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Advances in local ablation of malignant liver lesions

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Abstract

Local ablation of liver tumors matured during the recent years and is now proven to be an effective tool in the treatment of malignant liver lesions. Advances focus on the improvement of local tumor control by technical innovations, individual selection of imaging modalities, more accurate needle placement and the free choice of access to the liver. Considering data found in the current literature for conventional local ablative treatment strategies, virtually no single technology is able to demonstrate an unequivocal superiority. Hints at better performance of microwave compared to radiofrequency ablation regarding local tumor control, duration of the procedure and potentially achievable larger size of ablation areas favour the comparably more recent treatment modality; image fusion enables more patients to undergo ultrasound guided local ablation; magnetic resonance guidance may improve primary success rates in selected patients; navigation and robotics accelerate the needle placement and reduces deviation of needle positions; laparoscopic thermoablation results in larger ablation areas and therefore hypothetically better local tumor control under acceptable complication rates, but seems to be limited to patients with no, mild or moderate adhesions following earlier surgical procedures. Apart from that, most techniques appear technically feasible, albeit demanding. Which technology will in the long run become accepted, is subject to future work.

Key words: Local ablation; Liver; Microwave ablation; Hepatocellular carcinoma; Colorectal liver metastases; Navigation

Core tip: A wide variety of technical innovations enables us to use microwave as well as radiofrequency ablation, various image fusion technologies, magnetic resonance guidance for local ablation, navigation, robotics, and minimal invasive access to liver surgery in general in the 21st century. However, in comparison to data found in the current literature for conventional local ablative treatment strategies, virtually no single technology is able to demonstrate an unequivocal superiority. Most techniques appear technically feasible, albeit demanding. Which technology will in the long run become accepted, is subject to future work.

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COMMENTARY ON HOT TOPICS

Local ablation of liver tumors matured during the recent years and is now proven to be an effective tool in the treatment of malignant liver lesions. Advances focus on the improvement of local tumor control by technical innovations, individual selection of imaging modalities, more accurate needle placement and the free choice of access to the liver. Repeatedly, different elements of improving local ablation have been reported, including the use of microwaves instead of radiofrequency, magnetic resonance (MR) instead of computed tomography (CT) or ultrasound (US), navigation, robotics and minimal invasive surgical access routes instead of percutaneous or open surgical approaches. The following contribution is meant to illustrate some of the more recently envisioned developments with respect to the current literature.

TECHNICAL INNOVATIONS

The most important single step was certainly the spread of microwave coagulation therapy (MCT) largely replacing radiofrequency ablation (RFA) during the recent years. MCT is no real novelty, as first reports were available as early as 1994[1]. Microwaves emitted from a monopolar antenna lead to oscillation of water molecules in a dielectric surrounding such as liver tissue. Table 1 provides an overview displaying the cardinal characteristics of MCT in comparison to RFA, respectively. The renaissance of MCT is partly traced to better equipment with intelligent feedback controlled generators compared to the first devices[2], but as important seems to be, that MCT is meanwhile not considered yet another technique to generate heat in the same way like with RFA, but in contrast a completely distinct technology for thermal ablation with different and unique physical properties[3]. This leads eventually to an experimentally confirmed less susceptibility to heat sink phenomena[4,5], shorter treatment duration[6] and larger ablation areas[7]. So far, no clinical evidence supports the superiority of MCT to RFA; the only published randomized controlled trial revealed no statistically significant difference, and among 14 comparative cohort studies, only three found a significantly lower local recurrence rate (LR) following MCT[8-10]. The trend to shorter treatment times is however already clinically endorsed[11]. In general, RFA is believed to be most effective in tumors with a maximum diameter not larger than 3 cm. MCT promises to be successful also in the treatment of larger tumors[2], most probably when combined with transarterial chemoembolization (TACE)[12,13]. Sustained success may however also be achieved, if RFA is combined with TACE prior to or following the ablation[14]. At date, MCT - albeit promising - has not yet been convincingly confirmed to be superior to RFA.

Table 1 Differences comparing radiofrequency ablation to microwave coagulation therapy with regard to physical properties

	RFA	MCT
Electromagnetic waves	Radiowaves	Microwaves
Frequency	0.3-0.5 MHz	915-2450 MHz
Heating target	Ions	H_2O (approximately 50%)
Heat distribution	Convective	Direct heating (within field)
Alternating current	Closed circuit	Electromagnetic field
Applicator	Electrode	Antenna
Desiccation	Carbonization	Vaporization
Size of ablation area	Unaltered/slight increase	Marked shrinkage

RFA: Radiofrequency ablation; MCT: Microwave coagulation therapy.

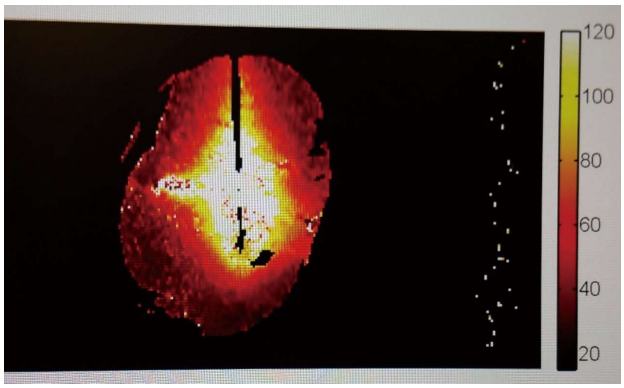

Figure 1 Thermal mapping using phase changes in magnetic resonance imaging, temperature code is depicted in the bar at the right margin of the image (values in degree Celsius). Courtesy of MedWaves Inc., San Diego, CA, United States.

IMAGING

US is presumably the most popular imaging modality in use for local ablation. Its value is undisputed; no differences to CT guidance are reported regarding success and time needed for needle placement. The widespread availability is considered a major advantage. In contrast, MR imaging is limited by shortcomings in organisation, number and cost of the required MR machines. MR offers in return several theoretical advantages in comparison to extant imaging modalities, including MR thermometry (Figure 1), absence of ionizing radiation and an impression of better imaging quality for soft tissues. The latter accounts for a significantly increased primary success rate following MR-guided RFA in comparison to CT-guided RFA (only 4% incomplete ablations *vs* 21%, *P*

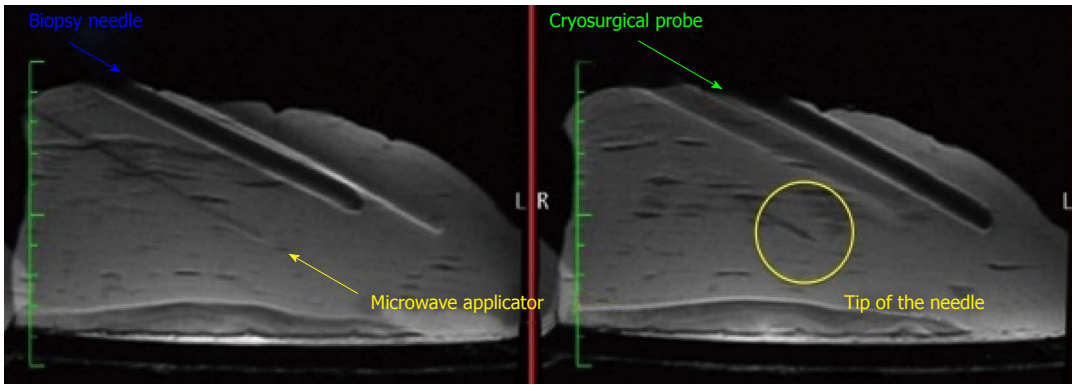

Figure 2 Magnetic resonance imaging of different devices for liver directed interventions. Of note is the inaccuracy in displaying the position of the needle shaft and tip with older devices and the complete absence of artifacts with the use of a novel microwave applicator. Courtesy of MedWaves Inc., San Diego, CA, United States.

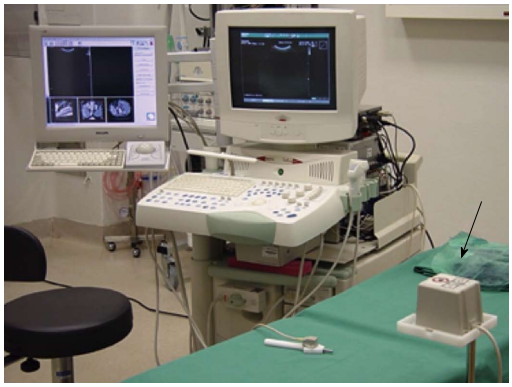

Figure 3 Clinical setup for ultrasound fusion imaging. The ultrasound machine is visible with an additional monitor for displaying the previously digitally acquired cross-sectional examination images. Meanwhile, there are also systems with a split screen. The arrow points at the electromagnetic reference point.

= 0.04), whereas the secondary success rate following a redo-ablation was not significantly different (4% *vs* 10%, P = 0.32)[15]. The former has been shown to be associated with an evolution of the interventional MR scanners from lower (*e.g.*, 0.2 T in 1997[16]) towards high field machines (*e.g.*, 1.5 T in 2008[17]). Nowadays, MR thermometry allows for an accurate prediction of size and geometry of an ablation area with a sensitivity of uniformly reported 87% using a threshold of 60 ℃[18,19]. The spatial resolution is however disappointing, and displaying the microwave applicator is cumbersome unless optimized hardware recently became available (Figure 2). In addition, no study exists comparing MR-guided interventions to US guidance except for an experimental evaluation of MR imaging by Chopra *et al*[20]. They found no differences in time to correct needle placement and number of required attempts. Dong *et al*[21] recently report on MR-guided MCT. Both experimental studies have in common the use of an open MR scanner instead of a closed or double doughnut system formerly used. An introduction into a clinically applicable surgical environment is not intended so far.

In contrast, intraoperative US is a clinical reality in most operation theaters, albeit some nodules are invisible in B-mode US. Additionally, mistargeting belongs to the crucial risk factors for local treatment failure[22]. A possible solution is registering the position of the US probe with a position tracking system and synchronizing the real-time US image with a previously recorded three-dimensional multiplanar imaging dataset derived from preoperatively obtained MR or CT scans (Figure 3), a method called Virtual Sonography or US Fusion Imaging (UFI). With UFI, technically successful RFA of hepatocellular carcinoma was achieved in 94.4%-100%, and local tumor progression occurred in 0%-8.3%[23]. In a recently published study from Japan[24], UFI was able to identify sonographically inconspicuous tumor nodules in 91.7% sufficiently for a successful RFA procedure, whereas by the application of US contrast media, the detection rate increased up to 96.7%. Local tumor control rate exceeded 90% after a follow-up of 3 years in nodules with a mean diameter of 14 mm (range 8 to 42 mm). The remaining tumors were treated by transarterial chemoembolization. The authors did not explain, why no other imaging modality was applied in order to perform a sufficient local ablation treatment.

So far, no evidence suggests superiority of one or the other imaging modality for guidance of local ablative therapies in the liver.

TARGETING Ⅰ: NAVIGATION

Registration and tracking are both technologies of image processing already mentioned above. Both are prerequisites for successful navigation. Three-dimensional visualization and navigation in deformable soft tissues like liver and lung is difficult to accomplish, if free movements of the patient's body due to breathing, intervention during mild sedation or comorbidities are not prevented. Stereotaxy was first evaluated and eventually introduced in neurosurgery, initially using a frame in order to limit the degree of freedom for movements of the target area in the

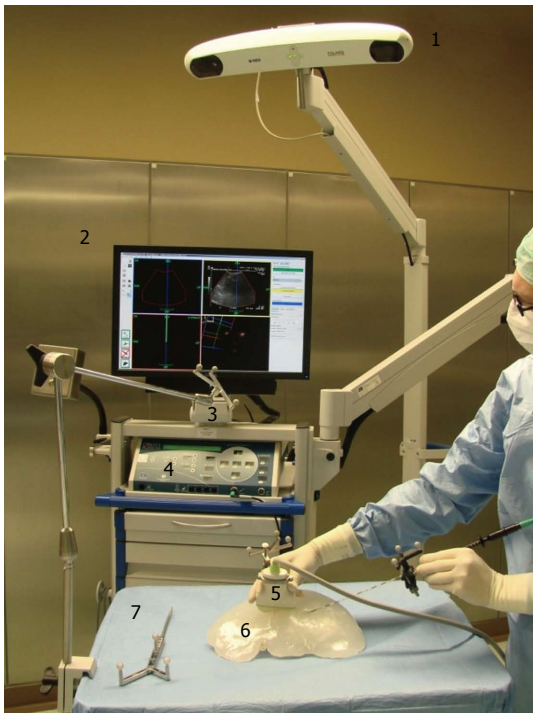

Figure 4 Example for a navigation device using optical tracking. The crucial elements are shown under intraoperative conditions with a phantom liver model. 1: Stereooptic camera; 2: Monitor with a horizontally and vertically split screen; 3: Reference point; 4: Radiofrequency generator; 5: Ultrasound probe; 6: Liver phantom; 7: Pointer.

central nervous system. Later, frameless navigation was available and evaluated in phantom experiments revealing deviations of 1.1 ± 0.4 mm for accurate needle placement with one commercially available system[25], ranging from 1.67 to 2.91 mm with two others under MR guidance[26]. Further research confirmed the high precision of yet another system with 1.1 ± 0.5 mm deviation[27]. Frameless stereotaxy opened the way for the application of navigation in the liver (Figure 4).

Navigation in liver directed surgery and interventions have been a subject of investigation for long. An overview is provided by Chopra *et al*[28] 2010. The authors describe a few single center experiences with optical and electromagnetic tracking, which after all reveal the disappointing result, that three-dimensional navigation seems to be feasible, but to date not yet superior to conventional two-dimensional biopsy US probes. Despite all obstacles, there are currently computer-assisted navigational systems commercially available. Similarities and differences among them are exhaustively discussed in an up-to-date paper[29] including a single center experience with one of the presented systems. The authors conclude, that working with the electromagnetic tracking system improved their performance compared to an ancient optical navigation device. Mean time to lesion acquisition was comparably short with only 3.5 min. Success rate with first-attempt passes was 93%. A direct comparison to conventional MCT procedures was not intended. The strategy for accurate liver intervention by an optical tracking system is outlined in a topical paper from Guangdong (China)[30]. The group suggests the use of fiducial markers to deal with the imminent inaccuracies of soft tissue navigation. So far, no vendor distributes such a technology.

TARGETING Ⅱ: ROBOTICS - A STEP FURTHER

If three-dimensional navigation increases the accuracy of the needle placement - at least under experimental *ex vivo* conditions, the complete elimination of the human component and probability for error by mechanical positioning will further improve the precision of an interventional treatment. Robotic surgery and ablation is emanating from this thesis. In phantom experiments, the use of a robot reduced Euklidic deviation from 2.2 to 1.9 mm and the mean standard distance from 1.8 to 1.6 mm[31]. The time for needle placement was however approximately 30 min. in comparison to approximately 18 min. without the roboter. A clinical study endorses the impression[32]: Robotic assistance required manual correction of the final needle position in more than 40% of all cases, resulting in a significantly decreased deviation of the active center of the microwave applicator from the tumor center (1.6 mm *vs* 3.3 mm). In addition, the exposure to radiation under fluoroscopy was significantly diminished in case of robotic needle placement. Methodological research with clinically applicable hard- and software was presented in 2010 by a group consisting in authors from the United States and China[33]. The data for accuracy of needle placement was within the previously mentioned range (positioning error between 1 and 2 mm), and the estimation of the created ablation area was except for a relative mean error of 5.6% correct. The projection of the ablation area is indeed the crucial point in robotic ablation, since it acts on the assumption of an ideal symmetric geometrical shape of the ablation area. Cai *et al*[34] describe nicely the mathematical functions and visualization backgrounds influencing the quality of predicting the ablation focus under conditions of unexpected soft tissue deformation, inhomogeneous heat conduction and undesired needle paths. The authors emphasize the demand for extensive training of the staff prior to the introduction of such techniques in a clinical environment. So far, no robotic application is set in clinical standard treatment protocols.

MINIMAL INVASIVE TREATMENT STRATEGIES

The goal of a local ablative treatment is complete tumor destruction with minimal side effects. In order to minimize adverse effects, miniaturization of the access to local ablation is intended. Occasionally, the

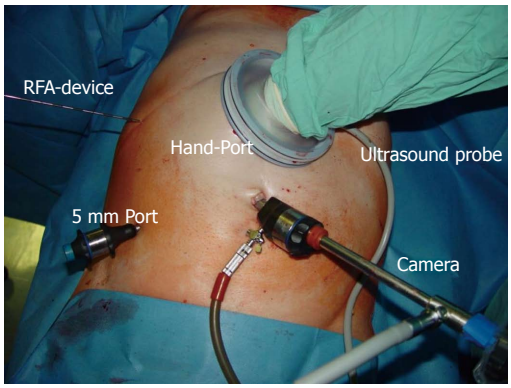

Figure 5 Clinical application of hand-assisted laparoscopic surgery. Intraoperative radiofrequency ablation using hand-assistance. The advantages of a minimal-invasive approach are preserved.

least invasive, percutaneous way is unsound or even dangerous[35]. In such cases, laparoscopic procedures are suggested[36]. Advantages of laparoscopy for thermoablation are related to the direct visualization of the abdominal cavity, which offers diagnostic features like better tumor staging using laparoscopic ultrasound (LUS) as well as the opportunity of detecting extra-hepatic intraabdominal tumor spread, and therapeutic implications in preventing thermal injury of abutting organs and structures, which are separated from the surface of the liver by the pneumoperitoneum itself or by distinct devices[36]. In addition, the combination of a thermoablation with laparoscopy results in specific additive effects. LUS works usually with higher frequencies and thus displays a higher resolution enabling a more accurate and precise needle placement besides the above mentioned diagnostic property. The pneumoperitoneum in turn decreases tissue perfusion and reduces convective heat sink phenomena, leading to larger ablation areas[37] and therefore preferably less local treatment failures. Clinical evidence for favourable outcome after laparoscopic RFA/MCT is scarce; a retrospective study recently presented a multivariate analysis of risk factors for local recurrence after US guided laparoscopic or percutaneous MCT[36]. Laparoscopic MCT was a statistically significant independent prognostic factor for better local tumor control. Since no randomized controlled trial is available, the conclusion of clinical superiority of laparoscopic compared to percutaneous MCT is drawn from this and other retrospective studies.

However, a large amount of indications to local ablation account for patients with recurrent disease following previous surgery. Adhesions frequently occurring after open surgery to a certain extent make laparoscopy difficult to accomplish if not impossible at all. Reluctance to offer open surgical access to local ablation in the liver is comprehensible. Hence, alternative approaches have been suggested including hand-assisted liver surgery (HALS, Figure 5)[38] and transthoracic local ablation[39]. Not a lot of experience is reported with both techniques worldwide. Besides technical remarks, no trial has ever been conducted showing superiority to more traditional procedures. Theoretic advantages encompass less risk of ascites and collateral injury to intraabdominal organs when comparing transthoracic ablation to open abdominal surgery, while local tumor control is reportedly superior to results obtained in percutaneous interventions, but no scientific evidence supports these postulations so far. With HALS, the advantages derived from the formation of pneumoperitoneum are preserved, albeit the open surgical part of the procedure imposes a similar risk to intraabdominal injury and consecutive morbidity upon the patient. In summary, except for proof of concepts, confirmation of improvements in local ablation using transthoracic approaches and/or HALS lacks.

Where are we now, and which prospects for the future may be drawn from the previous paragraphs? A wide variety of technical innovations enables us to use microwave as well as radiofrequency ablation, various image fusion technologies, MR guidance for local ablation, navigation, even robotics, and minimal invasive access to liver surgery in general in the 21st century. However, in comparison to data found in the current literature for conventional local ablative treatment strategies, virtually no single technology is able to demonstrate an unequivocal superiority. Hints at better performance of MCT compared to RFA regarding local tumor control, duration of the procedure and potentially achievable larger size of ablation areas favour the comparably more recent treatment modality; image fusion enables more patients to undergo ultrasound guided local ablation; MR guidance may improve primary success rates in selected patients; navigation and robotics accelerate the needle placement and reduces deviation of needle positions; laparoscopic thermoablation results in larger ablation areas and therefore hypothetically better local tumor control under acceptable complication rates, but seems to be limited to patients with no, mild or moderate adhesions following earlier surgical procedures. Apart from that, most techniques appear technically feasible, albeit demanding. It is a challenge to learn all novel treatment modalities and exhibit a satisfying command on it. So far, it remains an open question, which will eventually survive. In view of all mechanical and electronical support, there are some activities in our world, which are still best performed by humans, despite all highly sophisticated machines surrounding us.

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TOPIC HIGHLIGHT

2016 Alcoholic Liver Disease: Global view

New treatment options for alcoholic hepatitis

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Abstract

The burden of alcoholic liver disease has rapidly grown in the past two decades and is expected to increase further in the coming years. Alcoholic hepatitis, the most florid presentation of alcoholic liver disease, continues to have high morbidity and mortality, with significant financial and healthcare burden with limited treatment options. Steroids remain the current standard of care in severe alcoholic hepatitis in carefully selected patients. No specific treatments are available for those patients who are steroid ineligible, intolerant or unresponsive. Liver transplant has shown good short-term outcome; however, feasibility, ethical and economic concerns remain. Modification of gut microbiota composition and their products, such as lipopolysaccharide, nutritional interventions, immune modulation, increasing steroid sensitivity, genetic polymorphism and epigenetic modification of alcohol induced liver damage, augmenting hepatic regeneration using GCSF are potential therapeutic avenues in steroid non-responsive/ineligible patients. With better understanding of the pathophysiology, using "Omics" platforms, newer options for patients with alcoholic hepatitis are expected soon.

Key words: Alcoholic liver disease; Alcoholic steatosis; Alcoholic hepatitis; Gut microbiota; Lipopolysaccharide; Steroid non-response

Core tip: With better treatment options available for other liver diseases like viral hepatitis the proportion of alcoholic liver disease is on the rise. Alcoholic hepatitis is the most serious presentation of alcoholic liver disease with significant morbidity, mortality and health care burden. Treatment options in steroid non-responders and steroid ineligible patients of severe alcoholic hepatitis are limited. Newer treatment options for these patients are the need of the hour. The molecular and cellular targets have been discussed.

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INTRODUCTION

Better fortunes and increased stress have led to life style changes across the world. Alcohol has become the commonest and most socially acceptable hepatotoxin worldwide. Additionally, with the availability of better drugs and more treatment options for managing liver disorders related hepatotrophic viruses, the proportion and burden of alcoholic liver disease has grown, and is likely to increase in the coming years. Alcoholic hepatitis (AH) related hospital admissions continue to increase with substantial increase in healthcare cost and utilization[1,2]. The spectrum of alcoholic liver disease varies from fatty liver, steatohepatitis, compensated/ decompensated cirrhosis, to hepatocellular carcinoma. Alcoholic hepatitis is the most florid manifestation of alcoholic liver disease with substantial morbidity, mortality and financial burden. The treatment options of severe alcoholic hepatitis (Maddrey's discriminant score > 32) however, have not changed in nearly past three decades. Steroids, pentoxifylline and nutrition therapy remain the only accepted options available for the management of severe alcoholic hepatitis[3-5]. There is no advantage of combining or sequencing steroids and pentoxifylline in comparison with either alone[6-10]. No medical options are available to treat severe alcoholic hepatitis patients who are steroid unresponsive (Lille score > 0.45). Similarly no options are available for steroid ineligible patients (upper gastrointestinal bleed, impaired renal functions and/or sepsis) who far outnumber the patients who are eligible and respond to steroids. With advances in basic sciences and better understanding of the pathogenesis of alcoholic liver disease, significant advances in the management of severe alcoholic hepatitis are likely. This review discusses various aspects of the pathogenesis and targeted treatment options in patients with alcoholic liver disease.

PATHOGENESIS OF ALCOHOLIC HEPATITIS

Alcoholic steatosis is a complex process manifested through several mechanisms (Figure 1). The main pathogenetic factors underlying this process are increased fatty acid and triglyceride synthesis, enhanced hepatic influx of free fatty acids from adipose tissue and of chylomicrons from the intestinal mucosa, increased hepatic lipogenesis, inhibited lipolysis, and damaged mitochondria and microtubules, all of which result in accumulation of VLDL[11-16]. Lipogenic enzymes are overexpressed in alcoholics due to downregulation of peroxisome proliferator activated receptor α (PPARα) and the induction of sterol regulatory element binding protein (SREBP)[17,18]. AMP activated protein kinase (alters relative concentrations of intra cellular malonyl coenzyme A and long chain acyl coenzyme A) and the downstream pathways of fatty acid synthesis and degradation[19], the NADH reduction/oxidation potential in the liver and reduced microsomal triglyceride transfer protein activity are all implicated in the chronic alcoholism related fatty liver[20,21].

Some unclear triggering event is responsible for the initiation of steatohepatitis, in patients with alcoholic fatty liver who continue to consume excessive alcohol. Gut dysbiosis, increased gut permeability, altered alcohol metabolism, increased lipopolysaccharide release into portal circulation, genetic background, associated malnutrition and micronutrient deficiency have been implicated as the initiating event for inflammation[21,22] and decide the severity of alcoholic hepatitis and cellular injury[23-25]. Acetaldehyde (the major product of alcohol metabolism) adducts forming neoantigens leading to activation of adaptive immune system[26,27], impaired glutathione function, oxidative stress and apoptosis[28-30] lead to inflammation and liver injury.

Prolonged alcohol intake markedly upregulatesCytochrome P450 2E1 (CYP2E1) activity. CYP2E1 dependent microsomal electron transport system of the respiratory chain, NADH dependent cytochrome reductase and xanthine oxidase are involved in the production of reactive oxygen species[31-33] in addition to alcohol dehydrogenase and aldehyde dehydrogenase. Thus alcohol ingestion stimulates the generation of ROS and hydroxyl radicals[34]. These metabolites and ROS activate various downstream inflammatory pathways that involve nuclear factor-κB (NF-κB), signal transducer and activator of transcription (STAT)-Janus kinase (JAK) and cJun N terminal kinase (JNK) in hepatic resident cells, leading to local synthesis of inflammatory mediators, such as tumor necrosis factor (TNF), IL-17, CXC chemokines (including IL-8), as well as osteopontin[21,35-40].

Alcohol intake also induces dysbiosis in the gut and alters the gut permeability leading to increased levels of lipopolysaccharides in the portal circulation. This increase in lipopolysaccharide induces inflammatory activation of Kupffer cells *via* the CD14-Toll-like receptor (TLR) 4 pathway[41]. Finally, increased endoplasmic reticular stress due to impaired protein degradation forms Mallory Denk bodies (hepatocellular aggregates of cytokeratins)[42].

Robust involvement of innate and adaptive immune disturbances in patients with alcoholic hepatitis is well known but has been only partially explored. Exaggerated systemic inflammatory response but still increased susceptibility to bacterial infections is the characteristic of alcoholic hepatitis[43] meaning to say that their immune cells are stimulated but with impaired antibacterial functions[44]. Although precise immunological mechanisms of alcoholic hepatitis are not clear, both adaptive[45] (neoantigens) and innate immune activation[46] are known to drive the clinical out look of alcoholic hepatitis (Systemic inflammatory

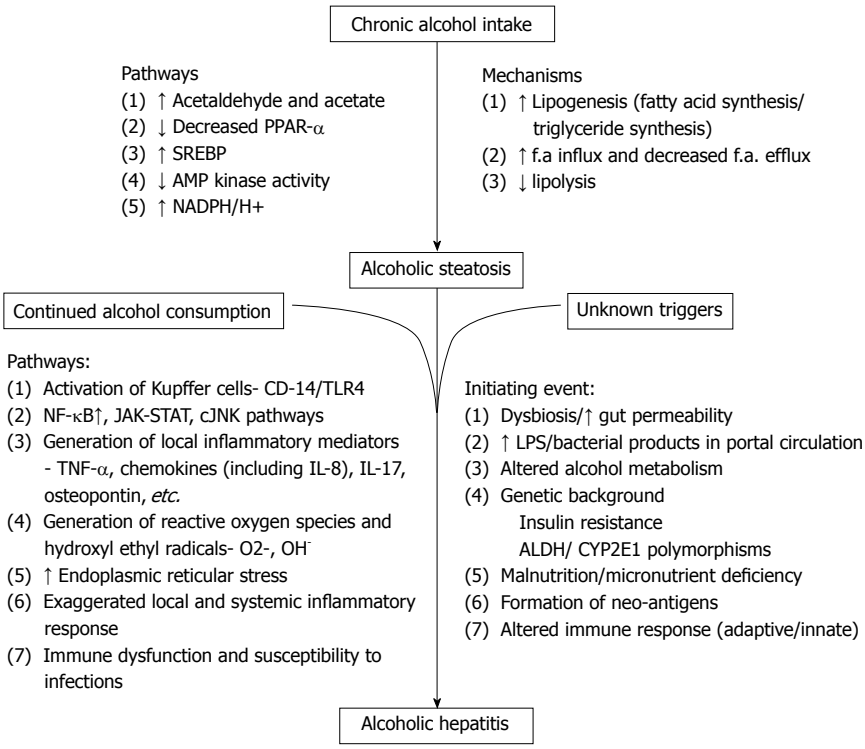

Figure 1 Pathogenesis of alcoholic hepatitis.

response). The precise mechanism or event that triggers the acute event in alcoholic hepatitis is still cryptic.

Some questions like, why only few chronic alcoholics develop liver disease and more so alcoholic hepatitis, why only few patients with alcoholic hepatitis respond to steroids and the standard of care and why only some of the alcoholic hepatitis patients are prone to develop renal impairment/ infections remain unanswered. The potential new treatments and key to better management of alcoholic hepatitis lie in the answers to the above questions.

METABOLOMIC PROFILING

Metabolomic profiling is one of the recently developed methods for identifying newer biomarkers. A recent study[47] compared serum metabolic profile between severe alcoholic hepatitis patients (n = 25) and alcoholic cirrhotics (n = 25). They have found altered levels of many biochemicals in subjects with severe AH and also demonstrated that metabolomic profiles separated the two cohorts with 100% accuracy. Severe AH was associated with enhanced triglyceride lipolysis, impaired mitochondrial fatty acid beta-oxidation, upregulated omega oxidation and decreased plasma membrane remodeling. While most measured bile acids were increased, low deoxycholate and glycodeoxycholate levels suggested intestinal dysbiosis in severe AH. Several changes in substrate utilization including increased glucose consumption by the pentose phosphate pathway, altered tricarboxylic acid (TCA) cycle activity, and enhanced peptide catabolism were noted. The same group has also demonstrated distinct lipidomic profile[48] in patients with severe alcoholic hepatitis in comparison with alcoholic cirrhosis with higher serum Resistin and plasma activation inhibitor-1 levels in and a decrease in serum Leptin levels in severe alcoholic hepatitis patients. Serum levels of the pro-lipolytic cytokines - tumor necrosis factor α, interleukin (IL)-6; IL-8 and IL-15 were found to be higher in severe alcoholic hepatitis patients in comparison to alcoholic cirrhosis. Serum IL-6 levels ⩾ 38.66 pg/mL most precisely identified deaths in severe alcoholic hepatitis patients (levels ⩾ 38.66 pg/mL had significantly decreased mean survival). Explosion of knowledge and understanding of metabolomic profiling (transcriptome, proteome and metabolome) in alcoholic hepatitis should lead to discovery of novel weak points in the pathogenesis. Future metabolomics studies should be able to identify novel specific therapeutic targets in the management of alcoholic hepatitis, which is the need of the hour.

