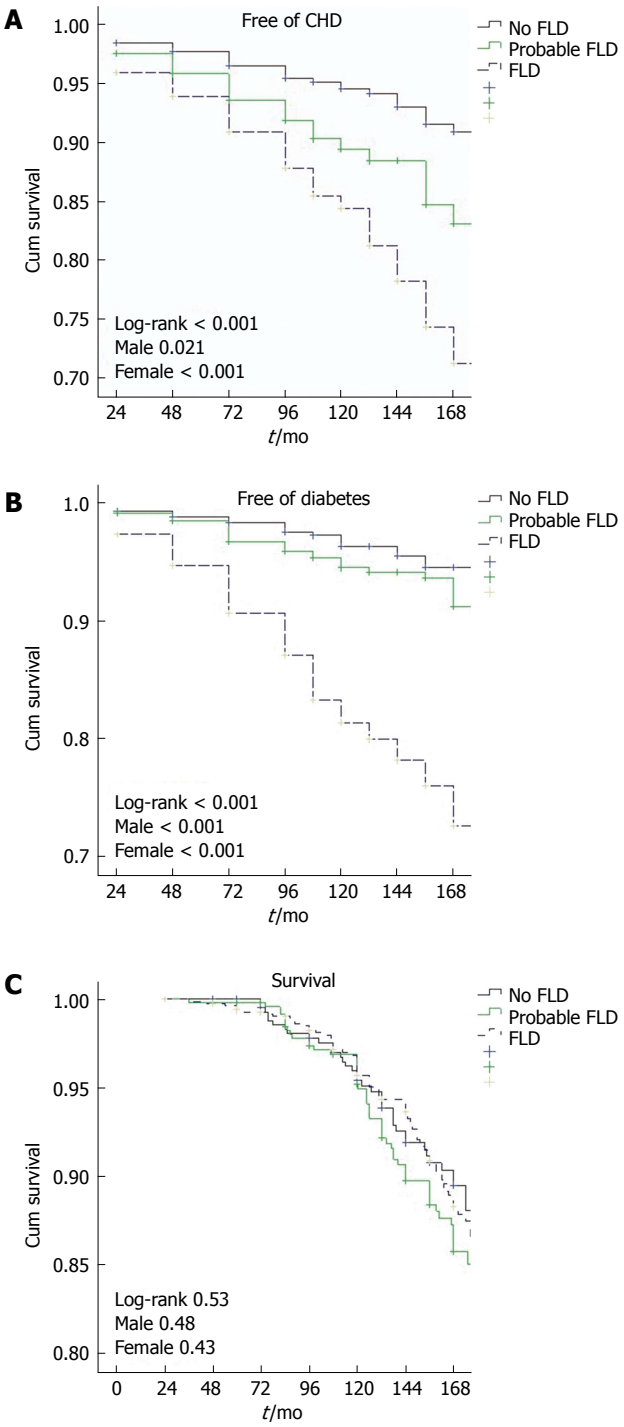

Figure 1 Diagram depicts Kaplan-Meier plots in the whole sample for survival and (exclusive of those with diabetes/coronary heart disease) survival free of diabetes/coronary heart disease. Significantly (Log-rank < 0.001) lower survival free of diabetes and of incident CHD are noted for participants with FLD at baseline. Subjects categorized as probable FLD separated from those with no FLD in regard to CHD. However, no significant difference was elicited (Log-rank 0.53) with respect to mortality. Log-rank values were similar in the sexes. BP: Blood pressure; CHD: Coronary heart disease; FLD: Fatty liver disease.

DISCUSSION

In this follow-up analysis of a cohort representative of middle-aged and elderly Turkish adults, we examined the independent predictive value of FLD, derived from a FLI, for the risks of type-2 diabetes, CHD, and overall mortality. The FLI was correlated with MetS traits as well as with markers of enhanced low-grade systemic inflammation (total cholesterol, apoB, uric acid, CRP, and SHBG levels). FLD was a powerful predictor of incident diabetes and disclosed a nearly 2-fold relative risk for CHD compared to participants without FLD.

Table 3 Adjusted Cox regression analyses of fatty liver disease for prediction of type-2 diabetes and incident coronary heart disease, by gender (and diabetic status)

	Total		Men		Women	
	HR	95%CI	HR	95%CI	HR	95%CI
Diabetes *n* =	203/1490[1]		110/707[1]		93/783[1]	
Gender, female	0.82	0.56; 1.20				
Age, 11 yr[2]	1.14	0.97; 1.34	1.13	0.90; 1.41	1.14	0.90; 1.46
Current smoking, *n* = 480	1.38	0.94; 2.00	1.35	0.84; 2.16	1.39	0.74; 2.69
Former smoking, *n* = 183	1.32	0.83; 2.08	1.22	0.72; 2.06	1.98	0.72; 5.48
HDL-cholesterol, 12 mg/dL	0.91	0.78; 1.06	0.96	0.77; 1.21	0.89	0.71; 1.10
Systolic BP, 25 mmHg	1.16	1.00; 1.38	1.28	1.03; 1.60	1.08	0.88; 1.35
Uric acid, 1.3 mg/dL	0.87	0.76; 0.996	0.81	0.67; 0.97	0.94	0.76; 1.15
Probable FLD, *n* = 423	1.44	0.80; 2.57	1.57	0.72; 3.43	1.28	0.52; 3.12
FLD, *n* = 673	4.93	2.98; 8.14	4.80	2.40; 9.59	5.33	2.53; 11.2
DM incid. per 1000 person-yr	16.6		18.8		14.5	
CHD: Diabetic sample *n* =	88/409[1]		45/192[1]		43/217[1]	
Gender, female	0.95	0.55; 1.63				
Age, 11 years	1.18	0.94; 1.49	1.27	0.90; 1.80	1.10	0.79; 1.56
Current smoking	1.12	0.64; 1.95	1.46	0.68; 3.15	0.66	0.25; 1.75
Former smoking	1.13	0.56; 2.31	1.36	0.59; 3.13	1.12	0.15; 8.52
Alcohol usage, yes/no	0.50	0.12; 2.10	0.58	0.14; 2.45		No user
HDL-cholesterol, 12 mg/dL	0.78	0.61; 1.00	0.90	0.62; 1.28	0.69	0.48; 0.99
Systolic BP, 25 mmHg	0.95	0.72; 1.28	0.98	0.60; 1.60	0.93	0.65; 1.31
Uric acid, 1.3 mg/dL	0.98	0.79: 1.23	0.73	0.54: 0.999	1.35	0.99: 1.84
Probable FLD	1.45	0.66; 3.18	1.68	0.56; 5.05	1.32	0.42; 4.11
FLD	3.59	1.78; 7.23	4.61	1.72; 12.4	3.12	1.17; 8.35
CHD inciden. per 1000 person-yr	25.3		27.4		23.4	
CHD: Whole sample *n* =	237/1505[1]		104/716		133/789[1]	
Gender, female	1.34	0.96; 1.86				
Age, 11 yr	1.48	1.27; 1.71	1.54	1.26; 2.00	1.43	1.17; 1.73
Current smoking	1.50	1.06; 2.13	1.80	1.12; 2.90	1.13	0.64; 1.98
Former smoking	1.02	0.64; 1.63	0.92	0.51; 1.64	1.82	0.79; 4.17
Alcohol usage, yes/no	0.80	0.37; 1.74	0.79	0.36; 1.75		Too few
HDL-cholesterol, 12 mg/dL	0.90	0.78; 1.02	1.04	0.84; 1.28	0.81	0.69; 0.98
Systolic BP, 25 mmHg	1.25	1.08; 1.42	1.31	1.12; 1.27	1.25	1.05; 1.45
Presence of diabetes	1.44	0.98; 2.13	1.81	1.04; 3.17	1.13	0.65; 1.95
Probable FLD	1.26	0.83; 1.92	1.79	0.92; 3.47	0.99	0.57; 1.73
FLD	1.72	1.17; 2.53	2.35	1.25; 4.43	1.42	0.86; 2.35
CHD incid. per 1000 person-yr	18.9		17.5		19.7	

[1]Number of cases/number at risk; [2]Referent was no FLD (*n* = 393 in the model for CHD). Mean age at baseline was 52.1 yr. Prevalent diabetes/coronary diseases were excluded. HDL: High-density lipoprotein; FLD: Fatty liver disease; CHD: Coronary heart disease; BP: Blood pressure.

All-cause mortality, however, was not independently related to baseline FLD, or to HOMA index, except for disclosing a tendency in prediabetic men and a tendency in women increasing in categories from normoglycemia to diabetes. These findings are in agreement with most previous reports and -regarding mortality- with studies on general population samples. We suspect the discrepancy between the relationship to outcomes (mortality *vs* cardiometabolic risk) in subjects with FLD is a consequence of (gender-modulated) circulating Lp(a) levels.

Correlation of FLI with MetS traits and low-grade inflammation markers

The close association between impaired glucose regulation and lipid metabolism with NAFLD is widely recognized[28]. It has been proposed that fatty liver represents a (novel) component of the MetS[2]. Correlation of the FLI with MetS traits in the present study is in line with this and other reports. The index was also significantly correlated with serum apoB, uric acid, and CRP and inversely correlated with circulating SHBG-all markers of proinflammatory state in our experience. Hence, male and female participants identified with FLD harbored both MetS components and markers of enhanced subclinical inflammation.

Prediction of diabetes and CHD by FLD

In these middle-aged and elderly adults, FLD prevailed in 48%, a substantially higher prevalence than in other population samples. In a slightly younger adult sample from the United States, ultrasonography-defined NAFLD prevalence was reported as 19.5%[16]. Current participants with FLD likely represent, moreover, a higher degree of fat accumulation and inflammation in the liver, as may be assessed from stronger HRs associated with incident cardiometabolic risk. NAFLD prevailed at a lower rate in other reports[4,5] as well.

We confirmed results of previous prospective studies documenting significant prediction of type-2

Table 4 Adjusted Cox regression analyses of fatty liver disease for prediction of overall mortality, by gender and diabetic status

	Total		Men		Women	
	HR	95%CI	HR	95%CI	HR	95%CI
Diabetic sample *n* =	30/118[1]		20/59[1]		10/59[1]	
Gender, female	0.71	0.25; 2.00				
Age, 11 yr	2.74	1.43; 5.22	2.61	1.19; 5.73	3.48	1.02; 12.0
Current *vs* non-smoker	0.94	0.30; 3.02	1.38	0.37; 5.18	*n* = 2	protecting
Former smoking, *n* = 170	1.17	0.40; 3.40	2.20	0.61; 7.85	*n* = 2	Too few
HDL-cholesterol, 12 mg/dL	0.81	0.55; 1.20	0.72	0.41; 1.24	0.77	0.43; 1.36
Systolic BP, 25 mmHg	1.16	0.74; 1.81	0.82	0.40; 1.72	1.25	0.62; 2.60
HOMA index, 2-fold	1.15	0.89; 1.49	1.03	0.75; 1.41	1.47	0.95; 2.27
Probable FLD, *n* = 25	0.48	0.15; 1.55	0.35	0.09; 1.34	*n* = 12	risk-
FLD, *n* = 66	0.74	0.24; 2.24	0.72	0.20; 2.57	*n* = 36	conferring
Death rate per 1000 person-yr	30.2		38.2		22.4	
Prediabetic sample[2] *n* =	32/268[1]		17/119[1]		15/149[1]	
HOMA index, 2-fold	1.25	0.93; 1.68	1.33	0.80; 2.23	1.32	0.87; 2.00
Probable FLD *n* = 57	1.05	0.25; 4.40	0.70	0.10; 4.77	1.19	0.10; 14.5
FLD *n* = 116	2.07	0.52; 8.19	2.69	0.42; 17.0	1.30	0.12; 14.7
Death rate per 1000 person-yr	14.0		17.2		11.8	
Normoglycemic sample *n* =	89/1268[1]		54/610[1]		35/658[1]	
Gender, female	0.77	0.42; 1.40				
Age, 11 yr	3.76	2.85; 4.97	3.91	2.69; 5.68	3.41	2.24; 5.17
Current *vs* never smoking	2.39	1.27; 4.51	2.99	1.36; 6.56	0.98	0.22; 4.38
Former smoking, *n* = 170	1.04	0.46; 2.34	1.16	0.47; 2.87	*n* = 15	Too few
HDL-cholesterol, 12 mg/dL	1.05	0.84; 1.31	1.07	0.78; 1.46	1.07	0.78; 1.51
Systolic BP, 25 mmHg	1.03	0.80; 1.31	1.13	0.80; 1.64	0.98	0.67; 1.38
HOMA index, 2-fold	1.02	0.88; 1.17	1.10	0.94; 1.29	0.79	0.62; 1.007
Probable FLD *n* = 312	0.89	0.47; 1.68	0.55	0.24; 1.31	2.02	0.75; 5.39
FLD *n* = 500	0.68	0.34; 1.36	0.58	0.24; 1.40	0.96	0.32; 2.88
Death rate per 1000 person-yr	8.1		10.0		6.3	
Whole sample *n* =	151/1654[1]		91/788[1]		60/866[1]	
Gender, female	0.73	0.46; 1.17				
Age, 11 yr[3]	3.38	2.69; 4.19	3.48	2.60; 4.65	3.34	2.36; 4.74
Current *vs* never smoking, *n* = 414	1.67	1.03; 2.70	2.01	1.13; 3.57	0.96	0.29; 3.17
Former smoking, *n* = 170	0.92	0.52; 1.64	0.99	0.52; 1.88	*n* = 22	Too few
Alcohol usage, yes/no, *n* = 116	0.84	0.37; 1.90	0.73	0.32; 1.67	*n* = 2	Too few
HDL-cholesterol, 12 mg/dL	1.02	0.87; 1.11	1.09	0.85; 1.38	0.94	0.73; 1.22
Systolic BP, 25 mmHg	1.03	0.86; 1.25	1.00	0.78; 1.31	1.08	0.84; 1.42
Prediabetes, *n* = 220	1.30	0.82; 2.07	1.08	0.56; 2.09	1.73	0.89; 3.35
Presence of diabetes, *n* = 104	2.70	1.73; 4.22	2.22	1.52; 4.86	2.76	1.32; 5.76
HOMA index, 2-fold	1.08	0.97; 1.21	1.12	0.99; 1.27	0.98	0.80; 1.20
Probable FLD, *n* = 394	0.79	0.48; 1.31	*0.58*	0.31; 1.11	1.42	0.57; 3.50
FLD *n* = 681	0.84	0.50; 1.39	0.83	0.44; 1.56	1.03	0.41; 2.59
Death rate per 1000 person-yr	10.8		13.3		7.9	

[1]Number of deaths/number at risk; [2]Adjusted also for sex, age, current smoking, systolic BP, HDL-cholesterol; [3]Referent was no FLD (*n* = 412). Mean age at baseline was 52.1 years. Missing HOMA index values limited by 15% the sample and deaths. HDL: High-density lipoprotein; FLD: Fatty liver disease; HOMA: Homeostatic assessment; BP: Blood pressure.

diabetes by NAFLD diagnosed by ultrasonography. In study samples exceeding 12000 subjects, Yamada *et al*[4] in Japanese and Sung *et al*[5] in South Korean people found over 5-year follow-ups that NAFLD independently predicted diabetes risk at about 2- to 2.5-fold HRs, respectively. In prior prospective studies on Japanese people with smaller sample sizes[29,30], the related HRs ranged between a non-significant value and 4.6. In this study, the predictive value of FLD for this association was over 5-fold that of individuals without FLD, independent of sex, age, smoking status, systolic blood pressure, serum HDL-cholesterol level, and uric acid level. This HR was similar to that found in French men but lower than that in women in the highest versus the lowest quartile of FLI[31].

NAFLD has been shown to predict incident CVD[6-9]. The prospective analysis over a 14-year follow-up in approximately 11600 participants of NHANES-Ⅲ demonstrated an independent association between NAFLD by ultrasonography and cardiovascular disease[9], similar to our current findings. However, the strong predictive ability of FLD for CHD among our diabetic subjects was substantially attenuated in the whole male sample when diabetes was included in the adjustments and was reduced to a non-significant level in female participants. It appears that in Turkish women who are prone to autoimmune activation NAFLD and diabetes, each conferring CHD risk, emerging bidirectional changes[1] mediate each other and attenuate this risk. Thus, both the substrate (prevalent CHD or diabetes) and gender modulate this risk. In fact, in Chinese patients with suspected CHD (*n*

Table 5 Adjusted Cox regression analyses of components of fatty liver index for prediction of type-2 diabetes, incident coronary heart disease and mortality, by gender

	Total		Men		Women	
	HR	95%CI	HR	95%CI	HR	95%CI
Diabetes						
Waist circumference, 12 cm	1.70	1.33; 2.15	1.64	1.13; 2.38	1.78	1.27; 2.46
Body mass index, 5 kg/m^2	1.10	0.91; 1.33	1.06	0.80; 1.40	1.11	0.86; 1.44
Triglycerides, 90 mg/dL	1.22	1.06; 1.40	1.09	0.91; 1.31	1.43	1.20; 1.71
γ-glutamyltransferase, 1.7-fold	2.19	1.61; 2.99	2.44	1.52; 3.92	2.22	1.44; 3.43
CHD						
Waist circumference, 12 cm	1.21	0.80; 1.82	1.21	0.20; 1.82	1.02	0.78; 1.33
Body mass index, 5 kg/m^2	1.01	0.84; 1.20	0.77	0.52; 1.14	0.98	0.80; 1.20
Triglycerides, 90 mg/dL	1.19	1.03; 1.35	1.20	1.01; 1.43	1.12	0.84; 1.31
γ-glutamyltransferase, 1.7-fold	1.52	0.94; 2.44	1.52	0.94; 2.44	1.64	1.16; 2.31
Mortality						
Waist circumference, 12 cm	1.31	1.00; 1.74	1.21	0.81; 1.80	1.38	0.90; 2.11
Body mass index, 5 kg/m^2	0.73	0.56; 0.96	0.68	0.45; 1.03	0.82	0.56; 1.20
Triglycerides, 90 mg/dL	1.12	0.96; 1.31	1.28	1.01; 1.57	0.91	0.70; 1.20
γ-glutamyltransferase, 1.7-fold	1.37	0.97; 1.93	1.28	0.79; 2.06	1.62	0.95; 2.77

Models were adjusted also to sex, age, smoking status, systolic BP, HDL-cholesterol. Further, uric acid protected men against diabetes and weakly tended to protect women against CHD.