POTENTIAL NEW AVENUES OF RESEARCH AND THERAPIES IN SEVERE ALCOHOLIC HEPATITIS

Prednisolone is the treatment of choice in the management of sub group of severe alcoholic hepatitis

Table 1 Potential new avenues for research and therapy in severe alcoholic hepatitis

Gut microbiota modification
Antibiotics (luminal, systemic)
Prebiotics and probiotics
Fecal microbiota transplantation (FMT)
Blockade of LPS and its downstream pathways *e.g.*, PD-1 and TIM-3 inhibition
Immune modulation
Chemokines *e.g.*, CCL20 inhibition
IL-8, IL-17 inhibition
Recombinant IL-22, recombinant human IL-10
Osteopontin inhibition
TNF-alfa superfamily receptor modulation
ADAMTS 13 enhancement
Inhibition of complement activation
Inhibition of inflammasome activation
Increasing steroid sensitivity
E.g., Basiliximab, Theophylline
Modification of genetic polymorphism of alcohol metabolizing enzymes
Epigenetic modification of alcohol induced liver damage
Liver regeneration and Early liver transplantation
Granulocyte colony stimulating factor (G-CSF)
Liver transplantation (DDLT/LDLT)
Setting up of alcohol units for post transplant support
Others
Extracorporeal liver support
Granulocytopheresis
Anti-oxidants - N-Acetyl Cysteine, S-Adenosyl Methionine

without any active infections or active UGI bleeding or renal impairment. Prednisolone is conventionally given at a dose of 40 mg per day for 4 wk followed by tapering over next 2 wk, with close monitoring for infections and other side effects of steroids. Despite the best standard of care and steroids, many patients either do not respond to steroids or are not eligible for steroids. Large majority of steroid intolerant, ineligible, unresponsive alcoholic hepatitis patients are thus left with no specific available treatment options. Better understanding of the pathogenesis of alcoholic hepatitis and advances in the basic sciences are opening new avenues for alcoholic hepatitis, as described below (Table 1).

Gut microbiota

Pre/probiotics and antibiotics: Several studies have shown that patients with liver disease have abnormal bowel flora overgrowth and thus probiotics, which help to restore normal bowel flora, have been proposed as a possible treatment for alcoholic liver disease[49,50]. Alcoholic patients who received probiotics (Bifidobacterium or Lactobacillus) for 5 d had improved AST, ALT and GGT levels in comparison to placebo[51]. Another study on alcoholic patients has shown that 4 wk probiotics (Bifidobacterium or Lactobacillus) improve and normalize neutrophil phagocytic capacity and decrease the endotoxin stimulated levels of soluble TNF-receptor-1, soluble TNF-receptor-2 and interleukin-10 *ex vivo* at the end of the study[52].

Rifaximin, a derivative of Rifamycin, with low systemic absorption and broad-spectrum activity against gastrointestinal tract micro-organisms that has been previously used to treat hepatic encephalopathy[53], is now being studied with regards to improving liver function in alcoholic cirrhosis. In a study, 28-d course of Rifaximin has been shown to decrease serum endotoxin levels in both systemic and splanchnic circulation along with significant improvement in HVPG in alcoholic cirrhotic patients[54].

Role of intestinal flora in the pathogenesis of alcoholic hepatitis is well known. However, targeting gut micro biome in the management of alcoholic hepatitis has rarely been attempted. This looks to be a promising area.

Lipopolysaccharide: Gut-derived microbial Lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria, has been known to have a central role in the pathogenesis of ALD[55,56]. Alcohol has been known to cause dysbiosis and as well disrupt the gut barrier function, consequently, promoting the translocation of microbial LPS from the lumen of the intestines to the portal vein, where it travels to the liver. In the Kupffer cell, LPS binds to CD14, which combines with toll-like receptor 4, ultimately activating multiple pro-inflammatory cytokine genes[57]. Therefore, probiotics, prebiotics, antibiotics, or transplantation of gut-microbiota may be proposed as possible treatment avenues for AH, by attenuation of the increase in LPS or normalising the healthy gut flora. Recently, fecal microbiota transplantation (FMT) has been successfully used in the treatment of life-threatening infections with Clostridium difficile. Gut bacteria being actively involved in the pathogenesis of alcoholic hepatitis, FMT might have a potential role in the management of alcoholic hepatitis. There is however, little data to support this proposition.

Michelena *et al*[58] have recently shown in 162 alcoholic hepatitis patients that blood LPS levels help in predicting progression to multiorgan failure, mortality and the response to steroids. Severe AH patients also have increased expression of TLRs (TLR 2, 4, and 9) in neutrophils along with impaired phagocytic function and increased secretion of CXC chemokines[44,59] suggesting that increased expression of TLRs can trigger neutrophils to display an inflammatory rather than phagocytic phenotype. Markwick *et al*[60] have recently shown that 2 known immunoinhibitory factors [*i.e.*, programmed death (PD-1) and T-cell immunoglobulin and mucin domain (TIM-3)] may play a role in the impaired immune function in AH. The authors found that antibacterial innate and adaptive immunity is severely dysfunctional in AH, differentiating these patients from stable alcoholic cirrhotics. Importantly, LPS or gut-derived endotoxin, which are typically elevated in sera of patients with AH, induced the overexpression of PD-1 and TIM-3 and their ligands PD-L1 and galectin-9 in all T-cell subsets.

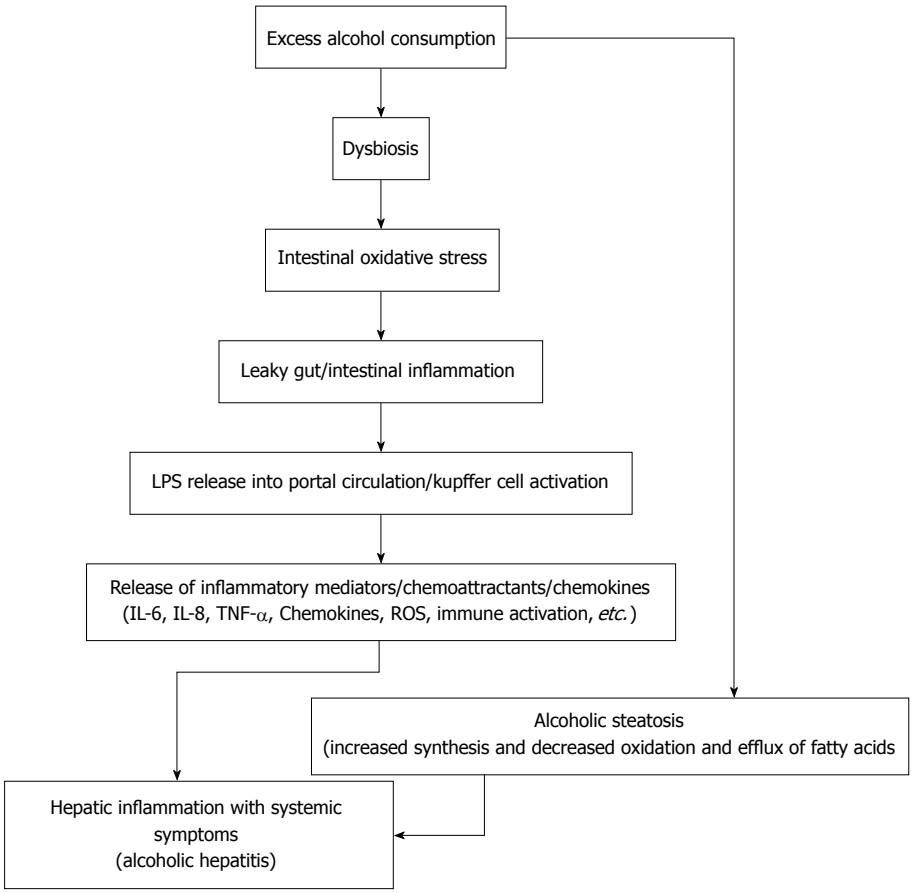

Figure 2 Potential steps in the initiation of alcoholic hepatitis, which can be modulated by targeting, the gut microbiome.

The blockade of these immunoinhibitory pathways restored normal lymphocyte (T-cell, natural killer/natural killer T cells, and T-regulatory cells) immunity and enhanced neutrophil antimicrobial activity. These results strongly suggest that LPS-induced expression of immunoinhibitory factors may play a role in the impaired antibacterial host immunity in AH. They also have shown that reduced interferon-γ/IL-10 ratios had a direct effect on PD1 and Tim3 expression on T-cell subsets. In another study, the soluble CD163, a specific marker of inflammatory macrophage activation was shown to be elevated in severe alcoholic hepatitis patients likely *via* LPS pathway (10 folds in comparison to controls and 3 folds in comparison to stable alcoholic cirrhotics)[61]. Hepatic macrophages may thus present a target for biological therapy of AH. Inflammatory activation of resident hepatic macrophages (Kupffer cells) by portal-derived lipopolysaccharide (LPS) has a primary role in hepatic inflammation in alcoholic hepatitis.

So, gut dysbiosis leading to increased gut derived bacterial products leads to immune-activation, exhaustion and paralysis in severe alcoholic hepatitis. Thus, changing the gut microbiome and/or altering the down stream inhibitory immune signals might improve outcomes in alcoholic hepatitis (Figure 2).

Immune modulation

Chemokines: Chemokines are known to play an important role in the pathogenesis of alcoholic hepatitis. Many of the chemokines have been shown to be upregulated in the livers of alcoholic hepatitis. Early studies revealed that the levels of several CXC subfamily members, including IL-8, Gro-α, CXCL5, CXCL6, CXCL10 and platelet factor 4, are significantly elevated in AH livers compared with normal healthy control livers, and correlate with neutrophil infiltration and the severity of portal hypertension and patient survival[36,38,62]. CC chemokine CCL2 is also upregulated[62]. Higher expression levels of IL-8, CXCL5, Gro-γ, and CXCL6 were associated with a worse prognosis[38]. Among these chemokines, CCL20 is one of the most upregulated chemokines in AH liver tissue[63]. Many inflammatory mediators such as LPS, TNF-α, and IL-1β induce CCL20 expression. The mechanism by which CCL20 contributes to the pathogenesis of AH remains unknown. The major function of CCL20 is to attract lymphocytes, dendritic cells, Helper T 17 (Th17) cells and monocytes and to a much lesser extent, attract neutrophils[62]. These cells then produce inflammatory mediators and chemokines that subsequently cause neutrophil infiltration in AH. So CCL20 has an important role in regulating adaptive

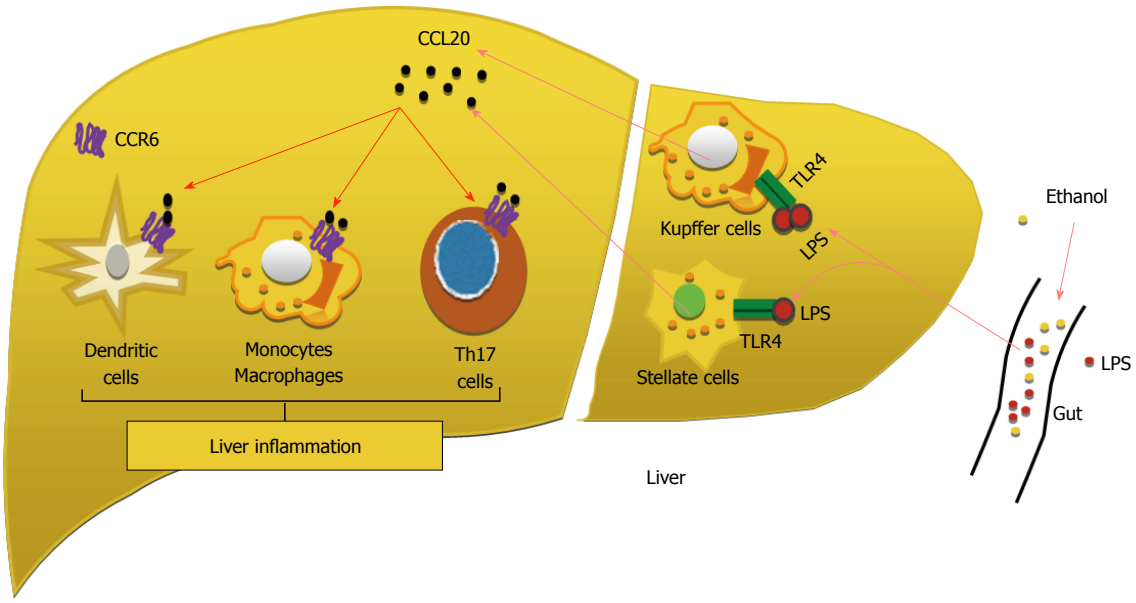

Figure 3 Model depicting the roles of CCL20 in alcoholic steatohepatitis. Excessive alcohol drinking results in elevated levels of bacterial products, such as lipopolysaccharides (LPS), in the portal blood. LPS, *via* targeting TLR4, stimulates Kupffer cells and hepatic stellate cells to produce CCL20. This chemokine activates dendritic cells (DCs) and monocytes/macrophages to produce proinflammatory cytokines by targeting CCR6, leading to liver inflammation[62].

immunity and autoimmunity (Figure 3). In their study Affò *et al*[63] have shown that the levels of CCL20 are elevated in patients with alcoholic hepatitis and correlate with the levels of endotoxemia, degree of fibrosis and portal hypertension. Macrophages and hepatic stellate cells (HSCs) were identified as the main CCL20 producing cell types. And also they have shown that silencing CCL20 *in vivo* reduces - LPS induced aspartate aminotransferase and lactate dehydrogenase serum levels and hepatic proinflammatory and pro-fibrogenic genes.

Further studies in future models and human livers of AH are required to determine if targeting CCL20 is an effective and safe therapeutic strategy to modulate the inflammatory response and liver injury in alcoholic hepatitis[64].

Several chemokine receptor antagonists are being developed. But so for only two drugs have been approved for non-inflammatory diseases: a CCR5 inhibitor used to treat HIV, and a CXCR4 antagonist which serves as a hematopoietic stem cell mobiliser[65]. Most common reason for failure is the receptor redundancy (one chemokine can target several receptors and *vice versa*). Development of chemokine receptor blockers which can block multiple receptors are needed to counter the receptor redundancy. Better translational and human studies are required to identify further key targets to block chemokine induced hepatic inflammation.

IL-8: Inhibition of neutrophil mediated hepatic injury might also be a potential therapeutic approach in managing AH. A higher level of IL-8 gene expression has been shown to correlate with poorer outcome in patients with alcoholic hepatitis[38]. IL-8 gene expression is also related to neutrophil hepatic infiltration as well as increased portal pressure[38]. IL-8 inhibition should lead to decrease in neutrophil infiltration of liver but the effect on bactericidal function of neutrophils could be a potential concern.

Th17: T cells producing Th17 have a central role in many inflammatory and autoimmune conditions[66]. IL-17 can act as a neutrophilchemotaxinand can also stimulate production of other chemotaxins such as IL-8 and CXCL1[67]. Serum and liver tissue IL-17 and IL-17 producing T cell are elevated in patients with alcoholic hepatitis and the number of infiltrating cells correlate with the Maddrey Discriminant Function. The increased levels of IL-17 within the liver are likely to act on hepatic stellate cells, which when stimulated with IL-17 increase chemotaxis of neutrophils[39].

Secukinumab, a humanised anti-IL-17A monoclonal antibody has shown some success in the phase 1 trials in the treatment of rheumatoid arthritis, psoriasis and uveitis[68]. To date no studies of secukinumab have been reported in patients with liver disease, which could be a potential agent.

IL-22/signal transducer and activator of transcription 3: IL-22 a member of IL-10 family of cytokines (produced by helper T17 and NK cells) has important role in bacterial infections and tissue repair[37]. IL-22 might be used to treat patients with ALD because of its antioxidant, antiapoptotic, antisteatotic, proliferative, and antimicrobial effects and it has been shown to work through activation of STAT3 in animal models of ALD. More over IL-22 receptor 1 expression is upregulated,

whereas IL-22 expression is undetectable, in patients with alcoholic hepatitis[40]. Støy *et al*[69] have found higher frequencies of IL-22 producing T-helper cells in alcoholic hepatitis patients (n = 21) in comparison with stable cirrhotics (n = 10) and healthy controls (n = 10). The frequency of IL-22-producing T helper cells was higher in AH patients and more so in those whose condition seemed to improve with improvement in alcoholic hepatitis severity. A study in a chronic ethanol-fed mouse model showed that treatment with recombinant IL-22 improves liver injury and hepatic oxidative stress[40] and another study in murine model of acute hepatitis, IL-22 receptors have been shown to be upregulated in the hepatocytes and blockade of IL-22 receptors has been shown to exacerbate the disease and administration of IL-22 to ameliorate the same[70].

So, IL-22 augmentation could be a potential therapeutic target in the management of alcoholic hepatitis. As IL-22 is produced by IL-17 cells, which also are involved in the production of pro inflammatory cytokines in the pathogenesis of alcoholic hepatitis, selective augmentation of IL-22 might be useful.

IL-10: IL-10 is a potent anti-inflammatory cytokine, which suppresses the production of many pro-inflammatory cytokines including TNF-α by Kupfer cells, monocytes and T-Helper cells and also from neutrophils[71]. IL-10 levels correlate with response to steroids and survival in patients of alcoholic hepatitis[72,73]. So far, recombinant human IL-10 (rhuIL-10) has failed to show any benefit in clinical trials on patients with Crohn's disease although well tolerated[74,75]. A pilot open label study of rhuIL-10 in combination with glucocorticoids in 8 patients with severe AH failed to show any changes to neutrophil-derived or serum IL-8 and TNF-α production or improvement in mortality or disease severity in comparison with the control group[76].

Osteopontin: Osteopontin is an extracellular matrix protein and is highly expressed in alcoholic hepatitis patients and the levels of osteopontin (OPN) are found to have very good correlation with the disease severity. OPN is reported to act as an immune modulator in a variety of manners-chemotaxis, immune cell recruitment and activation and modulation of apoptosis. In target cells, OPN binds to integrin and CD44 to promote profibrogenic and inflammatory actions[77]. There is growing evidence to suggest that OPN plays a major role in the wound-healing response to acute and chronic injury in many organs[78]. Hepatic expression and serum levels of OPN are markedly increased in AH, compared to normal livers and other types of chronic liver diseases, and its levels have been shown to correlate with short-term survival. Serum levels of OPN also correlated with hepatic expression and disease severity. OPN was mainly expressed in areas with inflammation and fibrosis. Two proteases that process OPN (thrombin and matrix metalloproteinase 7) and cleave OPN are increased in livers with AH. OPN synthesis is induced by lipopolysaccharide[78]. Fibrogenic mediators such as TGF-β are known to increase the OPN expression and also the alcohol mediated liver injury is attenuated in mice that lack OPN[21]. Human and experimental data suggest a role for OPN in the pathogenesis of AH. Further studies should evaluate OPN as a potential therapeutic target.

TNF superfamily receptors: Anti TNF-α agents like infliximab have been used with only limited clinical success and significant side effects[79,80]. Several members of the TNF receptor superfamily are markedly upregulated in patients with alcoholic hepatitis. TNF receptor superfamily member 12A (also known as Fn14 or the Tweak receptor) is markedly over expressed in these patients and its expression correlates with the severity of alcoholic hepatitis[21]. TNFRSF12A is mainly expressed in hepatic progenitor cells, which accumulate in patients with severe forms of alcoholic hepatitis. Therapeutic utility of this information needs further exploration. With the availability of metabolomics and transcriptomics, new therapeutic targets are expected. Affò *et al*[81], have identified increased expression of TNF-α superfamily receptors by transcriptome analysis of the liver tissue from alcoholic hepatitis patients. They have also shown that 207 genes are differentially expressed in patients with AH (> 5-fold) and revealed seven pathways differentially regulated including "cytokine-cytokine receptor interaction". Several tumor necrosis factor (TNF) superfamily receptors, but not ligands, were overexpressed in AH. Fn14, the receptor for TNF-like weak inducer of apoptosis, was selectively upregulated in patients with AH (n = 5) in comparison to normal controls (n = 7).

Intricate linkage analysis of the downstream signaling pathways might provide further insight in to the exact role and as well as therapeutic utility of the TNF and its receptors.

Proapoptotic molecules:Fas and Bcl-2: Oxidative stress stimulates the expression of Fas and Bcl2 in the livers of patients with alcoholic liver disease[82,83]. These targets are very appealing in the short-term management of acute alcoholic hepatitis.

ADAMTS13: von Willebrand factor cleaving protease: Activity of ADAMTS13 (a disintegrin and metalloproteinase produced by stellate cells) is decreased in alcoholic hepatitis due to pro inflammatory cytokines. This leads to accumulation of thrombi of unusually large von Willebrand factor leading to sinusoidal microcirculatory disturbances and subsequent liver injury[84,85]. Therapeutic potential of this fact needs

to further exploration.

Complement: Inflammation and systemic inflammatory response (SIRS) are an integral part of alcoholic hepatitis. Alcohol induced activation of complement system contributes to the pathophysiology of alcohol related liver injury[86]. In their study Shen *et al*[86] have shown significantly higher immunoreactivity intensity of C1q, C3, and C5 as well gene expression of C1q and C5 in patients with alcoholic hepatitis than that seen in normal controls. They have also shown the co-localization of C5a receptor (C5aR) in Mallory-Denk bodies (MDBs) forming balloon hepatocytes. C5aR was focally overexpressed in the MDB forming cells.Inhibition of complement activation could be a potential therapeutic option in the management of severe alcoholic hepatitis.

Inflammasomes: Recent studies indicate that the inflammasome activation plays important roles in the pathogenesis of AH. Nod-like receptor protein 3 (NLRP3) is a key component of the macromolecular complex that is so called the inflammasome that triggers caspase 1-dependent maturation of the precursors of IL-1β and IL-18 cytokines. It is expressed in myeloid cells and is a component of the innate immune system[87]. Inflammasome activation in AH liver biopsy specimen has been shown to correlate with Mallory Denk body (MDB) formation, suggesting that MDB could be an indicator of the extent of inflammasome activation.

So, new studies targeting inhibition of inflammaosome activation might discover some new treatment avenues for managing alcoholic hepatitis.

In vitro steroid sensitivity

A 48-h *in vitro* measure of steroid sensitivity, the dexamethasone inhibition of lymphocyte proliferation assay (DILPA), predicts 6-mo survival with 78% sensitivity[88]. The accuracy of the DILPA in predicting 6-mo survival, assessed by area under the receiver-operating characteristic (AUROC), was 0.86. Addition of the anti-IL-2 receptor (anti-CD25) monoclonal antibody, Basiliximab was shown to reverse the steroid resistance *in vitro* with improvement in lymphocyte proliferation count in 91% of the tested patients. Suggesting that intrinsic lack of steroid sensitivity may contribute to poor clinical response to steroids in severe AH and IL-2 receptor blockade represents a potential mechanism to overcome this. Basiliximab, the CD25 (IL-2 receptor) inhibitor which is used as single dose therapy to prevent transplant rejection, could reverse glucocorticoid resistance in peripheral blood mononuclear cells from patients with alcoholic hepatitis[88].

As T cells play a role in the recruitment of neutrophils and the perpetuation of inflammation in AH, Basiliximab may prove to be a useful adjunct to glucocorticoid therapy in patients who do not respond to this therapy.

Genetic polymorphism: Discoveries through genomic technologies and genome-wide association studies (GWAS), have increased evidence for genetic determinants of liver damage and progression to cirrhosis, and have implicated novel etiologic pathways. We know that not all heavy drinkers ever progress to cirrhosis. So the question remains why some people progress to cirrhosis, while others who drink to similar levels don't? Evidence from twin studies, variability in inter-ethnic ALD mortality rates and the recent association of PNPLA3 variant with alcoholic cirrhosis indicate that there is an underlying genetic basis that may account for the variability of liver damage observed in heavy drinkers, independent of alcohol dependence[89]. Genetic polymorphisms of ethanol metabolizing enzymes such as cytochrome p450 (CYP) 2E1 activation may change the severity of ASH.

Epigenetic modification: Ethanol consumption causes epigenetic changes that may contribute to alcohol-induced liver damage. Exposure to ethanol or its metabolite (acetate), up-regulates histone acetylation in macrophages, which causes up-regulation of transcription of several pro-inflammatory cytokines, leading to the development of alcoholic hepatitis. Therefore, epigenetic modifications can be new therapeutic target[90].

Liver regeneration through growth factors

Liver regeneration and stem cell therapy are active areas of current research in the field of hepatology. Ineffective liver regeneration has been postulated as one of the major reasons for progressive liver failure and non-recovery with conservative management in patients with alcoholic hepatitis. Recently Dubuquoy *et al*[91] have shown in explant livers from a small group of steroid non-responsive alcoholic hepatitis (n = 16) that their livers lack cytokine profile conducive for liver regeneration (TNF-α and IL-6) and also have shown insufficient hepatic progenitor cell differentiation tendency towards hepatocyte lineage.

In experimental models of alcoholic hepatitis, the administration of the cytokine granulocyte colony-stimulating factor (G-CSF) was found to mobilize the hematopoietic stem cells, induce liver regeneration, and improve survival[92,93]. In patients of alcoholic hepatitis, a 5 d G-CSF administration, (10 μg/kg/d, subcutaneously) mobilised $CD34^+$ stem cells, increased circulating hepatocyte growth factor and induced proliferation of hepatic progenitor cells in liver biopsy specimens[94]. A randomised placebo-controlled trial from our group using G-CSF (12 doses 5 μg/kg s.c. each over 1 mo) in patients with ACLF (57% had alcoholic hepatitis) has shown mobilization of $CD34^+$ stem cells, with significantly improved survival, and

decreased the risk of bacterial infection and kidney failure in G-CSF group[95]. In another recent study, Singh *et al*[96] have used G-CSF (5 μg/kg every 12 h for 5 d) in patients with severe alcoholic hepatitis and compared with Pentoxifylline (1200 mg/d) and have shown mobilization of $CD34^+$ cells and as well decreased infections, improved Maddrey's discriminant score and increased survival at 3 mo in patients receiving G-CSF. Other than stem cell mobilization and liver regeneration another potential mechanism of action of G-CSF in alcoholic hepatitis patients is postulated to be by stimulating the bactericidal activity of neutrophils[97-99] thus overcoming the immune paralysis.

It would be worthwhile to assess the role of G-CSF therapy in comparison to corticosteroids as none of the above studies have used steroids in their standard of care. G-CSF might improve the ineffective regeneration seen in those who fail to respond to the standard of care (*e.g.*, steroids) and may as well improve the neutrophil functionality and prevent infections. There is also need to study the role G-CSF in treating alcoholic hepatitis patients who are steroid ineligible or steroid unresponsive, in larger group of patients.

Early liver transplantation

The vast majority of transplant programs (85%) require 6 mo of abstinence[100] prior to transplantation commonly known as "6-mo rule". But there is a lack of evidence to support a 6-mo sobriety period. Patients who do not respond to steroids have a 6-mo survival of 25%-30%, and patients with hepatorenal syndrome (HRS) have a 3-mo mortality rate above 90%, unless treated with liver transplantation[101]. To date, nobody has been able to establish a certain period of abstinence, which ensures no future alcohol relapses; apart from this fact, in case of SAH, the 3-mo mortality rate is about 70%[102].

Currently there are very few options left in the management of severe alcoholic hepatitis patients, especially once they are unresponsive to steroids with Lille score > 0.45. Despite controversies, liver transplantation remains the sole major hope for such patients. Liver transplantation is more ethical in the case of living related donor transplantation as no other patient is deprived of the limited cadaveric resources and the emotionally attached relative wants to donate part of his liver to save his relative who may not survive to fulfill the current 6-mo abstinence rule. In a recently conducted public survey[103], majority of the respondents were neutral towards donating their organs for an early transplantation of a severe alcoholic hepatitis patient and only minority (26.3%) were hesitant to donate their organs to such patients. The scenario in a severe alcoholic hepatitis with a good family support and in the setting of living donor liver transplantation should be even better. So, early transplantation for carefully selected patients with acute alcoholic hepatitis may not be as controversial to the public as previously thought.

Emerging data has challenged the 6-mo abstinence rule as beneficial effects of early liver transplantation have been shown in select group of steroid unresponsive severe alcoholic hepatitis patients. In an elegant study, by Mathurin *et al*[104], twenty-six patients (median Lille score, 0.88) were selected and placed on liver transplantation list within a median of 13 d after nonresponse to steroid therapy. The cumulative 6-mo survival rate was higher among patients who received early transplantation than among those who did not (77% *vs* 23%, $P < 0.001$). This benefit of early transplantation was maintained through 2 years of follow-up (HR = 6.08; $P = 0.004$). The authors concluded that there are no major ethical barriers in transplanting patients affected by severe SAH, not responding to medical therapy. Presence of an Alcohol Addiction Unit (Alcohology unit) within a liver transplant center may significantly reduce the risk of alcohol relapse and the recurrence of disease after LT, and may allow liver transplantations in some selected patients, even in case of less than 6 mo of abstention[105]. Singal *et al*[106] have also shown a 5-year outcome in alcoholic hepatitis patients ($n = 11$) at par with alcoholic cirrhotic patients ($n = 33$) undergoing liver transplant from the UNOS database.

There is published data of seven severe alcoholic hepatitis patients with non-response to therapies, and hepatorenal syndrome, who were submitted to transjugular intrahepatic portosystemic stent shunt (TIPS), and then underwent liver transplantation[102,107]. Steroid therapy was contraindicated because of the presence of renal failure. All patients were followed up by the Alcohology Unit, and attended self-help groups. None of them had recidivism over the next 5 years.

Despite the promising results from many studies and over all public and professional concern and intent towards a shorter mandatory abstinence period prior to liver transplant after initial non-response to medical management, too many uncertainties exist. There is a need to define and form guidelines for setting universally suitable and logically acceptable norms to do liver transplant in this group of patients which should also address delicate issues like pre transplantation counseling, deceased *vs* living related donor liver transplantation, setting up of Alcohology units for post transplant support systems *etc*.