= 713), significant association between FLI and CHD was not detected[32].

Lack of prediction of mortality risk by NAFLD

Reasons for the paradoxical lack of NAFLD on mortality risk remain unclear. Using the NHANES-Ⅲ survey data, all-cause mortality for alanine transferase-defined NAFLD over a mean 8.7 year follow-up was marginally increased (HR = 1.37)[13], a risk confined to the age group 45-54. Women, Mexican Americans, non-smokers, and those with MetS or diabetes were more likely to have NAFLD. A more recent analysis of NHANES-Ⅲ survey data confirmed that ultrasonography-defined NAFLD did not increase the risk of mortality[33]. However, NAFLD with evidence of advanced fibrosis (only one out of 30 NAFLD cases) using non-invasive marker panels was associated with increased mortality, mainly attributable to cardiovascular causes. NAFLD fibrosis score was based on an algorithm using additional data on impaired fasting glucose/diabetes, as well as inflammation-related parameters such as aspartate aminotransferase/alanine aminotransferase ratio, platelet count, and serum albumin level.

GGT, a participant in the degradation of the antioxidant glutathione and, hence, capable of inducing pro-oxidant effect, is a major component of the FLI. GGT was shown to be independently and inversely associated with the mean low density lipoprotein (LDL) particle size in asymptomatic elderly subjects with dyslipidemia[34]. Though the FLI was found to predict all-cause mortality in the Cremona study, characterized by a cohort having a high prevalence of MetS and insulin resistance, it was the significant association of the HOMA index with the FLI that emerged as a mediator of mortality risk[15]. Lp(a) constitutes a typical example of small dense LDL and was documented elsewhere[35] and in the TARF study[36] to be inversely associated with HOMA index.

Our multivariable analysis with the four components of the FLI explains in part the lack of association with risk of death, insofar as BMI emerged (especially in men) as protective against mortality risk. Moreover, on our previous findings[37] showed that a disparate independent association existed among sexes between serum GGT and Lp(a) levels, with high Lp(a) levels in men and low levels in women (reflecting autoimmune activation), and this may have been pivotal for the associations of FLI with the risks of death and, in women, with incident CHD. Diabetic status, a major confounder of and interactor with an underlying autoimmune activation, may have, therefore, largely mediated FLD [and low Lp(a) concentrations] and attenuated the outcome of mortality.

Since FLD, HOMA index, and age in current prediabetic men had higher HRs and higher Lp(a) concentrations than in the remaining two categories, serum Lp(a) may not be involved in the autoimmune complex underlying its relation to FLD and HOMA index, which is in contrast to the relatively elevated HRs for mortality. The independent contribution to the risk of death, likely *via* cardiorenal disease, may well be reflected in the high HR of age. In women, the persistent increase in HRs of FLD and HOMA index may be a consequence of the increasing involvement of circulating Lp(a) in autoimmune activation from normoglycemia onwards.

Our observations in men support the view that the development of diabetes from prediabetes attenuates the independent risk of death for FLD[38,39]. Age-adjusted mortality in patients with NAFLD was, indeed, reported to be associated with IFG[40].

A critical role of serum GGT in the pathogenesis of IFG was suggested in a large Korean population-based

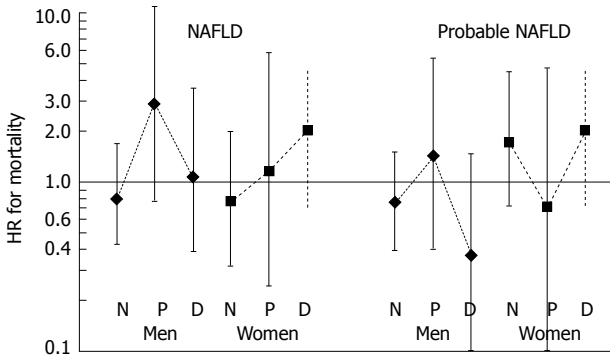

Figure 2 Diagram of multivariable adjusted hazard ratios are depicted for death of fatty liver disease in the 3 glucose categories. Though significant findings were not obtained, the risk of overall mortality among normoglycemic (N) individuals with fatty liver disease was lower than in subjects with no fatty liver disease. Risk increased in prediabetes (P) and tended so in diabetic (D) women, while declining in men with diabetes. NAFLD: Non-alcoholic fatty liver disease.

study that assessed the varying association of the enzyme level with BMI[41]. In our evaluation of subjects with IFG, we observed a parallel trend between serum Lp(a) and GGT in women regardless of the presence of MetS but in men in the absence of MetS alone[20].

Limitations and strengths

FLD was defined herein not by imaging methods or histology but by an algorithm based on obesity markers, fasting triglyceride, and GGT levels. This method has been validated in several epidemiologic studies[15,23], and other methods are costly and impractical to identify FLD in large epidemiologic studies. Collinearity between FLI and metabolic factors such as Lp(a) levels or obesity cannot be ruled out. The study sample size, long follow-up, and analysis stratified to gender are strengths of the study. Concomitant investigation of diabetes, CHD, and overall mortality in the same study sample is a major strength that allowed for the detection of emerging differences in the underlying pathogenesis.

FLD, defined by a FLI, was detected in one-half of a population-based cohort. FLD was a powerful predictor of incident diabetes and disclosed a nearly 2-fold HR for the risk of CHD, compared to participants without FLD. In essential agreement with most previous reports on general population samples, all-cause mortality, however, was not independently related to baseline FLD, or to HOMA index, except for a tendency in prediabetic men as well as prediabetic and diabetic women. Associations between BMI, GGT, and Lp(a) concentrations may herein be pivotal. Further research seeking the association between FLD and mortality risk should address the impact of circulating Lp(a) in the separate glycemic states in larger population samples.

COMMENTS

Background

Liver biopsy, ultrasonography, serum liver enzymes, and, more recently, an algorithm-based surrogate of fatty liver disease (FLD) have been commonly used in identifying the presence of non-alcoholic fatty liver disease (NAFLD), closely associated with features of the metabolic syndrome (MetS), and its relationship to adverse outcomes. NAFLD, a growing public health issue, has been demonstrated to be associated with MetS, type-2 diabetes cardiovascular events, and chronic kidney disease, but controversy exists on its predictive ability for overall mortality.

Research frontiers

Diabetic status, a recognized major confounder in the bidirectional relationship between FLD and cardiovascular morbidity and mortality, appears to be a major area requiring future research. Another hotspot that further research should be engaged, especially in population subgroups prone to metabolic syndrome, is the potential disparate independent association potentially existing among sexes between serum γ-glutamyltransferase (GGT) (a component of the FLI) and lipoprotein (Lp)(a) levels and their influence on outcome.

Innovations and breakthroughs

The lack of a relationship between NAFLD and risk of overall mortality as compared to its independent prediction of diabetes and cardiovascular disease has been intriguing. An algorithm consisting of body mass index, waist circumference, triglycerides, and GGT has been used elsewhere and herein to detect fatty liver. Confirmation in the present study that risk of death was essentially not predicted by FLI may be due to underlying involvement of circulating Lp(a) in autoimmune activation and the generally confounding role of diabetes, which may have largely mediated FLD and attenuated the outcome of mortality.

Applications

The previously proposed FLD index may reliably be utilized as a surrogate in population screening for the detection of fatty liver.

Terminology

Steatohepatitis designates fatty infiltration and inflammation of the liver.

Peer-review

The authors examined prospectively in over 1800 middle-aged Turkish adults the association of a surrogate of FLD, consisting of adiposity measures, triglyceride, and GGT levels, with type-2 diabetes, coronary heart disease (CHD), and all-cause mortality. Multivariably adjusted Cox regression analyses were used. Over an average 8-year follow-up, FLD was found as the major determinant of incident diabetes at a high relative risk. CHD was significantly and independently predicted by FLD in men alone and in the whole study sample. Despite these, and in line with several previous reports on the controversial topic, the authors detected no significant excess risk of death, though a tendency to increased risk was observed in the prediabetic state. Authors attributed the lack of prediction by FLI possibly to serum Lp(a) being involved in autoimmune activation and to a confounding role of the diabetic status mediating FLD.

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ORIGINAL ARTICLE

Prospective Study

Fecal calprotectin correlated with endoscopic remission for Asian inflammatory bowel disease patients

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Abstract

AIM: To evaluate the correlation between fecal calprotectin (fC), C-reactive protein (CRP), and endoscopic disease score in Asian inflammatory bowel disease (IBD) patients.

METHODS: Stool samples were collected and assessed for calprotectin levels by Quantum Blue Calprotectin High Range Rapid test. Crohn's disease endoscopic index of severity (CDEIS) and ulcerative colitis endoscopic index of severity (UCEIS) were used for endoscopic lesion scoring.

RESULTS: A total of 88 IBD patients [36 patients with Crohn's disease (CD) and 52 with ulcerative colitis (UC)] were enrolled. For CD patients, fC correlated with CDEIS (r = 0.465, P = 0.005) and CRP (r = 0.528, P = 0.001). fC levels in UC patients correlated with UCEIS (r = 0.696, P < 0.0001) and CRP (r = 0.529, P = 0.0005). Calprotectin could predict endoscopic remission (CDEIS < 6) with 50% sensitivity and 100% specificity (AUC: 0.74) in CD patients when using 918 μg/g as the cut-off. When using 191 μg/g as the cut-off in UC patients, calprotectin could be used for predicting endoscopic remission (UCEIS < 3) with 88% sensitivity and 75% specificity (AUC: 0.87).

CONCLUSION: fC correlated with both CDEIS and UCEIS. fC could be used as a predictor of endoscopic remission for Asian IBD patients.

Key words: Inflammatory bowel disease; Endoscopic score; Fecal calprotectin; Crohn's disease; Ulcerative colitis

Core tip: Mucosal healing is the goal in treating inflammatory bowel disease patients, and endoscopic examination remains the gold standard for evaluating mucosal status. Our results provide more evidence that fecal calprotectin (fC) correlates well with mucosal disease activity by using endoscopic disease score of ulcerative colitis endoscopic index of severity (UCEIS) and Crohn's disease endoscopic index of severity (CDEIS). The correlation of fC was higher in UCEIS than in CDEIS. fC could be used as a predictor of endoscopic remission for Asian inflammatory bowel disease patients.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory and destructive disease of the gastrointestinal tract. The chronic active inflammation causes ulcerations, stricture formations, and perforations and is a risk factor for the development of dysplasia and cancer[1]. To reduce longstanding IBD complications, the treatment goal in IBD patients is mucosal healing.

Certain classifications have been constructed to monitor the clinical parameters and treatment effects in IBD patients, such as the Crohn's disease activity index (CDAI) and the Montreal classification[2,3]. However, these scoring systems may be unreliable, because patients give subjective assessments of their symptoms, and clinicians are subjective in their assessments of patients. The gold standard for monitoring patients with IBD remains endoscopy, which is a time-consuming, expensive, and invasive procedure. In addition, the bowel cleansing that patients must undergo before endoscopic procedures is uncomfortable and inconvenient.

A stool sample analysis is noninvasive without the necessity of bowel preparation, relatively convenient, and much less time-consuming for the patient. Calprotectin, a calcium-binding protein, originates from neutrophils found in both plasma and stool[4]. It is markedly elevated in inflammatory processes, such as IBD, sepsis, and necrotizing enterocolitis[5]. There are many bacterial proteases in feces, however, calprotectin is resistant to these enzymes, and, therefore, it is possible to measure it in a fecal sample[6]. In addition, samples can be stored for several days and shipped at ambient temperatures. Thus, measurement of this biomarker provides a reliable method for the degree of inflammation in the bowel[7]. Recently, fecal calprotectin (fC) levels have been reported to be a predictive marker of mucosal healing in patients with IBD[5]. Its levels have been correlated with histological disease activity and could be used as a relapse marker and a disease activity marker for IBD patients[6,8,9].

There are two types of fC tests, including fully quantitative laboratory-based technologies [enzyme-linked immunosorbent assay (ELISA) platform], fully quantitative rapid tests, and semi-quantitative point-of-care tests [10]. The level of calprotectin is age-related. Two to 9 year-old children and adults above the age of 60 have significantly higher concentrations of calprotectin than 10 to 59 year-olds[11]. Furthermore, its level is higher in infants in rural areas than urban areas[12]. There are limited data regarding calprotectin levels in different races. Reports from Western countries show that fC levels significantly correlate with endoscopic disease activity in IBD[13]. In Japan, its levels were found to be correlated with endoscopic disease activity in pediatric and post-operative Crohn's disease (CD) patients[14,15]. For ulcerative colitis (UC), fC correlated with clinical activity in China[16]. We were unable to find any report

about evaluating fC, C-reactive protein (CRP), and endoscopic index in either Asian UC or CD patients. In order to define whether the fC could be used as a surrogate marker for mucosal healing, or even as a predictor for endoscopic remission, we examined the correlation between fC, CRP, and the endoscopic disease index in Asian IBD patients.

MATERIALS AND METHODS

Patients

All patients signed the informed written consent for participation in this study, which was approved by the institution's Ethics Committee. This was a prospective, multi-center study under the collaboration of the Taiwan Society of Inflammatory Bowel Disease. The inclusion criteria were: patients who were between 16 and 80 years of age; patients who were diagnosed with IBD based on standard endoscopic, radiological, and histological criteria; and outpatients who regularly followed up but not newly diagnosed, without acute illness under active medication adjustment. Exclusion criteria were: patients with possible concurrent gastrointestinal infection (fever and diarrhea) or malignancies, pregnant females, and patients who abused alcohol or regularly consumed aspirin, antibiotics, cytotoxic drugs, and non-steroidal anti-inflammatory drugs (more than two tablets per week). In Taiwan, when the patient's condition is stationary, the regular follow-up interval for the outpatient is 3 mo. Therefore, we invited those patients who were going to have their follow-up endoscope before their next visit to enroll in this study. In order to minimize inconvenience for patients, we educated the patients on how to collect stool samples and had them return the samples prior to or on the next visit. All invited patients who accepted these terms were included during the study period. In this way, the interval between calprotectin and CRP measurements and the endoscopic examination was within 3 mo. Serum CRP level was obtained from the clinical records (normal level was less than 0.8 mg/dL in all centers involved in this study).

Endoscopic scoring

All UC patients underwent complete colonoscopy, and all CD patients underwent ileocolonoscopy. CD endoscopic index of severity (CDEIS)[17] and UC endoscopic index of severity (UCEIS)[18] were used for the endoscopic lesions scoring. The CDEIS is based on the recognition of elementary lesions (non-ulcerated lesions, superficial and deep ulcerations) associated with the appreciation of their surface in five different segments (ileum, right colon, transverse, left colon, and rectum)[17]. The CDEIS ranges from 0 (normal) to 44 (most severe). The UCEIS is based on vascular patterns (2 levels), bleeding (3 levels), and erosions and ulcers (3 levels)[18]. The final score represents the sum of the components, with the UCEIS ranging from 0 (normal) to 8 (most severe). Endoscopic findings were recorded on color imaging and graded according to CDEIS and UCEIS by two specialist physicians who were blinded to the patient's clinical details.

Collection and measurement of fecal samples

Fecal samples (around 90 mg) were collected from all of the patients. Patients were instructed to collect the sample at home and have them stored in a refrigerator. Samples were delivered on the next day and transferred to the study laboratory for analysis. The laboratory personnel carrying out the analysis were blinded to the clinical history, data, and the endoscopic findings of the patients. The values quoted as normal in our laboratory were 100 μg/g. fC levels were assessed by using a commercially-available ELISA, Quantum Blue Calprotectin High Range Rapid Test (Buhlmann laboratories AG, Schonenbuch, Switzerland).

Statistical analysis

Each value is presented as a median with the inter-quartile range (IQR). For data analyses we used SPSS for Windows software 14.0 (Chicago, IL, United States). For correlation analyses, we used the nonparametric Spearman's rank order correlation (r). The Kruskal-Wallis test was used for exploring changes between related variables. Sensitivity and specificity were estimated by the receiver operating characteristic (ROC) curve analysis. The calprotectin cut-off values were selected by the Youden's index using ROC curves. The areas under the ROC curve (AUCs) were calculated for fC, and the combination of fC and CRP, respectively. All statistical testing was two-sided, and P values < 0.05 were considered statistically significant.

RESULTS

A total of 88 patients, 52 diagnosed with UC and 36 diagnosed with CD, were included in this study. The clinical characteristics of the 88 enrolled patients are listed in Table 1. The interval between the fC measurement and the endoscopy ranged from 4 to 80 d, with a mean of 36.5 d.