Others

Extracorporeal liver support: Extracorporeal liver support procedures, which have the ability to remove some potential damaging circulating molecules may, therefore, logically have a role in patients with severe AH. Survival in severe alcoholic hepatitis patients with non-response to medical care and renal impairment is very poor even with best of the available management. Despite great efforts[108,109], no clear benefits have been

Table 2 Ongoing registered studies on newer treatment for alcoholic hepatitis

Treatment	Type of molecule/intervention	Mechanism of action	Identifier
ELAD	Extracorporeal, human cell-based liver support system	Supplement hepatic function	NCT01829347, NCT00973817, NCT01471028
Obeticholic acid	Biliary acid	Affect bile acid abnormalities	NCT02039219
Corticosteroids + Bovine + Colostrum	Protein supplement	Improving immunity	NCT02473341
IMM 124-E	Hyperimmune bovine colostrum	Improving immunity	NCT01968382
G-CSF	Liver regeneration/ immunomodulation	improves liver regeneration and immunity in steroid non-responders	NCT02442180, NCT02451033, NCT01820208
Corticosteroids + N-acetyl cysteine	Anti-oxidant	Augments steroid function	NCT00863785
Amoxicillin + corticosteroid	Antibiotic	Decreases infections	NCT02281929
S-adenosyl-l-methionine	Antioxidant	decreasing oxidative stress	NCT02024295
Rifaximin	Luminal antibiotic	Improves gut dysbiosis	NCT02116556, NCT02485106
Ciprofloxacin	Antibiotic	Decreasing infections	NCT02326103
Emricasan/IDN-6556	Pan-caspase inhibitor	Reduces apoptosis	NCT01912404
Lactobacillus Rhamnosus GG	Probiotic	Improving dysbiosis	NCT01922895
MycophenolateMofetil and Rilonacept	Immunosuppressant and immune modulation	Decreasing hepatic inflammation	NCT01903798
Metadoxine	Anti-oxidant		NCT02161653
Fecal microbiota transplantation	Healthy microbiome replacement	Correction of dysbiosis	NCT02458079
Early liver transplantation	New liver	Liver transplant in patients unresponsive to medical treatment	NCT01756794
Anakinra	interleukin-1 receptor antagonist	Decreases hepatic inflammation	NCT01809132

proved using these complicated liver dialysis devices, which additionally are very expensive and have many issues to be answered before the utilization in the clinical setting. There are some motivating reports concerning albumin dialysis[110] as a support treatment in patients with severe AH which may potentially bridge recovery or liver transplantation who otherwise have no other options available especially those with renal impairment[111].

Granulocytapheresis: Granulocytapheresis, a technique that removes up to 60% of activated granulocytes and monocytes from circulating blood, is well tolerated and many case series exist in literature on its usefulness in severe alcoholic hepatitis patients. Some also mention their benefit in steroid non-responders[112,113]. Role of granulocytapheresis is still not proven in any good quality studies till date and only case series are available.

Anti-oxidants: Although oxidative stress is implicated in the pathogenesis of alcoholic hepatitis[114], several studies have negated any additional benefit of N-Acetyl cysteine in comparison to corticosteroids in the management of alcoholic hepatitis[115-117]. Only few of the studies have shown a short-term benefit of combination therapy with corticosteroid plus N-acetylcysteine with increased 1-mo survival among patients with severe alcoholic hepatitis, but without any improvement in 6 mo survival was noted[117]. A Cochrane review earlier had shown that use of S-Adenosyl-L-Methionine is not of any help in managing alcoholic hepatitis[118]. Conceptually anti-oxidants should potentially have a role in the management of alcoholic hepatitis but we need more data to establish the definitive role of N-Acetyl Cysteine or S-Adenosyl-L-Methionine in the management of severe alcoholic hepatitis. There remains a large void of treatment options for SAH. The ongoing clinical trials in treating alcoholic hepatitis are mentioned in Table 2. A major improvement in our understanding and a paradigm shift in the treatment approaches are required to improve the outcome of these patients.

CONCLUSION

The burden of alcoholic hepatitis is on the rise and the proportion of patients with alcoholic hepatitis is rapidly increasing with better treatment options of viral liver diseases. The morbidity, mortality and the treatment options for the management of alcoholic hepatitis have not significantly changed in the last many decades. Currently steroids, pentoxifylline and nutrition remain the only acceptable treatment options. A better and newer treatment options for alcoholic hepatitis are the need of the hour.Advances in the basic science, "Omics" platform and translational medicine have given a better insight into the pathogenesis and have opened up many potential new therapeutic avenues in the management of alcoholic hepatitis. Targeting gut microbes and their products, targeting hepatic inflammation and infections through immune modulation, improving liver regeneration by G-CSF and early liver transplantation for those not responding to the standard of care (Figure 4) are the most promising areas for research and future clinical trials should focus on these areas in developing new therapies in the management of alcoholic hepatitis.

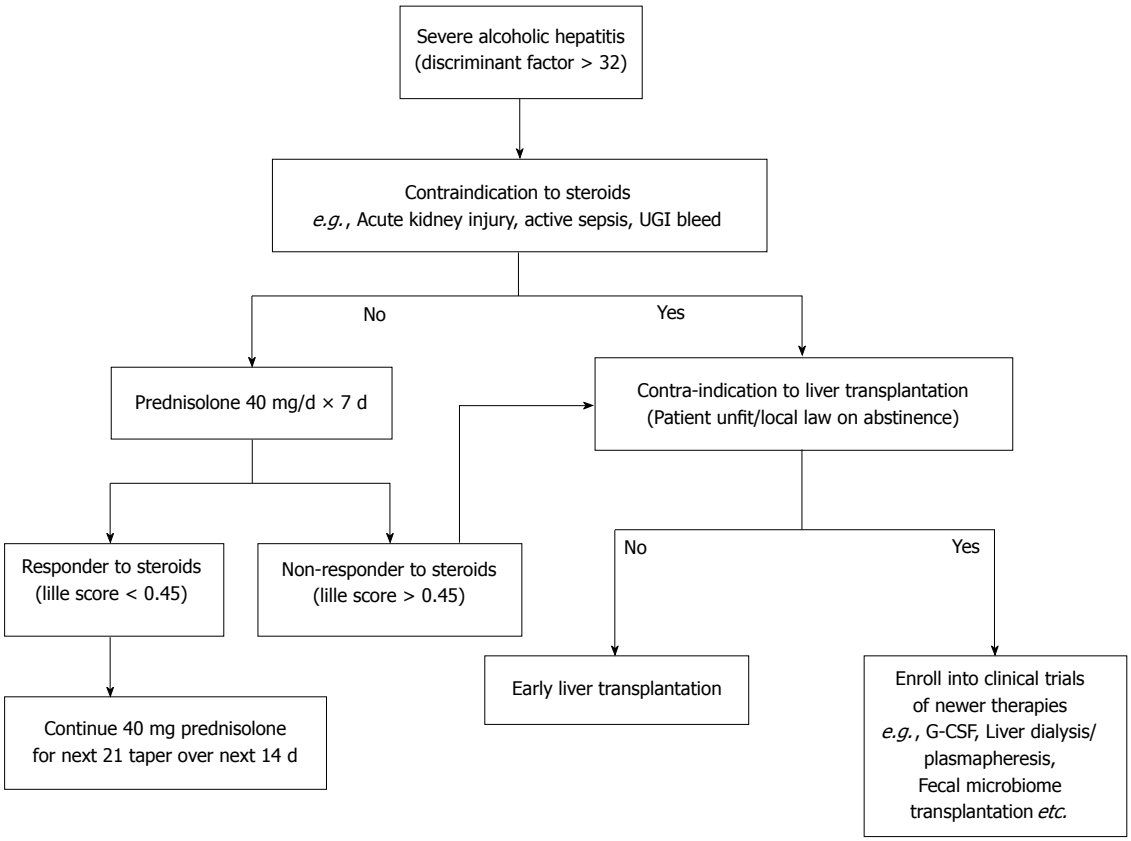

Figure 4 Approach to management of alcoholic hepatitis. G-CSF: Granulocyte colony-stimulating factor.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

MicroRNAs as possible biomarkers for diagnosis and prognosis of hepatitis B- and C-related-hepatocellular-carcinoma

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Abstract

Aim of the present review is to summarize the current knowledge about the potential relationship between miRNAs and hepatitis B virus (HBV)-hepatitis C virus (HCV) related liver diseases. A systematic computer-based search of published articles, according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis Statement, was performed to identify relevant studies on usefulness of serum/plasma/urine miRNAs, as noninvasive biomarkers for early detection of HBV and HCV-induced hepatocellular carcinoma (HCC) development, as well as for its prognostic evaluation. The used Medical Subject Headings terms and keywords were: "HBV", "HCV", "hepatocellular carcinoma", "microRNAs", "miRNAs", "diagnosis", "prognosis", "therapy", "treatment". Some serum/plasma miRNAs, including miR-21, miR-122, mi-125a/b, miR-199a/b, miR-221, miR-222, miR-223, miR-224 might serve as biomarkers for early diagnosis/prognosis of HCC, but, to date, not definitive results or well-defined panels of miRNAs have been obtained. More well-designed studies, focusing on populations of different geographical areas and involving larger series of patients, should be carried out to improve our knowledge on the potential role of miRNAs for HCC early detection and prognosis.

Key words: Hepatitis B virus; Hepatitis C virus; Hepatocellular carcinomas; Liver diseases; MicroRNAs; Review

Core tip: A systematic computer-based search of published articles was performed to identify relevant studies on usefulness of serum/plasma/urine miRNAs, as noninvasive biomarkers for early detection of hepatitis B virus and hepatitis C virus-induced hepatocellular carcinoma (HCC) development. Some serum/plasma miRNAs might serve as biomarkers for early diagnosis/prognosis of HCC, but, to date, not definitive results or well-defined panels of miRNAs have been obtained. More well-designed studies should be carried out to improve our knowledge on the potential role of miRNAs for HCC early detection and prognosis.

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INTRODUCTION

Hepatitis B (HBV) and Hepatitis C (HCV) viruses are well-known etiological factors for liver damage. It has been estimated that nearly 5% of world population is chronically infected with HBV (approximately 350 million of people)[1]. The global prevalence of HCV is about 2%, with 180 million people who persistently carrier this pathogen[2]. However, wide variations in HBV/HCV infection rates exist among different countries[3]. A significant percentage of chronic HBV and HCV carriers develop a necroinflammatory liver disease with different patterns of severity and course, ranging from persistent injury to cirrhosis, hepatic failure and hepatocellular carcinoma (HCC)[4]. Liver carcinogenesis is a multi-step process, which is characterized by the perturbation of several key and crucial cellular functions[5]. Cell-cycle control, apoptosis, senescence, growth, migration and energy production are the most important deregulated activities during cancer development both in liver and other organs[6,7]. HCC is the sixth most frequent malignancy in the world, and, irrespective of the improvement in diagnostic approaches and in treatment of this neoplasm, it still represents the second cause of cancer death, because of its poor outcome[8-10]. The high morbidity and mortality rates of this type of cancer require the adoption of more specific methods and more effective strategies for HCC diagnosis and treatment. To date, HCC detection is generally based on imaging techniques, including ultrasonography, Computed Tomography (CT) and Magnetic Resonance (MRI) in association of laboratory tests (serum α-feto protein) and/or histopathology (*i.e.*, liver biopsy)[11]. All these diagnostic tools present potentially limiting factors, including their costs, availability and reproducibility[12]. Therefore, in the last years, some serum or tissue biomarkers have been developed to be used in clinical practice, such as microRNAs (miRNAs). These molecules are small (19-23 nucleotides) single-stranded non-coding RNAs, able to silence endogenous messenger RNA (mRNA) transcripts[13,14]. MiRNAs modulate gene expression, by degrading or inhibiting mRNAs, therefore they decrease or suppress protein translations, at post-transcriptional level. In the last years, an increasing number of studies have investigated the role of miRNAs in the regulation of different cellular processes, including energy production, protein synthesis, proliferation, differentiation and apoptosis[15]. It is well-known that each natural tissue harbours peculiar profiles of miRNAs expression. In addition, characteristic perturbed miRNA patterns have been described in

different liver diseases, ranging from chronic hepatitis to cirrhosis and HCC[16-19]. The identification of subjects with HCC at early stages, before the development of clinical signs and symptoms, represents a pressing need to improve long-term prognosis of these individuals[20].

The aim of the study is to review the available data describing: (1) potential usefulness of serum/plasma/urine miRNAs that may serve as novel non-invasive biomarkers for early detection of HBV and HCV-induced HCC development, as well as for prognostic assessment in these patients; and (2) perturbation of miRNAs expression in liver tissue of HBV- and HCV-related HCC.

SEARCH STRATEGY AND SELECTION OF STUDIES

A systematic computer-based search of published articles, according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) Statement[21], issued in 2009, was conducted through Ovid interface, in order to identify relevant studies on the usefulness of serum/plasma/urine miRNAs that may serve as novel noninvasive biomarkers for early detection of HBV and HCV-induced HCC development, as well as for prognostic assessment in these patients.

The literature review was performed in March 2015. The following electronic databases were used: MEDLINE (January, 2000 to March, 2015) and the Cochrane Library (until the first quarter of 2015) for all relevant articles. The search strategy and the search terms were developed with the support of a professional research librarian. The search text words were identified by means of controlled vocabulary, such as the National Library of Medicine' s MESH (Medical Subject Headings) and Keywords. Our review assessed the perturbation of miRNAs expression in HBV and HCV related liver diseases. The used MESH terms and keywords were: "HBV", "HCV", "hepatocellular carcinoma", "microRNAs", "miRNAs", "diagnosis", "prognosis", "therapy", "treatment".

The inclusion criteria for our analysis were: (1) studies investigating liver-originated miRNAs expression in patients with HCC and performed with the aim to improve the diagnosis of this malignancy or to evaluate their potential role as tools for assessing prognosis and efficacy of treatment for patients, suffering from this neoplasm; (2) study samples were represented by serum/plasma, urine and hepatic tissue specimens and obtained in these studies directly from the investigated liver lesions or extracted from extra-lesional material (*e.g.*, plasma, sera); (3) each of included studies contained at least 10 subjects for group; and (4) articles which were reported in English, as peer-reviewed, full-text publications.

On the other hand, exclusion criteria were: (1) conference abstracts, case reports, editorials, articles not published as full reports; (2) duplicates; and (3) studies performed in cell lines or in animal models.

The PubMed "related articles" features and the reference lists of retrieved articles were also searched to find additional pertinent studies. If a study was considered potentially eligible by either of the two reviewers, the full-text of this study was further evaluated. Full-text assessment was performed according to eligibility criteria developed to systematically include studies into this review. Therefore, we excluded all trials, reporting patients with HBV or HCV and HIV co-infection.

STUDY SELECTION

Two authors (M.M.. and R.L.), independently and in a parallel manner, performed the literature search, identified and screened relevant articles, based on title or title and abstract. If a study was considered potentially eligible by either of the 2 reviewers, the full article of this research was collected for further assessment. Other two authors (M.Z. and L.M.) independently extracted and tabulated all relevant data from included studies by means of a standardized flow path, according to the Cochrane handbook section 7.3a checklist of domains. The following information was obtained from each study, by means of a predefined data extraction form, including: first author's name, study design, inclusion and exclusion criteria, year of publication, country of origin, ethnicity, matching criteria, number of cases and controls, diagnostic methods to detect each malignancy, HCV detection assays. The accuracy of data collection was checked by A.T. and A.D. and any disagreements concerning the results were settled by consensus between all authors. With the purpose to prevent multiple inclusions of the same data, we searched the presence of possible duplicates, examining the first author's name as well as the place and the period of subjects' enrolment. When different versions of the same study were detected, only the most recent one was considered.

Bearing in mind the purpose of our review, the characteristics and the wide heterogeneity of the identified reports (such as the difference in study designs as well as in end points and the limited number of screened miRNAs, recognized as potentially involved in HCC development) and the lack of a definite and appropriate knowledge of miRNAs profiles, associated with diagnosis and outcome of this malignancy, sensitivity and subgroup analyses of identified articles were considered inappropriate. Therefore, no qualitative analysis and quantitative assessment of these studies was performed and all articles, meeting the predefined inclusion criteria, were included in our review. We decided to search the miRNAs, that were reported at least five or more times in available studies.

NUMBER OF STUDIES REPORTING MIRNAS EXPRESSION IN HBV- AND HCV-RELATED HCC

The search of Medline and Cochrane Library identified a total of 2778 citations. Among these, 2579 were excluded after a preliminary review of the titles and/or abstracts. The full text of the remaining 199 articles was considered for a more detailed assessment. The full-text of these 199 articles was reviewed to determine whether they met our inclusion and exclusion criteria, 127 studies were excluded because of they were reviews, duplicates or not relevant to the miRNA expression in HCC. Finally, 72 reports were included in this systematic review and subdivided into three groups (Table 1, Table 2, Table 3, Table 4 and Table 5 and Supplementary Tables 1-3): (1) studies investigating miRNAs patterns in patients with only HBV-related HCC[22-64]; (2) studies showing miRNAs profiles in individuals with HBV and HCV-related HCC[65-85]; and (3) studies reporting miRNAs patterns in subjects with only HCV-related HCC[86-92].

The first subgroup included 43 articles (1): 36 performed in China, 3 in South Korea, one in Taiwan, one in India, one in Turkey and one in Italy; In the second subgroup 21 articles were available (2): 6 were carried out in Japan, 4 in China, 4 in Italy, 2 in United States, 2 in Taiwan, one in South Korea, one in France and one in Germany; and the subgroup consisted of 7 studies (3): 3 articles were performed in Egypt, 2 in Germany, one in China and one in Turkey.

Some studies enrolled only HCC patients, without comparison group, whereas most of them included controls as healthy subjects, patients with viral-related chronic hepatitis or cirrhosis, as well as hepatitis B surface antigen (HBsAg) positive subjects (defined as "asymptomatic carries" because of the evidence of liver active disease). Most of HCC patients included in these reports were male. In addition, a high heterogeneity among the studies is evident as reported in Tables 1-5 (and in Supplementary Tables 1-3), mainly due to differences in scope, end-points, reference control group, starting material and molecular techniques. In particular, some studies enrolled patients with HBV- or HCV-related HCC and the results, concerning the miRNA profiles, were not characterized on the basis of the viral infection etiology.

In Tables 2 and 4 we have tried to hypothesize two putative panels of deregulated miRNAs in HBV- and HCV-related HCCs, considering only miRNAs observed deregulated (with the same expression) in at least three papers. As shown in Table 2, in HCC patients with HBV-related infection, seven miRNAs (miR-221, miR-21, miR-222, miR-122a, miR-224, miR-18a and miR-223) have been observed as consistently up-regulated and only one miRNA (miR-101) has been described as down-regulated. Intriguingly, even if the vast majority of papers have obtained concordant results about the expression of these miRNAs, some studies have reported a different regulation of some of aforementioned miRNAs (miR-21, miR-222, miR-122a, miR-101)[22,28,61,93] (Table 2).

In Table 4, we have reported miRNAs observed deregulated in studies enrolling HCC patients with HBV and HCV-related infection in at least three papers. Two miRNAs (miR-21 and miR-224) were observed consistently up-regulated and two miRNAs (miR-130a and miR-195) as down-regulated (Table 4).

MIRNAS IN THE ASSESSMENT OF HCC DIAGNOSIS AND PROGNOSIS

Only a small number of circulating miRNAs has been assessed at least five or more times as potential and useful biomarkers in HCC diagnosis in the identified studies, because they have been reported as deregulated in cirrhosis and during development of hepatic malignancy. In particular, among the tested miRNAs profiles, miR-21, both in serum/plasma[45,49,56,61,63,82] and in liver cancerous tissue[24,29,72,77,83,87], miR-122 both in serum/plasma[43,49,50,55-57,71,88,93] and tissue samples[46,67,69,80,83], miR-125a/b in serum/plasma sample[30,43,90] and in tissue specimens[25,68,75,78,83,91], miR-199a/b in serum/plasma sample[50,65,76] and in tissue specimens[34,69,75,83,91], miR-221 both in serum plasma[49,52,88,94] and in hepatic specimens[29,63,68,69,83], miR-222 both in serum/plasma[49,52] and hepatic specimens[22,72,83], miR-223 both in serum/plasma[30,32,43,49] and in liver tissue[69], miR-224 both in serum plasma[45,57] and tissue samples[29,40,68,72,75] have been assessed more widely in comparison to other miRNAs. According to the reported results, these miRNAs represent the most important candidate biomarkers in term of diagnostic efficiency among the assessed ones, to compare circulating miRNA expression between HCC patients and healthy people as well as between subjects with liver malignancy and individuals with hepatic injury, such as persistent hepatitis or cirrhosis. Nevertheless, to date, no definitive conclusions may be drawn.

MIRNAS IN NON-VIRAL ASSOCIATED HUMAN HEPATOCARCINOGENESIS

Despite a wide series of efforts to investigate the roles of miRNAs both in malignant and in non-malignant liver diseases, little is known about the roles of miRNAs in non-viral associated human hepatocarcinogenesis, including non-alcoholic fatty liver disease (NAFLD) and/or non-alcoholic steatohepatitis (NASH), alcohol-related HCC, iron overload and primary biliary cirrhosis. Most of available studies have been performed in animal models, mimicking these pathological conditions. To date, only a small number of reports have been carried out to examine miRNA expression profiles and their potential impact, during the development

Table 1 miRNAs patterns in studies enrolling hepatocellular carcinoma patients with hepatitis B virus-related infection

References, period and state	Characteristics of the study	miRNAs Up-regulated	miRNAs Down-regulated	Conclusions
Bandopadhyay M, *BMC Cancer*, 2014 India Period: NR	Tissue samples obtained from: -16 healthy subjects -16 patients with advanced liver diseases (HBV positive cirrhosis and HCC)	ND	Decreased miR-21, miR-222 and miR-145 expression in patients with advanced liver diseases and HCC in comparison with healthy individuals	Differential modulation of miRNAs expression by HBx protein
Cheong JY, *J Korean Med Sci*, 2014 Korea Period: NR	Serum samples from: 1439 individuals with either past/present evidence of HBV infection: -HCC: 417; -LC: 305; -CHB: 313; -SR: 404.	NR	Higher rate of HBV persistence after infection subjects with miR-604 rs2368392 T allele in comparison with miR-604 rs2368392 C allele. Patients with miR-604 T allele may have a higher risk for HBV chronicity Higher rate of the miR-604 T allele in the chronic carrier without HCC	pre-miR-604 rs2368392 polymorphism might confer genetic susceptibility to the occurrence of HCC in HBV related chronic liver disease, and HBV persistence after HBV infection
Connolly E, *The American Journal of Pathology*, 2008 China Period: NR	Human HCC samples and matched non-tumor liver tissue (19 sets) were obtained from surgical resections of anonymous donors	Up-regulation of miR-17-92 (miR-17, miR-19a, miR-20, and miR-92) and miR-21 occurs in precancerous stages of liver disease and in HCC in comparison with normal liver	NR	The combination of assays presented in the present study supports a role for the miR-17-92 polycistron (all six members) or miR-21 in the maintenance of the malignant transformation of hepatocytes
Coppola N, *PLoS One*, 2013 Italy Period: April 2007 - March 2011	Tissue samples obtained from: twenty-seven consecutive HBsAg/anti-HBe/HBV-DNA-positive Caucasian patients who were naive to nucleos(t)ide analogues and interferon therapy	Higher miR-125a-5p liver concentrations observed in patients with HBV-DNA plasma levels > 10^3 IU/mL	NR	In HBsAg/anti-HBe-positive patients, the liver miR-125a-5p level correlated with liver and plasma HBV-DNA values and was associated to a more severe disease progression
Dang YW, *Asian Pac J Cancer Prev*, 2014 China Period: March 2010 and December 2011	89 pairs of HCC formalin-fixed paraffin-embedded and their adjacent tissue 74/89 pairs were obtained from HBV-related HCC samples	NR	Remarkably downregulation of miR-152 expression in HCC compared to that in adjacent hepatic tissues Lower expression was observed in HBV positive group than in the negative one	miR-152 underexpression is associated with hepatocarcinogenesis, acting as a tumor suppressor miRNA, its lack is related to the progression of HCC through deregulation of cell proliferation, motility and apoptosis

Fan MQ, *Journal of Experimental and Clinical Cancer Research*, 2013 China Period: 2002 -2007, patients were followed until December 2010	100 patients with HCC, undergoing LT 95/100 patients with HBV related cirrhosis Specimens obtained from formalin-fixed paraffin-embedded tissue	NR	Down-regulation of miRNA 20a	miR-20a is decreased in HCCs and correlates with HCC recurrence and prognosis. Its down-regulation increases the proliferation abilities of HCC cells. miR-20a may represent a novel Potential therapeutic target and biomarker for survival of HCC patients
Fu Y, *Oncol Letters*, 2013 China Period: NR	Serum and tissues (paired tissue specimens from 25 HCC tissues and adjacent noncancerous hepatic tissues (20 HBV-related HCC) were obtained from patients undergoing surgical resection and compared with 20 healthy subjects	miR-101 is upregulated in human HBV-related HCC serum	miR-101 is downregulated in human HBV-related HCC tissues	Serum miR-101 expression was closely associated with tumoral size of HCC-patients and provides a promising biochemical marker of HBV-related HCC
Gao P, *Hepatology*, 2011 Hong Kong Period: NR	Formalin fixed, paraffin embedded materials obtained from: -16 patients with dysplastic nodules -29 HCC nodules from 24 patients	Up-regulation of miR-224 in pre-malignant DNs Up-regulation of miR-10b, miR-21, miR-221, and miR-224 in the small HCCs	Down-regulation of miR-145 and miR-199b in pre-malignant DNs Down-regulation of miR-145 and miR-199b in the small HCCs	miRNA deregulation is an early event and accumulated throughout the various steps of HBV-associated hepatocarcinogenesis. Down-regulation of miR-145 and miR-199b and up-regulation of miR-224 were frequently observed in pre-malignant DNs and these changes persisted throughout HCC development miR-145 is a candidate tumor suppressive miRNA and may play an important role in HCC development
Giray BG, *Mol Biol Rep*, 2014 Turkey Period: NR	Plasma samples from: -66 HBV-positive patients (CHB: 24, cirrhosis: 22, HCC: 20) -28 healthy controls	mi125b-5p up-regulation in CHB, cirrhosis and HCC in comparison to healthy controls	miR-223-3p down regulation in CHB, cirrhosis and HCC in comparison to healthy controls	miR-125-5 p and miR-223 -3p could be used as novel non-invasive biomarkers of HBV-positive HCC in very early, even at CHB stage of liver disease
Gu H, *Mol Cell Biochem*, 2013 China Period: April 2001 - March 2009	Tissue samples obtained from 108 patients with HCC, undergoing surgical resection. HBsAg +: 92; HBsAg -: 16	Up-regulation of miR-372 associated with significant poorer recurrence-free survival and overall survival	NR	miR-372 may serve as a potent prognostic marker for tumor recurrence and survival of HCC patients as well as a promoter of tumorigenicity of HCC and may be a prospective therapeutic target for this malignancy

Gui J, *Clinical Science*, 2011 China Period: November 2008 - January 2010	Serum samples from: 25 HBV-positive patients (LC: 10, HCC: 15) -10 age-matched healthy controls	Up-regulation of miR-885-5p, miR-574-3p, miR-224, miR-215 and miR-146a in the HCC and LC patients	NR	miR-885-5p is significantly elevated in the sera of patients with liver pathologies miRNAs could serve as novel complementary biomarkers for the detection and assessment of liver pathologies
Han Y, *PLoS One*, 2013 China Period: -September 2009 - June 2010 -October 2009 to September 2011	Serum samples from: 1,012 healthy controls, 302 HBV natural clearance subjects, 316 ASCs, 316 patients with CHB, 358 HBV-infected patients with LC, and 1,021 HBV-infected patients with HCC Pri-miR-34b/c rs4938723 HBV-HCC patients: 311 HBV-infected subjects without HCC: 210 Pre-miR-196a2 rs11614913 HBV-HCC patients: 255 HBV-infected subjects without HCC: 170	NR	NR	Association of pri-miR-34b/c rs4938723 with a significant increased risk of HCC, mainly in women No statistically significant association of pre-miR-196a2 rs11614913 with HCC risk. pre-miR-196a2 rs11614913 may enhance the effect of primiR-34b/c rs4938723 in women rs4938723 CC genotype and rs11614913 TC genotype might predispose the host to immune selection of T1674C/G, and G1896A, respectively The rs4938723 effect on HCC risk can be seriously affected by the HBV mutations
Hou J, *Cancer Cell*, 2011 China Period: NR	Tissue samples obtained from 40 HCC patients with CHB	NR	Consistent miR-199a/b-3p decrease in HCC, and its reduction significantly correlates with poor survival of HCC patients	miRNomes of human liver and HCC and contributes to better understanding of the important deregulated miRNAs in HCC and liver diseases
Huang J, *Hepatology*, 2010 China Period: NR	20 HBV-related HCC tissues and the corresponding nearby noncancerous livers	NR	Down-regulation of miR-152 in human HBV-related HCC Tissues	Tumor suppressive role of miR-152 in the epigenetic aberration of HBV-related HCC and the potential development of miRNA-based targeted approaches for the treatment of HBV-related HCC

Huang YH, *PLoS One*, 2012 China Period: July 1998 - Aug 2004	Tissue samples obtained from: 228 patients with HCC, 12 with known better and poorer prognosis subjected for the first-step (pilot) study; 6 patients had a RFS time for more than 5 yr (better prognosis) and 6 had rapid relapse within six-month after operation (poorer prognosis)	High expression levels of miR-30c, miR-155, miR-432, miR-15b, and miR-30b associated with shorter RFS High miR-15a, miR-486-3p, and miR-381 expression significantly predicted a longer RFS High expression level of miR-29a, miR-486-3p, and miR-876-5p significantly predicted a longer OS	NR	Significant prognostic miRNA predictors identified through examination of miRNA expression levels in paraneoplastic liver tissues. Functional analysis of miR-155, suggested that the prognostic miRNA predictors identified under this strategy could serve as potential molecular targets for anticancer therapy
Jiang R, *Clin Cancer Res*, 2011 China Period: January 2001 - August 2009	Liver tissue obtained from: 116 HBV-related HCC patients 48 subjects with benign conditions	up-regulation of miR-22 in male tumor adjacent tissue	NR	Overexpression of miR-22 in male tumor adjacent tissue associated with down-regulated ERa expression, potentially causing the attenuation of the protective effect of estrogen and inducing increased IL-1a expression. These results may explain the high incidence of HBV-associated HCC in the male population
Kim HY, *J Med Virol*, 2014 South Korea Period: NR	Serum samples obtained from: 1439 Korean patients with either past or present HBV infection, -404 control subjects with spontaneous Recovery; -1035 subjects with chronic HBV (313 with chronic hepatitis B, 305 with liver cirrhosis, 417 with HCC)	NR	NR	Protective effect of miR-196a-2 rs12304647 CC genotype against development of HCC in comparison to the AA or AC genotypes in patients with chronic hepatitis and cirrhosis
Kwak MS, *PLoS One*, 2012 South Korea Period: January 2001 - August 2003	1439 Korean subjects with past or persistent HBV infection: SR: 404 CHB: 313 chronic LC: 305 HCC : 417	Micro RNAs-371-372-373 (miRNAs-371-373), originating from the same pri-miRNA transcript, are upregulated in HCC	NR	Among chronic carriers and liver cirrhosis patients, the A allele of rs3859501 and the haplotype pri-miRNAs-371-373_ht2 were more protective to HCC than other genotypes and haplotypes
Lan SH, *Hepatology*, 2014 Taiwan Period: NR	Tissue and specimens, obtained from patients from Taiwan patients with HCC	The level of autophagy was low and inversely Correlated with miR-224 expression only in HBV associated HCC	NR	A noncanonical pathway links autophagy, miR-224, Smad4, and HBV-associated HCC

Li J, *Biochemical and Biophysical Research Communications*, 2011 China Period: NR	Serum samples of HCC were obtained from 46 patient (30 HBsAg positive) The healthy sera were collected from 50 age-matched healthy individuals who serves as normal controls	Serum miR-221, up-regulation in HCC, correlates with tumor size, cirrhosis and tumor stage	NR	Serum miR-221, upregulated in HCC, can provide predictive significance for prognosis of HCC patients
Li L, *Digestive Diseases and Sciences*, 2012 China Period: NR	Serum samples obtained from: HCC: 101 (HBsAg +) CLD and cirrhosis: 30 Healthy controls: 60	miR-18a significantly up-regulated in HBV-related HCC, chronic hepatitis or cirrhosis than those in healthy Controls	NR	Significant increase of elevated serum miR-18a in the patients of HBV-related HCC. It might serve as a novel noninvasive biomarker to distinguish patients with HBV-related HCC from healthy subjects, and further from those with HBV-related chronic hepatitis or cirrhosis
Li LM, *Cancer Research*, 2010 China Period: September 2007 - July 2008	Serum samples from: -120 HCC-affected individuals; -135 HBV carriers; -48 HCV carriers; -210 controls	Serum up-regulation of miR-375, miR-92a, miR-10a, miR-223, miR-423, miR-23b/a, miR-342-3p, miR-99a, miR-122a, miR-125b, miR-150, and let-7c. in HBV positive patients with HCC in comparison with healthy controls	NR	The expression profile of serum miRNAs can serve as novel non-invasive biomarkers for the diagnosis of HBV infection and HBV positive HCC. The use of 3 miRNAs: miR-25, miR-375, and let-7f could be used to separate HCC cases from controls, miR-375 alone had high specificity and sensitivity in HCC prediction
Li T, *Oncology Reports*, 2014 China Period: NR	Tissue and plasma obtained from: 31 patients with HBV-related HCC 31 age- and gender-matched CHB patients	Tissue miRNA-21, miRNA-221, miRNA-148b and miRNA-186 over-expression	Tissue miRNA-99a, miRNA-27b, miRNA-378a, miRNA-378e and miRNA-30 down-regulation Tissue and plasma miRNA-139 down-regulation in HCC *vs* non-HCC patients	miRNA-139 is downregulated in both cancerous tissue and plasma of HCC. The plasma miRNA-139 is a possible diagnostic biomarker for identifying HCC patients while combined with other biomarkers, it is also a prognostic factor for indicating patient survival

Li W, *Int J Cancer*, 2008 China Period: NR	Specimens obtained obtained from: 78 human primary hepatocellular carcinoma (68 HBsAg +) and matched noncancerous liver tissues	84 miRNAs identified with deregulated expression in tissues from HCC patients. 69/84 miRNAs resulted differentially expressed in normal or non-tumour liver tissue *vs* cancerous hepatic tissue with 29 miRNAs up-regulated -Noncancerous *vs* normal liver tissue: 27 miRNAs differentially expressed, with 14 up-regulated in noncancerous liver specimens -HCC *vs* normal tissue: 55 differentially expressed miRNAs, with 29 up-regulated in HCC tissues	84 miRNAs identified with deregulated expression in tissues from HCC patients. 69/84 miRNAs resulted differentially expressed in normal or non-tumour liver tissue *vs* cancerous hepatic tissue with 40 miRNAs down-regulated Noncancerous *vs* normal liver tissue: 27 miRNAs differentially expressed, with 13 down-regulated in noncancerous liver specimens -HCC *vs* normal tissue: 55 differentially expressed miRNAs, with 26 down-regulated in HCC tissue	miRNA signature identified as a HCC diagnostic discriminator from both noncancerous and normal liver tissues. This is the first report to identify single miRNA correlated to the HCC prognosis, *i.e.*, miR-125b as a HCC survival predictor
Liu AM, *BMJ Open*, 2012 China Period: 1990-2007	Serum and Cancerous/non tumors tissue samples collected from: - 96 cirrhotic patients with HCC (84 HBsAg positive) undergoing hepatectomy (exploration phase) -29 hepatitis B carriers, 57 patients with HCC and 30 healthy controls (validation phase)	Exploration phase in resected tumour/ adjacent non-tumour tissues: Upregulated miR in the AFP-low (< 400 ng/mL) HCC subgroup: miR-9, -9*, -15b, -21, -34c, -96, -130b, -183, -188, -196b, -216, -224, -301 and -324-5p Upregulated miR in all HCC samples of varying serum AFP levels: miR-15b, -21, -130b, -183, -224 and -301	Decreased serum miR-224 and miR-301 levels in HCC patients post-surgery in comparison with pre- surgery. Slight reduction of serum miR-15b and miR-130b levels in HCC patients post-surgery in comparison with pre- surgery	The combined miR-15b and miR-130b classifier is a serum biomarker with clinical value (high sensitivity and accuracy) for HCC screening. This classifier also identified early-stage HCC cases that could not be detected by AFP
Liu Y, *PLoS One*, 2012 China Period: January 2006-December-10 Controls screened for the HBV/HCV markers in 2004 and 2009	Serum samples collected from: - 1300 HBV positive HCC cases, -1344 HBV persistent carriers; - 1344 subjects with HBV natural clearance people These patients were matched to the HCC cases on age and gender	NR	NR	A genetic variant in the promoter region of miR-106b-25 cluster might provide a protective effect against chronic HBV infection but an increased risk for HCC in HBV persistent carriers by affecting the expression of miR-106b-25 cluster A to G base change of rs999885 may have a protective effect on the probability to develop chronic HBV infection, but increased the risk of HCC in HBV persistent carriers

Liu Y, *J Med Virol*, 2014 China Period: April 2008 - November 2011 Newly diagnosed HCC patients included from January 2006 - December 2010 HBV carriers and patients with signs oif past HBv infection, screened from 2004 to 2009	Samples obtained from: 29 pairs of HCC and adjacent noncancerous liver tissues	NR	In noncancerous liver tissues, subjects with a CA genotype exhibited significantly lower expression level of pri-miR-122 than those carrying the CC genotype. Positive or inverse correlation between the expression levels of pri-miR-122 and mature miR-122 were observed in HCC tissues or oncancerous tissues, respectively	The C to A base change of rs4309483 may alter the expression of miR-122, thus providing protective effect from chronic HBV infection but an increased risk for HCC in HBV carriers
Meng FL, *Med Oncol*, 2014 China Period: January 2009 - December 2011	Tissue obtained from: 84 patients with HBV-related HCC 31 with CLDs 46 with healthy controls	Tissue miR-24-3p over-expression in HCC in comparison with healthy controls and CLD	NR	The combination of serum miR-24-3p and AFP improves the diagnostic accuracy for HCC prediction compared to each biomarker alone. High serum miR-24-3p level is an independent predictor of overall survival and disease free-survival. In patients with HBV-related HCC
Qi, F, *PLoS One*, 2014 China Period: NR	Serum samples obtained from 331 patients with HBV-related HCC in either intermediate or advanced stage of disease without surgery	NR	NR	miR-106b-25 cluster plays oncogenic roles in cancers through influencing tumor growth, cell survival, and angiogenesis. rs999885 is associated with prognosis of intermediate or advanced HBV-related hepatocellular carcinoma (HCC) . rs999885 variant could influence miR-106b-25 expression and the expression levels of miR-106b-25 were significantly higher in AG/GG carriers than that in AA carriers G allele of rs999885 may provide a protective effect on the prognosis of intermediate or advanced HCC in Chinese

Qi P, *PLoS One*, 2011 China Period: NR	Study divided into four phases. Serum samples obtained from: (Ⅰ phase) -10 HBV-positive HCC patients and -10 age- and sex-matched healthy subjects; (Ⅱ phase) before surgery, sera from another 48 HBV-positive HCC patients, from 48 chronic HBV infection patients without HCC and 24 age- and sex-matched healthy subjects; (Ⅲ phase) 14 HCC patients before and after surgery, (Ⅳ phase) correlation between the expressions of candidate serum miRNAs with clinical parameters of HCC patients	Up-regulation of serum miR-122, miR-222 and miR-223 in HCC patients in comparison with healthy controls	Down-regulation of serum miR-21 in HCC patients in comparison with healthy controls	Serum miR-122 might serve as a novel and potential biomarker for detection of HCC in healthy subjects and it might serve as a novel biomarker for liver injury but not specifically for detection of HCC in chronic HBV infection patients
Tan Y, *PLoS One*, 2014 China Period: August 2010 - June 2013	Serum samples obtained from: HCC: 261, LC: 133; Healthy controls:173	up-regulation: miR-190b; miR-141-3p; miR-4532; mir-6127; miR-99b-3p; miR-1228-5p between HCC and healthy controls up-regulation: miR-206, mir-1285-1-p5, miR-10a-5p ,miR-511-5p, miR-433-3p between HCC and cirrhosis groups	Down-regulation: miR-30a-3p; miR-199a-5p ; let-7f-5p ; miR-122-5p ; miR-192-5p; miR-98-5p; miR-574-3p; miR-30e-3p; miR-6852-5p between HCC and healthy controls Down-regulation: miR-100-5p; miR-483-5p, miR-584-5p; miR-28-5p miR-30b-5p; miR-30c-5p ; miR-26a-5p; miR-4454; let-7e-5p; let-7c-5p; miR-4433b-5p between HCC and cirrhosis groups	A serum panel of miRNA with considerable clinical value in HCC diagnosis was identified. miR-206, miR-141-3p, miR-433-3p, miR-1228-5p, miR-199a-5p, miR-122-5p, miR-192-5p, and hsa-miR-26a-5p as potential circulating markers for HCC diagnosis
Wei X, *Cellular Signalling*, 2013 China Period: NR	Serum and tissues (paired tissue specimens from HBV-related HCC tissues and adjacent noncancerous hepatic tissues)	NR	miR-132 is more frequently downregulated in HBV-positive HCCs tumor tissues than in adjacent noncancerous tissues and has a significant inverse correlation with HBx expression in HBV-related HCCs	miR-132 may play a tumor-suppressive role in HBV-related HCC development. Serum miR-132 levels are closely correlated with miR-132 expression levels in tumor tissues. miR-132 may be a promising biochemical marker and may have therapeutic applications in HBV-related HCC

Wen Y, *Int J Cancer*, 2015 China Period (3 phases): December 2010- December 2011 January 2010- December 2012 2004-2005	Multicenter, three-phase study to screen liver-originated HCC-associated plasma miRNAs in both plasma and tissue samples The training set consisted of 35 HCC cases and 50 cancer-free HBV carriers who were frequency matched for age and sex, whereas the validation set consisted of 32 HCC cases and 32 matched cancer-free HBV carriers	Up-regulation of miR-221, miR-222, miR-31	Down-regulation of miR-126, and miR-122a miR-223	miR-223 may represent a potential target in cancer therapy because it regulates Stathmin 1
Xiang Y, *Mol Biol Rep*, 2012 China Period: December 2009 - February 2011	Specimens obtained from: -100 patients with HCC (73 HBV positive); -100 patients with CHB; -100 healthy subjects	NR	NR	miRNA 499 polymorphisms is associated with susceptibility in HBV-related HCC in Chinese population. The risk of HCC development is increased in patients with miR-399 C/C was higher in comparison with subjects with miR 499 T/T
Xie Y, *Cancer Biology and Therapy* China Period: NR	Specimens and tissue samples obtained from: -67 HBV-HCC patients, -61 HBV-LC patients, -79 CHB patients, -30 Healthy subjects	Elevated miR-101 levels in the sera and liver tissues of HBV-LC patients and decreased in HBV-HCC patients	NR	Serum miR-101 as a potential biomarker for monitoring the development of HBV-HCC from HBV-LC and the development of HBV-LC from CHB
Xing TJ, *Genetics and Molecular Research*, 2014 China Period: NR	Serum samples obtained from: HCC: 20 patients; LC: 20 patients; CHB: 29 patients; ASC: 20 patients; Healthy controls: 20	Increased miRNA-122 levels in patients with HCC and CHB *vs* patients with HC, LC, and ASC	lower miRNA-29 serum levels in LC patients than those in the healthy controls	The elevation in miR-122 was correlated with liver damage in CHB patients and with the pathogenesis of liver cancer in HCC patients. The decrease in miR-29 expression was related to the incidence of liver fibrosis
Xu J, *Molecular Carcinogenesis*, 2011 China Period: NR	Serum samples obtained from: -101 patients with advanced primary HCC (78 HBsAg +), -48 patients with CHB, -89 healthy controls	Higher serum miR-21, miR-122, and miR-223 levels in patients with HCC or CHB, compared with healthy controls	NR	Serum miR-21, miR-122 and miR-223 are elevated in patients with HCC or chronic hepatitis and these miRNAs have strong potential to serve as novel biomarkers for liver injury but not specifically for HCC

Zhang H, *WJG*, 2012 China Period: NR	Serum samples obtained from patients with: -34 CHB, -20 NASH -34 healthy donors Serum samples from 10 CHB patients and 10 controls were subjected to miRNA microarray analysis to obtain serum miRNA profiles	Up-regulation of miR-122, miR-138, miR-638, hsv1-miR-H1, miR-575, miR-572, kshv-miR-K12-3, miR-1915, miR-623, miR-1268, miR-939, miR-498	Down-regulation of: miR-421, miR-598, miR-155, miR-424, miR-23b, miR-195, miR-487b, miR-224, miR-495, miR-181c, miR-654-3p, let-7e, miR-382, miR-171, miR-128, miR-625, miR-30e1, miR-139-5p, miR-30c, miR-744, miR-374b, miR-376c	Serum levels of miR-122, -572, -575, -638 and -744 are deregulated in patients with CHB or NASH. The levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH
Zhang T, *Neoplasia*, 2013 China Period: NR	Samples obtained from cancerous tissues of thirty-three patients with HBV-related HCC and their corresponding nearby nontumorous liver tissues	NR	HBx-mediated downregulation of miR-205 through the induction of miR-205 promoter hypermethylation	HBx is able to inhibit tumor suppressor miR-205. miR-205 may be useful in the treatment of HCC
Zhang ZZ, *WJG*, 2011 China Period: NR	miRNA expression profiles obtained from 78 HCC patients from Gene Expression Omnibus study	8/ 10 differentially expressed miRNAs common to the AHB infection and HCC datasets were inversely changed, only 3/8 differentially expressed miRNAs common to the chronic HBV infection and HCC datasets exhibited opposite alterations	8/ 10 differentially expressed miRNAs common to the AHB infection and HCC datasets were inversely changed, only 3/8 differentially expressed miRNAs common to the chronic HBV infection and HCC datasets exhibited opposite alterations	miRNA level is correlated in HBV infection and HCC
Zhao Q, *PLoS One*, 2014 China Period: February 2012 - January 2013	Serum and cancerous and non-tumors tissue samples obtained from: -66 patients with HBV-related HCC patients -11 hepatic hemangioma Patients	Up-regulation of miR-545/374a cluster in HBV-HCC tissue	NR	The overexpression of miR-545/374a cluster is partially responsible for a poor prognosis, and monitoring sera levels of miR-545/374a may be a useful diagnostic marker for HCC
Zhou J, *J Clin Oncol*, 2011 China Period: August 2008 - June 2010	934 blood samples, from healthy subjects patients with CHB, cirrhosis or HCC	High expression levels of miR-192, miR-21, and miR-801 in patients with HCC compared with those in the control group	Low expression levels of miR-122, miR-223, miR-26a, and miR-27a observed in patients with HCC compared with those in the control group	miR panel with considerable clinical value in diagnosing early-stage of HBV-related HCC
Zhu HT, *PLoS One*, 2012 China Period: January 2004 - December 2008	Tissue obtained from:	Up-regulation in microdissected HCC tissue with early recurrence: miR-29a-5p, miR-27b*, miR-204, miR-29c, miR-10b, miR-	Down-regulation in microdissected HCC tissue with early recurrence:	In the multivariate analyses, miR-29a-5p was identified as an independent factor for tumor recurrence. miR-29a-5p might be a useful marker for the prediction of early tumor recurrence after HCC resection, especially in BCLC 0/A stage HCCs

	266 patients, undergoing curative liver resection for HCC 48 patients subdivided into: -group with early recurrence (24) -group without early recurrence (24) 218 patients enrolled into: training (106) and validation (112) cohort	196b, miR-216a, miR-217, miR-517a, miR-518e, miR-518f, miR-518b, miR-519a, miR-519d, miR-522, miR-486-5p, miR-181c, miR-210, miR-215 Up-regulation in microdissected non-tumorous liver tissue with early recurrence: miR-486-5p, miR-181c, miR-193b*, miR-643, miR-409-3p, miR-424*, miR-139-3p, miR-766	miR-193b*, miR-643, miR-22, miR-15b, miR-505, miR-107, miR-142-5p, miR-135a, miR-34c-5p, miR-98, miR-483-5p Down-regulation in microdissected non-tumorous liver tissue with early recurrence: miR-210, miR-215, miR-22, miR-409-5p, miR-200a*, miR-10b*

ABH: Acute B hepatitis; ASCs: Asymptomatic HBsAg carriers; BCLC: Barcelona clinic liver cancer staging system; CHB: Chronic hepatitis B; CLD: Chronic liver disease; ER-α: Estrogen receptor-α; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; HC: Healthy controls; HCA: Hepatocellular adenoma; LC: Liver cirrhosis; LT: Liver transplantation; NR: Not reported; OS: Overall survival; RFS: Recurrence-free survival; SR: Spontaneously recovered.

Table 2 miRNA observed deregulated in studies enrolling hepatocellular carcinoma patients with hepatitis B virus-related infection in at least three papers

miRNA	Type of deregulation (number of papers)	Type of Sample (number of papers)	Ref.
miR-221	Upregulated (6)	Tissue (5), serum (1)	Gao P, *Hepatology*, 2011 Li J, *Biochemical and Biophysical Research Communications*, 2011 Li T, *Oncology Reports*, 2014 Li W, *Int J Cancer*, 2008 Wen Y, *Int J Cancer*, 2015 Zhang ZZ, *WJG*, 2011
[1]miR-21	Upregulated (5)	Tissue (4), serum (1)	Bandopadhyay M, *BMC Cancer*, 2014 Connolly E, *The American Journal of Pathology*, 2008 Gao P, *Hepatology*, 2011 Li T, *Oncology Reports*, 2014 Xu J, *Molecular Carcinogenesis*, 2011
[2]miR-222	Upregulated (4)	Tissue (3), serum (1)	Li W, *Int J Cancer*, 2008 Qi P, *PLoS One*, 2011 Zhang ZZ, *WJG*, 2011 Wen Y, *Int J Cancer*, 2015
[3]miR-122a	Upregulated (4)	Serum (4)	Li LM, *Cancer Research*, 2010 Qi P, *PLoS One*, 2011 Xing TJ, *Genetics and Molecular Research*, 2014 Xu J, *Molecular Carcinogenesis*, 2011
miR-224	Upregulated (4)	Tissue (3), serum (1)	Gao P, *Hepatology*, 2011 Gui J, *Clinical Science*, 2011 Li W, *Int J Cancer*, 2008 Zhang ZZ, *WJG*, 2011
[4]miR-101	Downregulated (4)	Tissue (4)	Fu Y, *Oncol Letters*, 2013 Li W, *Int J Cancer*, 2008 Xie Y, *Cancer Biology and Therapy*, 2014 Zhang ZZ, *WJG*, 2011
miR-18a	Upregulated (3)	Tissue (2), serum (1)	Li L, *Digestive Diseases and Sciences*, 2012 Li W, *Int J Cancer*, 2008 Zhang ZZ, *WJG*, 2011
miR-223	Upregulated (3)	Serum (3)	Li LM, *Cancer Research*, 2010 Qi P, *PLoS One*, 2011 Xu J, *Molecular Carcinogenesis*, 2011

[1]miR-21: in one paper by Zhou [Zhou J, *J Clin Oncol* 2011] starting from serum samples, miR-21 was observed as down-regulated; [2]miR-222: in one paper by Bandopadhyay [Bandopadhyay M, *BMC Cancer* 2014] starting from tissue samples, miR-222 was observed as down-regulated; [3]miR-122a: in two papers by Tan *et al* and Zhou *et al* [Tan Y, *PLoS One* 2014; Zhou J, *J Clin Oncol* 2011] both starting from serum samples, miR-122 was observed as down-regulated; [4]miR-101: in paper by Fu *et al*, miR-101 was observed as down-regulated in tissue but up-regulated in serum [Fu Y, *Oncol Letters* 2013].

Table 3 miRNAs patterns in studies enrolling hepatocellular carcinoma patients with hepatitis B virus and hepatitis C virus-related infection

Ref.	Characteristics of the study	miRNAs Up-regulated	miRNAs Down-regulated	Conclusions
Chung GE, *Oncol Rep*, 2010 Korea Period: 2001-2004	Tissue samples obtained from twenty-five pairs of primary HCC (18 HBV positive patients, 2 HCV positive subjects, 5 HBV/HCV negative) and adjacent non-tumor liver tissues were evaluated in this study	Up-regulation of miR-15b, miR-105 and miR-339, let-7d, miR-107, miR-103, miR-210, miR-25, let-7a, miR-93, miR-345, miR-30d, miR-423, miR-320	miR-422b, miR-22, miR-497, miR-195, miR-199a*, miR-199[a], miR-130a	miR-15b expression in HCC tissues may predict a low risk of HCC recurrence. In addition, the modulation of miR-15b expression may be useful as an apoptosis-sensitizing strategy for HCC treatment
Cong N, *Tumor Biol*, 2014 China Period: January 2007 - February 2012	Serum samples obtained from: -206 patients with HCC -217 controls	NR	NR	The miR-146a GG genotype and G allele carried an increased risk of HCC HBV-positive subjects carrying but not in HCV-infected patients
Coulouarn C, *Oncogene*, 2009 USA Period: NR	Specimens obtained from 64 HCC tissues (18 pts HBsAg +, 13 HCV +, 3 with HBV and HCV coinfection, 30 with different etiologies) and 28 matched non-tumor surrounding liver tissues from patients undergoing partial hepatectomy as treatment for HCC	NR	Down-regulation of miRNA-122 in HCC	miR-122 as a diagnostic and prognostic marker for HCC progression
Diaz G, *Int J Cancer*, 2013 Italy Period: NR	Tissue samples obtained from: -HCV-associated HCC (HCC), -HCC-associated non-tumours cirrhosis (HCC-CIR), -HCV- associated cirrhosis without HCC (CIR), -HBV-associated acute liver failure (ALF), -normal liver tissue surrounding angioma (NL), -normal liver from liver donors (LD)	Up-regulation of: miR 221, miR-224 and miR-224-3p, miR-452, miR-1269	Down-regulation of: miR-125a-5p , miR-130a, miR-139-5p, miR-139-3p, miR-195, miR-199a-5 and miR-199a-3p, miR-214, miR-424-3p, miR-497	18 miRNAs exclusively expressed in HCV-associated HCC and characterized by high specificity and selectivity *vs* all other liver diseases and healthy conditions were identified Among the 18 HCC-exclusive miRNAs identified in this research, miR-221 and miR-224, miR-199a-5p, miR-195, miR-214, miR-199a-3p, miR-125a-5p, miR-139-5p, miR-130a, miR-199b-3p, miR-139-3p, miR-224-3p and miR-452 were already reported in previous studies miR-497, miRNA-1269 miR-424-3p were never described in previous reports

Gramantieri L, *Cancer Research*, 2007 Italy Period: NR	Tissue obtained from: 60 patients (HBV: 5, HCV: 31, HBV+ HCV: 5, HCV+ pas tHBV: 5, past HBV: 1; HBV + ethanol: 1, HCV + ethanol: 2, ethanol: 3)	Up-regulation of: miR-221	Down-regulation of : let-7a-1, let-7a2, let-7a3, let-7b, let-7c, let-7d, let-7e, let-7f2, let-7g, miR-122a, miR-124a, miR-130a, miR-132, miR-136, miR-141, miR-142, miR-143, miR-145, miR-146, miR-150, miR-155, miR-181a-1, miR-181a-2, miR-181c, miR-195, miR-199a1-5p, miR-199a2-5p, miR-199b, miR-200b, miR-214, miR-223	The aberrant expression of a restricted panel of miRNAs could participate in the molecular events leading to HCC development
Hao YX, *Asian Pac J Cancer Prev*, 2013 China Period: January 2010 - April 2012	Serum samples from: -285 patients with HCC 133 HBsAg-positive 36 anti-HCV positive 8 with coinfection -Residents without HCC who entered the hospital for health check-ups were enrolled into control group Each control was pair-matched by sex and age (± 5 yr) to a patient with HCC	NR	NR	miR-196a2 CC genotype and C allele have an important role in HCC risk in Chinese patients, especially in HBV infection carriers No significant association observed between miR-146aG>C and miR-499A>G genetic polymorphisms and HCC risk
Köberle V, *Eur J Cancer*, 2013 Germany Period: February 2009 - July 2012	Serum samples obtained from: 195 patients with HCC (33 HBV+; 87 HCV +, 14 NASH, 65 Alcohol, 8 Haemochromatosis, 9 Cryptogenic) 54 patients with liver cirrhosis (2 HBV +; 41 HCV +, 0 NASH, 16 Alcohol, 0 Haemochromatosis, 0 Cryptogenic)	Longer OS in patients with higher miR-1 and miR-122 serum levels	Reduced OS in patients with lower miR-1 and miR-122 serum levels	At age-, sex-, tumor stage and treatment-adjusted multivariate Cox regression analysis miR-1 serum levels were independently associated with OS, whereas serum miR-122 was no. t miR-1 may improve the predictive value of classical HCC staging scores
Ladeiro Y, *Hepatology*, 2008 France Period: 1992-2004	109 liver samples, collected from 93 patients surgically treated. Analysed cases: HCC: 28, HC: 13, FNH: 5, non-tumor liver samples: 4 Two sets of samples were considered:(first set of samples (n = 50, 16 HBV positive, 9 HCV positive) and validation set of samples (n = 59, 18 HBV positive, 17 HCV positive)	- miR-96 overexpressed in HBV tumors - miR-21, miR-222, miR-10b overexpression in HCC - miR-224 overexpression in HCC *vs* benign tumours	-miR-422b, miR-122a down-regulation both in benign and malignant tumors - miR-200c and miR-203 underexpression in benign tumours - low expression of miR-375 in both HCA and HCC mutated for β-catenin	Hepatocellular tumors may have a distinct miRNA expression fingerprint according to malignancy, risk factors, and oncogene/tumor suppressor gene alterations

Liu YX, *BioMed Research International*, 2014 China Period: January 2004 - December 2008	Tissue obtained from: -207 HCC liver tissue and patients and adjacent noncancerous tissue samples. HBV + : 174 patients; HCV+ : 3 patients	miR-24 up-regulation in HCC tumor tissues relative to adjacent noncancerous tissue samples	NR	High expression of miR-24 could promote AFB1-DNA formation and increase adducts mount. High expression of miR-24 was significantly correlated with larger tumor size, higher microvessel density, and tumor dedifferentiation
Liu WH, *Gastroenterology*, 2009 Taiwan Period: 2002-2006	Tissue obtained from: -80 HCC patients (40 HBV+ and 40 HCV +), -16 focal nodular hyperplasia cases -7 adenoma cases	Specifically increased miR-18a miRNA in samples from female HCC patients. miR-18a expression in tumor tissues not different from the non-tumoral tissues in either male or female patient FNHs and adenomas	NR	miR-18a prevents translation of ER, potentially blocking the protective effects of oestrogen and promoting the development of HCC in women
Lu CY, *Genes Chromosomes and Cancer*, 2013 Taiwan Period: NR	Tissue and sera obtained from: -41 patients with HCC (19 HBV positive, 11 HCV positive, 2 HBV/HCV positive, 8 HBV/HCV negative and 1 not determined) -8 patients with cirrhosis (6 HBV positive, 2 HCV positive, 1 HBV/HCV positive), -10 Healthy subjects	In 39/41 HCC, the methylation levels of miR-129-2 were significantly increased in tumor tissues compared with adjacent normal tissues miR-129-2 methylation was detectable in plasma samples from HCC patients, but not in plasma samples from healthy individuals or patients with liver cirrhosis	NR	miR-129-2 methylation is highly accurate in distinguishing HCC patients from cirrhosis patients and healthy individuals, implying its potential utility as an early diagnostic marker for HCC
Murakami Y, *Oncogene*, 2006 Japan Period: NR	miR expression profiles in 25 pairs of hepatocellular carcinoma (HCC) and adjacent nontumorous tissue (NT) and nine additional CHB specimens was performed, using a human miRNA microarray HBV +: 6; HCV +: 26	Major expression of miR-18, precursor miR-18, and miR-224 in HCC *vs* non-cancerous tissue	Minor expression of miR-199a*, miR-195, miR-199a, miR-200a, and miR-125[a] in HCC *vs* non-cancerous tissue	Higher expression of three miRNAs in the HCC samples *vs* NT samples, demonstrated lower expression of five miRNAs in the HCC samples *vs* NT samples

Qu KZ, *J Clin Gastroenterol*, 2011 USA Period: NR	283 subjects studied: -105 patients with HCC (20 HBV +, 66 HCV +, 1 with coinfection); -107 individuals with CLDs; -71 healthy controls	NR	Significantly lower serum levels of miR-16 and miR-199a in HCC than in CLD patients or control subjects	Measurement of serum levels of miR-16 improves differentiation of HCC from non-HCC CLD. The combination of miR-16, AFP, AFP-L3, and DCP yielded greater sensitivity and specificity for HCC detection than any other single marker or marker combination examined
Salvi A, *Intern J Oncol*, 2012 Italy Period: NR	Tissue specimens obtained from: human HCC samples, corresponding peritumoral and non-tumor samples (resected 1-2 cm from the malignant tumor) 15 HCV + patients, 10 HBV+ patients 4 HBV+/HCV + subjects 7 HBV -/ HCV - patients 5 patients with no available informations (25 cirrhosis, 15 hepatitis, 1 steatosis)	Up-regulation of: miR-21 in HCC tissues *vs* the corresponding peritumoral tissues, particularly in non-cirrhotic HCC	Down-regulation of: miR-24 and miR-27a in HCCs from cirrhotic liver tissues in comparison to those from non-cirrhotic liver tissues. The downregulation of miR-24 was correlated with poorer prognosis in patients with HBV and HCV virus infections	Differential expression of miRNAs in cirrhotic and non-cirrhotic HCCs, thereby contributing to advances in the discovery and validation of novel molecular biomarkers of HCC progression
Sato F, *PLoS One*, 2011 Japan Period: January 1997 - March 2007	73/639 patients with HCC and satisfying enrollment criteria, underwent hepatic resection Patients HBsAg +: 12; Patients HCV +: 51	Recurrence-related miR in tumor tissues: miR22, miR99a, miR99b, miR100, miR 125a-5p, miR125b, miR129-5p, miR 140-3p, miR145, miR195, miR221, miR378, miR497	Recurrence-related miRNA in non-tumor tissues: miR18a, miR18b, miR21, miR23a, miR24, miR27a, miR96, miR103, miR 107, miR126, miR142- 3p, miR 148a, miR 191, miR 222, miR362-3p, miR 425, miR378, miR1202, let 7e, let-7f	miRNA profiling can predict HCC recurrence in Milan criteria cases miR-96 in non-tumor tissues is the most strongly associated with HCC recurrence
Shigoka M, *Pathology International*, 2010 Japan Period: NR	Serum and tissue samples obtained from: -22 HCC cases (6 HBV +, 10 HCV +, 6 non-HBV/HCV) -5 pairs of fresh HCC and non-tumorous LCD samples surgically resected from HCC patients -10 healthy subjects	Higher levels of miR-92a expression in HCC sections *vs* adjacent LC sections	Decreased ratio of miR-92a to miR-638 in the plasma samples from the HCC patients compared with that from the normal donors	Deregulation of miR-92 expression in cells and plasma could be implicated in the development of HCC