The UC patients were predominantly male (65%), with an age range of 17 to 76 years (median age of 44). The correlation between readers for UCEIS was good, with an intraclass correlation coefficient of 0.8980. They had a median (IQR) UCEIS of 3 (1-4), a median (IQR) CRP level of 0.17 (0.04-0.51) mg/L, and a median (IQR) fC level of 447 (116-1700) μg/g. When separated by disease extent, as shown in Table 2, the median level of fC in UC was 116 μg/g in proctitis, 478 μg/g in left-sided UC, and 611 μg/g in extensive UC. The fC level in UC patients was not statistically different between age groups more or less than 60 years old (mean ± SD: 331 ± 638 and 1033 ± 2038,

Table 1 Clinical characteristics of the 88 inflammatory bowel disease patients *n* (%)

	UC (*n* = 52)	CD (*n* = 36)
Gender (M/F)	34/18	19/17
Age (yr)	44 (17-76)	34 (21-71)
Disease extent/location		
E1/L1	13 (25)	4 (11)
E2/L2	15 (29)	8 (22)
E3/L3	24 (46)	24 (67)
EIS [median (IQR)]	3 (1-4)	8 (5.2-21)
Calprotectin (μg/g) [median (IQR)]	447 (116-1700)	309 (100-1130)
CRP [median (IQR)]	0.17 (0.04-0.51)	0.15 (0.05-0.91)

For UC: E1: Proctitis; E2: Left-sided colon; E3: Total colon; for CD: L1: Ileum; L2: Colon; L3: Ileocolon; UC: Ulcerative colitis; CD: Crohn's disease.

Table 2 Fecal calprotectin, C-reactive protein, and ulcerative colitis endoscopic index of severity in ulcerative colitis patients separated by the extent of disease

	Fecal calprotectin (μg/g)		UCEIS		CRP	
	Median	Range	Median	Range	Median	Range
E1	116	100-620	3	1-4	0.06	0.04-0.17
E2	478	214.5-1249	3	1-4	0.19	0.04-0.45
E3	611	172-1800	3.5	3-4.5	0.3	0.05-1.04

EIS: Endoscopic index of severity; CRP: Serum C-reactive protein; E1: Proctitis; E2: Left-sided UC; E3: Extensive; UCEIS: Ulcerative colitis endoscopic index of severity.

respectively; $P = 0.37$). The median level of CRP in UC was 0.06 mg/dL in proctitis, 0.19 mg/dL in left-sided UC, and 0.30 mg/dL in extensive UC. The median (IQR) UCEIS was 3 (1-4) in proctitis, 3 (1-4) in left-sided UC, and 3.5 (3-4.5) in extensive UC. For each biomarker of calprotectin, there was no significant difference among proctitis, left-sided UC, and extensive UC patients.

Of the 36 CD patients enrolled, there were slightly more males than females (52% male), and the age ranged from 21 to 71 years, with a median age of 34. The correlation between readers for CDEIS was also good, with an intraclass correlation coefficient of 0.9632. They had a median (IQR) CDEIS of 8.0 (5.2-21), a median (IQR) CRP level of 0.15 (0.05-0.91) mg/L, and a median (IQR) fC level of 309 (100-1130) μg/g. The fC level in CD patients was not statistically different between patient groups more or less than 60 years old (mean ± SD: 3250 ± 35436 and 1369 ± 2257, respectively; $P = 0.115$). When separated by disease location, as shown in Table 3, the median (IQR) level of fC in CD was 2693 (1071-5010) μg/g in ileum (L1), 176 (100-573) μg/g in colon (L2), and 279 (100-2175) μg/g in ileocolon (L3) subtypes. The median (IQR) level of CRP in CD was 0.56 (0.13-1.09) mg/dL in L1, 0.05 (0.03-0.15) mg/dL in L2, and 0.17 (0.08-1.27) mg/dL in L3 subtypes. The median (IQR) CDEIS was 9 (8-19) in L1, 3.9 (3.2-9.2) in L2, and 8 (6-28) in L3 subtypes. For each biomarker of calprotectin, there was no significant difference among the L1, L2, and L3 patients.

Table 3 Fecal calprotectin, Serum C-reactive protein and Crohn's disease endoscopic index of severity divided by location of the disease in patients with Crohn's disease

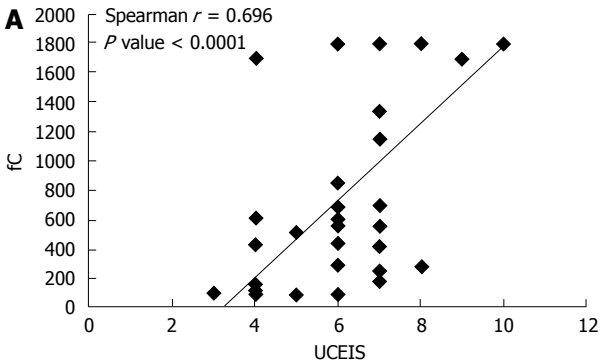
	Fecal calprotectin (μg/g)		CDEIS		CRP	
	Median	Range	Median	Range	Median	Range
L1	2693	1071-5010	9	8-18.5	0.56	0.13-1.09
L2	176	100-573	3.9	3.2-9.2	0.05	0.03-0.15
L3	279	100-2175	8	6-27.5	0.17	0.08-1.27

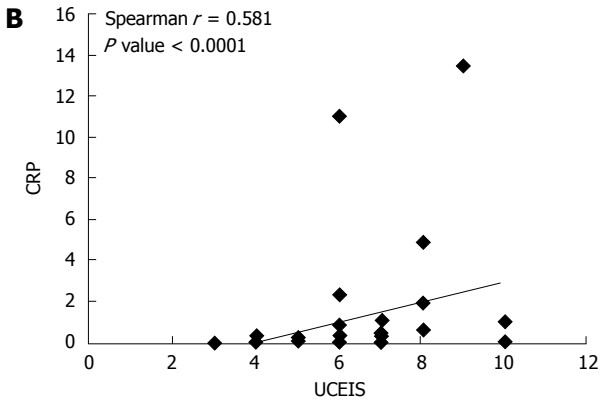
EIS: Endoscopic index of severity; CRP: Serum C-reactive protein; L1: Ileum; L2: Colon; L3: Ileocolon; CDEIS: Crohn's disease endoscopic index of severity.

In UC patients, UCEIS correlated significantly with fC level, with a Spearman correlation coefficient of 0.696 ($P < 0.0001$) (Figure 1A). UCEIS also correlated with the levels of CRP (Spearman correlation coefficient of 0.581, $P < 0.0001$) (Figure 1B). fC correlated with CRP, with a correlation coefficient of 0.529 ($P = 0.005$) (Figure 1C). Although there was a trend suggesting that fC increased from E1 (proctitis) to E3 (total colitis), there was no correlation between the extent of disease and fC, CRP, and UCEIS. In the CD patients, CDEIS correlated with fC (Figure 2A) as well as with CRP (Figure 2B), with Spearman correlation coefficients of 0.465 ($P = 0.005$) and 0.582 ($P = 0.0002$) for calprotectin and CRP, respectively. Moreover, the correlation coefficient was 0.528 ($P = 0.001$) for CRP and fC (Figure 2C). There was no correlation between the location of disease and fC ($P = 0.060$), CRP ($P = 0.153$) and CDEIS ($P = 0.081$).

In CD, where the threshold for endoscopic remission has been set by a systemic review study as a CDEIS < 6[19], the best discriminative cutoff was obtained at a calprotectin threshold of 918 μg/g, which could predict endoscopic remission with 50% sensitivity and 100% specificity (AUC of 0.74). In the UC group, where the threshold for endoscopic remission was set as a UCEIS < 3, the ROC curve analysis (AUC of 0.87) revealed a cut-off calprotectin level of 191 μg/g, which could predict mucosa healing with a sensitivity and specificity of 88% and 75%, respectively. As shown in Figure 3, when using the combination of CRP and fC, the predicting ability of endoscopic remission increased, but it did not significantly increase when compared with fC alone (*P* value for CD: 0.07 and for UC: 0.28).

When we divided the results by mean interval (36 d) of fC measurement and endoscopy, the Spearman correlation coefficient of fC and UCEIS was 0.810 ($P = 0.008$) for those with the shorter interval (< 36 d), and the correlation coefficient of fC and UCEIS decreased to 0.388 ($P = 0.342$) for those with the longer interval (> 36 d). However, there was no such difference for the CD patients. The Spearman correlation coefficient of fC and CDEIS was 0.509 for the shorter interval (< 36 d) ($P = 0.0631$), and the correlation coefficient of





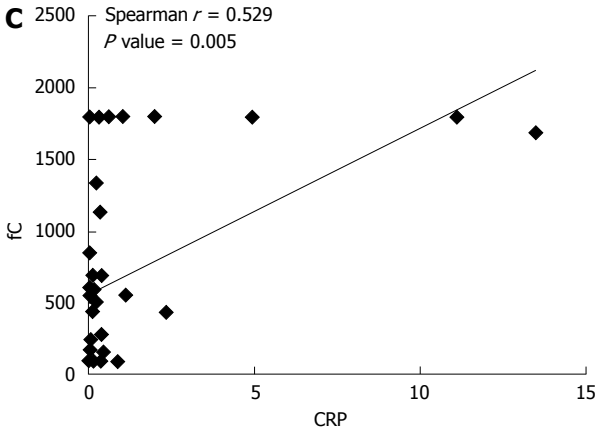
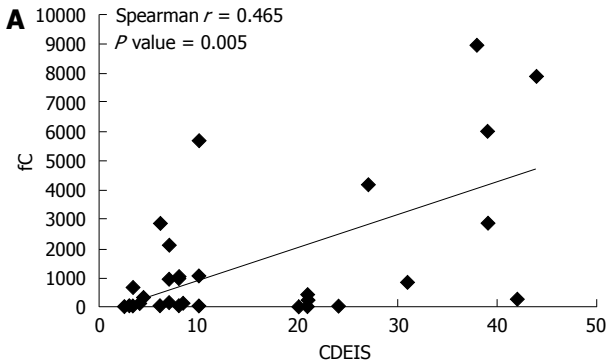
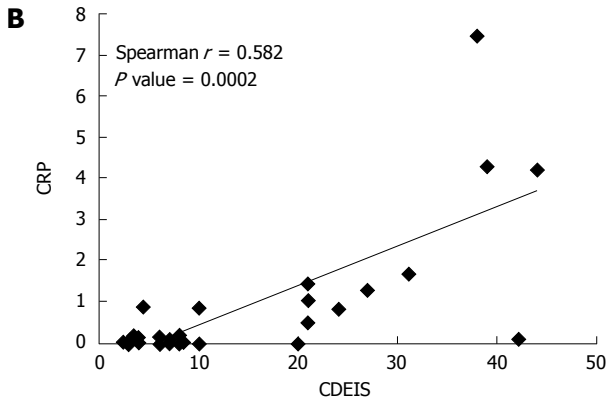


Figure 1 Correlations between (A) UCEIS and fecal calprotectin, (B) UCEIS and CRP, and (C) fecal calprotectin and CRP in ulcerative colitis patients. UCEIS: Ulcerative colitis endoscopic index of severity; CRP: C-reactive protein; fC: Fecal calprotectin.





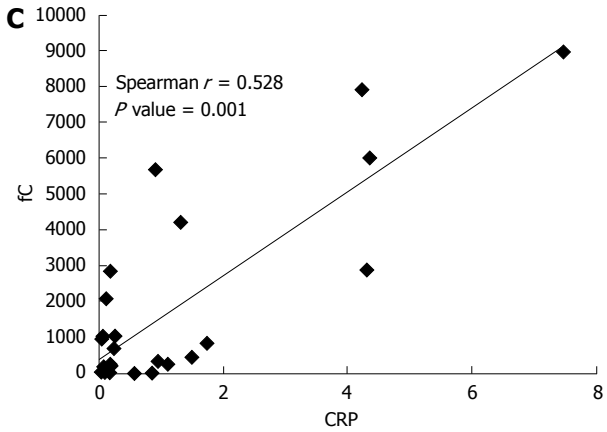


Figure 2 Correlations between (A) CDEIS and fecal calprotectin, (B) CDEIS and CRP, and (C) fecal calprotectin and CRP in Crohn's disease patients. CDEIS: Crohn's disease endoscopic index of severity; CRP: C-reactive protein; fC: Fecal calprotectin.

fC and CDEIS was 0.511 (P = 0.0179) for the longer interval (> 36 d) group.

DISCUSSION

The fC test is a simple, non-invasive, and reproducible test. To date, there have been few studies on the relationship between fC and endoscopic indices (mucosal status) in the Asian population. In this study, we assessed the association between fC, CRP level, and endoscopic disease activity. Our results demonstrated that the concentration of fC in Asian IBD patients was significantly correlated with the endoscopic index, which was evaluated by the UCEIS for UC and CDEIS for CD patients.

In our study, a calprotectin level below 191 μg/g was associated with mucosal healing in UC (UCEIS < 3), whereas a calprotectin level below 918 μg/g in CD reflected endoscopic remission (CDEIS < 6). This result is consistent with a Denmark study[20] that showed the cutoff level of fC was 192 μg/g for predicting endoscopic mucosa healing in UC, as assesed using both the UCEIS and Mayo Endoscopic Score. This finding confirmed that fC reflects endoscopic disease activity with different endoscopic scores. Another European report[13] observed that an fC level below 250 μg/g was predictive for endoscopic remission (CDEIS ⩽ 3) in CD patients. In UC, an fC level above 250

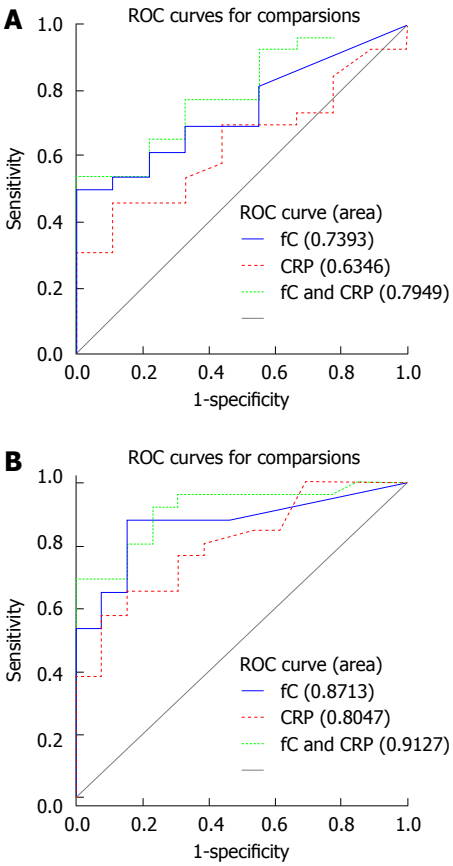


Figure 3 Comparison of receiver operating characteristic curves for Crohn's disease (A) and ulcerative colitis (B) patients. ROC: Receiver operating characteristic. CRP: C-reactive protein; fC: Fecal calprotectin.

μg/g indicated active mucosal disease activity (Mayo Endoscopic Score > 0). Here, we provide additional necessary evidence that fC levels correlate significantly with endoscopic disease activity in Asian IBD patients.

There is evidence that fC levels correlate more closely with histological evaluation than macroscopic findings, suggesting that this biological marker is more sensible than endoscopy in evaluating IBDs activity[9,20,21]. In combination with our results, the fC levels could help us identify not only endoscopic but also histologic healing. Therefore, fC could be used as a surrogate marker for mucosal status in both CD and UC patients. Although it has been reported that fC tended to be higher in patients more than 60 years old[11], we found no statistically significant difference between patients more or less than 60 years old for both UC and CD.

In clinical practice, these cut-off points could be used to guide treatment decisions, where patients with calprotectin levels higher than these cut-off points will need treatment intensification or escalation. We found different studies reporting various cut-off levels of fC with endoscopic disease activity, which might be due to differences in patient selection, the extent of disease, and the remission duration[22]. Based on these studies, the use of serial fC measurements to detect endoscopic recurrence could potentially overcome the imperfect sensitivity of individual measurements and was better than using a cut-off level of fC[23]. Although the combination of fC and CRP increased the predictive ability, it did not reach statistical significance, which may be due to an underpowered sample.

In previous studies[24-31], the correlation coefficients ranged from 0.48-0.83 for fC with endoscopic disease activity and from 0.46-0.61 for CRP in CD patients. In UC, the correlation coefficient ranged from 0.51-0.83 for fC with endoscopic disease activity. In our study, we found that the correlation between fC concentration and endoscopic disease activity was higher in patients with UC. We also found that the fC can rapidly reflect the mucosal condition, as demonstrated by the stronger correlations when the intervals between fC measurement and endoscopy were shorter. Costa and colleagues reported a study describing that a high concentration of fC was associated with a 14-fold relapse risk in patients with UC and a 2-fold relapse risk in patients with CD, indicating that a high concentration of fC may be a more accurate predictive marker of relapse in UC than in CD[32]. In contrast to UC, patients with CD have stronger CRP responses[33]. This finding may be related to the fact that transmural inflammation is seen in patients with CD, whereas the inflammation in UC patients is confined to the mucosa. Therefore, fC levels seem to represent a stronger biomarker for monitoring endoscopic activity in UC patients. For patients with CD, increased levels of both CRP and fC are likely to be sufficient to identify an active mucosal disease.

As for the disease extent in IBD, ileocolonic and colonic CD significantly correlated with positive calprotectin tests and the probability of relapse[22,33]. It was reported that patients with ileal CD had significantly lower fC levels than those with ileocolonic disease, even in the presence of large ulcers[34]. In UC patients, a previous study showed that the fC increased significantly with disease extent in proctitis and left-sided colitis but the difference was not statistically significant[20]. In our study, we did not find a correlation between disease location and fC in UC or CD patients. However, fC, CRP levels, and CDEIS were higher in patients with ileum involvement than in CD patient with other sites affected. In UC, the extensive type had higher fC, CRP levels, and UCEIS but without statistical significance.

In conclusion, fC correlated with both the CDEIS for CD patients and the UCEIS for UC patients. Our results provide additional evidence that fC correlates well with mucosal disease activity. Therefore, fC levels could be used as a surrogate marker for monitoring mucosal status as well as predicting endoscopic remission of not only European but also the Asian IBD patients.

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Bioinformatics and Statistical Center, Training Center, and Pharmacogenomics Laboratory. We thank the second Core Laboratory of the Department of Medical Research of the National Taiwan University Hospital for technical assistance.

COMMENTS

Background

Mucosal healing is the goal in treating inflammatory bowel disease (IBD) patients, and endoscopic examination remains the gold standard for evaluating the mucosal status. Recently, fecal calprotectin (fC) levels have been reported to be a predictive marker of mucosal healing in IBD. The aim of this study was to evaluate the correlation between fC, C-reactive protein, and endoscopic disease score.

Research frontiers

Previously, several studies have reported that fC is a stronger predictive marker of relapse in ulcerative colitis (UC) than in Crohn's disease (CD) with colon involvement but not ileal CD. However, the effect of fC is less known in Asian patients. Therefore, we performed this head-to-head comparison for CD and UC.

Applications

Those results demonstrated that the concentration of fC in Asian IBD patients was significantly correlated to the endoscopic index that was evaluated by the UC endoscopic index of severity for UC and CD endoscopic index of severity for CD patients. In agreement with the previous studies, when comparing fC level with endoscopic activity index in CD and UC, the correlation was higher in UC than in CD.

Terminology

fC is an extremely stable protein, and it can be found unaltered in stool for longer than 7 d. Thus, measurement of this biomarker provides a reliable method for determining the degree of inflammation in the bowel.

Peer-review

The study is certainly interesting and well designed. It sheds some light on the important issue of non-invasive evaluation of treatment effect in IBD patients.

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CASE REPORT

Rare long-term survivors of pancreatic adenocarcinoma without curative resection

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Institutional review board statement: The study was reviewed and approved by the Virginia Mason Medical Center Institutional Review Board (IRB). Informed consent from individual patients was not required by the IRB given the nature of the study (retrospective review of medical records) and the lack of identifiable patient information in the study.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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Abstract

Long-term outcome data in pancreatic adenocarcinoma are predominantly based on surgical series, as resection is currently considered essential for long-term survival. In contrast, five-year survival in non-resected patients has rarely been reported. In this report, we examined the incidence and natural history of ⩾ 5-year survivors with non-resected pancreatic adenocarcinoma. All patients with pancreatic adenocarcinoma who received oncologic therapy alone without surgery at our institution between 1995 and 2009 were identified. Non-resected ⩾ 5-year survivors represented 2% (11/544) of all non-resected patients undergoing treatment for pancreatic adenocarcinoma, and 11% (11/98) of ⩾ 5-year survivors. Nine patients had localized tumor and 2 metastatic disease at initial diagnosis. Disease progression occurred in 6 patients, and the local tumor bed was the most common site of progression. Six patients suffered from significant morbidities including recurrent cholangitis, second malignancy, malnutrition and bowel perforation. A rare subset of patients with pancreatic cancer achieve long-term survival without resection. Despite prolonged survival, morbidities unrelated to the primary cancer were frequently encountered and a close follow-up

is warranted in these patients. Factors such as tumor biology and host immunity may play a key role in disease progression and survival.

Key words: Non-resected pancreatic cancer; Long-term survival; 5-year survival; Chemotherapy; Cholangitis; Second malignancy; Malnutrition

Core tip: Five-year survival in patients with pancreatic cancer without curative resection is rare and has not been well described in the literature. At our institution from 1995 to 2009, non-resected ⩾ 5-year survivors represented 2% (11/544) of all non-resected patients with pancreatic adenocarcinoma, and 11% (11/98) of ⩾ 5-year survivors. These patients were mostly younger than 70 years of age, had excellent performance status and responded favorably to chemotherapy but suffered significant morbidities such as biliary sepsis. We speculate that tumor biology and host immunity play important roles on disease progression and survival.

Oh SY, Edwards A, Mandelson MT, Lin B, Dorer R, Helton WS, Kozarek RA, Picozzi VJ. Rare long-term survivors of pancreatic adenocarcinoma without curative resection. *World J Gastroenterol* 2015; 21(48): 13574-13581 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13574.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13574

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States and has one of the worst five-year survival rate of any cancer[1]. Several large population-based studies indicated an overall 5-year survival rate less than 5% and median overall survival of 3 to 6 mo[2-5]. Although a recent Surveillance, Epidemiology, and End Results (SEER) database demonstrated a steady increase in the 5-year survival rates from 3.1% to 4.4% to 6.9% over the three decades from 1981 to 2010[6], long-term survival rate compared with other malignancies still remains very low.

Currently, complete surgical resection is considered essential for potential cure for pancreatic cancer and management is benchmarked on the outcomes of resection. As a result, the vast majority of long-term survivors are described in surgical series, and the reported 5-year actual survival rate after resection ranges from 12% to 27%[7-23].

Although the prognosis for patients with unresectable disease is dismal with a median overall survival of 9 mo for locally advanced disease and 3 to 6 mo for metastatic disease[24], there is some evidence of long-term survival for patients with non-resected pancreatic cancer in the literature. The original paper published by the Gastrointestinal Tumor Study Group examining the role of chemoradiation in locally advanced pancreatic cancer suggested the possibility of long-term survival without surgery for a small subset of patients[25]. Furthermore, the most recent SEER database also displayed a small (2.1%) 5-year survival rate for stage Ⅳ pancreatic cancer patients[26]. Unfortunately, clinical details about such patients are lacking from the medical literature.

In this case series, we report the incidence and natural history of non-resected ⩾ 5-year survivors with biopsy-proven pancreatic adenocarcinoma treated with oncologic therapy alone without curative resection between January 1995 and December 2009.

CASE REPORT

Eleven non-resected ⩾ 5-year survivors represented 2% (11/544) of all non-resected patients with pancreatic adenocarcinoma who received treatment at our institution from 1995 to 2009, and 11% (11/98) of all ⩾ 5-year survivors (Figure 1).

Histopathological evaluation

The original histopathology slides of all patients were re-reviewed by a single faculty gastrointestinal pathologist to confirm the presence of pancreatic adenocarcinoma. Additionally, autoimmune pancreatitis, neuroendocrine tumors and tumors arising from the bile duct, gallbladder and duodenum were excluded. Endoscopic ultrasound-guided FNA (EUS-FNA) for cytology of a pancreatic mass (4) or lymph node (1) was performed in 5 patients, endoscopic retrograde cholangiopancreatography (ERCP) brushing of the pancreatic/bile duct for cytology in 2, computed tomography (CT)-guided core needle biopsies of a pancreatic mass in 2 and laparoscopic biopsies of a liver mass (1) and a pyloric implant (1) in 2.

Clinical characteristics

Clinical characteristics of the 11 non-resected patients are described in Tables 1-3. The median age was 62.7 years (54.2-69.5). All of them had an ECOG score of 0 or 1, normal serum albumin level and tumor in the head of the pancreas. Neutrophil-to-lymphocyte ratio (NLR) in the peripheral blood ranged from 1.5 to 14.2. Pain, weight loss or jaundice were present in about half of the patients. Nine patients with localized tumor were treated with combined chemoradiation, of whom 6 received the Virginia Mason protocol therapy consisting of interferon-alpha-2b, 5-fluorouracil (5-FU), cisplatin and radiotherapy. Two patients with metastatic disease were treated with gemcitabine and docetaxel[26].

Six patients suffered from significant morbidities leading to frequent hospitalizations. Recurrent cholangitis occurred in 5 patients (patients 4, 6,

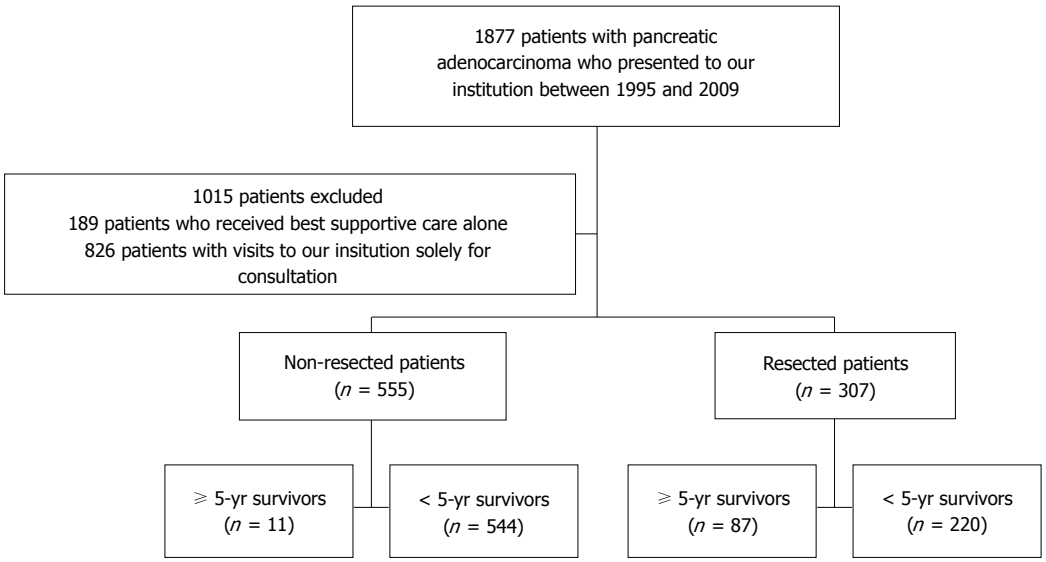

Figure 1 Treatment for pancreatic adenocarcinoma from 1995 to 2009, and 11% of all ⩾ 5-yr survivors.

Table 1 Clinical characteristics of 11 non-resected ⩾ 5-year survivors *n* (%)

Baseline characteristics	
Age (yr)	62.7 (54.2-69.5)
Male gender	7 (64)
ECOG ⩽ 1	11 (100)
BMI (kg/m^2)	27.2 (24.2-27.9)
DM	2 (18)
Smoking[1]	6 (55)
Pancreatic head tumor	11 (100)
Biliary stent[2]	7 (64)
Presenting symptoms and signs[2]	
Pain[3]	5 (45)
Weight loss[4]	6 (55)
Jaundice	6 (55)
Laboratory values[2]	
Ca 19-9 (U/mL)	72 (33.9-397.1)
Abnormal Ca 19-9	8 (73)
Bilirubin (mg/dL)	4.1 (0.5-11.4)
Abnormal bilirubin	6 (55)
Albumin (g/dL)	4.2 (4.0-4.9)
Abnormal albumin	0 (0)
Resectability[5]	
Resectable	0 (0)
Borderline resectable	2 (18)
Unresectable	7 (64)
Metastatic	2 (18)
AJCC staging[5]	
1	0 (0)
2	2 (18)
3	7 (64)
4	2 (18)

[1]History of cigarette smoking with 5 yr of diagnosis; [2]At the time of initial diagnosis; [3]Persistent abdominal or back pain; [4]Self-reported weight loss of > 10%; [5]Staging CT scan was re-reviewed by an experienced pancreas cancer surgeon who had no involvement in the original care of the patients. Continuous variables are shown as median (interquartile range). Categorical variables are shown as % (number). BMI: Body mass index; CT: Computed tomography.

7, 8 and 11 in Tables 2 and 3). These patients underwent frequent biliary drainage *via* ERCP and/or percutaneous transhepatic biliary drainage (PTBD). Two patients developed small bowel perforations (patient 7 due to an erosion at the site of previously placed duodenal stent, and patient 9 following ERCP). Two patients developed gastric outlet obstruction during the course of the disease (patients 4 and 7), thought to be due to therapy-induced peritumoral scarring. Both were treated with endoscopic duodenal stent placement and/or bypass surgery. One of them (patient 7) had a minimal response to endoscopic and surgical interventions and subsequently developed severe malnutrition, necessitating long-term enteral feeding and total parenteral nutrition. The patient also had an issue with chronic pain and was treated with narcotics administered *via* intrathecal pump. Second malignancies were observed in 2 patients, one with bladder cancer (patient 8), and the other with breast cancer as well as hypernephroma metastatic to the bone (patient 6). Five remaining patients (patients 6, 7, 8, 9 and 11) had uneventful progress in the setting of indolent disease.

Disease progression

Disease progression was observed in 6 patients (1 with local progression only, 5 with systemic with or without local progression). Five of those patients experienced disease progression within the first 3 years after diagnosis. Local tumor bed was the most common site of progression. To date, no patient has survived longer than 10 years and the longest overall survival is 8.6 years.

DISCUSSION

To our knowledge, this is the first series of non-resected ⩾ 5-year survivors with pancreatic adenocarcinoma. There are 3 case reports of patients with unresectable pancreatic cancer surviving at least 4 years[27-29]

Table 2 Clinical characteristics of 11 non-resected ⩾ 5-year survivors

Patient	Age (yr)	Gender	ECOG	BMI (kg/m^2)	Pain	Weight loss	Jaundice	DM	Smoking	Biliary stent	CA19-9 (U/mL)	NLR	Tumor location	AJCC Staging	Initial therapy	Duration of initial therapy (mo)
1	52.3	Female	0	20.8	Yes	No	No	No	No	No	2183.1	2.5	Head	2	VM CRT, GD	9
2	61.6	Male	0	28	No	Yes	Yes	No	No	Yes	200	3.4	Head	3	GD, VM CRT	12
3	62.7	Female	0	24.1	Yes	Yes	No	No	Yes	No	72	1.5	Head	3	GD[1], 5-FU + R[1]	8[1]
4	48.3	Male	0	26.7	No	No	No	No	No	No	9.8	2.2	Head	3	VM CRT, DT	10
5	51.7	Male	0	28	No	Yes	Yes	No	No	Yes	38.8	14.2	Head	3	GD, VM CRT	4
6	66.6	Female	0	21.7	Yes	Yes	Yes	Yes	Yes	Yes	661	4.7	Head	3	VM CRT, GD	8
7	56	Female	0	24.3	Yes	Yes	Yes	No	Yes	Yes	29	1.2	Head	3	VM CRT, GD	10
8	65.4	Male	0	27.8	No	No	Yes	No	Yes	Yes	156.8	10.1	Head	2	GD[1], 5-FU + R[1]	6[1]
9	81	Male	0	27.8	No	Yes	No	No	Yes	Yes	19.3	7.7	Head	4	GD	12
10	72.5	Male	0	27.2	Yes	No	No	No	Yes	Yes	40	2.7	Head	3	5-FU + R	6
11	76.5	Male	0	29.1	No	No	Yes	Yes	No	Yes	594.2	6.4	Head	4	GD	12

[1]Neoadjuvant therapy. NLR: Peripheral blood neutrophil-to-lymphocyte ratio; GD: Gemcitabine and doxetaxel; 5-FU + R: 5-fluorouracil and radiotherapy; VM CRT: Virginia Mason Protocol chemoradiation regimen consisting of interferon-alpha-2b.

and a report of a long-term survivor treated with 57 courses of gemcitabine where the exact survival was not reported[30]. The paucity of such reports is related to the fact that non-resected long-term survivors are exceedingly rare and the literature on long-term survival is focused heavily on patients undergoing resection.

Our study shows that there is a small subset of patients with unresectable pancreatic cancer who can survive at least 5 years. The 11 ⩾ 5-year survivors represent only 2% of all non-resected patients undergoing treatment for pancreatic adenocarcinoma from 1995 to 2009 at our institution, and 11% of all ⩾ 5-year survivors (Figure 1). The majority of our long-term survivors were younger than 70 years of age, exhibited excellent performance status and had tumors in the head of the pancreas. These were previously identified as favorable prognostic factors in pancreatic cancer[31-33]. Most of our patients were also overweight or obese, supporting the fact that obesity is associated with increased risk of developing pancreatic cancer[34-36]. On the other hand, obesity has also been shown to confer a survival advantage to patients with diseases associated with wasting, such as pancreatic cancer[37,38].

The majority of our non-resected survivors had locally advanced unresectable disease, and all of these patients received chemoradiation (Tables 2 and 3). Chemoradiation in locally advanced pancreatic cancer is thought to improve local disease control but has been investigated with conflicting results[25,39-42]. A selective strategy of first providing chemotherapy followed by chemoradiation has been proposed to minimize radiation-induced toxicities in patients with rapidly progressive tumors during initial chemotherapy and some studies have shown improved outcomes in patients who complete initial chemotherapy and subsequent chemoradiation[38,42,43]. At our institution, induction chemotherapy with gemcitabine and docetaxel followed by consolidative chemoradiation consisting of interferon-alpha-2b, 5-FU, cisplatin and radiotherapy has been used for patients with locally advanced unresectable disease who do not experience disease progression during induction therapy. The interferon-based regimen is also used as adjuvant therapy in resected patients, which we have previously reported[26].

It is noteworthy that 6 survivors suffered significant morbidities including recurrent cholangitis, liver abscess, malnutrition, second malignancy and small bowel perforation (Tables 2 and 3). In 4 out of 6 patients who died, death was not related directly to tumor: patient 6 died from a perforated duodenal ulcer and sepsis unrelated to tumor; patients 7 and 8 had recurrent biliary sepsis; patient 10 developed sepsis due to bacterial peritonitis in the setting of ascites. Long-term survivors often possess structural alterations to the pancreaticobiliary anatomy, are frequently exposed to endoscopic interventions and receive immunosuppressive therapies, making them susceptible to treatment-induced complications and infections. Hence, a close long-term follow-up is often indicated in these patients.