Spaniel C, *PLoS One*, 2013 Japan/USA Period: NR	Tissue samples obtained from: a) Paired tumor and nontumor tissues collected from 26 patients undergoing surgical resection of HCC: -16 with concomitant chronic HCV infection, -10 infected with HBV b) 9 with non-infected 'normal' liver tissue collected from patients undergoing resection of metastases of non-hepatic primary cancers	Possible miR-191 increased expression in HBV-associated HCC	Significant reduction of miR-122 abundance in HBV associated HCC in comparison with "normal" liver tissue, but not in liver cancer associated with HCV. Significant differences in miR-122 expression exist in non-tumor tissue, with miR-122 abundance reduced from "normal" in HCV- but not HBV-infected liver	miR-122 abundance varies between HBV- and HCV-related liver HCC as well as in non-tumor tissue
Toffanin S, *Hoshida Cabellos Gastroenterology*, 2011 Italy Period: NR	Tissue samples obtained from: - 89 fresh-frozen HCC samples (surgical resection or LT); - Formalin-fixed paraffin-embedded tissues of 165 HCCs (validation set) caused by HCV, HBV, alcohol, and others Subjects subdivided into: Training set: 79 patients Validation set: 161 patients Training set:, HCV +: 79/79 patients Validation set: HCV +: 74/161; HBV +: 43/161; Alcohol: 12/161; Other: 25/161	Up-regulation of 23 miRNAs in cluster C2 (miR-517a, miR-517b, miR-517c, miR-520g, miR-520h, miR-519b, miR-519d, miR-516-5p, miR-519a,miR-520c, miR-520b, miR-520f, miR-526b, miR-524, miR-516-1, miR-526b, miR-519e, miR-512-3p, miR-522, miR-526a, miR-518f, miR-518b, and miR-525 Up-regulation of 16 miRNAsin cluster C3 (miR-376a, miR-494, miR-409-3p, miR-376b, miR-377, miR-368, miR-382, miR-369-3p, miR-410, miR-432, miR-154, miR-379, miR-299-5p, miR-431, miR-381, miR-495)	Down-regulation of miR-26a and miR-26b in cluster C2 and C3	miRNA-based classification of 3 subclasses of HCC is proposed. Among the proliferation class, miR-517a is an oncogenic miRNA, promoting tumor progression A rationale for developing therapies that miRNA 517 for patients with HCC is proposed A hierarchical clustering of miRNA data identified 3 main clusters of HCC: clusters A (32/89), B (29/ 89) and C (28/89). The C cluster divided into 3 sub-clusters with distinct miRNA expression patterns: C1 (15/89), C2 (8/89) and C3 (5/89)
Tomimaru Y, *J Hepatol*, 2011 Japan Period: January2010 - February 2010	Serum samples obtained from: -10 patients before and after curative resection of HCC (HBV/HCV-: 1, HBV: /3/ HCV+: 6/); -126 patients with HCC, -30 patients with CLD, -50 healthy volunteers	Significantly higher plasma miR-21 level in the HCC patients in comparison with CLD patients and healthy volunteers	Significantly diminished plasma miRNA-21 levels after surgery compared with the pre-operative values	Plasma miRNA-21 level is a promising biochemical marker for HCC

Ura S *Hepatology*, 2009 Japan Period: 1999-2004	12 patients with HBV-related HCC 14 patients with HCV-related HCC	NR	Commonly repressed miR in CH-B, CH-C, HCC-B, and HCC-C compared with normal liver: miR-219, miR-320, miR-154, miR-29c; miR-338; miR-26a; miR-126; miR-325	miRNAs as important mediators of HBV and HCV infection as well as liver disease progression, they could be potential therapeutic target molecules Major miRNAs expression in HCC *vs* CLD: miR21, miR-98, miR183, miR221, miR222, miR301. Minor miRNAs expression in HCC *vs* CLD: miR17-3p, miR30a-3p, miR30e, miR92, miR 99a, miR122, miR125b, miR130a, miR139, miR187, miR199a, miR200a, miR200b, miR223, miR326
Zhou B *Tumor Biol*, 2014 China Period: January 2010 - February 2012	Serum samples obtained from: -266 patients with HCC -281 Healthy controls	NR	NR	Subjects with miR-146a GG and G allele had an enhanced risk of HCC in comparison with homozygote CC genotype. Individuals with miR-196a2, TT and T allele significantly decreased the risk of HCC in comparison with CC genotype. miR-196a2C>T polymorphisms associated with a decreased risk of HBV-related HCC, but not in HCV-related HCC cases

ABH: Acute B hepatitis; ASCs: Asymptomatic HBsAg carriers; BCLC: Barcelona Clinic Liver Cancer staging system; CHB: Chronic hepatitis B; CLD: Chronic liver disease; ER-α: Estrogen receptor-α; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; HC: Healthy controls; HCA: Hepatocellular Adenoma; LC: Liver cirrhosis; LT: Liver transplantation; NR: Not reported; OS: Overall survival; RFS: Recurrence-free survival; SR: Spontaneously recovered.

of HCC in this different spectrum of human diseases. Concerning NAFLD/NASH, most of available articles have studied the circulating or tissue miR signature associated with NAFLD progression and predictive power, as well as the role of miRs in disease biology and the relationship between circulating miRNA and features of the metabolic syndrome. In particular, a report has assessed tissue miRNA patterns in patients with NASH in comparison with normal controls. Forty-six miRNAs have resulted to be differentially expressed in these two distinct groups, 23 miRNAs were up-regulated (miR-125b, miR-16, miR-21, miR-23a, miR-23b, miR-24, miR-27b, miR-34a, miR-99b, miR-100, miR-127, miR-128a, miR-128b, miR-146b, miR-181b, miR-199a, miR-199a*miR-200a, miR-214, miR-221, miR-222, miR-224, miR-455) and 23 down-regulated (miR-126, miR-28, miR-26b, miR-30d, miR-122, miR-361, miR-574, miR-92b, miR-768-5p, miR-375, miR-203, miR-223, miR-145, miR-671, miR-139, miR-191*, miR-563, miR-188, miR-601, miR-765, miR-198, miR-641. miR-617)[95]. MiR-122 levels were significantly decreased in subjects with NASH. Estep

Table 4 miRNA observed deregulated in studies enrolling hepatocellular carcinoma patients with hepatitis B virus and hepatitis C virus-related infection in at least three papers

miRNA	Type of deregulation (number of papers)	Type of Sample (number of papers)	Ref.
miR-130a	Downregulated (4)	Tissue (4)	Gramantieri L, *Cancer Research*, 2007 Diaz G, *Int J Cancer*, 2013 Chung GE, *Oncol Rep*, 2010 Oksuz Z, *Mol Biol Rep*, 2015
miR-21	Upregulated (3)	Tissue (2), serum (1)	Ladeiro Y, *Hepatology*, 2008 Salvi A, *Intern J Oncol*, 2012 Tomimaru Y, *J Hepatol*, 2011
miR-224	Upregulated (3)	Tissue (3)	Diaz G, *Int J Cancer*, 2013 Ladeiro Y, *Hepatology*, 2008 Murakami Y, *Oncogene*, 2006
miR-195	Downregulated (3)	Tissue (3)	Gramantieri L, *Cancer Research*, 2007 Diaz G, *Int J Cancer*, 2013 Chung GE, *Oncol Rep*, 2010

Table 5 miRNAs patterns in studies enrolling hepatocellular carcinoma patients with hepatitis C virus-related infection

Ref.	Characteristics of the study	miRNAs Up-regulated	miRNAs Down-regulated	Conclusions
Abdalla MA, 2012 Egypt Period: NR	Urine samples collected from: -32 patients with HCC post-HCV infection, -74 patients with chronic HCV infection --12 normal individuals	Up-regulation of: - miR-765, miR200a and miR-610, in the HCC-post HCV group; - miR-335, miR-618, miR-625, miR-532, miR-7 were in both the HCC-post HCV positive group and in the HCV positive group, relative to the control group	Down-regulation of: -miR-765, miR200a and miR-610 in the HCV positive group; - miR-323, miR-449, miR-502d, miR-92b, miR-516-5p and miR-650 in both the HCC-post HCV positive group and in the HCV positive group, relative to the control group	The predictive sensitivity and specificity values of miR-618/650 in tandem for detecting HCC among HCV-positive individuals were 58% and 75%, respectively. These values were higher, compared to the traditional α-feto protein (AFP) level-based detection method
Bihrer V, *PLoS One*, 2011 Germany Period: NR	Tissue and serum samples obtained from: -CHC: 62; -CHC plus HCC: 29; -Healthy subjects: 29; An -Independent cohort of 47 CHC patients	ND	ND	The serum miR-21 level is a marker for necroin-flammatory activity, but does not differ between patients with HCV and HCV-induced HCC
El-Garem H, *WJG*, 2014 Egypt Period: March-June 2012	Serum samples obtained from: -30 with chronic HCV alone (CH); -30 with HCV-related cirrhosis (LC); -30 with HCV-related HCC; -10-age and gender-matched healthy volunteers	Up-regulation of miR-122, miR-221	NR	Serum miR-221 has a strong potential to serve as one of the novel non-invasive biomarkers of HCC

Elhelw DS, *Biomedical Reports*, 2014 Egypt Period: NR	Serum, liver tissues and peripheral mononuclear cells samples obtained from patients infected with genotype 4-HCV: -72 patients chronically infected with HCV - 22 age-matched controls. The patients classified as: 24 naïve-patients, 11 SVRs pre-treatment, 15 SVRs post-treatment, 12 NRS pre-treatment and 10 NRS post-treatment	*miR-181a* significantly higher in the serum of naïve patients compared to controls, no difference in *miR-181a* expression observed in the liver tissues and PBMCs of patients compared to controls up-regulation of *miR-181a* post-interferon/ribavirin treatment in the serum of SVRs compared to non-responders and treatment-naïve SVR	NR	The up-regulation of miR-181a in the serum of HCV patients as an indication of good prognosis Any decrease during follow-up may be an early marker for progression to HCC
Oksuz Z, *Mol Biol Rep*, 2015 Turkey Period: NR	Serum samples obtained from: -26 patients with CHC; -30 patients with HCV-related cirrhosis; -8 patients with HCV-positive HCC; -28 patients with control group	Deregulated miR-30a-5p, miR-30c-5p, miR-206, miR302c-3p in CHC Deregulated miR-17-5p, miR-30c-5p, miR-93-5p, miR-130a-3p, miR-223-3p, miR-302c-3p, miR-302c-5p	Deregulated miR-17-5p, miR-30c-5p, miR-223-3p, miR-302c-3p in cirrhosis and HCC	miR-17-5p, miR-30c-5p, miR-223-3p, miR-302c-3p could serve as novel non-invasive biomarkers in the early phases of HCV-related HCC and in the cirrhosis stage of liver disease
Varnholt H *Hepatology*, 2008 Germany Period: 1995-2007	Tissue samples obtained from: 52 primary liver tumors from 39 patients induced by HCV infection	Increased expression of miR-9, miR-10a, miR-15a, miR-16, miR-299, miR-370 miR-326, miR-let-7g, miR-100, miR-125b in HCC in comparison with normal liver	Decreased expression of miR-198, miR-302b, miR-302b, miR-145, miR-368, miR-218, miR-330, miR-137, miR-147, miR-104, miR-9, miR-106a, miR-204, miR-159a, miR-134, miR-29c, miR-95, miR-199b, miR-185 in HCC in comparison with normal liver	A subset of miRNAs are aberrantly expressed in primary liver tumors, serving both as putative tumor suppressors and as oncogenic regulators
Zhang Y *Hepatology*, 2012 China Period: NR	Tissue specimens obtained from: -Healthy controls: 7; -NASH:12; -Chronic HCV infection; patients: 34; -HCV-HCC patients:10	Up-regulation of: miRNA-155	NR	HCV-induced miR-155 expression promotes hepatocyte proliferation and tumorigenesis by activating Wnt signaling

BCLC: Barcelona Clinic Liver Cancer staging system; CLD: Chronic liver disease; ER-α: Estrogen receptor-α; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; HC: Healthy controls; HCA: Hepatocellular adenoma; LC: Liver cirrhosis; LT: Liver transplantation; NASH: Nonalcoholic steatohepatitis; ND: Not detected; NR: Not reported; NRS: Non-responder; OS: Overall survival; RFS: Recurrence-free survival; SR: Spontaneously recovered; SVRs: Sustained viral responses.

et al[96] have studied miRNA expression in the visceral adipose tissue of patients with non-alcoholic fatty liver disease. A total of 113 species of miRNAs were differentially expressed in the visceral adipose tissue of NASH patients compared with those with non-NASH type of NAFLD. After multiple test correction, a significant down-regulation in the expression of seven miRs (miR-132, miR-150, miR-433, miR-28-3p, miR-511, miR-517a, miR-671) was detected[96]. Functional analysis of these seven miRNAs differentially expressed in NASH showed significant association with paths involved in the liver carcinogenesis. In addition, two miRNAs (miR-197 and miR-99), were significantly associated with pericellular fibrosis in NASH patients. A significant correlation was detected between serum and hepatic miR-122 expression in a series of 67 patients with NAFLD[97]. Patients with mild steatosis (< 33%) had significantly lower levels of hepatic miR-122 in comparison with subjects with severe steatosis (> 33%). Hepatic and serum miR-122 levels were significantly higher in patients with mild fibrosis than in those with severe fibrosis. The serum miR-122 level has resulted to be a useful predictive marker of liver fibrosis in patients with NAFLD, but no correlation was assessed between miR-122 and risk of HCC development[97]. A further study has evaluated serum miRNA profiles in patients with NASH[98]. This paper shown that miR-122, miR-192, miR-19a/miR-19b, miR-125b, and miR-375 were up-regulated > 2-fold either in simple steatosis or NASH and that, at a regression analysis for an ordinal multinomial distribution, miR-122, miR-192 and miR-375 were significantly associated with the histological disease severity and significantly up-regulated in NASH compared with patients, suffering from simple steatosis. In addition, few data are available, concerning expression pattern of miRNAs in alcohol-related liver cancer. Only some studies has evaluated miRNA profiles in a small number of patients with liver cancer and history of alcohol abuse, but some of them had a coexisting HBV- or HCV- infection[69,81]. Ladeiro *et al*[72] found that miR-21, miR-222, and miR-10b were significantly over-expressed in patients with HCCs, associated with both viral- and non-viral risk factors, but only under-expression of miR-126* was specifically related to alcohol abuse. Primary biliary cirrhosis (PBC) is also characterized by an altered expression pattern of miRNAs in comparison with healthy individuals. In particular, in liver tissue miR-346, miR-145, miR-328, miR-371, miR-299, miR-374, miR-506, miR-202, miR-186, miR-341, miR-25 were up-regulated, whereas miR-122a, miR-23b, miR-26a, miR-192, miR-126, miR-130b, miR-192, miR-194, miR-24, miR-107, miR-455-3p, miR-16, miR-193b, miR-103, miR-100, miR-27b, miR-19b, let-7d, miR-99a, miR-30c, miR-422b, miR-30e-5p, miR-92, miR-101b resulted down-regulated[99], whereas, in serum, miR-1273g-5p, miR-33a-5p, miR-3960 were up regulated and miR-766-5p, miR-505-3p, miR-30b-3p, miR-139-5p, miR-197-3p, miR-500a-3p were down-regulated[100]. However, no data exist, concerning the roles of miRNAs in hepatocarcinogenesis in patients with this pathology. In addition, no results are available, concerning a specific miRNA signature and liver cancer in subjects with hemochromatosis. In conclusion, no definite miRNA patterns, associated with an increased risk of HCC development in these non-viral diseases, have been described.

DISCUSSION

It is well-known that miRNAs act as key factors in several biological processes, such as growth, cell proliferation, differentiation, apoptosis and carcinogenesis. To date, most of the current diagnostic approaches for cancer screening are invasive, not specific as well as generally little effective or unable to detect malignancies in the early phases of development. Accumulating evidence indicates that miRNAs are perturbed both in non-cancerous human diseases and in the course of human carcinogenesis, from the early- to the late-phases of this process, in a large series of malignancies, including lung, colon, stomach, kidney and prostate and breast tumours. HBV- and HCV-related HCC development and progression is also associated with a significant and important deregulation of serum/plasma and liver tissues profiles of miRNAs, as it has been widely reported by several studies. Therefore, this evidence makes miRNAs potential non-invasive biomarkers for diagnosis, staging, progression, prognosis and response to treatment not only in non-cancerous diseases, but also in different malignancies. In particular, whether a definitive and reliable correlation between specific miRNAs levels and/or profiles in body fluids and HCC could be defined, these molecules might become a very useful tool for early detection of this type of neoplasm, in particular in the pre-symptomatic phases of its development. Therefore, in the last years, a large series of studies has been performed with these purposes. Ideal biomarkers should allow to diagnose and to monitor a disease, with an adequate sensibility and specificity, to define its stage as well as to permit an easy and reproducible screening in the general population, with a low cost. MiRNAs possess some peculiar and usefulness characteristics, including the possibility to detect these molecules in serum/plasma samples, that may be easily collected, and their high stability, even in conditions that are generally known to induce RNAs degradation, such as fluctuations in temperature and pH levels as well as long-term storage[101-103]. Unfortunately, several factors may strongly influence and decrease the possible helpfulness and benefit of miRNAs use in diagnostic and prognostic assessment of patients with cancers, such as bioptic or surgical procedures for

samples collection, methods of specimens freezing and RNA detection, aetiology of neoplasms and changing miRNAs profiles in the different phases of carcinogenetic process. Taking advantage from these elements, several Authors have evaluated miRNAs expression patterns in serum/plasma of patients with HBV- and or HCV-related HCC and compared these profiles with those detectable in serum/plasma of subjects with HBV- and/or HCV-positive hepatitis or cirrhosis as well as of healthy individuals with the aim to assess their potential role in the early diagnosis, prognosis and treatment outcome of patients at high risk of HCC development. In our review we specifically focused on these reports to summarize the available knowledge on this topic. Although the results of these studies seem to suggest that the use of miRNAs might be a feasible tool for the diagnosis of HCC, to date several important questions remain unresolved and incompletely defined. Therefore the utility and feasibility of miRNAs employment in clinical practice is still debatable and no definitive conclusions may be drawn. A doubtful point emerges from available results and has to be taken into account: the extreme heterogeneity among the different available studies. In particular the following factors have to be considered.

Study design and end-points. A high number of reports include a small sample sizes, very low number of screened miRNAs as well as a poor research methodology. In particular, in a high series of studies, only one or two miRNAs were considered for data analysis.

Most reports (in particular, studies investigating miRNAs patterns in HBV-related HCC) were carried out in China or in South East Asia, such as South Korea and Taiwan, in people of Asian ethnicity. These countries are high HBV-endemic areas, although, in the last years, long-term vaccination programs have contributed to decrease HBsAg positivity rate in the general population. On the other hand, only a small number of studies have been carried out in Europe, America and Africa. Therefore, the substantial variation in serum prevalence of HBV-related antigens/antibodies (*i.e.*, the antibodies patterns of HBsAg negative individuals, with signs of past HBV infection, a frequent conditions, at least in subjects of Southern Europe), as well as difference in geographical distribution of HBV genotypes, should be taken into account. All these factors, mainly for HBV, might have a substantial impact on the results obtained by available reports and could limit their validity.

It is possible that several miRNAs with potential important roles in HCC development have not been yet identified and validated as possible specific and useful biomarkers in the process of liver carcinogenesis and their activity has not yet been assessed in the available scientific works.

The potential inter-relations and cooperation among host- and viral-miRNAs, during the development of this malignancy, the potential cooperation between viruses and host in the process of liver carcinogenesis requires further evaluations. Cellular miRNAs may directly affect replication and pathogenesis of HBV and HCV viruses. It has been reported that miRNA-122 is essential for maintaining the adult phenotype in hepatocytes[104]. Moreover miR-122 is able to modulate the activities of genes controlling some important liver functions, as metabolism of lipid and cholesterol[105]. MiR-122 is also able to facilitate HCV replication, by targeting a 50 non-coding region of the viral genome[106]. HBV genome includes targets for human encoded miRNAs, as miR-7, miR-196b, miR-205, miR-345, miR-433, miR-511 and also miR-122. This last binds to the region of HBV pregenomic RNA, which codes both for the viral polymerase and for the 3' untranslated region and for the core protein. Therefore, HBV gene expression and replication are negatively modulated[107]. It has been reporteded that several miRNAs (miR-7, miR-196b, miR-433 and miR-511) interact with some viral DNA sequences and influence their activities[108]. A study by Novellino *et al*[109] has showed that, during HBV chronic infection, circulating HBsAg particles represent the carriers of selective pools of hepatocellular miRNAs (miR-27a, miR-30b, miR-122, miR-126 and miR-145), with specific liver functions.

Actually it has not yet been defined which is the best human biological sample (*i.e.*, serum, plasma, urine or tissue) to establish both which is the type of miRNAs and the range of their levels useful to an early diagnosis of HCC diagnosis or the risk of its recurrence. It should be considered that also different starting material could lead to different miRNA expression results. For example, a miRNA found as up-regulated in tissue specimen could be observed as not-deregulated (or down-regulated) in serum sample (*e.g.*, miR-122, Table 2).

Distinct molecular techniques are used in different studies, as microarrays, reverse-transcription polymerase chain reaction-based assays and next-generation sequencing, making it difficult to compare the results from different studies.

It should be considered that different studies have compared miRNA expression profiles of patients with several different control groups (*e.g.*, non-neoplastic liver, non-neoplastic liver adjacent to the tumor, chronic hepatitis, hepatic tissue from alcoholic cirrhosis). The selection of reference control group is still a big issue also in miRNA studies performed in other tumors (*e.g.*, in brain neoplasia[110]). This variability in selection of reference groups is another reason explaining the different expression profiles of some miRNAs observed throughout different studies (Tables 1-5 and Supplementary Tables 1-3).

Therefore, our knowledge of this field of search is still far from complete. Taken into account the results available in literature, although some studies have pointed out the potential role of some serum/

plasma miRNAs, including miR-21, miR-122, miR-125a/b, miR199a/b, miR-221, miR-222, miR-223, miR-224, as biomarkers for an early diagnosis of HCC development as well as for the assessment of its prognosis in HBV- or HCV- positive patients with this type of malignancy, their efficiency and usefulness require further evaluation and several issues have to be addressed to establish circulating miRNAs as definite reliable and useful diagnostic and prognostic tools. It is conceivable that different panels of miRNAs should be defined to obtain this end-point. Because of extreme biological complexity of miRNAs system, where each single miRNA may not only possess many targets and may modulate several pathways but also it may be influenced by a large series of distinct miRNAs, it is very improbable that a single miRNA may be sufficient for this purpose. Therefore, more well-designed and well-adjusted studies, focusing on populations of different geographical areas and involving larger series of patients, should be carried out to improve our knowledge on the potential role of miRNAs in HCC detection and allow us to define efficient panels of miRNAs. These trials should also allow us to establish which is the better type of sample and of test to be used for miRNAs search. In addition, these studies might provide the opportunity to design new treatments and anticancer approaches as well as to assess the efficacy and the side effects of these therapies.

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Missing metastases as a model to challenge current therapeutic algorithms in colorectal liver metastases

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Abstract

In oncosurgical approach to colorectal liver metastases, surgery remains considered as the only potentially curative option, while chemotherapy alone represents a strictly palliative treatment. However, missing metastases, defined as metastases disappearing after chemotherapy, represent a unique model to evaluate the curative potential of chemotherapy and to challenge current therapeutic algorithms. We reviewed recent series on missing colorectal liver metastases to evaluate incidence of this phenomenon, predictive factors and rates of cure defined by complete pathologic response in resected missing metastases and sustained clinical response when they were left unresected. According to the progresses in the efficacy of chemotherapeutic regimen, the incidence of missing liver metastases regularly increases these last years. Main predictive factors are small tumor size, low marker level, duration of chemotherapy, and use of intra-arterial chemotherapy. Initial series showed low rates of complete pathologic response in resected missing metastases and high recurrence rates when unresected. However, recent reports describe complete pathologic responses and sustained clinical responses reaching 50%, suggesting that chemotherapy could be curative in some cases. Accordingly, in case of missing colorectal liver metastases, the classical recommendation to resect initial tumor sites might have become partially obsolete. Furthermore, the curative effect of chemotherapy in selected cases could lead to a change of paradigm in patients with unresectable liver-only metastases, using intensive first-line chemotherapy to intentionally induce missing metastases, followed by adjuvant surgery on remnant

chemoresistant tumors and close surveillance of initial sites that have been left unresected.

Key words: Colorectal; Liver; Metastases, Surgery; Chemotherapy; Missing

Core tip: Surgery is considered as the only potentially curative option for patients with colorectal liver metastases, while chemotherapy alone is considered as a palliative treatment. Recent data shown that colorectal liver metastases disappearing after chemotherapy, so-called missing metastases, could not reappear on the long-term, suggesting that systemic treatments might be curative in selected cases. Accordingly, we propose that classical recommendation to limit surgery only when all initial tumor sites could be resected might have become partially obsolete. Furthermore, when missing liver metastases have been induced, adjuvant surgery targeting the resistant part of the disease could represent a new strategy.

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INTRODUCTION

The current oncosurgical approach in patients with isolated colorectal liver metastases (CRLM) is primarily driven by the concept that surgery represents the only potentially curative option. Retrospective series regularly report 5-year overall survival (OS) rates superior to 50% after surgical resection[1-5], reaching 75% in selected groups[6,7], while rates of cure, defined as disease-free survival (DFS) greater than 10 years[8], may reach 35%[9]. In contrast, in non-resected patients treated with chemotherapy only, median OS is still limited to 30 mo[10,11] and survival exceeding 10 years remains exceptional[12-14]. Such a dichotomy in the respective potentials of surgery and chemotherapy has major implications for establishment of strategic management plans in which resectability plays a central role in therapeutic decisions. Currently, surgical resection is the standard of care in patients with resectable CRLM (defined as patients in whom radical R0 resection is possible), irrespective of tumor load and tumor biology[15]. At the opposite end of the spectrum, in patients with diffuse metastatic liver infiltration that will never be amenable to surgery, chemotherapy is given in a strict palliative setting which aims to provide global cancer control and maintain an acceptable quality of life rather than induce an optimal tumor response. However, it can be argued that this vision is overly simplified, and is, at least partially, dogmatic rather than evidence-based. For example, it is clear that technical resectability does not optimally categorize all patients with CRLM. In some patients, rapid postoperative recurrence despite curative-intent resection casts doubt upon the benefits of surgery[16]. Moreover, no randomized study is currently available comparing surgery and chemotherapy in similar patients and the interpretation is massively biased when comparing the results of surgery in patients with limited disease and favorable prognostic factors to those of chemotherapy in patients with massive tumor burden. For obvious ethical reasons, it is not possible to conduct a trial that randomizes surgery and chemotherapy in patients with resectable CRLM and, therefore, arguments to challenge current therapeutic algorithms can only arise from indirect observations. The constant improvement in survival rates in patients receiving surgery for CRLM in recent years may represent the first evidence that challenges the current view. This has been achieved in the context of 2 major evolutionary changes. At the surgical level, sophisticated techniques now allow curative-intent surgery in patients with advanced metastatic disease who would have been previously ineligible for surgery. In parallel, the efficacy of chemotherapeutic regimens has continuously improved, resulting in significantly increased tumor response rates[11]. It is postulated that it is the combination of these 2 factors that is responsible for major improvements in post-surgical outcomes. A paradox is that the chances of long-term survival after resection have increased despite extension of the oncological indications. From a surgical point of view, it is unlikely that technical progress has improved the oncological efficacy of liver resection. This brings up the possibility that better results observed in these patients may be attributable to improved performance of chemotherapy, usually combined with surgery in complex cases. One could hypothesize that these results in patients with advanced CRLM may rely on the capacity of modern systemic regimens to clear occult metastatic disease[17-19]. Along this same line, the prognostic factors for postoperative outcomes have changed notably in the last few years. Previously, significant prognostic factors were mainly related to tumor stage, at primary and secondary levels, and to the possibility for radical surgery[20,21]. In the current era of efficient multimodal treatments, the predictive value of these factors has substantially decreased, replaced by prognostic markers that define the intrinsic tumor biology and potential interactions between the cancer and systemic treatments. Accordingly, several retrospective studies have indicated that the response to preoperative chemotherapy has a major prognostic impact and, particularly, that the chances of cure are significantly increased in patients with complete

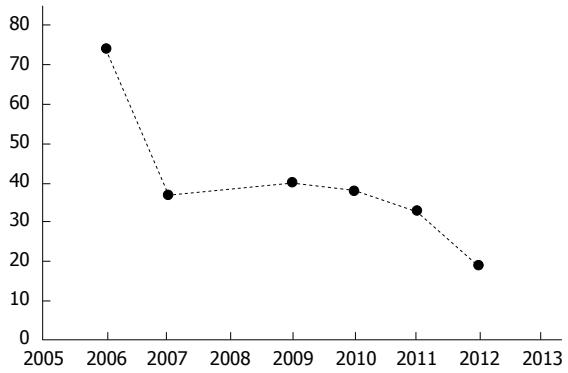

Figure 1 Rates of local recurrence of non-resected missing liver metastases according to the time of reporting. Local recurrence rates of unresected MLM according to the time of reporting, in 6 studies in which the in situ recurrence rates of MLM left in place could be calculated, in 2006[40], 2007[41], 2009[42], 2010[43], 2011[44] and 2012[47].

pathological response (CPR) in resected metastases as compared with patients with minor tumor response or progressive disease[5-7,16,22-24]. These data were not systematically confirmed[25] and should be interpreted with caution due to the retrospective nature of the studies and the variability of treatment regimens. Moreover, these observations do not constitute proof of the benefit of preoperative chemotherapy, as CPR may represent a surrogate marker of favorable tumor biology and/or genetics. Therefore, excellent outcomes in these patients may be related to surgery only in individuals with favorable tumor biology, identified by their positive response to chemotherapy. Still, and despite these reservations, if preoperative chemotherapy may contribute to improved post-operative survival, its effect is not expected to be dependent on its efficacy at the level of the responding metastasis itself, as it will be subsequently resected, but rather to active tumoricidal effects on occult disease. The potential capacity of modern therapeutic agents to eliminate microscopic disease could also explain why the predictive value of surgical margins regularly decreases. Classically, resectability was defined as the possibility of achieving a 1 cm negative margin and surgery was considered to be beneficial only when radical[26-28]. These principles are now challenged by several works showing that neither margin width, nor R1 resection have a significant impact on long-term survival in multivariate analyses and, therefore, an anticipated R1 resection should not be considered to be an isolated contraindication for surgery anymore[17,29-35]. Modern surgical transection methods, using ultrasonic dissectors and aspiration devices and high energy coagulation systems, may play a role in this phenomenon, transforming macroscopic R0 resections into R1 pathological resections[30] and R1 anatomical resections into R0 resections in situ. Perioperative chemotherapy may also play a role, potentially through elimination of residual cancer cells at resection margins. This is suggested by retrospective studies showing that the differences in survival between R0 and R1 resection are abolished in patients with optimal responses to chemotherapy[17,18,36], while R1 resection still carries a poor prognosis in patients with suboptimal responses[37]. Taken together, these observations support the hypothesis that new chemotherapeutic regimens might have become potentially curative at a cellular level. If this is true, the next step would be to evaluate whether chemotherapy might be curative on a macroscopic tumor and how to integrate this concept into new therapeutic strategies. Taking into account current established therapeutic algorithms, this simple hypothesis is extremely difficult to verify in a clinical model. However, the particular situation where liver metastases disappear after chemotherapy represents a unique opportunity to address this question. When these so-called missing liver metastases (MLMs) are left unresected, their long-term surveillance provides the chance to verify whether such complete radiological response (CRR) could correspond to a cure.