With regard to disease progression, 6 of the 11 non-resected long-term survivors experienced disease progression. Five of these received second-line therapy and responded favorably, even though 3 of these patients were given a regimen identical to the initial therapy (Tables 2 and 3). These tumors may exhibit characteristics that render them susceptible to cytotoxic effects of chemotherapy. Interestingly, tumor progression occurred most commonly in the local tumor bed which is similar to what has been observed in resected patients - when disease recurrence occurs 5 years after the surgery, it is more often local than distant[18]. Long-term survivors, whether or not resected, may have less propensity to develop distant

Table 3 Clinical characteristics of 11 non-resected ≥ 5-year survivors

Patient	Time to initial progression (mo)[1]	Site of initial progression[1]	Therapy used for progression	Alive	Overall survival (yr)[2]	Cause of death	Clinical progress
1	15.3	Supraclavicular lymph node	5-FU + R	Yes	8.6	N/A	Recurrence in the supraclavicular lymph node was treated with 5-FU and radiotherapy. No further therapy was required and the patient remains alive 8.6 yr after diagnosis
2	37.1	Peritoneum	GD	Yes	5.9	N/A	Prophylactic double bypass surgery was performed at diagnosis. Initial chemotherapy was interrupted by ischemic bowel but completed subsequently. Peritoneal recurrence was treated with GD intermittently over 2.5 yr. The patient remains alive 5.9 yr after diagnosis
3	N/A	N/A	N/A	Yes	5.7	N/A	Resection was attempted however aborted due to occluded common hepatic artery. No major medical issues following initial chemoradiation was noted and the patient remains alive 5.7 yr after diagnosis
4	N/A	N/A	N/A	Yes	5.6	N/A	After initial therapy, patient developed gastric outlet and biliary obstruction requiring endoscopic and radiology interventions. Patient then received salvage therapy for 4 mo with gemcitabine-based combination therapy. This was later stopped as imaging demonstrated stable disease. The patient remains alive 5.6 yr after diagnosis
5	N/A	N/A	N/A	Yes	5.5	N/A	Patient has a remarkable family history of cancers in multiple family members and *BRCA2* gene mutation. Initial therapy was stopped prematurely due to financial issue. GD was re-commended as maintenance therapy for 5 mo. No further therapy was required afterwards and the patient remains alive 5.5 yr after diagnosis
6	N/A	N/A	N/A	No	8.4	Perforated duodenal ulcer and sepsis	Patient's progress was hampered by recurrent cholangitis requiring multiple biliary interventions and second malignancies (breast cancer at 5 yr after the initial diagnosis of pancreatic cancer, recurrent hypernephroma metastasizing to the bone at 7 yr after the initial diagnosis)
7	87.3	Local, mesenteric lymph node, peritoneum	Palliation	No	7.5	Biliary sepsis	After initial therapy, patient developed gastric outlet and biliary obstruction treated with endoscopic stent placement and double bypass surgery. The patient suffered from chronic pain and malnutrition necessitating supplemental feeding and narcotics *via* intrathecal pump, respectively. Seven years after the diagnosis, the patient developed bowel perforation at the site of previously placed duodenal stent and died from sepsis
8	N/A	N/A	N/A	No	6.4	Biliary sepsis	The tumor was resectable but cirrhosis discovered incidentally at laparoscopy precluded patient from resection. The patient developed bladder cancer (2 yr after diagnosis) treated with resection and chemotherapy, and chronic renal failure due to glomerulonephritis (6 yr after diagnosis) requiring hemodialysis. The patient underwent multiple interventions for recurrent cholangitis
9	37.1	local, liver	GD	No	6.4	Disease progression	ERCP at the time of diagnosis was complicated by perforated duodenal diverticulum requiring surgery for the repair of perforation and double bypass surgery at the same time. GD was used intermittently to treat disease progression over 5 yr until death
10	33.6	local	GD	No	6.2	Sepsis due to ascitic fluid infection/ peritonitis	Prophylactic double bypass surgery was performed at diagnosis. Patient was later treated with GD and investigational monoclonal antibody therapy for local and peritoneal recurrence, respectively
11	27.8	Lung	GD	No	5.2	Disease progression	Patient experienced recurrent cholangitis and liver abscess requiring multiple biliary interventions

[1]Disease progression was defined as the presence of recurrent tumor in the pancreas (local) or development of new metastatic focus in a distant organ (systemic) coinciding with patient's symptom and/or rise in Ca 19-9. Time to initial progression was measured from initial diagnosis (the date with the first pathological evidence of pancreatic adenocarcinoma) to the date of first progression; [2]Overall survival was calculated from initial diagnosis to the date of death. The actual date of death was confirmed in all patients by assessing the cancer registry. N/A: Not applicable; GD: Gemcitabine and doxetaxel; 5-FU + R: 5-fluorouracil and radiotherapy.

metastases.

Long-term survival in our non-resected patients implies that the survival may be determined by factors other than the stage of the cancer and treatment, such as tumor biology and host immunity. The tumors in long-term survivors may have a lower number of cancer-initiating cells (aldehyde dehydrogenase-high), cells that possess properties of self-renewal, tumor initiation, and differentiation[44]. Other studies have shown a poorer prognosis in patients who have more mesenchymal tumors, which express vimentin, fibronectin, and N-cadherin[45,46], suggesting that long-term survivors may have tumors with more epithelial features. Although many gene amplifications and deletions, epigenetic changes and proteomic abnormalities have been identified, the biology of pancreatic cancer is still poorly understood. Of particular interest in this regards is that patient 5 (Tables 2 and 3) had a remarkable family history of breast and gastrointestinal cancer and a BRCA2 mutation, a phenotype known to possess unusual sensitivity to PARP-1 inhibitors and platinum based drugs[47,48].

On the other hand, there has been increased interest in the role of host immunity in the outcomes of pancreatic cancer. α-Enolase (ENO1) is an enzyme expressed on the surface of pancreatic cancer cells responsible for cell migration and metastasis. A recent study showed that there was a decreased number of ENO1-specific T helper 17 cells, which have anti-tumor effector function, in patients with pancreatic cancer. Conversely, elevated levels of ENO1-specific regulatory T cells, which lead to the inhibition of the effector T cells and promotes tumor progression, were noted in pancreatic cancer patients[49]. These results may be relevant for the design of novel immunotherapies for pancreatic cancer, include passive immunotherapeutic approaches, such as the use of effector cells generated in vitro, and active immunotherapeutic strategies, whose goal is to stimulate an antitumor response in vivo, by means of vaccination[50].

In addition, the presence of a systemic inflammatory response has been postulated as having prognostic significance in malignancy and high NLR was independently associated with worse outcome in pancreatic cancer[51]. In our small cohort of survivors, a wide range of NLR was observed and further validation in a large population of patients with pancreatic cancer would be useful to determine its predictability in long-term survival.

There are several limitations to our study. Firstly, as our institution is a tertiary referral center, patients may not be an accurate representation of the average patient population. There may be a tendency for patients who are younger with good performance status to be referred and selected for treatment (referral and selection bias). Secondly, histology subtypes and lymphocyte infiltrations in the tumors of the long-term survivors are unknown due to the inadequacy of FNA samples. However, all specimens from the 11 non-resected survivors were re-reviewed by a pathologist to confirm the presence of adenocarcinoma and exclude autoimmune pancreatitis, neuroendocrine tumors and cancers arising from the bile duct, gallbladder and duodenum. In addition, every CT scan used to clinically stage patients in the 11 non-resected survivors was re-reviewed by an experienced pancreas cancer surgeon who had no involvement in the original care of the patients. Thirdly, our study population was predominantly Caucasian and additional studies in patients of other ethnic background are warranted. Lastly, we do not have detailed data for non-resected patients who survived less than 5 years for comparison, which may allow us to identify predictors of long-term survival.

In conclusion, a rare subset of patients with pancreatic adenocarcinoma achieve long-term survival without resection. However, morbidities unrelated to disease were frequently encountered and a close long-term follow-up is warranted in these patients. Our series implies that factors other than the stage of the disease such as tumor biology and host immunity may play a key role in the outcome of patients afflicted with pancreatic cancer. Continued investigation of clinical and molecular prognostic markers is therefore needed in the future.

COMMENTS

Case characteristics

The authors report eleven patients with pancreatic adenocarcinoma who did not undergo curative resection, were treated with oncologic therapy alone and survived 5 years or more.

Clinical diagnosis

Patients presented with pain, weight loss and/or jaundice. Seven patients required biliary stent placement due to obstructive jaundice at the time of initial presentation.

Laboratory diagnosis

The median serum Ca 19-9 was 72 U/mL and 8 patients had a raised Ca 19-9 at the time of diagnosis. Serum bilirubin was raised in 6 patients at the time of diagnosis.

Imaging diagnosis

Staging computed tomography (CT) scan was performed in all patient. CT scans were re-reviewed by an experienced pancreas cancer surgeon who had no involvement in the original care of the patients.

Pathological diagnosis

Patients had biopsy-proven pancreatic adenocarcinoma. The original histopathology slides of all patients were re-reviewed by a single faculty gastrointestinal pathologist to confirm the presence of pancreatic adenocarcinoma.

Treatment

All patients received chemotherapy or chemoradiation, including gemcitabine and docetaxel combination, 5-fluorouracil (5-FU) and radiotherapy or Virginia Mason Protocol chemoradiation regimen consisting of interferon-alpha-2b, 5-FU, cisplatin and radiotherapy. Six patients experienced disease progression, in whom 5 received second-line therapy and responded favorably. Six patients suffered from significant morbidities including recurrent cholangitis, second

malignancy, malnutrition and bowel perforation. These patients required frequent endoscopic and radiological interventions.

Experiences and lessons

A rare subset of patients with pancreatic adenocarcinoma achieve long-term survival without resection but morbidities unrelated to the primary cancer were frequently encountered and a close long-term follow-up is warranted in these patients.

Peer-review

This manuscript report clinical features of long-term survivors of pancreatic adenocarcinoma without curative resection. This case series are well written. The features of long-survivors are detailed.

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CASE REPORT

Case report of Graves' disease manifesting with odynophagia and heartburn

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Author contributions: Ivashkin V designed the report; Evsyutina Y was attending doctor for the patient; Trukhmanov A, Storonova O and Godjello E were performed image diagnosis; Ivashkin V organized the report; and Evsyutina Y wrote paper.

Institutional review board statement: This case report was approved by the Sechenov First Moscow State Medical University Institutional Review Board.

Informed consent statement: The patient involved in this study gave his written informed consent authorizing use and disclosure of his protected health information.

Conflict-of-interest statement: All the authors have no conflicts of interests to declare.

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Abstract

Graves' disease is an autoimmune disease, which can manifest with a variety of extrathyroidal clinical syndromes like ophthalmopathy, pretibial myxedema (dermopathy), acropathy, cardiomyopathy, and encephalopathy. Though quite rare, this disease can also manifest with gastrointestinal symptoms such as dysphagia, heartburn, nausea, vomiting and diarrhea. We report a clinical case of Graves' disease manifesting with dysfunction of the esophagus and heartburn in a 61-year-old man. In the muscular layer of the esophagus we found dystrophic changes led to its atony, which was documented by endoscopy and high-resolution manometry. The pathology features of esophageal symptoms were: focal proliferation of the basal cells, vascular distension, and dystrophy of the epithelial cells. Antithyroid treatment led to decrease of all clinical symptoms after 5 d of Thiamazole administration. Complete restoration of peristalsis in the esophagus, according to manometry, was observed in 1 mo after initiation of treatment.

Key words: Graves' disease; Heartburn; Odynophagia; Esophagopathy; Dysfunction

Core tip: We report a clinical case of Graves' disease manifesting with dysfunction of the esophagus and heartburn in a 61-year-old man. In the muscular layer of the esophagus we found dystrophic changes led to its atony, which was documented by endoscopy and

high-resolution manometry.

Evsyutina Y, Trukhmanov A, Ivashkin V, Storonova O, Godjello E. Case report of Graves' disease manifesting with odynophagia and heartburn. *World J Gastroenterol* 2015; 21(48): 13582-13586 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13582.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13582

INTRODUCTION

Graves' disease (diffused toxic goiter) is an autoimmune disease which is characterized by the presence of circulating autoantibodies that bind to and stimulate the TSH receptor, which eventually leads to hyperthyroidism and goiter[1]. It's a rare disease which can manifest with gastrointestinal symptoms like dysphagia, nausea, vomiting and diarrhea[2]. We present a clinical case of Graves' disease manifesting with odynophagia and heartburn in a 61-year-old man.

CASE REPORT

A 61-year-old-man was admitted to the Clinic of the First Moscow State Medical University on May 2014 with the following complaints: odynophagia arising on swallowing of both solid and liquid food, severe heartburn, tachycardia, dyspnea, general weakness, and weight loss of 15 kg over the past year.

From the medical history, it was noted that the patient was sick since June 2013, when he first noted the appearance of heartburn, which manifested in 15-20 min after eating and odynophagia, which arose on swallowing solid food. Based on the results of the conducted esophagogastroduodenoscopy (EGD), he was diagnosed with distal esophageal reflux and was prescribed therapy with proton pump inhibitor (PPI) -Omeprazole 20 mg per day and antacids. The prescribed therapy did not produce any positive effect as the patient noted an increase in the severity of the heartburn and odynophagia, which began to worsen further and emerged even while swallowing liquid food. Following a comprehensive survey performed in October 2013, a complete blood analysis was done, all his blood parameters were within normal values, in the general analysis of urine and feces-all indicators were normal too. A re-esophagogastroduodenoscopy was done which revealed distal reflux esophagitis, on basis of which the diagnosis was changed to refractory gastroesophageal reflux disease and the dose of omeprazole was increased to 20 mg 2 times a day. However, the patient's condition progressively worsened-with heartburn and odynophagia the patient also experienced general weakness, tachycardia and dyspnea on exertion. In January 2014 after a re-endoscopy performed on the patient it was decided to change the PPIs from omeprazole to rabeprazole 20 mg 2 times a day and to this was added itopride 50 mg three times a day. This change of therapy did not produce any positive clinical response, the patient noted a strong heartburn after every meal, in addition to the above mentioned symptoms, and the patient also lost 15 kg of weight during the course of the disease.

On admission to the clinic, the general condition of the patient was relatively satisfactory. BMI was 19.6 kg/m^2. Skin and visible mucous layers-physiological color and moist. On auscultation, vesicular breathing, no pathologic lung sounds were heard. Cardiac sounds were rhythmic. Pulse 90 beats per minute. BP 130/70 mmHg. Upon abdominal palpation-soft and painless in all areas. Liver could be palpated along the edges of the right costal arch. The thyroid gland was not enlarged, nodules were not palpable. The patient tested positive for symptom Marie (tremor of the fingers of the hand). No edema.

On examination: in complete analysis of blood-hemoglobin 143 g/L, erythrocytes 5.27 × 10^{12}, leukocytes 6.34 × 10^9, platelets 258 × 10^9, ESR 10 mm/h. In the biochemical analysis of blood: total protein 76.0 g/L, albumin 47.0 g/L, creatinine 64.0 U/L, glucose 5.9 mmol/L, sodium 145.0 mmol/L, potassium 4.8 mmol/L, calcium 2. 53 mmol/L, total cholesterol 4.9 mmol/L, creatine kinase 50.0 U/L, AST 24.0 U/l, ALT 40.0 U/L, total bilirubin 14.5 U/L, iron 17.63 mmol/L. Overall urine analysis and stool showed no abnormalities. According to the electrocardiogram-sinus tachycardia with a heart rate of 96 beats per minute, intervals PQ 0.18', QRS 0.08', QT 0.34' and normal electrical axis of the heart. Given tachycardia and dyspnea on exertion for a long period of time, the patient underwent echocardiography, the results of which revealed atherosclerosis of the aorta, mitral regurgitation of 1st degree, contractile function of which is satisfactory (ejection fraction 61%, stroke volume 80 mL). According to the results of the endoscopy it was noted that the patient suffered from esophageal dysmotility problem with severe hypotonia of the upper and middle third and segmental spasm in the lower third of the esophagus. Based on the results of the endoscopy and long-term course of the disease a decision was taken to do multiple biopsies of the esophagus. Morphological examination of the biopsy specimens revealed the following changes: Focal proliferation of the basal cells, plethora of vessels and stasis of erythrocytes in the vessels of lamina propria papillae, and dystrophy of the epithelial cells (Figure 1A and B).

The patient also underwent high-resolution manometry, the results of which: the resting pressure upper esophageal sphincter (UES) 47 mmHg (normal 46-81 mmHg), resting pressure lower esophageal sphincter (LES) 29 mmHg (normal 10-35 mmHg), the opening of the UES and LESs in response to swallowing of water was normal. In thoracic esophageal peristaltic contractions were absent. This marked an increase

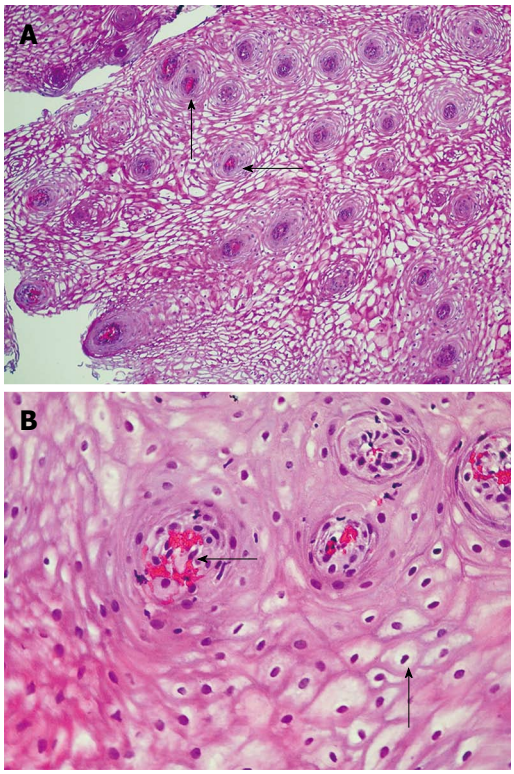

Figure 1 Stratified squamous non-keratinized epithelium stained with haematoxylin and eosin. A: Focal proliferation of the basal cells (arrow, magnification × 100); B: Plethora of vessels and stasis of erythrocytes in the vessels of lamina propria papillae (arrow), dystrophy of the epithelial cells (arrow, magnification × 400).

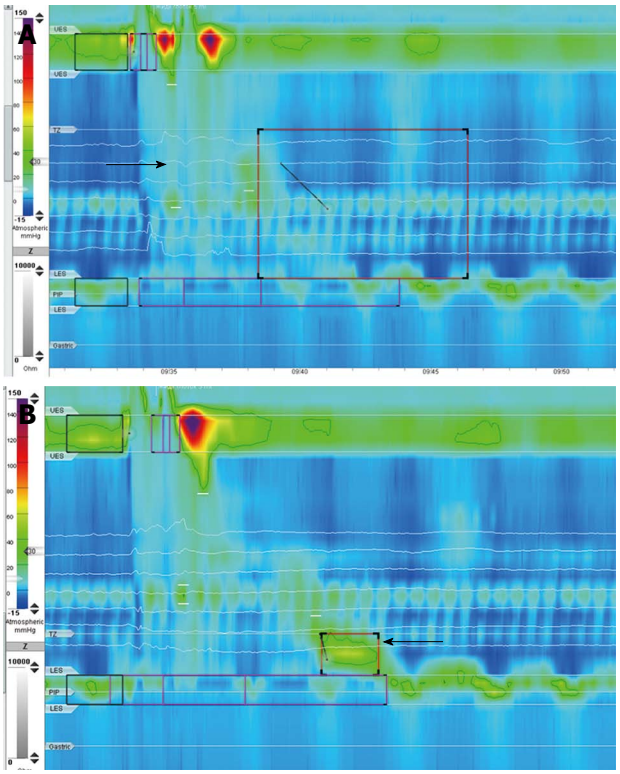

Figure 2 High-resolution manometry. A: Lack of peristaltic contractions in the thoracic esophagus (arrow); B: The intraesophageal pressure was increased during the passage of the liquid bolus and 1-2 s later in the distal part of the esophagus was registered the spastic contraction (arrow).

in the intraesophageal pressure at the time of the passage of the liquid bolus and 1-2 s later in the distal part of the esophagus was registered spastic contraction with low amplitude (19 mmHg) (Figure 2A and B).

Taking into account the patient's complaints: tachycardia, dyspnea, general weakness, weight loss, as well as positive symptom of Marie, the patient' s blood was checked for the levels of the thyroid hormones. The analysis revealed the following changes: thyrotropin-releasing hormone 0.0025 mU/L (normal 0.4-4.0 mU/L), triiodothyronine 14.3 pmol/L (normal 3.5-6.5 pmol/L), thyroxine 39.3 pmol/L (normal 11.5-23.2 pmol/L), antibodies to the TSH receptor 7.6 IU/L (normal 0-1 IU/L), anti-thyroid peroxidase antibodies 39 IU/L (normal 0-30 IU/L). Thus, the patient showed signs of marked thyrotoxicosis. In order clarify the exact causes of thyrotoxicosis an ultrasound of the thyroid gland was done, the results of which showed: enlargement of the thyroid gland, the right lobe 22 mm × 23 mm × 53 mm, left lobe 21 mm × 23 mm × 51 mm isthmus 4.5 mm, thyroid volume 24.6 cm^3; the contours of the gland capsule were clear, smooth, and the structure of the parenchyma was heterogeneous with the formation of zones reduced and increased echogenicity. To eliminate the diagnosis of toxic adenoma was done thyroid scintigraphy with 99th Tc-pertechnetate. The intake of radioactive isotope of the thyroid gland were significantly higher than normal (15.1%) (normal 3%-7%), which was of uneven-diffused character; was observed asymmetric distribution of radiopharmaceuticals between the lobes, with some of its predominance in the right lobe; local sources of hypo-and hyperfixation indicator were not detected.

Based on the results of this survey, the patient was diagnosed with 1st degree Graves' disease of average severity in the stage of decompensation and was prescribed treatment with antithyroid drug Thiamazole 10 mg 3 times a day, and β-blocker Bisoprolol 2.5 mg in the morning. Following the therapy after 5 d, the patient noted a decrease in heartburn and odynophagia, and a complete disappearance of the above mentioned symptoms after a month.

The patient underwent a re-manometry a month later. There was a complete restoration of peristalsis in the thoracic region of the esophagus; he showed no signs of esophageal spasm (Figure 3).

The patient continues to receive antithyroid drug therapy at a dose of 10 mg 3 times a day, on achieving the euthyroidic state, its planned to reduce the daily dose by 5 mg every week to a maintenance dose of 10 mg per day.

DISCUSSION

Graves' disease can manifest with a variety of extra-

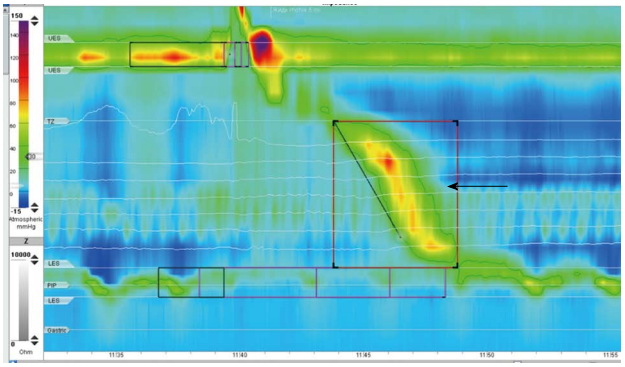

Figure 3 High-resolution manometry (against the background of antithyroid drug therapy) in the thoracic region of the esophagus is noted the normal esophageal peristalsis in response to swallowing of water (arrow).

thyroidal clinical syndromes like ophthalmopathy, pretibial myxedema (dermopathy), acropathy[3], cardiomyopathy[4], encephalopathy[5]. Though quite rare, this disease can also manifest with gastrointestinal symptoms such as dysphagia, heartburn, nausea, vomiting and diarrhea[2].

The lesion of the esophagus in patients with Graves' disease may be caused by various reasons. The first being extrinsic enlargement of the thyroid gland, however, for our patient, his thyroid gland was not that enlarged, eliminating the above mentioned mechanism. On the other hand, the excess production of thyroid hormones can lead to the dysfunction of bulbar muscles, which is manifested by symptoms such as dysphagia, dysarthria and dysphonia[6,7]. As for our patient, none of the above mentioned symptoms were present, indicating a completely different mechanism of esophageal complaints.

In the present case, its most likely that there is is a direct effect of excess amount of thyroxine and triiodothyronine on the mitochondria of the muscle cells of the esophagus, leading to the dissociation of oxidative phosphorylation, which in turn causes a disruption of synthesis of the ATP and deenergization tissues[8]. Triiodothyronine and thyroxine are *mitochondria*-targeting protonophores that can cross the lipid bilayer membrane and transmit the protons through the inner membrane back to the matrix. It leads to loss of *electrochemical gradient* and cessation of ATP synthesis[8]. Due to the reduction of oxidative phosphorylation *dystrophic lesions in esophageal muscles and atonic esophagus were* developed.

In this patient dystrophic changes in the muscular layer of the esophagus led to its atony, which was confirmed by endoscopy and manometry, and clinical equivalent of this changes was odynophagia.

Another key symptom of the patient was strongly expressed heartburn. In this case, it could be due to the ineffective esophageal motility-the patient had a complete absence of peristaltic contractions in the thoracic region of the esophagus-resulting in slower bolus clearance (the time required for the esophagus to empty the contents which had entered it by means of primary and secondary peristalsis). The slower bolus clearance is accompanied by a longer delay in the esophageal reflux which creates a pressure high enough for the LES and as a result is clinically accompanied by heartburn.

The histology of esophageal symptoms were degenerative changes in the esophageal mucosa, which were expressed by focal proliferation of the basal cells, plethora of vessels and stasis of erythrocytes in the vessels of lamina propria papillae, and dystrophy of the epithelial cells.

Therefore striking confirmation of the fact that the esophageal symptoms with such a nature of occurrence, had a rapid clinical response to antithyroid drug therapy and there was a complete recovery of the esophageal peristalsis which was confirmed by the results of the esophageal manometry.

In conclusion, it should be noted that Graves' disease can manifest not only with the thyrotoxic cardiomyopathy, encephalopathy, opthalmopathy, dermopathy and acropathy, but also with thyrotoxic esophagopathy. Odynophagia can be revealed on the thyrotoxic esophagopathy as well as previously described dysphagia and heartburn.

COMMENTS

Case characteristics

A 61-year-old-man with odynophagia arising on swallowing of both solid and liquid food, severe heartburn, tachycardia, dyspnea, general weakness, and weight loss of 15 kg over the past year.

Clinical diagnosis

Graves' disease, 1st degree of average severity in the stage of decompensation.

Differential diagnosis

Toxic multinodular goiter, Toxic adenoma, Hashimoto Thyroiditis.

Laboratory diagnosis

The patient showed signs of thyrotoxicosis.

Imaging diagnosis

Peristaltic contractions were absent in thoracic esophageal, the intake of radioactive isotope of the thyroid gland were significantly higher than normal.

Pathological diagnosis

The focal proliferation of the basal cells, plethora of vessels and stasis of erythrocytes in the vessels of lamina propria papillae, and dystrophy of the epithelial cells.

Treatment

The antithyroid drug Thiamazole 10 mg 3 times a day, β-blocker Bisoprolol 2.5 mg a day.

Related reports

Graves' disease can manifest with a variety of extrathyroidal clinical syndromes. Though quite rare, this disease can also manifest with gastrointestinal symptoms such as dysphagia, heartburn, nausea, vomiting and diarrhea.

Term explanation

Thyrotoxic esophagopathy is a rare extrathyroidal syndrome of Graves' disease, that can manifests with dysphagia, odynophagia, and heartburn.

Experiences and lessons

Graves' disease can manifest not only with the thyrotoxic cardiomyopathy, encephalopathy, opthalmopathy, dermopathy and acropathy, but also with thyrotoxic esophagopathy. Odynophagia can be revealed on the thyrotoxic esophagopathy as well as previously described dysphagia and heartburn.

Peer-review

This is a case report about rare clinical manifestations of Graves' disease. The whole manuscript is simple but interesting, including the consultation process, examination results, and treatment outcome.

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CASE REPORT

Novel endoscopic over-the-scope clip system

Elia Armellini, Stefano Francesco Crinò, Marco Orsello, Marco Ballarè, Roberto Tari, Silvia Saettone, Franco Montino, Pietro Occhipinti

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Author contributions: Armellini E and Crinò SF wrote the paper and contributed equally to the manuscript; Armellini E, Orsello M, Crinò SF and Occhipinti P performed the endoscopic procedures; Ballarè M, Tari R and Saettone S collected and alalysed the data; we are indebted to Montino F for editing the movie clips.

Institutional review board statement: This case report was exempt from the Institutional Review Board standards at Azienda Ospedaliero Universitaria "Maggiore della Carità" in Novara.

Informed consent statement: The patients involved in this study gave them written informed consent authorizing use and disclosure of them protected health information.

Conflict-of-interest statement: All the authors have no conflicts of interests to declare.

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Abstract

This paper reports our experience with a new over-the-scope clip in the setting of recurrent bleeding and oesophageal fistula. We treated five patients with the over-the-scope Padlock Clip™. It is a nitinol ring, with six inner needles preassembled on an applicator cap, thumb press displaced by the Lock-It™ delivery system. The trigger wire is located alongside the shaft of the endoscope, and does not require the working channel. Three patients had recurrent bleeding lesions (bleeding rectal ulcer, post polypectomy delayed bleeding and duodenal Dieulafoy's lesion) and two patients had a persistent respiratory-esophageal fistula. In all patients a previous endoscopic attempt with standard techniques had been useless. All procedures were conducted under conscious sedation but for one patient that required general anaesthesia due to multiple comorbidities. We used one Padlock Clip™ for each patient in a single session. Simple suction was enough in all of our patients to obtain tissue adhesion to the instrument tip. A remarkably short application time was recorded for all cases (mean duration of the procedure: 8 min). We obtained technical and immediate clinical success for every patient. No major immediate, early or late (within 24 h, 7 d or 4 wk) adverse events were observed, over follow-up durations lasting a mean of 109.4 d. One patient, treated for duodenal bulb bleeding from a Dieulafoy's lesion, developed signs of mild pancreatitis 24 h after the procedure. The new over-the-scope Padlock Clip™ seems to be simple to use and effective in different clinical settings, particularly in "difficult" scenarios, like recurrent bleeding and respiratory-oesophageal fistulas.

Key words: Therapeutic endoscopy; Over-the-scope

clip; Non variceal gastrointestinal bleeding; Endoscopic hemostasis; Respiratory-oesophageal fistula; Fistula closure

Core tip: We report our experience with the novel over-the-scope Padlock Clip™, applied in five patients in the clinical settings of oesophageal fistulas and refractory gastrointestinal bleeding. The Padlock Clip™ has shown reliable closure of wall defects in a porcine survival study, although clinical usage remains limited. As yet, there are no published data regarding its application in the management of non-variceal gastrointestinal bleeding. We provide a comprehensive iconographic documentation and two videos showing its application. We also accurately describe the device and its release system, pointing out the differences with the well-known over-the-scope clip system.

Armellini E, Crinò SF, Orsello M, Ballarè M, Tari R, Saettone S, Montino F, Occhipinti P. Novel endoscopic over-the-scope clip system. *World J Gastroenterol* 2015; 21(48): 13587-13592 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i48/13587.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i48.13587

INTRODUCTION

Given improvements in therapeutic endoscopy techniques and devices, endoscopists are being confronted with clinical complex situations such as perforations, anastomotic leakage and fistulas.

The incidence of perforations in diagnostic endoscopies is approximately 0.03%, with a mortality rate of up to 17% and a morbidity rate of up to 40%[1]; when interventional procedures are included, the incidence of perforations increases to 5%[2]. It is well known that a surgical approach to perforations, as well as to respiratory-esophageal fistulas, has relevant morbidity and mortality. This is the reason that prompted endoscopists to deal with such conditions[3].

Another complex and demanding condition is non-variceal gastrointestinal bleeding (NVGIB), which cannot be managed using conventional endoscopic therapies. Deeply penetrating fibrotic ulcers are an example of such a condition. Despite adequate initial endoscopic therapy, recurrent upper gastrointestinal (GI) bleeding can occur in up to 24% of high-risk patients[4]. Surgery or selective radiological embolization may represent the salvage therapeutic option, but surgical management in such patients is often difficult, time-consuming and related to non-negligible levels of morbidity and mortality[5].

In past years, an over-the-scope clip system (OTSC, Ovesco Endoscopy AG, Tubingen, Germany) has been tested in large studies for closing GI defects[6] and in smaller studies for refractory bleeding[7], so its clinical efficacy has been established. Recently a new OTS clip (Padlock Clip™, Aponos Medical, Kingston, NH, United States) has been developed and tested in porcine models for closing wall defects[8,9]. As of yet, data regarding its application in clinical practise are lacking.

The aim of this series is to describe our experience with the novel OTS Padlock Clip™.

CASE REPORT

The Padlock Clip™ is a nitinol ring, with six inner needles preassembled on an applicator cap (available for 9.5-11 mm scope tips), thumb press displaced by the Lock-It™ delivery system (Aponos Medical, Kingston, NH, United States). The trigger wire is located alongside the shaft of the endoscope, and does not require the working channel (Figure 1, Video 1).

This design allows for more efficient suction that is sufficient to ensure tissue adhesion to the instrument tip into the cap, not requiring other instruments. Moreover, the suction of blood and secretions is always ensured.

When the clip is deployed, it instantly springs back to its original form, an 11 mm hexagonal ring, and gathers, folds and compresses the tissue. Proprietary radial compression technology provides even 360° tissue compression. Precise tissue controllers limit penetration and moderate tissue-on-tissue pressure. Effective tissue closure is obtained that is resistant to GI pressures, while still maintaining blood flow to promote healing.

In this series, we included all of the patients treated using the new Padlock Clip™ between October 2014 and December 2014. An overview of the patient demographic data, etiology, clinical condition, comorbidities and previous treatment attempts is listed in Table 1.

Case 1: Post-mucosectomy delayed bleeding

A 61-year-old patient with multiple comorbidities underwent a colonoscopy for positive faecal blood; a large laterally spreading tumor in the rectum was diagnosed and removed by piecemeal endoscopic mucosal resection (EMR). One week later, massive rectal bleeding and hypotension occurred. After an unsuccessful conventional endoscopic hemostasis (adrenaline injection plus endoscopic clipping), we decided to apply the Padlock Clip™ to obtain immediate control of the bleeding (Video 2). Clip retention was documented during the three-month endoscopic control (Figure 2) without clinical consequences.

Case 2: Solitary rectal ulcer bleeding

A solitary rectal ulcer showing overt bleeding was diagnosed in an 80-year-old male with hemorrhagic shock. Due to the large, deeply penetrating hard base

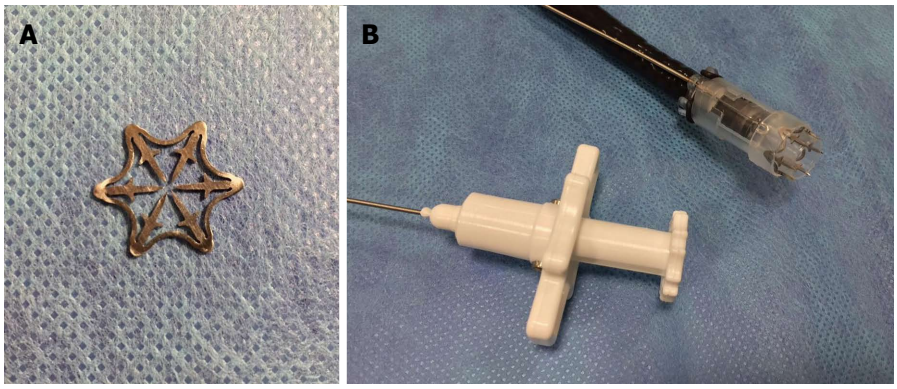

Figure 1 Hexagonal Padlock Clip™ (A) and the push-bottom Lock-It™ delivery system (B).