MISSING LIVER METASTASES AS AN OPPORTUNITY TO DEMONSTRATE THAT CHEMOTHERAPY COULD BE CURATIVE

MLMs, or disappearing or vanishing metastases, refer to liver metastases that become undetectable upon imaging after administration of chemotherapy. As radiological response to chemotherapy can be inhomogeneous[38] and as MLMs may be reported as the percentage of patients having at least 1 MLM or as the ratio of MLMs compared to the total number of liver metastases, the exact incidence of this phenomenon remains difficult to evaluate. Yet, consistent with the increased response rates to modern chemotherapies and despite the increasing sensitivity of liver imaging techniques, a trend toward an increasing rate of MLMs has been described in the last years, ranging from 6% to 10% in initial studies to 10% to 24% in more recent works (Figure 1)[39-47]. The definition of MLM critically depends on imaging technique performance. Currently, most authors prefer magnetic resonance imaging (MRI) over computed tomography scans[48] but optimally use both techniques to confirm the disappearance of the lesions[49,50]. Additionally, in 10% to 50% of the cases, MLMs on preoperative imaging are still found at surgery, using visual and manual inspection and intraoperative ultrasound (IOUS)[39,40,42-46,51,52], this rate reaches 80% when contrast-enhanced IOUS is used[51]. After accurate preoperative imaging and intraoperative exploration, some MLMs remain undetectable and should therefore be considered to be true MLMs. The main predictors for development of MLMs are small initial tumor size, low carcinoembryonic antigen (CEA) level, rapid normalization of CEA after chemotherapy, duration of chemotherapy, and use of hepatic arterial

Table 1 Incidence of missing liver metastases and rates of local cure as defined by complete pathological response or absence of *in situ* recurrence on follow-up

Ref.	Year	Incidence[1]	CPR[2]	Sustained local clinical response[3]
Elias *et al*[39]	2004	10%	-	-
Benoist *et al*[40]	2006	6%	20%	26%
Elias *et al*[41]	2007	-	-	62%
Tanaka *et al*[42]	2009	-	100%	59%
Auer *et al*[43]	2010	9%	65%	62%
van Vledder *et al*[45]	2010	24%	39%	53%
Ferrero *et al*[46]	2012	11%	-	39%
Ono *et al*[47]	2012	-	100%	82%

[1]Incidence calculated as the number of patients with at least 1 missing liver metastases (MLM) among the total group of patients evaluated in the study; [2]Complete pathologic response as defined by the absence of residual cancer cells when the initial site of MLM was resected; [3]Sustained clinical response as defined as the absence of local recurrence at the initial site when MLM was left unresected.

infusion of chemotherapy (HAI)[40-43]. The central question concerning MLMs is whether they correspond to a false negative result of preoperative and intra-operative staging or represent a true complete response and, potentially, a cure of the lesion. This question is critical for its strategic implications in cases when MLMs were accidentally induced but also for evaluation of whether intentional induction of MLMs could become part of a therapeutic plan. One possible answer to this question comes from pathologic analysis when the site of an MLM was resected during surgery, either when included in the planned resection or when a blind hepatectomy was performed. Among resected MLMs, the rate of complete pathologic response (CPR), defined as the absence of residual viable cancer cells, varies widely in the literature, from 20% to 100% (Table 1)[39-47]. In different series, the predictive factors for a correlation between CRR and CPR are the use of HAI, the absence of steatosis, low body mass index, an MRI-based diagnosis, the normalization of CEA level during chemotherapy, and the use of a modern chemotherapy regimen[41-43,47]. A second, and more convincing answer, is provided by the long-term local follow-up of MLMs when they were left unresected. When such specific follow-up could be performed, most of the local recurrences appear rapidly, within 20 mo after chemotherapy withdrawal[43]. Among unresected MLMs, the rate of sustained clinical response (SCR), defined as the absence of local recurrence on follow-up, varies massively in literature, from 25% to 80% (Table 1)[39-47]. Interestingly, this rate tends to progressively increase in recent reports, potentially related to improved sensitivity of imaging and improved efficacy of perioperative chemotherapies (Table 1)[39-47]. Particularly, adjuvant chemotherapy and adjuvant HAI could be determinants, as re-growth of MLMs appears substantially increased in patients who do not receive postoperative treatments[41,43]. In an early study, Benoist *et al*[40] reported that, in the large majority of cases, residual cancer cells were still present at pathology when original sites of MLMs were resected and that local recurrences were almost certain to occur when they were left unresected. This led to the view that MLMs are an undesirable event resulting from preoperative overtreatment and precluding the chances for radical surgical resection. Accordingly, the consensus recommendation was, and remains, to systematically resect all original sites of MLM whenever technically feasible[40,49,53]. Currently however, apart from the surgical difficulty of such blind resections, this recommendation should probably be reevaluated, as recent studies suggest that a significant proportion of MLMs would not reappear in the long term.

PROPOSAL FOR A NEW THERAPEUTIC STRATEGY RELYING ON THE INTENTIONAL INDUCTION OF MLMS

There are 3 categories of patients at the time of presentation of CRLM: Patients with resectable metastases, patients with borderline or potentially resectable metastases, and patients with metastases considered to be definitively unresectable. The therapeutic options are well-defined for the 2 first categories. Patients with resectable CRLM should undergo surgery, giving them, most probably, the best chance for cure (Figure 2). In these cases, the place for perioperative systemic treatments remains under discussion and is currently decided according to associated risk factors. Patients with borderline CRLM are those potentially amenable to radical surgical resection, provided significant downsizing after neoadjuvant chemotherapy. In these cases, first-line intensive chemotherapy is indicated, to maximize the chances of response, followed by surgery with curative intent when allowed by tumor-response (Figure 2). It is in the third group of patients that new therapeutic options can be developed. In the current therapeutic algorithm, these patients receive chemotherapy in a palliative setting, favoring long-term tolerance. If we can concede that disappearance of CRLM after chemotherapy corresponds to a cure in selected cases, a new option in these patients could be to intentionally induce MLMs. In this objective, the initial decision should be, therefore, to modify the first-line chemotherapy from a palliative to an intensive regimen, including possibly HAI, to elicit a maximal tumor response and to enhance the chances of obtaining MLMs. Under these conditions, if MLMs are induced, adjuvant surgery and/or local destruction with radiofrequency, targeted to remnant visible disease could represent an acceptable option when safely feasible (Figure 2). To reasonably develop such an exploratory approach, patient selection is pivotal. First, selection should rely on surgical aspects, reserving this type of approach for patients with tumor distributions that are potentially amenable to surgical resection if

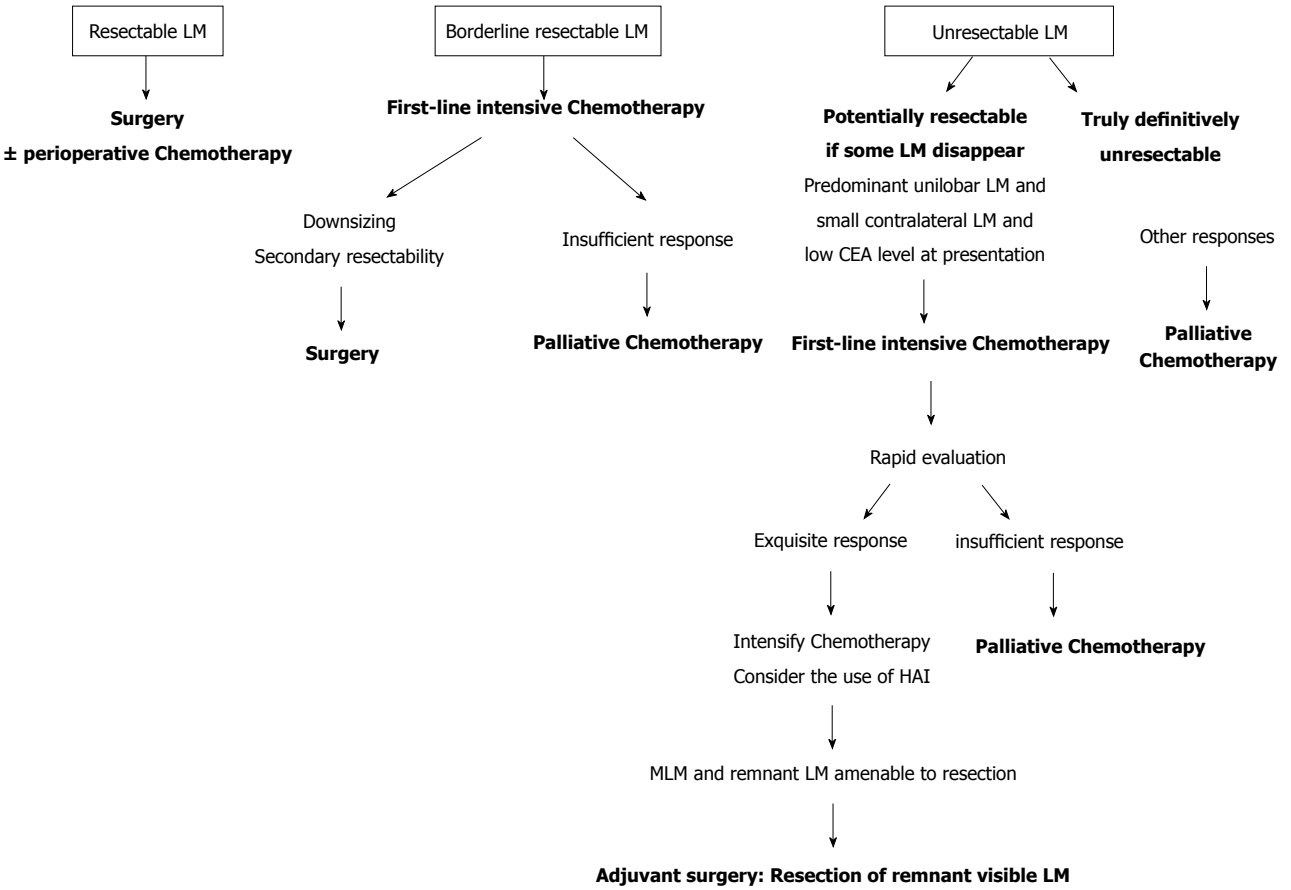

Figure 2 Therapeutic strategy in patients with patients with colorectal liver metastases. The potential role for adjuvant surgery. CEA: Carcinoembryonic antigen; MLM: Missing liver metastase; HAI: Hepatic arterial infusion of chemotherapy; LM: Liver metastases.

some lesions would disappear after chemotherapy (Figure 2). Other selection criteria may include the predictive factors for development of MLMs, such as low CEA level and small tumor size. In these cases, rapid evaluation of tumor chemosensitivity, using evolution of CEA levels and metabolic imaging[54], would be critical, to reinforce first-line chemotherapy or to shift to a palliative regimen in cases of poor initial response. In addition, in such an approach, the use of adjuvant systemic chemotherapy or HAI should be considered to enhance the chances of long-term SCR[41,43].

CONCLUSION

Classically, MLMs were judged to be false negative results of imaging and recurrence at those sites was considered to be a near certainty. Now, however, taking into account the increased sensitivity of imaging and the improved efficacy of chemotherapy, this view should be reevaluated. Better identification of the factors which may lead to disappearance of liver metastases and better knowledge of their long-term evolution may allow for consideration of the induction of MLMs as a potential therapeutic option, to convert unresectable into macroscopically resectable disease. In patients with initially resectable CRLM, the accidental generation of MLMs during preoperative treatment remains a globally unfavorable event as it may complicate a subsequent curative-intent surgery. However, in these cases, the classical recommendation to resect all initial metastatic sites[40,49,53] might have become partially obsolete with regard to the substantial chances for SCR in these cases. Therefore, when such resections are hazardous, a watch-and-wait strategy now appears to be a reasonable alternative. In patients with unresectable CRLM, the recent demonstration that some MLMs are cured challenges the dogma that curative potential is exclusively reserved to surgery, and may lead to new therapeutic options. In these strategies, the classical roles of chemotherapy and surgery would be modified, using first-line intensive chemotherapy to obtain maximal tumor response, followed, if MLMs were induced, by resection and/or RF destruction of the chemoresistant part of the disease. This may lead, in fact, to the identification of a new subgroup of patients, defined as those with initially unresectable but potentially resectable metastases if MLMs are induced by chemotherapy, and to the new concept of adjuvant surgery, defined as surgery targeting the remnant visible metastases after CRR to chemotherapy of other lesions. The future development of such complex strategies will critically depend upon the identification of accurate predictive factors for

response to chemotherapy at the single-tumor level, on the determination of the best chemotherapeutic regimen and on close, multidisciplinary collaborations in therapeutic decisions at the presentation of the disease and during follow-up.

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MINIREVIEWS

Technical tips for endoscopic ultrasound-guided hepaticogastrostomy

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Abstract

Interventional procedures using endoscopic ultrasound (EUS) have recently been developed. For biliary drainage, EUS-guided trans-luminal drainage has been reported. In this procedure, the transduodenal approach for extrahepatic bile ducts is called EUS-guided choledochoduodenostomy, and the transgastric approach for intrahepatic bile ducts is called EUS-guided hepaticogastrostomy (EUS-HGS). These procedures have several effects, such as internal drainage and avoiding post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis, and they are indicated for an inaccessible ampulla of Vater due to duodenal obstruction or surgical anatomy. EUS-HGS has particularly wide indications and clinical impact as an alternative biliary drainage method. In this procedure, it is necessary to dilate the fistula, and several devices and approaches have been reported. Stent selection is also important. In previous reports, the overall technical success rate was 82% (221/270), the clinical success rate was 97% (218/225), and the overall adverse event rate for EUS-HGS was 23% (62/270). Adverse events of EUS-biliary drainage are still high compared with ERCP or PTCD. EUS-HGS should continue to be performed by experienced endoscopists who can use various strategies when adverse events occur.

Key words: Endoscopic ultrasound; Endoscopic ultrasound-guided hepaticogastrostomy; Endoscopic ultrasound-guided biliary drainage; Endoscopic retrograde cholangiopancreatography

Core tip: Endoscopic ultrasound-guided hepaticogastrostomy (EUS-HGS) has been developed as an alternative biliary drainage method. The reported technical success rate of EUS-HGS ranges from 65% to 100%, and the clinical success rate ranges from 87% to 100%. Furthermore, the overall technical success rate was 82%, and the overall clinical success rate was 97%. Based on the currently available literature, the overall adverse event rate for EUS-HGS is 23%. EUS-HGS has high rate of adverse events that are sometimes fatal. Therefore, EUS-HGS should continue to be performed by experienced endoscopists who can

use various strategies when adverse events occur.

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INTRODUCTION

Biliary drainage under endoscopic retrograde cholangiopancreatography (ERCP) guidance has been well established and widely performed[1,2]. The technical success rate of this procedure is high according to previous reports. If ERCP fails, percutaneous transhepatic biliary drainage (PTBD) is conventionally attempted. PTBD is also established as an alternative drainage method. However, this procedure has several disadvantages, such as catheter dislodgement, pneumothorax, external drainage, and cosmetic problems[3-5]. Recently, interventional procedures using endoscopic ultrasound (EUS) have been developed. For biliary drainage, EUS-guided transluminal drainage has been reported (EUS-guided biliary drainage; EUS-BD). The transduodenal approach for extrahepatic bile ducts is called EUS-guided choledochoduodenostomy (EUS-CDS)[6-8], and the transgastric approach for intrahepatic bile ducts is called EUS-guided hepaticogastrostomy (EUS-HGS). For EUS-BD, EUS-HGS can be performed if the duodenal bulb is obstructed due to malignant tumor. The technical success rate has been high, however, the rate of adverse events has also been reported to be high.

Table 1 shows an overview of recent published reports of EUS-HGS (over 10 cases, and excluding insufficient data)[9-19]. In this paper, previous reports are reviewed, and technical tips for EUS-HGS to ensure successful performance and avoid adverse events are presented.

INDICATIONS

To date, EUS-BD is seen as a consistent alternative drainage method. Therefore, as well as other EUS-BD procedures, EUS-HGS should also be indicated for failed ERCP due to surgical anatomy and an inaccessible ampulla of Vater. Although EUS-CDS is contraindicated in patients with surgically altered anatomy, such as a Roux-en-Y anastomosis or duodenal bulb obstruction caused by tumor invasion, EUS-HGS can be performed because this procedure is performed from the stomach. With respect to a biliary stricture, if the hepatic hilum is obstructed, EUS-HGS may be contraindicated, because with stent placement in the left intrahepatic bile duct, the right hepatic bile duct cannot drain**.** Recently, EUS-BD for right hepatic biliary obstruction has been reported as an expanding indication[20,21]. Park *et al*[20] reported that, among 6 patients who had isolated right hepatic bile duct obstruction, EUS-guided access successfully resulted in antegrade bypass stenting in 2 patients, antegrade transanastomotic stenting in 1 patient, antegrade transanastomotic balloon dilation in 1 patient, and a cholangiogram as a roadmap in 1 patient. We also reported[21] that, among 11 patients with right hepatic bile duct obstruction, EUS-BD was successfully performed from the left hepatic approach (bridging method) in 7 patients and from the right hepatic approach (locking stent method) in 4 patients. Remarkably, no adverse events were reported in both papers. Therefore, EUS-HGS may be indicated for hepatic hilar obstruction. However, because this technique is challenging, the right hepatic approach using EUS-BD should be performed for limited cases.

Recently, Khashab *et al*[22] reported a comparative evaluation of EUS-BD and PTCD in patients with distal malignant biliary obstruction. In this report, although the technical success rate was higher in the PTCD group (100% *vs* 86.4%, $P = 0.007$), clinical success and stent patency were the same. In addition, the adverse event rate (70.6% *vs* 18.2%, $P < 0.001$) and total charges were higher in the PTCD group ($9.072 ± 3.817 *vs* $18.261 ± 16.021, $P = 0.003$). Therefore, they concluded that EUS-BD should be selected if the procedure can be performed by experienced endoscopists. However, this study has several limitations, such as a small number of patients in a single center with a single operator. To determine whether EUS-HGS or PTCD should be performed as an alternative drainage method, a multicenter, prospective, randomized, controlled trial is needed.

Hence, the following are the indications for EUS-HGS: (1) Failed ERCP; (2) Inaccessibility of the ampulla of Vater, including due to surgical anatomy and tumor invasion; and (3) Contraindications for PTCD such as ascites and possibility of self-tube removal.

On the other hand, compared with PTCD, metallic stent placement is performed in EUS-HGS; therefore, if a small amount of ascites is present between the stomach and liver, EUS-HGS may be indicated. However, if massive ascites is present, preventing the formation of a fistula between the stomach and the liver, EUS-HGS is not indicated. For patients with unresectable gastric cancer, because the stomach volume is decreased due to tumor growth, the EUS-HGS stent might be pulled into the stomach (Figure 1).

Hence, the following are the contraindications for EUS-HGS: (1) Massive ascites between the stomach and the liver; and (2) Unresectable gastric cancer.

DEVICE SELECTION AND TECHNICAL TIPS

Puncture of the intrahepatic bile duct

To visualize the left intrahepatic bile duct, EUS should

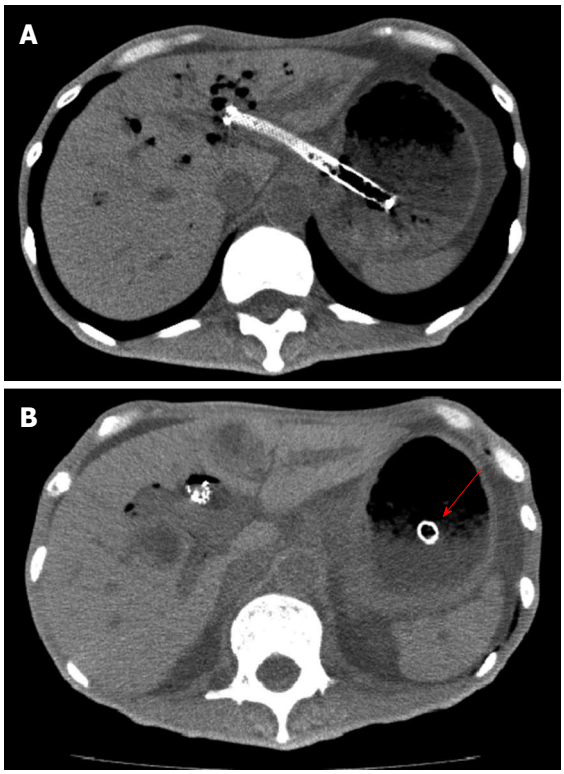

Figure 1 Dislocation of endoscopic ultrasound-guided hepaticogastrostomy metallic stent. (A) EUS-HGS was performed for gastric cancer patient. (B) Because of tumor growth, dislocation of EUS-HGS metallic stent was seen (arrow). EUS-HGS: Endoscopic ultrasound-guided hepaticogastrostomy.

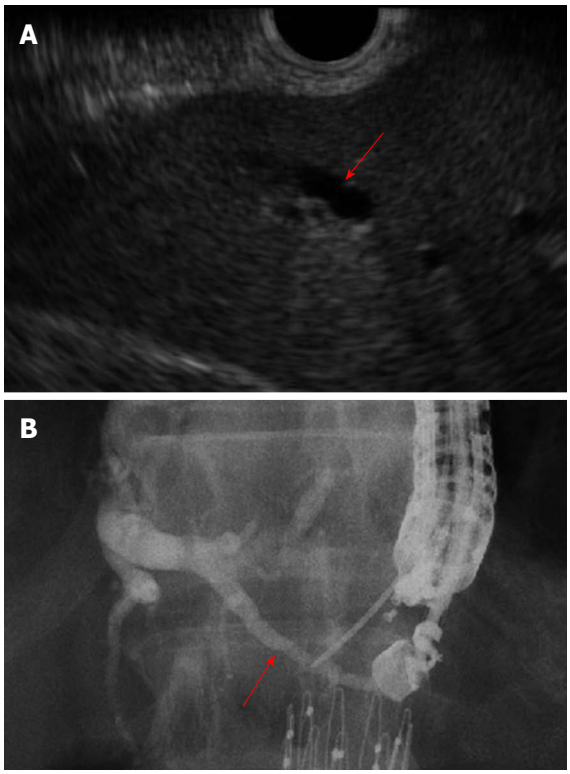

Figure 2 Technical tips of puncture on endoscopic ultrasound-guided hepaticogastrostomy. (A) To advance the guidewire toward the hepatic hilum, the bile duct that runs from the upper left to the lower right on EUS imaging should be punctured (arrow) (B) fluoroscopic imaging (arrow). EUS-HGS: Endoscopic ultrasound-guided hepaticogastrostomy.

be advanced into the stomach. Then, using slight counter clockwise rotation, the left hepatic lobe can be visualized. A 19G-FNA needle is better than a 22G-FNA needle. A stiffer guidewire can be inserted through the FNA needle, because a dilation fistula is more needed to insert the stent delivery system than with EUS-CDS. If segment 2 (B2) is punctured, because each device is passed through the mediastinum when puncturing from the esophagus, severe adverse events, such as mediastinitis or pneumomediastinum, may occur. Therefore, on EUS-HGS, segment 3 (B3) should be initially selected as the puncture site. To puncture the intrahepatic bile duct, there are two important points. One is the angle of the bile duct, and the other is the volume of liver parenchyma.

To advance the guidewire toward the hepatic hilum, the bile duct that runs from the upper left to the lower right on EUS imaging should be punctured (Figure 2). Furthermore, to avoid stent migration, sufficient volume of liver parenchyma is needed, as for the PTCD procedure**.** Therefore, for these reasons, B3 is better for puncturing the biliary tract.

Guidewire insertion into the hepatic hilum or common bile duct

Guidewire insertion is one of most important procedures during EUS-HGS. If the guidewire is advanced into the peripheral biliary tract, the next step, such as dilation device or stent delivery system insertion, cannot be performed. To successfully advance the guidewire toward the hepatic hilum, as described in the puncture of the intrahepatic bile duct section, the biliary tract running from the upper left to the lower right on EUS imaging should be punctured. If the guidewire is advanced into the peripheral biliary tract, the guidewire should be pulled, and then one should try to advance the guidewire into the hepatic hilum. However, during this procedure, the guidewire is sometimes kinked with the FNA needle. To avoid this adverse event, the liver impaction method is useful[23].

Various types of guidewires are available. A 0.025-inch guidewire with a highly flexible tip, sufficient stiffness, and easy seeking ability (VisiGlide, Olympus Medical Systems, Tokyo, Japan) is preferable for the EUS-guided procedure. After the guidewire is inserted along with other devices, it is important to keep visualizing the other devices on EUS imaging during various EUS-guided procedures to fit the axis.

Devices used to dilate the fistula

To insert the stent delivery system, the bile duct and stomach wall must be dilated. To date, various techniques of dilating a fistula have been reported (Table 1). According to previous reports, a graded dilation technique using a dilator or a 4-mm balloon catheter is used by many authors. The dilator (6 to 10 Fr; Soehendra biliary dilation catheters, Cook Medical), balloon catheter (4-8 mm; MaxForce or Hurricane

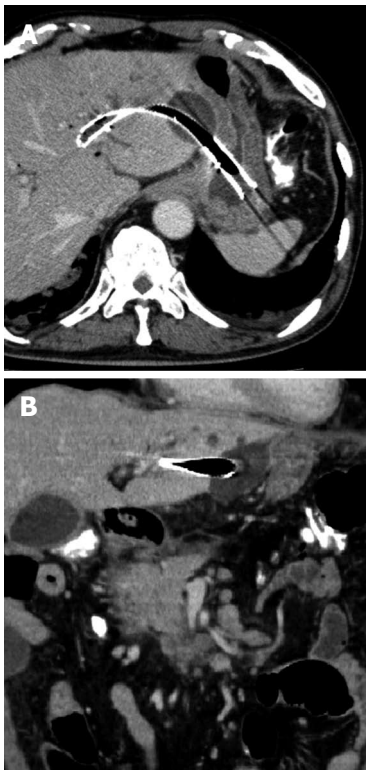

Figure 3 Biloma after endoscopic ultrasound-guided hepaticogastrostomy. Long procedure time was needed, therefore, bile leak was increased.

RX; Boston Scientific), and needle knife (Microtome, Boston Scientific) are most used by many authors.

Park *et al*[9] reported that, among total 57 consecutive patients, post procedure adverse events occurred in 11 patients (bile peritonitis *n* = 2, mild bleeding *n* = 2, self-limited pneumoperitoneum *n* = 7). On multivariate analysis, using a needle knife was the only risk factor for post procedure adverse events of EUS-BD (P = 0.01, HR = 12.4, 95%CI: 1.83-83.5). They concluded that a needle knife should not be used as a dilation device if possible. To avoid this risk, a diathermic dilator (Cysto Gastro Set; Endoflex, GmbH, Voerde, Germany) has recently become available. This device is always coaxial with the guidewire. Although this device has clinical impact as a dilation device, a burning effect can occur. When the bile duct is punctured avoiding small vessels of the stomach or bile duct wall, bleeding may occur due to the burning effect of the diathermic dilator.

On the other hand, a graded dilation technique using a balloon or dilator catheter may be safe. Park do *et al*[24] reported that graded dilation using a 4-Fr cannula and 6-Fr and 7-Fr bougie dilators is safe. In their study, technical success of EUS-CDS was relatively high, with a low rate of adverse events. In our previous report[25], we successfully performed EUS-HGS using an ERCP catheter and a 4-mm balloon catheter without using a needle knife or cystotome. This technique may be associated with a lesserfrequency of bleeding caused by the burning effect of a needle knife or diathermic dilator, but bile leakage may easily occur during graded dilation. Recently, novel techniques and dilation devices for EUS-BD have been reported. Paik *et al*[15] reported a simplified fistula dilation technique. After the biliary tract was punctured using a 19G FNA needle, direct insertion using a 4-mm balloon catheter (Hurricane RX; Boston Scientific) was performed. In 28 patients, technical success of creating a dilation fistula using this technique was 96% (27/28). In addition, no early adverse events were seen. We also reported a simplified fistula dilation technique using a novel balloon catheter[26,27]. As an even more novel technique, a one-step stent placement technique has been reported[19]. In this study, 32 patients with malignant biliary obstruction were enrolled, and EUS-BD using a novel metallic stent was attempted. The introducer for this novel stent has only a 3-Fr-tip-4-Fr tapered. Technical success of one-step stent placement without any additional dilation was 88% (14/16). In addition, the procedure time was short in the one-step stent placement group. With a longer procedure time, the possibility of bile leakage may be increased (Figure 3). Indeed, in their study, compared with the graded dilation group, although significant differences were not seen, early adverse events were uncommon in the one-step dilation group (31.3% *vs* 6.3%, P = 0.172). Although randomized, clinical trials and additional cases are needed for which dilation technique or devices are more suitable, simplified dilation technique or one-step stent placement technique using novel metallic stents may decrease the frequency of adverse events such as bile leakage.

Stent selection

According to previous reports, a fully covered self-expandable metallic stent (FCSEMS) was mainly used (Table 1). An FCSEMS may be more suitable for EUS-HGS than a plastic stent for the following reasons: (1) If a large fistula is created to insert the stent delivery system, bile leakage is less likely from the gap between the stent and fistula[28-30]; (2) Longer stent patency may be obtained; (3) A tamponade effect of the FCSEMS itself will occur if there is bleeding from the stomach wall[17].

Also, there are several disadvantages of FCSEMS, as follows: (1) Expensive; (2) Stent shortening must be considered, especially the luminal portion to prevent stent migration[17]; and (3) Side branches of the left hepatic biliary tract may be obstructed.