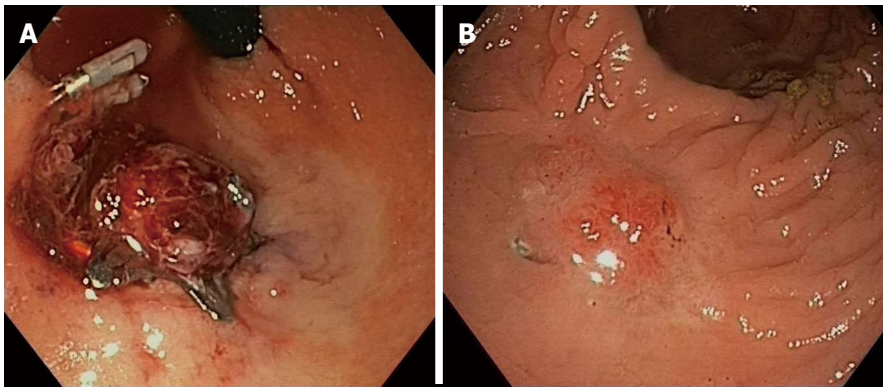

Figure 2 Post-polypectomy delayed bleeding. Permanent hemostasis was achieved by Padlock Clip™ application (A). At three-month endoscopic follow-up, persistence of the Padlock Clip™ under hyperplastic mucosal proliferation (B).

Table 1 Patient demographic and clinical information

No.	Age, yr	Etiology	Clinical condition	Comorbidities	Previous treatment
1	61	Endoscopic mucosal resection	Delayed rectal bleeding	Hypertension, HF, RF	Endoclip, injective therapy
2	80	Solitary rectal ulcer	Rectal bleeding	Hypertension	Endoclip, injective therapy
3	85	Duodenal Dieulafoy lesion	Duodenal bleeding	Hypertension, prosthetic aortic valve, HF	Injective and thermal therapy
4	53	Mediastinal lymphoma	Broncho-esophageal fistula	None	Endoclip
5	66	Post-laringectomy radio-chemoteraphy	Tracheo-esophageal fistula	COPD, HIV+, hypertension, HF	Endoclip, salivary stent

HF: Chronic heart failure; RF: Renal failure; COPD: Chronic obstructive pulmonary disease.

of the ulcer, we considered that using a conventional through-the-scope clip would likely be unsuccessful. Injective and thermal devices were insufficient for controlling bleeding and, therefore, a Padlock Clip™ was deployed obtaining durable hemostasis (Figure 3).

Case 3: Duodenal Dieulafoy lesion

The patient was referred for shock due to massive gastrointestinal bleeding on the third day after major cardiac surgery for prosthetic thoracic aorta replacement. An inferior duodenal bulb wall Dieulafoy lesion was treated with adrenalin injection and argon plasma coagulator. Twenty four hours later, massive rebleeding occurred and was successfully controlled by a Padlock Clip™.

Case 4: Broncho-esophageal fistula

A 53-year-old male exhibiting recent onset of a cough and fever underwent a thoracic and abdominal computed tomography (CT) scan that revealed a bulky mediastinal lymphoma complicated by a broncho-esophageal fistula. An upper endoscopy was performed and after a failed attempt with traditional clipping, the absence of esophageal stenosis and the adequate accessibility of the esophageal edge prompted us to use the Padlock Clip™ system (Video 3). A tracheal prosthesis was then placed. One month later, persisting the bulky lymphoma, two new fistula

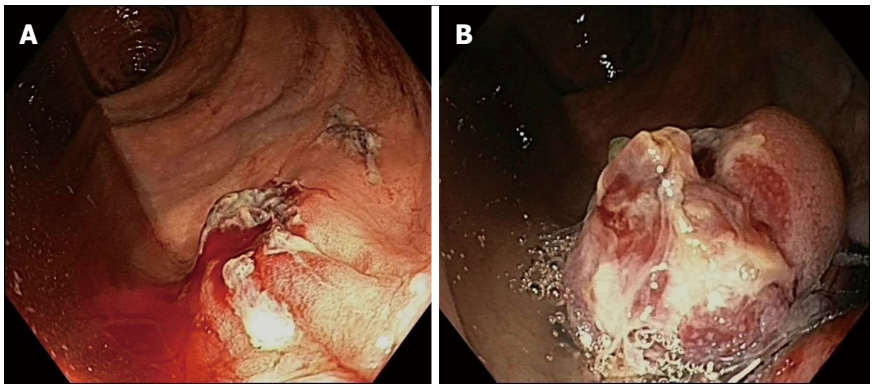

Figure 3 Rectal ulcer before (A) and after Padlock Clip™ application (B).

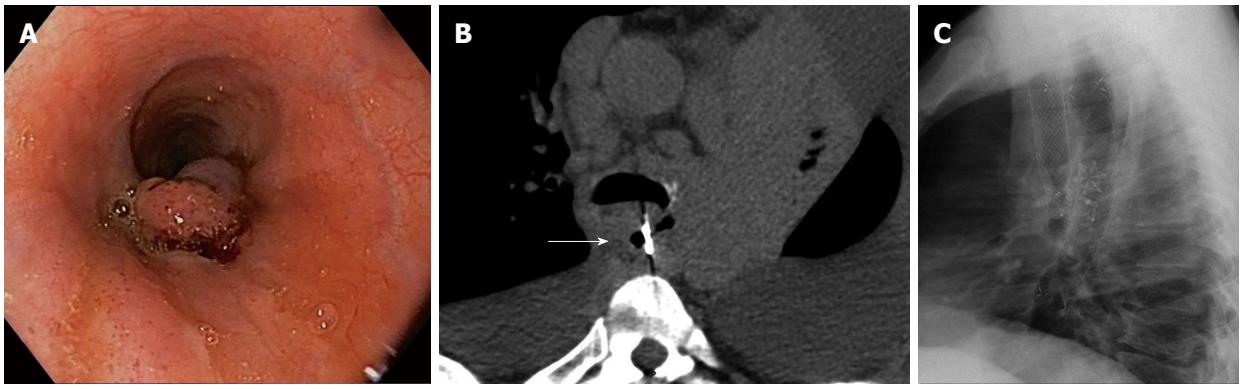

Figure 4 Endoscopic view after the Padlock Clip™ displacement (A). Computed tomography control (B) showing the clip (white arrow) positioned at the esophageal edge of the fistula. Rx control (C) after the esophageal self-expandable metal stent release.

tracts developed proximally and distally to the first one; we accordingly implanted a fully covered, self-expandable metal stent (Figure 4). No clip retention was documented during follow-up.

Case 5: Tracheoesophageal fistula

A 65-year-old male received a laryngectomy, followed by radio-chemotherapy for laryngeal cancer. He was re-admitted to our hospital because of recurrent bronchitis and cough. A diagnosis of a tracheoesophageal fistula was achieved. After several therapies failed, we successfully used the Padlock Clip™ at the esophageal edge of the fistula.

DISCUSSION

All patients were male with a mean age of 69 years. Conscious sedation was used for four out of the five patients; one (case 3) required general anaesthesia due to multiple comorbidities. All patients had already been treated with standard endoscopic techniques, which had failed. We used one Padlock Clip™ for each patient in a single session. Simple suction was enough in all of our patients to obtain tissue adhesion to the instrument tip. A remarkably short application time was recorded for all cases (mean duration of the procedure: 8 min). We obtained technical and immediate clinical success for every patient (Table 2). Bleeding control was persistent. No rescue therapies, surgeries or interventional radiologies were necessary. The fistulous tract was closed in both of our patients. However, in patient number 4, we observed two new broncho-esophageal fistulas after one month due to the persistence of the mediastinal colliquated nodes. It is worth noting that the recurrences occurred in close proximity, below and on top of the first opening of the fistula, which showed the persistence of the fully deployed Padlock Clip™. Therefore, we decided to place an esophageal fully covered, self-expandable metal stent. No major immediate, early or late (within 24 h, 7 d or 4 wk) adverse events were observed, over follow-up durations lasting a mean of 109.4 d. Patient number 3, treated for duodenal bulb bleeding from a Dieulafoy lesion, developed signs of mild pancreatitis 24 h after the procedure. An abdominal CT-scan did not show transmural penetration of the clip.

In the case of rectal post-polypectomy delayed bleeding, the 3-mo endoscopic control revealed a mucosal proliferation hiding the Padlock Clip™. A biopsy of the redundant tissue was performed and revealed a foreign body granuloma (Figure 2).

The increasing number of gastrointestinal endoscopic examinations and more advanced operative techniques, including EMR and endoscopic submucosal

Table 2 Results of Padlock Clip™ application

No.	Technical success	Operation time (min)	Treatment outcome	Salvage procedure (surgery, interventional radiology)	Adverse events/morbidity	Follow up (d)
1	Yes	11	Persistent control of the bleeding	No	None	102
2	Yes	6	Persistent control of the bleeding	No	None	142
3	Yes	7	Persistent control of the bleeding	No	Mild pancreatitis	90
4	Yes	6	New fistulas development	No	None	60
5	Yes	8	Fistula healing	No	None	153

dissection (largely applied in the field of oncology) has yielded a larger number of adverse events. Perforation during upper and lower GI endoscopies is relatively rare, but its occurrence is associated with significant morbidity and potential mortality for the affected patient.

Furthermore, given recent advancements in equipment, the role of therapeutic endoscopies in dealing with complex clinical conditions, such as GI perforations and acquired fistulas, is being increasingly tested on an every-day basis.

In terms of GI bleeding, mechanical hemostatic methods have been shown to achieve a higher rate of permanent hemostasis than injections or thermal methods alone[10] and endoclips have been increasingly adopted. Unfortunately, the rebleeding rate after endoclipping ranges from 7% to 10%. The application of a standard through-the-scope clip on a large fibrotic ulcer base is often technically difficult or impossible. The OTSC clip has advantages of a larger jaw width and a greater strength[7].

To date, the most commonly used over-the-scope system is the OTSC, which has already been tested in large series for closure of GI defects[6] and in a smaller series for refractory bleeding. It has demonstrated good technical and clinical success due to its stronger closure than regular through-the-scope clips, due to its wider mouth and ability to grip larger amounts of tissue. The OTSC is associated with only a few adverse events[7].

In our series, we used the new Padlock over-the-scope system in three patients with refractory GI bleeding and two patients affected by esophageal fistulas, according to the indications already tested in large series for the OTSC. Although the working mechanism of the systems are similar, these two systems differ in some relevant details. Both are made of nitinol and do not preclude magnetic resonance imaging. However, the OTSC "bear claw" design differs from the round-hexagonal Padlock Clip™. Both are preloaded on an applicator cap. The OTSC is available in three diameters (11, 12, and 14 mm), two working depths (3 and 6 mm) and three types of teeth (atraumatic, traumatic and gastrostomy closure). On the other hand, the Padlock Clip™ is produced as a single model. Another important difference is in the delivery system. The OTSC deployment, similar to a band ligator, is achieved by triggering a string wire connected to a rotating hand wheel that is fastened to the entrance port of the working channel.

Invaginating the tissue inside the cap is obtained by suction alone or by the additional use of the OTSC Twin Grasper or the OTSC Anchor (both Ovesco Endoscopy AG), inserted through the operative channel. While the string wire alone does not hamper the suction trough the operative channel, this may be hampered by such additional devices.

The novel Padlock Clip™ is displaced with a thumb press using the Lock-It™ delivery system, which is located parallel to the endoscope, and does not require the working channel at all (Figure 1). No tissue grasper device is available for this over-the-scope clip.

The target tissue is invaginated inside the cap by simple suction, which allows also for continuous removal of blood and secretions.

The push-button system is not connected to the scope and the possibility of easy release by a nurse is necessary, but it prevents the endoscopists from having to remove their hands from the scope.

Up to now the novel Padlock Clip™ has been tested in survival porcine models for the closure of wall defects[8,9].

We used the Padlock Clip™ for the management of clinical conditions that have been largely tested to date with the OTSC: refractory NVGIB and GI wall defects. Standard endoscopic therapy was attempted in all of our cases; no major adverse events have been observed.

The OTSC remains attached longer than through-the-scope clips, likely due to its attachment to the submucosa and muscularis propria. In a recent series, the mean retention time of the OTSC was 28 d (range: 0-42 d)[11]. In a larger retrospective study of the OTSC system, clip retention occurred in approximately 2/3 of patients[12].

We documented Padlock Clip™ retention for three months in patient number 1 and for one month in patient number 4. We did not determine the mean retention time in the other patients.

In patient number 3, we used the Padlock Clip™ on a Dieulafoy lesion of the duodenum with technical and clinical success at stopping the bleeding. However, 24 h later, we observed the onset of mild pancreatitis, which was resolved with medical therapy. The connection between this event and the application of the clip is not clear in a critically ill patient, although the strict time

relationship may suggest a cause-effect relation; deep transmural penetration of the clip was excluded by an abdominal CT-scan.

Described cases, while confirming a role for the OTS systems in operative endoscopy (compression of large tissue areas, non-variceal bleedings difficult to control and lesions or perforations of the GI tract), show the Padlock Clip™ to be safe and efficient, with a short application time.

Particularly it has been effective in "difficult" clinical settings like recurrent bleeding and respiratory-oesophageal fistulas. Although other studies are needed, in our experience the new over-the-scope Padlock Clip™ seems to be simple to use and effective. This system may represent a new and alternative therapeutic option for the management of different clinical scenarios.

COMMENTS

Case characteristics

Three patients presented with overt gastrointestinal bleeding (one upper and two lower) and two patients presented with dysphagia and cough.

Clinical diagnosis

Bleeding patients had hypotension and hemorrhagic shock; fistula patients had pathologic thoracic physical examination.

Differential diagnosis

Other causes of upper and lower gastrointestinal bleeding (peptic ulcer, variceal bleeding, diverticulas); mediastinal malignancy, esophageal cancer.

Laboratory diagnosis

Severe anaemia in three patients affected by refractory bleeding and elevated white cells count and C-reactive protein in patients affected by respiratory-esophageal fistulas.

Imaging diagnosis

For fistula patients, thoracic computed tomography-scan and X-ray confirmed the fistulous tract.

Treatment

The authors treated all patients using the Padlock Clip™.

Related reports

Endoscopic over-the-scope clips (OTS) systems have showed good performance in the treatment of recurrent bleeding and oesophageal fistulas. Few experience with the new over-the-scope Padlock Clip™ are reported.

Term explanation

OTS are endoscopic devices loaded on the tip of the scope and not passed through the scope as for traditional endoscopic clips.

Experiences and lessons

OTS is effective in different clinical settings, particularly in “difficult” scenarios like recurrent bleeding and respiratory-oesophageal fistulas.

Peer-review

The study reports initial experiences with the new OTS Padlock Clip™ system, with comprehensive documentation supported by videos.

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CASE REPORT

Widespread lymph node recurrence of major duodenal papilla cancer following pancreaticoduodenectomy

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Abstract

Major duodenal papilla cancer (MDPC) represents the primary type of duodenal cancer, and is typically considered a periampullary carcinoma as most tumors arise in this region. This report describes an extremely rare case involving a patient with rapidly and extensively recurrent MDPC following pancreaticoduodenectomy, who achieved complete response by concurrent image-guided radiation and intravenous oxaliplatin plus oral capecitabine therapies. The patient was a 50-year-old female who was admitted to our hospital 6 wk after resection for MDPC for evaluation of a nontender and enlarged node in the left side of her neck. After clinical work-up, the patient was diagnosed with postoperatively recurrent MDPC with widespread lymph node metastases at the bilateral cervix, mediastinum, abdominal cavity, and retroperitoneal area. She was administered whole field image-guided radiation therapy along with four cycles of the intravenous oxaliplatin plus oral capecitabine regimen. A complete response by positron emission tomography with 18-fluorodeoxyglucose was observed 4 months after treatment. The patient continues to be disease-free 2 years after the diagnosis of recurrence.

Key words: Chemoradiotherapy; Complete response; Neoplasm recurrence; Periampullary cancer; Pancreatico-

duodenectomy

Core tip: Major duodenal papilla cancer (MDPC) is a rare malignancy, and there are limited data regarding its recurrence after radical resection. This report describes a case of recurrent MDPC with widespread lymph node involvement at the bilateral cervix, mediastinum, abdominal cavity, and retroperitoneal area, 6 wk after pancreaticoduodenectomy. The patient experienced a complete response to image-guided radiation therapy and a concomitant regimen of intravenous oxaliplatin plus oral capecitabine, and remains disease-free 2 years after the diagnosis of recurrence. This, to our knowledge, is the first case to demonstrate the role of chemoradiotherapy with improved survival in extensively recurrent MDPC.

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INTRODUCTION

Major duodenal papilla cancer (MDPC), despite representing approximately 60% of all small bowel malignancies, remains a very rare disease that comprises less than 0.5% of all gastrointestinal cancers[1]. The primary treatment is surgical resection, with pancreaticoduodenectomy or Whipple procedure as the preferred approach for resectable lesions[2]. However, recurrent disease following radical surgery has been inadequately documented. Particularly, there is very little data in the literature regarding the treatment of relapse settings involving multiple lymph node (LN) metastases. This report describes a case involving widespread LN recurrence of an adenocarcinoma of the major duodenal papilla after pancreaticoduodenectomy in which complete response was achieved by image-guided radiation therapy (IGRT) and a concomitant regimen of intravenous oxaliplatin plus oral capecitabine (XELOX).