Recently, a novel metallic stent and several efforts to prevent stent migration have been reported. With the use of the standard metallic stent, some authors reported that a double pigtail plastic stent can be placed inside the metal stent, with the pigtail functioning as an anchor[31]. To prevent proximal stent migration, sufficient stent length is needed. We also previously reported that EUS-HGS could be safety performed using a long and partially covered metallic stent[25,32]. More recently, Song *et al*[14] published a preliminary report on a hybrid metal stent for EUS-

Table 1 Overview of recent published reports on endoscopic ultrasound-guided hepaticogastrostomy (over 10 cases, excluding insufficient data) *n* (%)

Ref.	Technical success	Clinical success	Dilation devise	Stents	Adverse events (*n*)
Park *et al*[9], 2011	31 (100)	27 (87)	4Fr cannula, 6Fr, 7Fr biliary dilator, needle-knife	PS, Fully CSEMS	Pneumoperitoneum (6)
Vila *et al*[10], 2012	22 (65)	22 (100)	NA	NA	Bleeding (3), biloma (4), perforation (4), liver hematoma (2), abscess (1)
Attasaranya *et al*[11], 2012	11 (85)	11 (100)	ERCP cannula, 6Fr, 7Fr biliary dilator, needle-knife	Pig PS Fully CSEMS	Minor adverse events (5) Major adverse events (1)
Prachayakul *et al*[12], 2013	NA	NA	Tapered tip Teflon catheter	Fully CSEMS	NA
Kawakubo *et al*[13], 2013	19 (95)	NA	Biliary dilation catheter, dilating balloon, cautery dilator	Straight PS, CSEMS	Bile leakage (2) Stent migration (2) Bleeding (1) Cholangitis (1) Biloma (1)
Song *et al*[14], 2014	10 (100)	10 (100)	6Fr, 7Fr biliary dilator, needle-knife, 4-mm dilating balloon	Hybrid metal stent	Pneumoperitoneum (2) minor bleeding (1)
Paik *et al*[15], 2014	27 (96)	24 (89)	4-mm balloon	Dual-flap Fully CSEMS	Migration (1) Pseudoaneurysm (1)
Artifon *et al*[16], 2015	24 (96)	22 (91)	Dilating catheter, needle-knife	Partially CSEMS	Minor bleeding (3) Biloma (1) Bacteremia (1)
Umeda *et al*[17], 2015	23 (100)	23 (100)	Standard or tapered catheter, cautery dilator, 8Fr dilation catheter, 4-mm balloon	8Fr single-plastic stent	Bleeding (1), self-limited abdominal pain (3)
Poincloux *et al*[18], 2015	65 (99)	61/65 (94)	5.5Fr needle-knife, 6Fr, 7Fr dilation catheter	10Fr PS Fully CSEMS Two fitting CSEMS Half covered SEMS	Pneumoperitoneum (2) Intrahepatic hematoma (1) Bile leakage (5) Sever sepsis (2)
Park *et al*[19], 2015	20 (100)	18/20 (90)	Without dilation, 4-mm balloon catheter, dilation catheter	CSEMS with dedicated stent introducer, fully CSEMS	Mild adverse events (2) Moderate adverse events (3)

PS: Plastic stent; CSEMS: Covered self-expandable metallic stent; NA: Not available.

BD. The distal portion of this novel stent, which is 3.5 mm long, is composed of silicone-covered nitinol wire to prevent bile leakage. In addition, proximal and distal of the covered site there are anti-migration flaps to prevent stent migration. This novel stent has the uncovered site on the proximal site, which is 1.5 to 5.5 mm long, to prevent obstruction of side branches. In their study using this novel stent, EUS-HGS was successfully performed for all patients (n = 10), and, in addition, stent migration or bile leakage was not seen.

On the other hand, EUS-HGS using a newly designed plastic stent has been reported by Umeda *et al*[17]. In their study, an 8-Fr single-pigtail plastic stent, which is a pus-type stent that is usually not possible to retract, with a total of 20 cm and an effective length of 15 cm with 4 flanges, was used. The proximal end has a pigtail stricture**,** and the distal end is tapered. EUS-HGS using this novel plastic stent was successfully performed in 23 patients (technical success rate 100%). Although bleeding or abdominal pain was seen in 4 patients (17.4%), no severe adverse events such as stent migration or dislocation were seen during follow-up (median 5.0 mo). Stent patency was 4.0 mo (range 0.5-12.5 mo). This result was clinically encouraging, but, as the author described, additional long-term studies with a large number cases are needed to evaluate the clinical impact of using this stent for EUS-HGS.

SUCCESS RATE

The reported technical success rate of EUS-HGS ranges from 65% to 100%, and the clinical success rate ranges from 87% to 100% (Table 1). Furthermore, the overall technical success rate was 82% (221/270), and the overall clinical success rate was 97% (218/225). Compared with EUS-CDS, the technical success rate was relatively lower. This may be due to the difficulty puncturing the biliary tract and inserting the guidewire. To increase the technical success rate, devices should be improved. EUS-HGS should be still performed by experienced endoscopists at high-volume centers, because the adverse events of EUS-HGS are sometimes fatal.

ADVERSE EVENTS

Adverse events of EUS-BD are still high compared with ERCP or PTCD. Based on the currently available

literature (Table 1), the overall adverse event rate for EUS-HGS is 23% (62/270), and these adverse events, include: (1) Bleeding; (2) Pneumoperitoneum; (3) Biloma, bile leakage; (4) Infection (cholangitis, abscess, bacteremia); (5) Hematoma; (6) Perforation; (7) Abdominal pain; (8) Pseudoaneurysm; and (9) Stent migration

Among them, stent migration is recognized as a severe adverse event that is sometimes fatal. Okuno *et al*[33] reported stent migration that was treated by surgery. They used an 8-cm-long FCSEMS, and stent migration occurred immediately. Martins *et al*[34] also reported that EUS-HGS was successfully performed using an 8-mm-long, partially covered SEMS, but after 5 days, stent migration with the proximal end located within a large biloma occurred. Although conservative treatment was performed, this patient died. We also reported stent migration within the stomach wall after 3 d[25]. In addition, we also reported that stent length in the luminal portion is an important factor to reduce the adverse events of EUS-HGS[32]. Therefore, considering stent shortening, over 10 cm or a novel SEMS such as previously reported should be used to prevent stent migration[32,33].

CONCLUSION

EUS-HGS has wide indications and clinical impact as an alternative biliary drainage method. However, EUS-HGS also has a high rate of adverse events that are sometimes fatal. Therefore, EUS-HGS should continue to be performed by experienced endoscopists who can use various strategies when adverse events occur.

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ORIGINAL ARTICLE

Basic Study

Gallic acid induced apoptotic events in HCT-15 colon cancer cells

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Abstract

AIM: To investigate the inhibitory action of diet-derived phenolic compound gallic acid (GA) against HCT-15 colon cancer cells.

METHODS: The antiproliferative effect of GA against colon cancer cells was determined by performing thiazolyl blue tetrazolium bromide (MTT) assay. The colony forming ability of GA treated colon cancer cells was evaluated using the colony forming assay. The cell cycle changes induced by GA in HCT-15 cells were analyzed by propidium iodide staining. Levels of reactive oxygen species (ROS) and mitochondrial membrane potential of HCT-15 exposed to GA was assessed using 2',7'-dichlorfluorescein-diacetate and rhodamine-123 respectively, with the help of flow cytometry. Morphological changes caused by GA treatment in the colon cancer cells were identified by scanning electron microscope and photomicrograph examination. Apoptosis was confirmed using flow cytometric analysis of GA treated HCT-15 cells after staining with Yo-Pro-1.

RESULTS: MTT assay results illustrated that GA has an inhibitory effect on HCT-15 cells with IC_{50} value of 740 μmol/L. A time-dependent inhibition of colony formation was evident with GA treatment. Cell cycle arrest was evident from the accumulation of GA treated HCT-15 cells at sub-G1 phase (0.98 ± 1.03 *vs* 58.01 ± 2.05)

with increasing exposure time. Flow cytometric analysis of GA treated HCT-15 cells depicted early events associated with apoptosis like lipid layer breakage and fall in mitochondrial membrane potential apart from an increase in the generation of ROS which were in a time dependent manner. SEM and photomicrograph images of the GA-treated cells displayed membrane blebbing and cell shrinking characteristics of apoptosis. Further apoptosis confirmation by Yo-Pro-1 staining also showed the time-dependent increase of apoptotic cells after treatment.

CONCLUSION: These results show that GA induced ROS dependent apoptosis and inhibited the growth of colon cancer cells.

Key words: Gallic acid; Colon cancer; Apoptosis; Cell cycle; Reactive oxygen species; Lipid layer break

Core tip: This article describes the inhibitory effect of gallic acid (GA), against colon cancer cells. GA treatment suppressed the proliferation and colony formation of HCT-15 cells and the anti-cancerous effect of GA was found to follow reactive oxygen species dependent apoptosis. Early events associated with apoptosis like lipid layer breakage and fall in mitochondrial membrane potential were induced by GA treatment in HCT-15 cells along with cell cycle arrest. Further, morphological changes like membrane blebbing and cell shrinkage were seen in the colon cancer cells after GA. Therefore, our results propel the role of GA as a possible anticancer agent.

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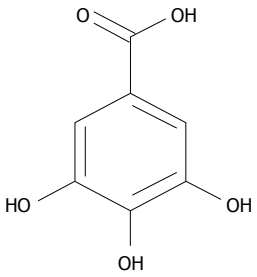
INTRODUCTION

Gastrointestinal (GI) diseases account for substantial morbidity, mortality and cost in both developed and developing countries. Gastrointestinal diseases refer to diseases involving the gastrointestinal tract[1]. The GI tract is essentially a long tube extending from the mouth to the anus with a number of specialized regions. Gastrointestinal cancers usually develop in the large intestine or the small intestine[2]. Colorectal cancer is a malignant tumor arising from the inner wall of large intestine, which is the third most common type of cancer[3]. The American Cancer Society's estimates 93090 new cases and 49700 deaths due to colorectal cancer in the United States for 2015[4]. Colon cancer arises out of the conversion of the normal functioning colonic epithelium to adenomatous polyps. Its etiology is known to be a combination of hereditary, environmental, dietary factors and lack of physical activity[5]. Several anticancer agents were found to exert their effect by inducing apoptosis. Various lines of evidence suggest that apoptosis provides a protective mechanism against neoplasia by removing genetically damaged stem cells from the epithelium before they can undergo clonal expansion. Some of these anticancer agents were also found to occur in our diets. These diets include majorly flavonoids and phenolic compounds[6]. In this scenario, research communities explore more diet-derived compounds to treat colon cancer as the lining-epithelial cells are chronically exposed to these dietary agents[7].



3,4,5-trihydroxybenzoic acid
$C_6H_2(OH)_3COOH$

Figure 1 Chemical structure of gallic acid.

Gallic acid is one such diet-derived phenolic substance being surveyed. GA is a 3,4,5-trihydroxybenzoic acid ($C_6H_2(OH)_3COOH$), a type of phenolic organic compound found in many plants and food substances. The chemical structure is shown in the Figure 1. GA is found in free as well as part of hydrolyzable tannins and easily freed from gallotannins by oxidation. GA is a phytochemical in oak, Drosera, golden root, stinging nettle, Chinese mahogany and dietary substances like bearberry, blackberry, hot chocolate, common walnut, Indian gooseberry, raspberry, clove, vinegar, wine, witch hazel and green tea[8].

Previous studies have demonstrated a range of biological activities of GA, including anticancer, antioxidant and anti-inflammatory properties[9]. The various *in vitro* assays examining the anticancer property showed that the GA is active against several types of cancer cell lines[10]. Particularly, the studies showed that GA induced cell death in colon cancer lines COLO 205, HCT-15, HCT 116[11]. However, the mechanism induced by GA against colon cancer is not yet elucidated. Thus, this research proposes a study of the antiproliferative activity of GA as well as, intends to find the events associated with apoptotic effect of GA in HCT-15 colon cancer cells.

MATERIALS AND METHODS

Chemicals

The Roswell Park Memorial Institute medium (RPMI-1640) cell culture medium, fetal bovine serum (FBS), additional sources like sodium pyruvate, nonessential amino acids, L-glutamine, vitamin solution, penicillin and streptomycin were purchased from Life Technologies, Inc., Grand Island, United States. Reagents like as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT), propidium iodide, mercury orange, RNase and GA were obtained from Sigma-Aldrich (United States). Supplementary stains such as merocyanine - 540 and YO-PRO-1 were acquired from Invitrogen Inc, United States.

Cell culture

Human colorectal adenocarcinoma cell line HCT-15 (Organ: Colon, Disease: Colorectal adenocarcinoma; Organism: Human; procured from National Centre for Cell Science, Pune, India) was grown in RPMI medium, while 10% FBS, sodium pyruvate, penicillin, L-glutamine, nonessential amino acids and vitamin solution was given as supplements. Adherent monolayer cultures of HCT-15 were preserved in T-25 flasks and incubated at 37 ℃ in 5% carbon dioxide (CO_2). The cultures were free of mycoplasma and maintained no longer than 12 wk after recovery from frozen stocks.

Cell viability assay

Cell numbers or cell proliferation inhibition by GA was determined by thiazolyl blue tetrazolium bromide (MTT) assay. In brief, colon cancer cells were trypsinized, counted and 1000 cells were seeded per well in 96-well plate. The subsequent day, 100 μL of the medium containing the preferred concentration of GA was added to the appropriate wells. The cells were then maintained at 37 ℃ in 5% CO_2 for the desired length of time. The untreated cells kept for 72 h was used as control for this experiment. At this moment, 100 μL of (5 mg/mL) MTT reagent was added to each well, and the plate was sustained at 37 ℃ in the incubator for 2 h. After aspirating the supernatant, 200 μL of dimethyl sulfoxide was added to each well to solubilize the formazan crystals formed in viable cells. The optical density was spectrophotometrically measured at 570 nm using enzyme-linked immunosorbent assay plate reader[12].

Colony forming assay

In order to assess the colony forming ability of GA treated colon cancer cells, the colony formation assay was executed. The cultured HCT-15 cells were treated with GA at a concentration of 740 μmol/L for definite time periods of 12 h, 24 h, 48 h and collected by trypsinization. The cells were counted and seeded again in triplicate on a 6-well tissue culture plate with 3000 cells/well. Following 15-d incubation at 37 ℃, colonies were stained with 0.5% crystal violet in methanol and the number of colonies was counted[13]. Control used in this experiment was untreated cells kept for 72 h. For all the experiments performed below, control cells remained untreated and kept for the same duration as the longest time point of the respective experiment.

Cell cycle analysis

Cell cycle analysis is an exceptional type of test that involves the flow cytometry and the fluorescent propidium dye and distinguishes the different phases of the cell cycle. The sub-G_1 fraction of the cell cycle was used as a measure of the apoptotic cells. After the appropriate treatment with GA, HCT-15 cells were washed with phosphate-buffered saline (PBS), then re-suspended in 50 μg/mL of propidium iodide stain containing 0.1% sodium citrate with 0.1% Triton X-100 for 20 min at 4 ℃. Analysis was performed in linear amplification mode in case of cell cycle analysis using FACScan; Becton Dickinson Immunocytometry Systems. Remaining experiments of flow cytometry were performed in the logarithmic amplification mode unless otherwise stated[14].

Determination of mitochondrial membrane potential

MMP ($\Delta\Psi_m$) levels of GA treated HCT-15 cells were measured by the rhodamine-123 fluorescent dye. The HCT-15 colon cancer cells were treated with GA (740 μmol/L) for different time points. Then cells were harvested and re-suspended in 1 mL of rhodamine-123 (5 μg/mL) for 1 h and maintained at 37 ℃. The intensity of fluorescence from rhodamine-123 was measured by flow cytometry[12]. Obtained fluorescence values were normalized with respect to control as 100%.

Detection of membrane lipid organization

The lipid bilayer breaks when the drugs induce an antiproliferative effect on the cancerous cells. To estimate the lipid layer breakage the fluorescent dye merocyanine-540 is used. The cultured HCT-15 cells were treated with GA at concentration of 740 μmol/L for different time points. Cells were harvested and re-suspended in 1 mL of merocyanine-540 (10 μg/mL) for 15 min at 37 ℃. The intensity of fluorescence was measured by flow cytometry[13].

Estimation of ROS generation

GA treated HCT-15 cells (740 μmol/L) were harvested using trypsin/EDTA and re-suspended in PBS. Working solution (20 μmol/L) of Dichlorofluorescein-diacetate (DCFH-DA) was directly added to the cells and then it was incubated at 37 ℃ for 15 min. DCFH-DA was cleaved by the intracellular nonspecific esterase to form DCFH. DCFH are oxidized by ROS to form the fluorescent compound DCF. Cells were

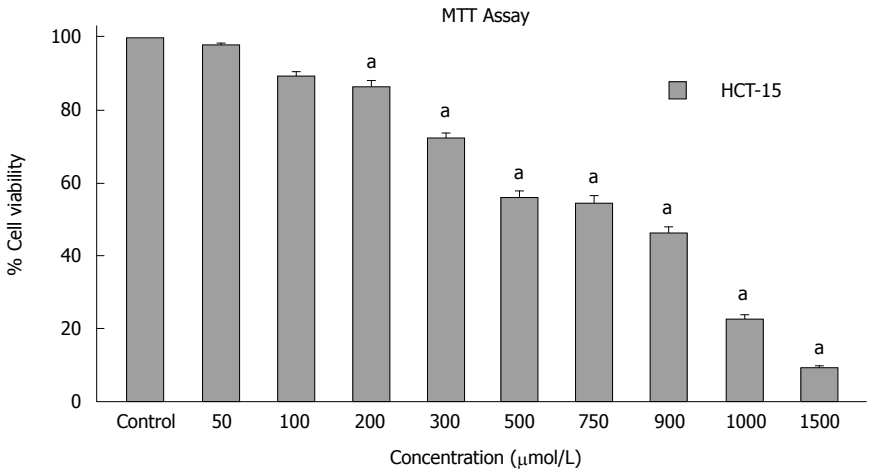


Figure 2 Cell proliferation inhibition by gallic acid of colon cancer cells. Human colorectal carcinoma HCT-15 cells grown in 96-well plate were treated with various concentration of gallic acid (GA) (0-1500 μmol/L) for 72 h. Percentage of mean cell viability along-with the SD is indicated (n = 3). Mean differences are significant compared with untreated control cells (P < 0.05 *vs* untreated control cells). Statistical analysis showed that GA treatment results in significant inhibition (^aP < 0.05) compared with untreated control cells starting at 200 μmol/L for HCT-15 cells. Data represents mean ± SD.

washed before re-suspending in PBS and kept on ice immediately before analyzing by flow cytometry[12]. The fluorescence intensity of DCF was measured and correlated with the ROS generated in the GA treated colon cancer cells.

Yo-Pro-1 staining

Yo-Pro-1 staining helps in the analysis of apoptotic cells without interfering cell viability. The stain targets the nucleic acids of the cells and emits a green fluorescence that is detected. The HCT-15 cells after treatment with GA (740 μmol/L), were seeded in the cell pellets and mixed with 1 μmol/L Yo-Pro-1 for 20 min at room temperature. After incubation, the fluorescent intensity was measured using flow cytometry[14].

Morphological assessment after GA treatment

Fixed amount of HCT-15 cells were seeded in a sterilized glass slide and incubated for 24 h. GA at a concentration of 740 μmol/L was added for 72 h time interval. After incubation, cells were harvested by using trypsin/EDTA and centrifuged for 5 min at room temperature. Then the supernatant was decanted and pellet was dried. Pellet was treated with 2.5% glutaraldehyde in distilled water for 45 min in hybrid oven shaker at 37 ℃. Cells were washed thrice with PBS for 5 min and then dehydrated by ethyl alcohol of different concentration (30%, 50%, 70%, 95% and 100%) for 5-10 min. Cell fixation was done with hexamethyl disilazane and the sample was taken for scanning electron microscope analysis. As well as the photomicrograph images of HCT-15 cells were also acquired using light microscope.

Statistical analyses

The results are expressed as the mean ± SD and repeated at least three independently (biological triplicates). The data were analyzed using Instat software (GraphPad Prism, San Diego, CA). The statistical significance was found using one-way analysis of variance.

RESULTS

Antiproliferative activity of gallic acid

The antiproliferative effect of GA on the cancer cells was assessed by MTT assay. The cell viability assay was performed on the GA treated cells after 72 h of treatment. GA inhibited the growth of HCT-15 colon cancer cells in a dose-dependent manner. The HCT-15 cell growth were inhibited significantly with an IC_{50} of around 740 μmol/L (Figure 2). However, the growth of GA treated HCT-15 cells were fairly affected even at higher concentrations. Statistical analysis showed that GA treatment results in significant inhibition (P < 0.05) compared with untreated control cells at 200 μmol/L for HCT-15 cells (Figure 2).

Colony forming assay

The HCT-15 cells found to form 192 colonies after 12 h treatment with GA (740 μmol/L). The colony numbers reduced with increase in the period of exposure to GA. The GA treated HCT-15 cells showed a maximum of 155 and 110 colonies after 24 h and 48 h of treatment respectively. In contrast, the untreated HCT-15 cells were found to produce a maximum of 210 colonies after 48 h. This is graphically represented in Figure 3. The figure depicts the time-dependent inhibition of colony formation by GA on the colon cancer cells. There was a significant reduction (P < 0.05) in the number of colonies formed under the various time intervals examined when compared with corresponding untreated cells (Figure 3).

Cell cycle analysis

The effect of GA on the different phases of cell cycle

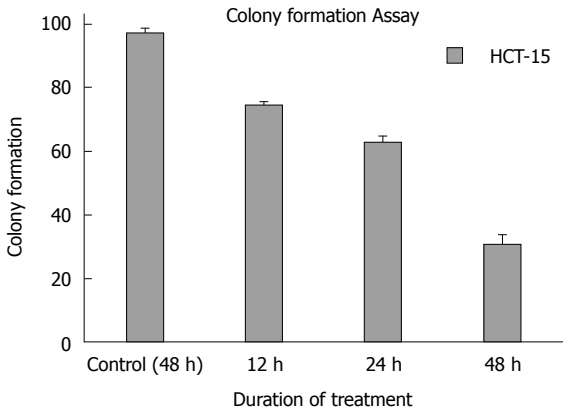


Figure 3 Colony inhibitory activity of gallic acid against colon cancer cells. After various incubation periods of gallic acid (GA) treatment, colonies formed were stained with 0.5% crystal violet and counted, and percentage of survival was calculated by normalizing the values. Data reported is the mean ± SD from three different observations. Mean differences are significant at 12 h, 24 h and 48 h compared with untreated control cells ($P < 0.05$ *vs* untreated control cells).

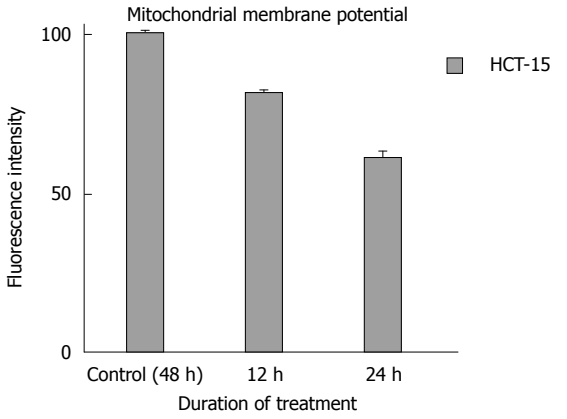


Figure 4 Effects of gallic acid on MMP (ΔΨm) in HCT-15 cells. Human colorectal carcinoma (HCT-15) cells were treated with GA for specified time-periods and then mitochondrial membrane potential were determined using rhodamine-123 by flow cytometry. Mean differences are significant at 12 h and 24 h compared with untreated control cells ($P < 0.05$ *vs* untreated control cells).

of HCT-15 cells was estimated for time intervals of 24 h, 48 h and 72 h. The mean percentage of cells at various phases like sub-G_1, G_0/G_1, S and G_2/M phases are tabulated in Table 1. The Table showed an increase in the sub-G_1 phase arrest from 0.98% (control) to 58.01% after 72 h. Statistical analysis of the sub-G_1 column indicated a significant increase ($P < 0.05$) of cells in the sub-G_1 phase. Besides this, the time dependency on the cell cycle arrest induced by GA treatment on HCT-15 cells was also inferred.

Mitochondrial membrane potential after GA treatment

The HCT-15 cells were treated with GA of concentration at 740 μmol/L at various time intervals (12 h and 24 h) and then stained with rhodamine-123 dye. The Figure 4 shows the mean fluorescent intensity of the control and GA treated HCT-15 cells. The untreated HCT-15 cells are considered to have maximum fluorescent

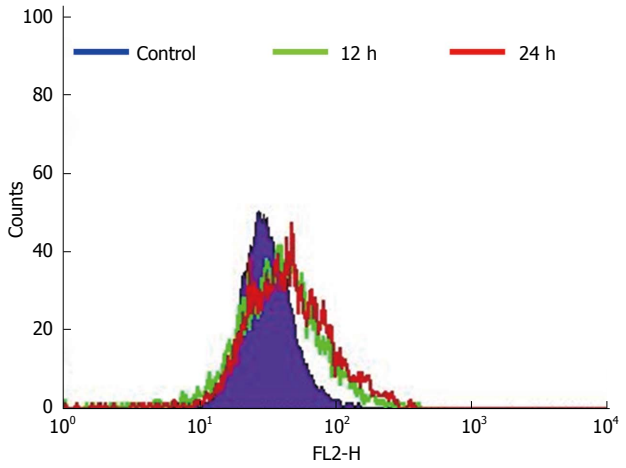


Figure 5 Lipid layer break provoked by gallic acid. HCT-15 cells were treated with gallic acid (GA) and evaluated using merocyanine-540 to quantify the lipid layer breaks (LLBs). The fluorescence intensity was estimated using flow cytometry. Data is representative of three independent experiments, mean differences are significant at 12 h and 24 h compared with untreated control cells ($P < 0.05$ *vs* untreated control cells).

Table 1 Percentage distribution of HCT-15 cells after gallic acid treatment among various cell cycle stages

Time (h)	Sub G_1[1]	G_0/G_1	S	G_2/M
Control	0.98 ± 1.03	42.82 ± 3.70	8.03 ± 2.37	40.07 ± 2.81
24	6.80 ± 2.73	32.50 ± 2.04	3.89 ± 1.78	42.25 ± 4.52
48	27.67 ± 1.56	25.51 ± 1.68	2.50 ± 3.54	29.12 ± 1.75
72	58.01 ± 2.05	12.89 ± 2.56	3.52 ± 1.09	15.26 ± 3.08

[1]Mean differences are significant at $P < 0.05$. Data represents mean ± SD.

intensity. In contrast, the normalized percentage of mean fluorescent intensity decreased to 81 ± 0.577 and 61 ± 1.528 compared to the untreated cells (100% after 24 h) after 12 h and 24 h of GA treatment respectively. There was also a statistically significant reduction ($P < 0.05$) of potential at the estimated intervals compared to untreated cells (Figure 4).

Membrane Lipid organization after GA treatment

The HCT-15 cells were treated with GA (740 μmol/L) at intervals of 12 h and 24 h. The fluorescent intensities detected from the merocyanine-540 stained cells were measured using flow cytometry. The untreated HCT-15 cells displayed a maximum mean fluorescence intensity at 40 after 24 h. The GA treated HCT-15 cells showed maximum intensity at 62 and 68 after 12 h and 24 h respectively. The maximum mean fluorescence intensities of the GA treated cells are given in Figure 5. It is evident from the above results that the GA treated HCT-15 cells displayed an increase in the lipid layer breaks with an increase in exposure time.

ROS generation after GA treatment

The ROS generated by the HCT-15 cells after GA (740 μmol/L) treatment was estimated at various

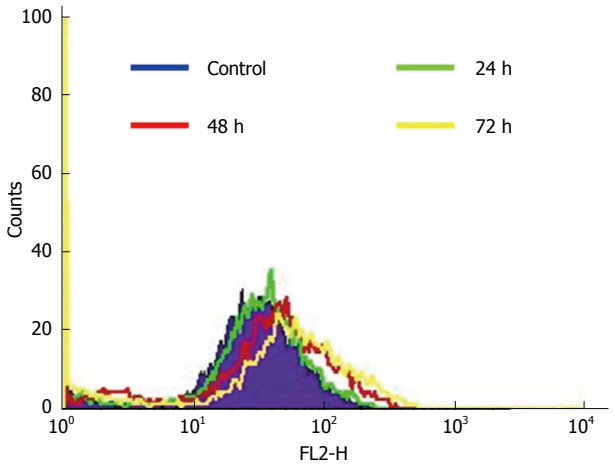


Figure 6 Gallic acid induced reactive oxygen species generation. HCT-15 cells were cultured in the presence or absence of gallic acid (GA) for the specified time points. DCFH-DA fluorescence intensity was detected by using flow cytometry. Data represented is the maximum of three independent experiments. Mean differences are significant at 24 h, 48 h and 72 h compared with untreated control cells ($P < 0.05$ *vs* untreated control cells).

time intervals such as 24 h, 48 h and 72 h. The ROS generated by the cells oxidized the DCFH-DA to dichlorofluoresin and the fluorescence emitted was measured. The fluorescent intensity obtained by flow cytometry is shown in the Figure 6. The maximum mean fluorescent intensity was found to be 112, 140, and 182 during 24 h, 48 h and 72 h respectively. Whilst, the maximum intensity of control HCT-15 cells was about 98 after 72 h. Moreover, the differences in the ROS levels at various hours examined were significant (Figure 6). The ROS generation from the HCT-15 cells was increased by GA treatment with respect to the increase in time of exposure.

Morphological assessment of GA treated cells

Photomicrograph images of untreated and GA treated colon cancer cells (72 h) were acquired. The obtained images are shown in Figure 7A. In comparison to the untreated cells, the characteristic changes such as membrane blebbing and cell shrinkage are visible in the GA treated cells. These changes embrace the induction of apoptosis after GA treatment. Along with the digital microscopic image obtained, the HCT-15 cells were also examined with a scanning electron microscope to provide topographical or morphological images with high resolution. The images of untreated HCT-15 cells and GA treated HCT-15 cells (72 h) are given in the Figure 7B. It is evident from the images that the GA treated cells showed typical signs of apoptosis like membrane blebbing and shrinkage. In contrast, normal cells did not show any marked shrinkage. Hence, the microscopic images further corroborated the signs of apoptosis induced by GA. The images given the Figure 7 is representative of three independent experiments.

Yo-Pro-1 staining

The HCT-15 cells were treated with GA (740 μmol/L) at various intervals of time such as 48 h and 72 h. The green fluorescence emitted by the Yo-Pro-1 stain from the GA treated HCT-15 cells is measured using flow cytometry. The result of fluorescence detected from the colon cancer cell line is given in the Figure 8. The maximum mean fluorescence intensity of GA treated HCT-15 cells at M2 phase was found to be 25.32 and 54.61 after 48 h and 72 h. In contrast, the maximum mean fluorescence intensity of control cells at M2 phase was found to be 7.75 after 72 h.

DISCUSSION

Diet is thought to have a major role in the etiology of colorectal cancer. Similar studies also proved that a phytochemical-rich diet, which is absorbed by the body from fruit and vegetable sources, could decrease the risk of developing colon cancer[15]. Previous work revealed various biological properties of phenolic content in the diet that we consume regularly. These phenolic compounds are commonly known for their anticancer property. The phenolic compound GA exhibited an antiproliferative effect on the HCT-15 colon cancer cell lines in a dose dependent manner which was similar to the effect of GA on the human hepatoma SMMC-7721 cell proliferation in *in vitro* condition[16]. Hence it can be inferred that GA treatment led to lysis of HCT-15 cells with increasing concentrations either by apoptosis or necrosis. Cell growth was inhibited significantly with an IC_{50} of around 740 μmol/L and this was parallel to the results obtained in the recently conducted study of the antiproliferative effect of GA on HCT-15 by Yumnam *et al*[17]. In a particular study, the oral consumption of six cups of black tea resulted in 344 mg GA[18]. However, about 25 mg of GA is enough to yield about 750 μmol/L in the colonic volume of 200 mL. This shows that the IC_{50} obtained in our study lies within the range of biological availability. Moreover, the effect of GA on intestinal epithelial cells (IEC) was investigated. It was seen that about 85% of cells were viable when treated with 3.5 mmol/L showing that the GA treatment was non-toxic to normal cells (results not shown). These results depict that the phenolic compound GA has insignificant inhibitory activity against colon cancer cells at even a very high concentration.