CASE REPORT

An otherwise healthy, 50-year-old Chinese female had previously consulted another hospital in January 2013 with significant upper abdominal pain. At that time, she underwent a pancreaticoduodenectomy and lymphadenectomy, and was diagnosed intraoperatively with MDPC. Postoperative pathology revealed a well-differentiated adenocarcinoma of the major duodenal papilla. Surgical margins and four LNs were negative for tumor tissue. The patient's postoperative course was uneventful and she was discharged 2 wk after the operation.

Six weeks following the surgery, in March 2013, she became aware of a hard, palpable nodule in the left supraclavicular area, and was referred to our hospital. Upon initial examination, she had no clinical symptoms, such as hoarseness, abdominal pain, or melena. The Karnofsky performance status was 90%. Physical examination of the cervical region showed a fixed, nontender, left supraclavicular LN, approximately 2 cm in diameter. A total body computed tomography (CT) scan revealed swollen LNs at the bilateral cervix, mediastinum, abdominal cavity, and retroperitoneal area (Figure 1A-C). At this time, the patient's serum level of carbohydrate antigen (CA)19-9 was 1046 U/mL (normal range, < 34 U/mL). Metastatic adenocarcinoma was confirmed by ultrasonographic-guided fine-needle biopsy of the left neck. Subsequent fluorodeoxyglucose-positron emission tomography/CT (PET/CT) fusion imaging revealed hypermetabolic foci at the above-mentioned sites with mean standard uptake values of 2.8 (bilateral cervix), 2.4 (mediastinum), 1.8 (abdominal cavity) and 1.8 (retroperitoneal area) (Figure 2A). Based on these findings, postoperative widespread LN recurrence of MDPC was the likely diagnosis. After multidisciplinary consultation, a multimodality therapeutic strategy was planned consisting of whole-field IGRT and four concomitant cycles of the XELOX regimen [intravenous oxaliplatin (130 mg/m^2) on day 1 and oral capecitabine (750 mg/m^2) twice daily on days 1-14 every 4 wk]. Specifically, the dose prescriptions of IGRT were 6444 cGy/25 plus a boost of 1745 cGy/7 fractions to the left cervical nodes, 6170 cGy/25 plus a boost of 1754 cGy/7 fractions to the right cervical nodes, 6170 cGy/25 plus a boost of 1730 cGy/7 fractions to the mediastinal nodes, and 5744 cGy/25 fractions to the celiac nodes. The dosimetric values for target coverage and organ-at-risk sparing were defined according to the criteria of the International Commission on Radiation Units and Measurements guidelines. The gross tumor volume was defined as the resection site and the suspicious LNs visualized on CT imaging, including the bilateral cervical, mediastinal, and retroperitoneal areas (Figure 3). Prescribed doses were the minimum for the gross tumor volume. The outlined organs at risk were the uninvolved bilateral lungs, esophagus, spinal cord, bilateral kidneys, and small bowel (Figure 3). IGRT planning was performed using the Varian Medical Systems Eclipse 11.0.47 (Varian Medical Systems, Inc., Palo Alto, CA, United States). There were no interruptions or delays in chemoradiotherapy.

One and a half months after treatment, a re-evaluation with CT scan was carried out. Remarkably, the patient achieved a near complete remission

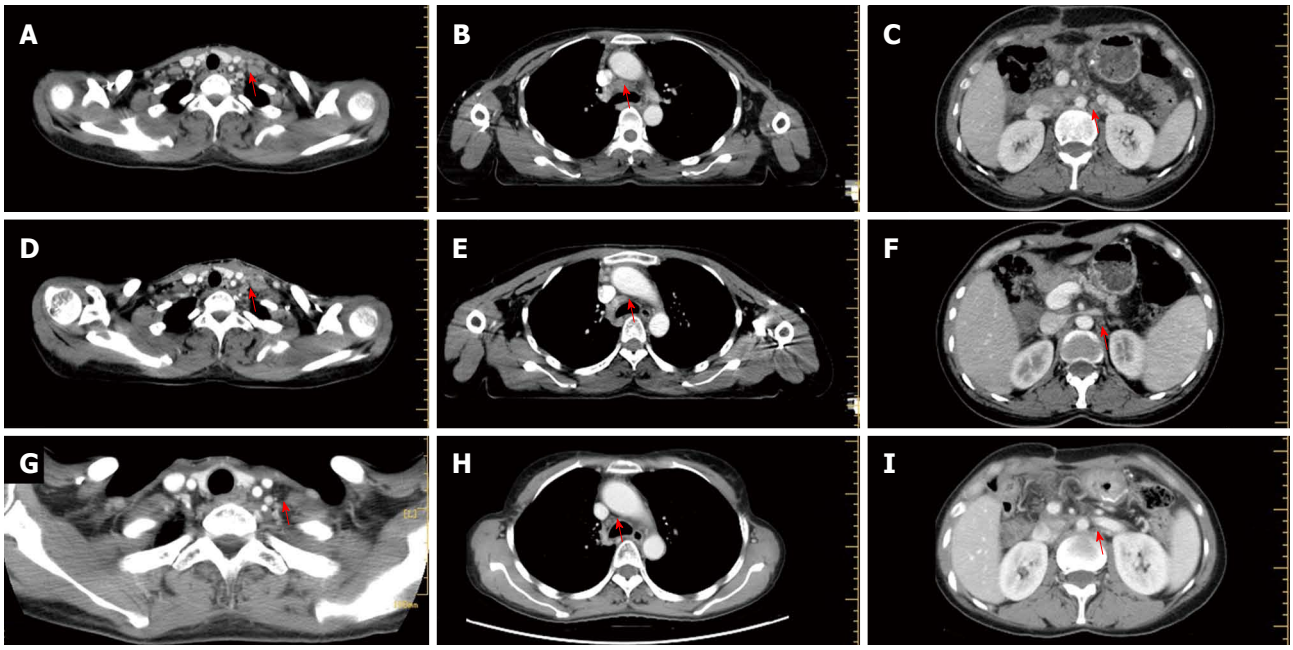

Figure 1 Computed tomography images of the recurrent lymph node metastases (red arrows). A-C: At the time of recurrence (A: Cervical region; B: Mediastinal region; C: Retroperitoneal region); D-F: Images taken 1.5 mo after chemoradiotherapy (D: Cervical region; E: Mediastinal region; F: Retroperitoneal region); G-I: Images taken 2 years after treatment (G: Cervical region; H: Mediastinal region; I: Retroperitoneal region). The recurrent lymph nodes dramatically decreased in size 1.5 mo after treatment, and nearly disappeared 2 years after treatment.

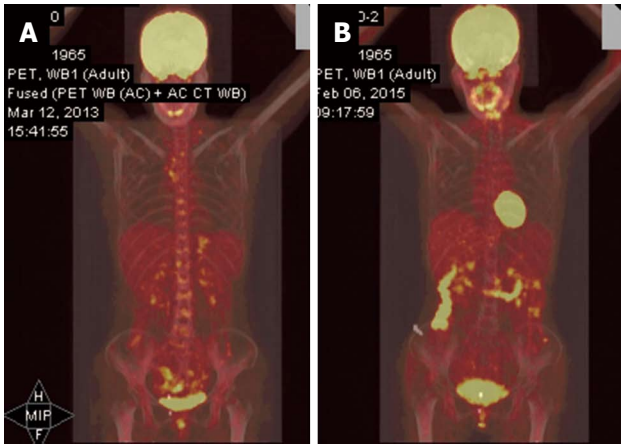

Figure 2 Positron emission tomography/computed tomography images of the patient. A: Before treatment; B: Two years after chemoradiotherapy.

(Figure 1D-F), and her serum CA19-9 level returned to normal.

The patient completed chemoradiotherapy in late July 2013. No other adverse effects with clinical significance were observed, except grade 3 diarrhea and grade 2 skin reactions. A subsequent 2-year follow-up showed no evidence of disease recurrence (Figure 1G-I). Furthermore, a recent fluorodeoxyglucose-PET examination showed almost total disappearance of the known uptake areas (Figure 2B).

DISCUSSION

Recurrent disease after curative resection represents a major challenge for effective treatment of MDPC, which has a median survival of less than 1 year after the diagnosis of recurrence[3,4]. These studies also report a median time to tumor recurrence of 14.5-29 mo, with locoregional (tumor bed and regional LNs) and/or distant (liver, peritoneum, lung, and supraclavicular LNs) recurrence patterns. The predominant site of relapse is the operative bed, followed by the liver and retroperitoneal LNs. Widespread metastatic LN failure, however, is extremely rare[5,6]. Moreover, there are limited reports concerning treatment decisions for cases of recurrent MDPC following pancreaticoduodenectomy, especially for rapid, widespread LN recurrence. The current case involves a patient who experienced early and extensive LN recurrence along with an elevated CA19-9 level less than 2 mo following surgery. Complete response was achieved with concurrent therapies of IGRT and a XELOX regimen. This, to our knowledge, is the first case to demonstrate the role of chemoradiotherapy and a corresponding survival improvement in extensively recurrent MDPC.

Given the rare occurrence of MDPC, the role of chemotherapy, radiotherapy, or combined chemo-radiotherapy remains unclear, particularly for recurrent disease after surgery. For patients with inoperable or metastatic small bowel adenocarcinoma (SBA), chemotherapy can improve overall survival when compared with no chemotherapy[4,7-9]. These studies used regimens of capecitabine combined with oxaliplatin (XELOX or CAPOX), or administered them as an adjuvant therapy after curative or palliative surgery for patients with MDPC[10]. Pharmacologically, capecitabine is a prodrug of the cytotoxic agent 5-FU, which selectively exerts its anticancer effects

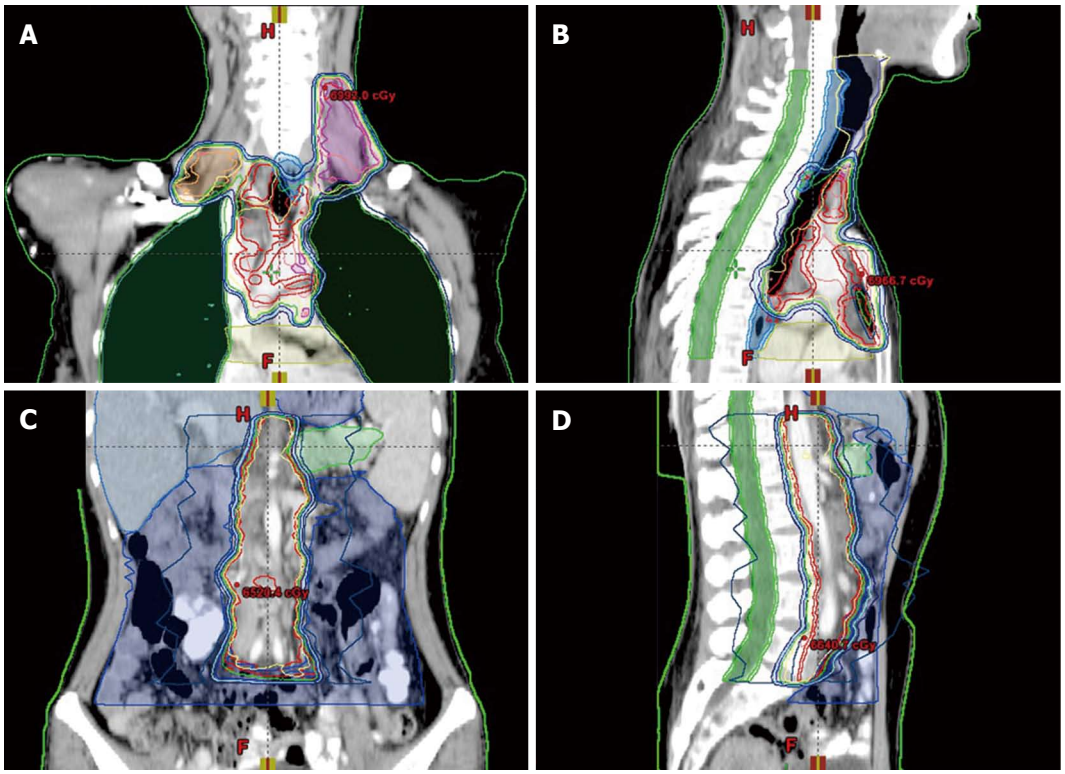

Figure 3 Initial radiation treatment plan showing isodose distribution images of the body. A: Bilateral cervical area; B: Bilateral supraclavicular area; C: Mediastinal area; D: Retroperitoneal area.

preferentially in tumor cells *via* the up-regulation of thymidine phosphorylase. Furthermore, capecitabine is orally administered and clinically tolerable during the treatment process. The combination of capecitabine with oxaliplatin reduces the need for intravenous drug administration and associated visits to the clinic. As advanced and recurrent MDPC are similar in tumor biology, the XELOX regimen was selected for treatment of the patient in the case presented here.

The role of radiation therapy (RT) is also not well defined. Available studies are either retrospective or involve a small number of patients, and report either only a slight increase in 1-year survival or no significant survival advantage for patients receiving adjuvant RT after resection of primary tumors[2,11]. Nevertheless, retrospective studies have also reported high rates of in-field control when adjuvant RT was administered to high-risk patients with positive margins, LN metastases, and locally aggressive tumor biology[5]. Thus, there is general agreement on the value of RT in reducing local failure of MDPC following radical resection. IGRT has emerged as a safe and effective technique to precisely deliver conformal RT in real time, while sparing critical organs[12,13]. Indeed, successful use of this technique in other malignancies indicated an additional benefit of IGRT in the treatment of recurrent MDPC[14,15]. Consequently, IGRT was chosen concurrent with the XELOX regimen to treat the patient described in this case report, which had a successful outcome as observed by her survival for more than 2 years from the diagnosis of recurrence.

Some side effects have previously been reported for the same therapies used to treat the patient in the present case. These include diarrhea, nausea, vomiting, fatigue, abdominal pain, hand-foot syndrome, and peripheral sensory neuropathy for the XELOX regimen, and subcutaneous fibrosis, edema, and joint stiffness for IGRT[16]. Indeed, the patient in the present case experienced grade 3 diarrhea due to the oral capecitabine, which required temporary parenteral nutrition. Additionally, acute grade 2 dermatitis and pneumonitis occurred due to IGRT, though the patient tolerated these adverse effects well without discontinuing treatment.

The current AJCC guideline recommends that six is the minimum number of LNs to be examined for duodenal cancer, including MDPC, though it has been questioned whether the threshold should be raised[17]. Recent data from the Surveillance, Epidemiology, and End Results database demonstrated that increasing the number of dissected LNs correlates to improved survival in stage Ⅱ SBA[18]. Other studies also reported that the removal of at least 15 LNs improved prognostic discrimination by the pathologic N category, and that increasing the total number of LNs assessed markedly improved prognostication for patients with stage Ⅰ, Ⅱ, and Ⅲ SBA who underwent resection[18,19]. The patient in the present case had only four LNs dissected, which may explain the rapid and widespread recurrence of the disease. Nevertheless, there are also

molecular mechanisms that may be associated with the aggressiveness of invasion and metastasis early in the course of the disease[20].

Another aspect worth mentioning is the sharply increased level of the tumor marker CA19-9, which, in general, is significantly associated with a diagnosis of pancreatic and colorectal adenocarcinoma[21,22]. However, there is less data concerning the correlation between serum CA19-9 levels and postoperative survival in patients with SBA, though a retrospective multicenter study found that serum CA19-9 values were independent prognostic factors for progression-free and overall survival of such patients[23]. It is unclear whether the CA19-9 concentration is a prognostic indicator in patients with MDPC, though it can potentially be used to survey for the recurrence of MDPC. As observed in our patient, there was a sudden rise in CA19-9 levels 40 d postoperatively, which may have been indicative of the widespread recurrence of disease.

In conclusion, our experience demonstrates that XELOX with concomitant IGRT is a potential treatment for MDPC patients with widespread LN recurrence after radical pancreaticoduodenectomy. Therefore, future studies should further evaluate the efficacy of the regimen in this very rare setting.

ACKNOWLEDGMENTS

We appreciate Professor Qiang Li for the critical reading of the manuscript and fruitful discussion.

COMMENTS

Case characteristics

A 50-year-old female with recurrent major duodenal papilla cancer (MDPC) following pancreaticoduodenectomy.

Clinical diagnosis

Postoperatively recurrent MDPC with widespread lymph node metastases at the bilateral cervix, mediastinum, abdominal cavity, and retroperitoneal area.

Differential diagnosis

Periampullary cancers (pancreas, duodenum, distal common bile duct, and ampulla of Vater).

Laboratory diagnosis

Serum carbohydrate antigen 19-9 was 1046 U/mL (normal range, < 34 U/mL).

Imaging diagnosis

Positron emission tomography with 18-fluorodeoxyglucose/computed tomography showed hypermetabolic foci at the bilateral cervix, mediastinum, abdominal cavity, and retroperitoneal sites with mean standard uptake values of 2.8, 2.4, 1.8 and 1.8 respectively.

Pathological diagnosis

Metastatic adenocarcinoma was confirmed by ultrasonographic-guided fine-needle biopsy of the left neck.

Treatment

Intravenous oxaliplatin plus oral capecitabine regimen concurrent with image-guided radiation therapy.

Term explanation

MDPC is typically described as periampullary carcinoma because most tumors arise in the periampullary region.

Experiences and lessons

A regimen of intravenous oxaliplatin plus oral capecitabine concomitant with image-guided radiation therapy is a potential treatment for MDPC patients with widespread lymph node recurrence after radical pancreaticoduodenectomy.

Peer-review

In a case presentation, the authors wrote that a patient was operated on for major duodenal papilla cancer. The paper is interesting and innovating.

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