Colony formation is one of the characteristic features of cancer cells, which was inhibited by GA treatment in a time dependent manner which is alike to the results obtained by the clonogenic assay on the GA treated A549 human lung adenocarcinoma cells[19]. The cancer cells grow rapidly and multiply uncontrollably therefore, one of the fundamental features expected to be present in the anticancer drug, is the ability of the drug to affect the cell proliferation.

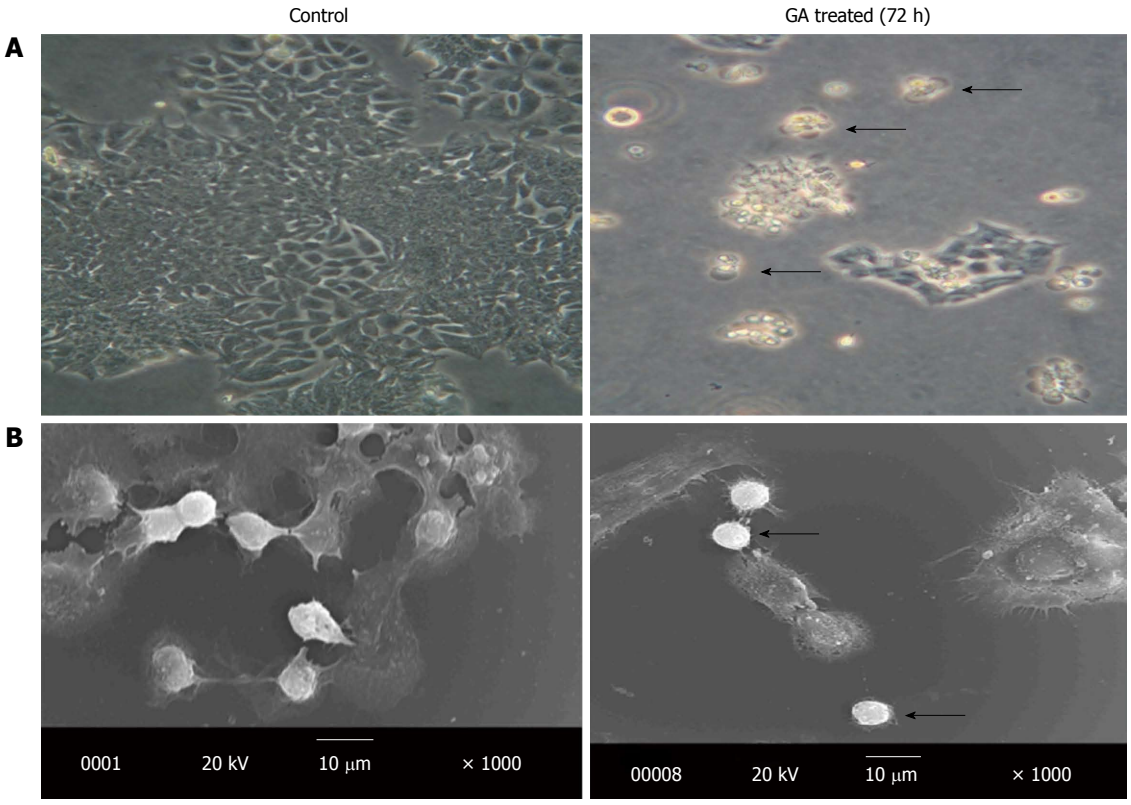


Figure 7 Representative photomicrograph and scanning electron microscopic images of three independent experiments. A: Photomicrograph images of untreated and gallic acid (GA) treated HCT-15 cells. The arrow mark indicates cell death after GA treatment; B: Scanning electron microscopic images of untreated and GA treated HCT-15 cells. The arrow mark represents the rounding up of HCT-15 cells after GA treatment.

Nevertheless, GA is found to evidently affect the colony formation of HCT-15 cells. The morphological changes such as cell shrinkage and membrane blebbing was visible in the colon cancer cells exposed to GA, which agrees to the earlier experimentation of Yumnam *et al*[17] on the GA treated HCT-15 cells[17]. These cellular changes witnessed in GA treated cells are similar to characteristic changes in the cellular organelles during apoptosis[20]. Thus, the microscopic examinations show GA induces apoptosis in HCT-15 cells.

In normal biological systems, ROS is continuously generated and eliminated as well as plays an important role in driving various regulatory pathways. The cell balances the generation of ROS thereby controlling it. However, abundant generation of ROS during oxidative stress may affect the lipids, cellular proteins. In our study, GA promoted the generation of ROS in the colon cancer cell lines depending on the duration of exposure, which is analogous to the antiproliferative effect of GA against MiaPaCa-2 human pancreatic cancer cells[21]. As the raise in ROS generation is said to cause apoptosis through extrinsic or intrinsic pathways in the cancer cells, the promotion of ROS generation in GA treated HCT-15 cells supports the antiproliferative effect of GA[22]. Other significant pro-apoptotic event is lipid layer breakage, which is said to favor the interaction between the drug tested and the other cell organelles Lipid layer breakage was enhanced by GA treatment in the colon cancer cell lines. This finding is supported by GA-induced lipid layer breaks in the HSC-2 human oral cancer cells[23]. The lipid layer breakage is an optimistic event that favor the interaction between the drug tested and the other cell organelles[24]. Hence, the effect of GA on the lipid layer of HCT-15 cells may be related to the apoptosis-inducing ability of GA. A great increase in ROS has been associated with reduced cancer cell proliferation by induction of cell cycle arrest. The GA treatment caused a time-dependent cell cycle arrest at the sub-G_1 phase in HCT-15 cells, which was similar to activity of GA on HL-60 human leukemia cells[25]. However, the Sub-G_1 phase is related to measurement of apoptosis or programmed cell death[13]. During apoptosis, the DNA is degraded and the content becomes less than that the DNA content in healthy cells undergoing cell cycle[26]. The increase in the amount of cells at Sub-G1 phase infers that GA treatment of HCT-15 cells may be ascribed to programmed cell death in a time dependent manner.

Mitochondrial malfunction is another key event that occurs during apoptosis. Mitochondrial membrane potential (MPP) of GA treated cells showed decreasing intensity, with an increase in the exposure time that is similar to the result acquired during the investigation of effect of GA on A375S2 human melanoma cells by Lo *et al*[27]. Various anticancer drugs cause MPP

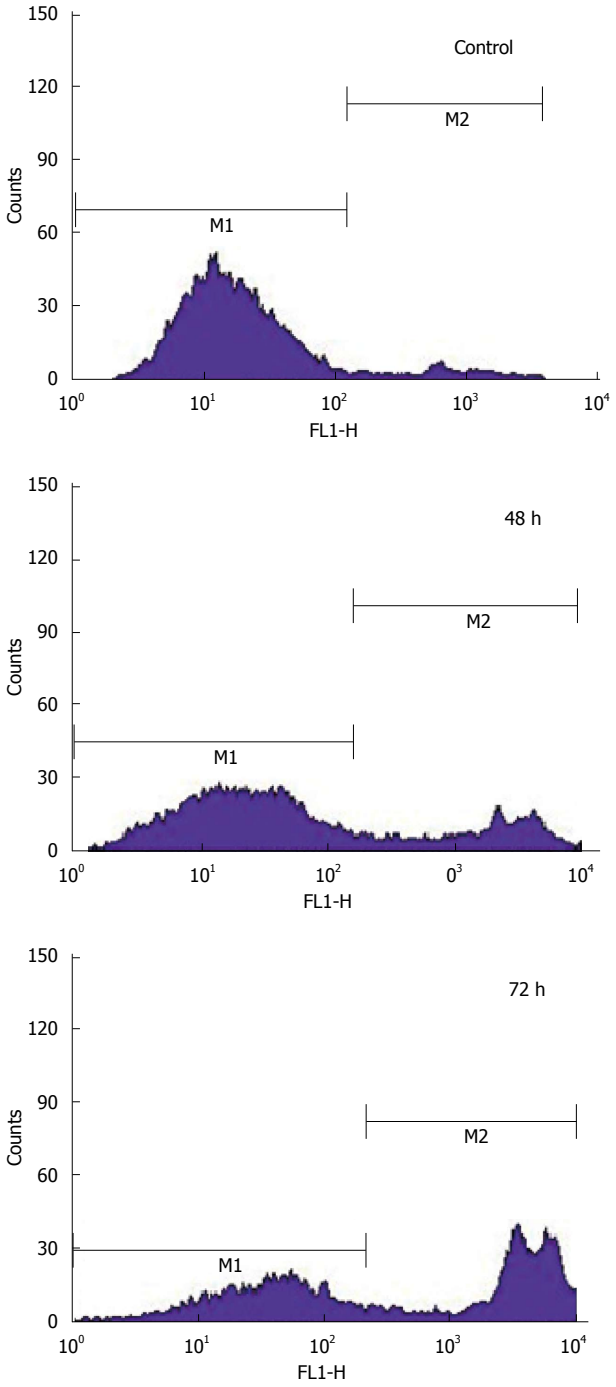


Figure 8 Apoptosis assessment using Yo-Pro-1 dye by flow cytometry. HCT-15 cells was treated with gallic acid (GA) for specified time points. The distribution of the cell population changed according to the exposure time as indicated by M1 and M2. Data represented is the maximum of three independent experiments and the differences in the values of M2 were significant at 48 h and 72 h compared to untreated control cells ($P < 0.05$ *vs* untreated control cells).

fluctuations and induce death of cancer cells[24]. Furthermore, the changes identified in the level of MPP in may be related to its inhibitory effect of GA against HCT-15 cells. Some recently concluded researches utilized Yo-Pro-1 as an effective agent in confirming apoptosis. The Yo-Pro-1 staining used to detect apoptosis induced by anticancer agents as it analyses the apoptotic cells without interfering cell viability[14]. Apart from the early and later events indicating the occurrence of apoptosis, Yo-pro-1 staining confirmed the apoptosis after GA treatment.

Although apoptosis is confirmed by Yo-Pro-1 staining it would be more interesting to study the various pro-apoptotic and anti-apoptotic protein level in GA treated HCT-15 cells. The analysis of cyclin/CDK, p53, Bax, Bad, Bcl-2 and Bcl-xL protein levels at different time intervals would give more information regarding the apoptosis induced by GA. Hence in future research, this interesting points will be addressed. However, further development of this research work would be *in vivo* experimentation with GA. This would need a proper understanding of the degree to which GA is absorbed or becomes available at the site of physiological activity after administration. As the half-maximal inhibitory concentration of GA obtained in our studies lies within the range of biological availability, the *in vivo* experimentation can be preceded. Nevertheless, proper experimentation using humans with risk of colon cancer in a larger group may validate the anticancer activity of GA more precisely.

In conclusion, the phenolic compound GA inhibited the growth of HCT-15 colon cancer cells. GA exhibited antiproliferative effect on both colon cancer cell lines along with notable morphological and biochemical changes. The anti-cancerous effect of GA followed ROS dependent apoptosis in HCT-15 colon cancer cell lines. Early events associated with apoptosis like lipid layer breakage and fall in MPP were induced by GA treatment. The cell cycle progression was arrested at the sub-G1 phase by GA treatment. Morphological changes like membrane blebbing and shrinkage in the GA treated cells was depicted by SEM and photomicrograph images. In conclusion, GA induced apoptosis in HCT-15 cells through the ROS - mitochondrial pathway in a time dependent manner. These results propel the role of GA as a possible anticancer agent. However, further experiments in preclinical and clinical settings are needed to promote GA as a likely candidate for chemotherapy of colon cancer.

COMMENTS

Background

Many surveys have shown that all types of cancers have a link with the diets the authors consume. Especially, in the case of colon cancer, diet plays a crucial role as the colonic epithelial cells are exposed to diets directly. Because of this reason, scientists explore various natural compounds present in food substances to treat colon cancer. Some studies have already shown that these natural compounds are absorbed by the body and have the potential to reduce the risk of colon cancer. The current study deals with examining the growth inhibitory effect of the dietary phenolic phytochemical gallic acid (GA) against HCT-15 colon cancer cells.

Research frontiers

Despite the several anticancer drugs that are available for colon cancer, scientists continuously search for a novel anticancer drug with enhanced

efficacy. Previous experiments have investigated the anticancer effect of GA against colon cancer, while the various reactions induced by GA on colon cancer cells have never been examined.

Innovations and breakthroughs

The study emphasizes the mechanism related to the anticancer effect of GA against colon cancer cell. GA was found to inhibit the growth of colon cancer cells through reactive oxygen species (ROS)-mediated apoptosis with notable morphological changes.

Applications

A series of events associated with the anticancer activity of GA are clearly depicted. Moreover, in-depth experimentation in preclinical and clinical settings is needed to promote GA as a plausible candidate for chemotherapy of colon cancer. Further, an in-depth proteomics study in relation to the GA induced apoptosis would be of great interest.

Terminology

Apoptosis or programmed cell death, the death of cells that occurs as a normal and controlled part of an organism's growth or development which can be Apoptosis can be induced either by a stimulus, such as irradiation or toxic drugs, or by removal of a repressor agent.

Peer-review

Phytochemicals modulate key cellular signaling pathways and have proven anticancer effects. In the past, a large number of substances derived from plants have been studied in antitumor research fields and many have proven to exhibit chemopreventive properties which could be used as adjuvant chemotherapy. In this manuscript, the authors found that diet-derived phenolic compound GA inhibited the proliferation and induced the apoptosis of HCT-15 cancer cells through increased generation of ROS. The study is important and may advance the field of chemoprevention.

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ORIGINAL ARTICLE

Basic Study

Norcantharidin combined with ABT-737 for hepatocellular carcinoma: Therapeutic effects and molecular mechanisms

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Author contributions: Ren J and Ye T designed research; Ren J, Li G, Zhao W and Ye T performed research; Li G, Zhao W and Lin L contributed new reagents/analytic tools; Ren J, Lin L and Ye T analyzed data; and Ren J and Ye T wrote the paper.

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Conflict-of-interest statement: We declare that there is no conflict of interest to disclose in our study.

Data sharing statement: We state that no additional data are available to share.

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Abstract

AIM: To study the therapeutic effect of norcantharidin (NCTD) combined with ABT-737 on hepatocellular carcinoma cells and the molecular mechanism.

METHODS: Two hepatocellular carcinoma (HCC) cell lines, HepG2 and SMMC-7721, were selected. ABT-737 and NCTD were allocated into groups to be used alone or in combination. HepG2 and SMMC-7721 cells were cultured *in vitro*. Liver cancer cells in the logarithmic phase of growth were vaccinated and cultured to the cell wall stage; these cells were treated for 48 h with different concentrations of NCTD, or ABT-737, or NCTD combined with ABT-737. The cell proliferation inhibition rate was detected by methyl thiazolyl tetrazolium. The expression of Mcl in HCC cells was detected by Western Blotting, and the cells in each group after treatment had apoptosis detected by flow cytometry. The proliferation inhibition rate, the expression of Mcl-1 in cells and the apoptosis inducing effect of treatment were observed in each group, and the effect of NCTD on ABT-737 in the treatment of HCC and its mechanism of action were analyzed.

RESULTS: As the concentration of NCTD increased, the cell proliferation inhibition rate gradually decreased; and the treatment effect of ABT-737 1-3 μm combined with NCTD on cell proliferation inhibition was stronger than that of ABT-737 alone. The difference was statistically significant ($P < 0.05$). In observing the expression of Mcl-1 in cells after the treatment of different concentrations of NCTD, this was partially

inhibited after treatment with NCTD 15 μm, and the expression of Mcl-1 was almost undetectable after treatment with NCTD 30 μm and 60 μm. The effect on inducing apoptosis with the treatment of ABT-737 or NCTD alone for 48 h was lower than that of the control group. The difference was not statistically significant ($P > 0.05$). The effect on inducing apoptosis in HepG2 and SMMC-7721 cells with the treatment of ABT-737 combined with NCTD for 48 h was greater than that of ABT-737 or NCTD alone. The difference was statistically significant ($P < 0.05$).

CONCLUSION: NCTD combined with ABT-737 has a positive role in the treatment of HCC, and it has great value in clinical research.

Key words: Norcantharidin; Hepatocellular carcinoma cell; Mcl-1

Core tip: The effects of ABT-737 and norcantharidin (NCTD) alone or in combination on HepG2 and SMMC-7721 cells were tested by methyl thiazolyl tetrazolium, Western blot and flow cytometry. We found that as the concentration of NCTD increased, the cell proliferation inhibition rate gradually decreased; and the treatment effect of ABT-737 1-3 μm combined with NCTD on cell proliferation inhibition was stronger than that of ABT-737 alone ($P < 0.05$). The effect on inducing apoptosis in HepG2 and SMMC-7721 cells with the treatment of ABT-737 combined with NCTD for 48 h was greater than that of ABT-737 or NCTD alone ($P < 0.05$). NCTD combined with ABT-737 has a positive role in the treatment of HCC.

Ren J, Li G, Zhao W, Lin L, Ye T. Norcantharidin combined with ABT-737 for hepatocellular carcinoma: Therapeutic effects and molecular mechanisms. *World J Gastroenterol* 2016; 22(15): 3962-3968 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/3962.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.3962

INTRODUCTION

Myeloid cell leukemin-1 (Mcl-1) is a special B-cell lymphoma 2 (Bcl-2) family protein. It can not only control cell survival and death, but also plays an important role in regulating apoptosis signaling[1-3]. Several studies have shown that Mcl-1 generally has a high expression in hepatocellular carcinoma (HCC) and other malignant tumors[4-6], and this has become a cancer research focus of molecular targeted therapy. ABT-737 is a novel cancer therapeutic agent that has good prospects for clinical application[7]. However, ABT-737-mediated apoptosis is limited when there is high expression of Mcl-1 in liver cancer and other solid tumors[8-10], and this has become a major obstacle point in clinical application. Research has shown the treatment sensitivity of tumor cells to ABT-737 can be enhanced by its combination with other chemotherapy drugs[11-14].

Norcantharidin (NCTD) is a derivative of the Chinese medicine cantharidin, which has good anti-tumor effects[15-17]. Studies have reported that the anti-tumor effect of NCTD may be related to the role of Bcl-2 family members[18], which can inhibit Mcl-l expression in HCC cells[19]. Therefore, this study aims to investigate the therapeutic effects of NCTD combined with ABT-737 on HCC cells, and to preliminarily analyze its mechanism of action for the future development of anticancer drugs, aiming to provide theoretical guidance for clinical applications.

Table 1 List of the main reagents and instruments in the experiments

Primary reagents	Source
ABT-737	Biochempartner
NCTD	Nanjing Zelang Medical Technology Co., Ltd.
DMSO	Hyclone
96-well and 6-well cell culture plates	Costar, United States
Methyl thiazolyl tetrazolium (MTT)	Sigma, United States
Trypsin	Hangzhou Gino Biomedical Technology Co., Ltd.
CO2 Incubator	Thermo Scientific, United States
Multiskan MK3 microplate reader	Thermo Scientific, United States
Flow cytometer	BD Biosciences
TUNEL Assay Kit for Apoptosis Detection	Nanjing KeyGEN BioTECH

MATERIALS AND METHODS

Materials

HCC cell lines: HepG2, SMMC-7721 (purchased from Cell Bank of Beijing Concord Technical Institute). Reagents and equipment details are displayed in Table 1.

Experimental methods

Cultured cell lines: Hepatoma cell lines HepG2 and SMMC-7721 were cultured *in vitro*, placed in RPMI-1640 medium containing 10% fetal bovine serum, and placed in an incubator with 5% CO_2 at 37 ℃.

Cell proliferation inhibition detection by methyl thiazolyl tetrazolium assay: HepG2 and SMMC-7721 hepatoma cells in the logarithmic growth phase were seeded into 96-well plates and cultured. Cells were divided into the following groups when they attached to the wall: ABT-737 monotherapy group, NCTD monotherapy group, ABT-737 combined with NCTD group, control group, and apoptosis group; and each group had 3 parallel wells. After treatment,

culture was continued for 48 h. Then, 20 μL of methyl thiazolyl tetrazolium (MTT) solution was added into each well, and incubated for 4 h in an incubator. The supernatant was discarded, 150 μL of DMSO was added into each well, and they were placed in the incubator for 10 min. Optical density (OD) value was measured with an enzyme mark instrument. Measurements were repeated three times, and the average value was obtained. Proliferation inhibitory rates of the drug-treated groups were calculated as follows: inhibition rate (%) = [1 - (average OD value of drug-treated groups - average OD value of the apoptosis group)/(average OD value of the control group - average OD value of the apoptosis group)] × 100%.

Western blot detection of Mcl expression in hepatoma cells: Before initiation of the experiment, 4×10^5 hepatoma cells were seeded in 6-well plates. After cell adhesion, they were treated with NCTD alone, ABT-737 alone, and NCTD combined with ABT-737, and placed in an incubator for 24 h. After drug-treated cells were trypsinized, cells were collected by centrifugation, total protein was extracted, then protein was quantified by Bradford assay. Western blot detection was carried out as follows: (1) loading volume: the sample injection volume per well was 20 μg, boiled in water for 5 min, centrifuged 5 min, and the supernatant sample was obtained; (2) SDS-PAGE electrophoresis, electrophoresis procedure: 100 V for 15 min and 180 V for 45 min; (3) electricity facing: 45 V for 35 min, 100 V for 10 min and blocked, then the membrane was washed for 5 min twice; (4) the primary antibody was added, incubated at 4 ℃ overnight and membrane was washed for 5 min three times, the secondary antibody was added, the membrane was washed twice; and (5) development and fixing: the membrane was fixed, chemiluminescent was added, wrapped in plastic wrap after drying, washed after exposure for 1-3 min, then scanned and protein bands analyzed.

Apoptosis detection by flow cytometry: After treatment, cells in the NCTD group, ABT-737 group, and NCTD combined with ABT-737 group were trypsinized, collected, centrifuged, washed, and resuspended. Then, flow cytometry detection and analysis were performed according to the TUNEL apoptosis kit manufacturer's instructions.

RESULTS

Cell proliferation inhibition rate in each group

After treatment of HepG2 and SMMC-7721 cells with different concentrations of NCTD for 48 h, cell proliferation inhibition rates detected by MTT were as follows: when concentrations of NCTD were increased, the cell proliferation inhibition rate became smaller (Figure 1A and B); meanwhile, the effect of ABT-737 1-3 μm and NCTD combined treatment on cell proliferation inhibition was stronger than ABT-737 alone. The difference was statistically significant ($P < 0.05$) (Figure 2A and B).

Mcl-1 expression in cells after different concentrations of NCDT treatment

After HepG2 and SMMC-7721 cells were treated with NCTD 15 μm, the expression of Mcl-1 was partially inhibited; and when the concentration of NCTD was 30 and 60 μm, the expression of Mcl-1 was almost undetectable (Figure 3).

Effect of NCTD combined with ABT-737 on cytochrome C

Results showed that the expression of cytochrome C was not detected in cells in the control group or the ABT-737 monotherapy group, and that a low expression of cytochrome C was detected in cells in the NCTD monotherapy group. Cytochrome C was highly expressed in cells in the ABT-737 combined with NCTD group (Figure 4A and B).

Apoptosis detection by flow cytometry

In the control group, ABT-737 3 μm monotherapy group, NCTD 30 μm monotherapy group, or ABT-737 combined with NCTD group, after the 48 h treatment of HepG2 and SMMC-7721 cells, cell apoptosis detection by flow cytometry showed the following: in the monotherapy groups, cells showed an small increase in apoptosis induction compared with the control group, and the difference was not statistically significant ($P > 0.05$); while after the combination treatment for 48 h, HepG2 and SMMC-7721 cells had a greater amount of apoptosis compared with ABT-737 and NCTD monotherapy, and the difference was statistically significant ($P < 0.05$) (Figure 5A and B).

DISCUSSION

ABT-737 is an antagonist of small molecule Bcl-2[20], and a novel anti-cancer drug that induces tumor cell apoptosis without causing damage to normal cells[21-23]; it has broad prospects for development. However, ABT-737 is inhibited in the induction process of apoptosis in hepatocellular carcinoma and some solid tumors that have high expression of Mcl-1[24,25]. Therefore, determining how to reduce the expression of Mcl-1 in cells to increase the efficiency of the therapeutic effect of ABT-737 for liver cancer would be a breakthrough. Studies have reported norcantharidin treatment for cancer can inhibit the expression of Mcl-1[26-28]. Therefore, norcantharidin combined with ABT-737 was used in this study to analyze its effect in the treatment of liver cancer, and to explore its mechanism.

The results are as follows: when HepG2 and

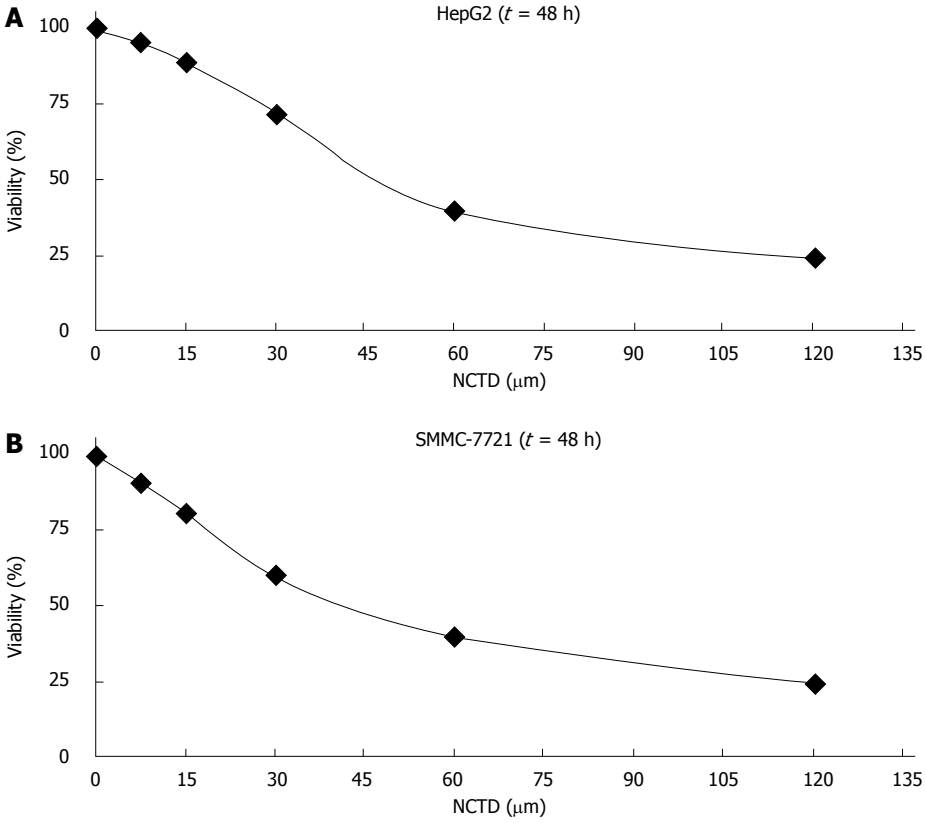

Figure 1 Change in cell proliferation inhibition rate after 48 h of treatment with different concentrations of norcantharidin. A: The change of cell proliferation inhibition rate in HepG2 cells; B: The change of cell proliferation inhibition rate in SMMC-7721 cells.

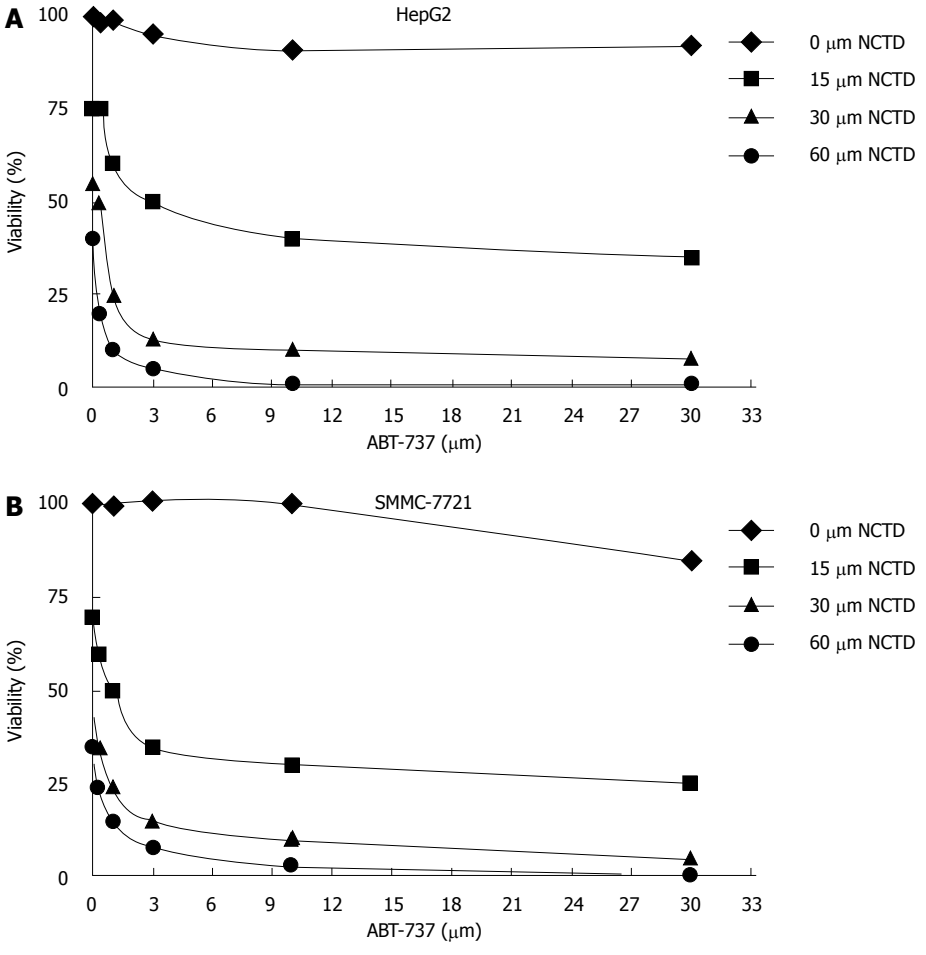

Figure 2 Change in cell proliferation inhibition rate after 48 h of treatment with different concentrations of ABT-737 or with ABT-737 combined with different concentrations of norcantharidin (15 μm, 30 μm, 60 μm). A: The change of cell proliferation inhibition rate in HepG2 cells; B: The change of cell proliferation inhibition rate in SMMC-7721 cells.

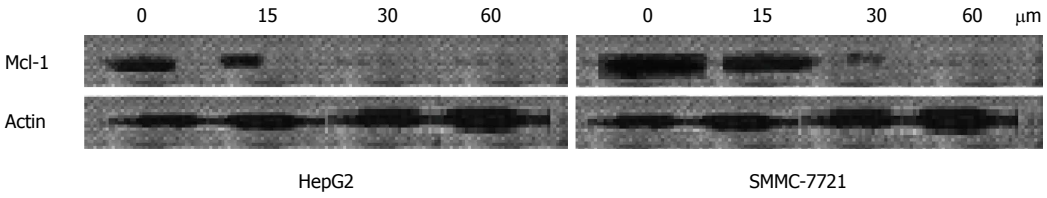

Figure 3 Expression of Mcl-1 in cells after treatment with different concentrations of norcantharidin.

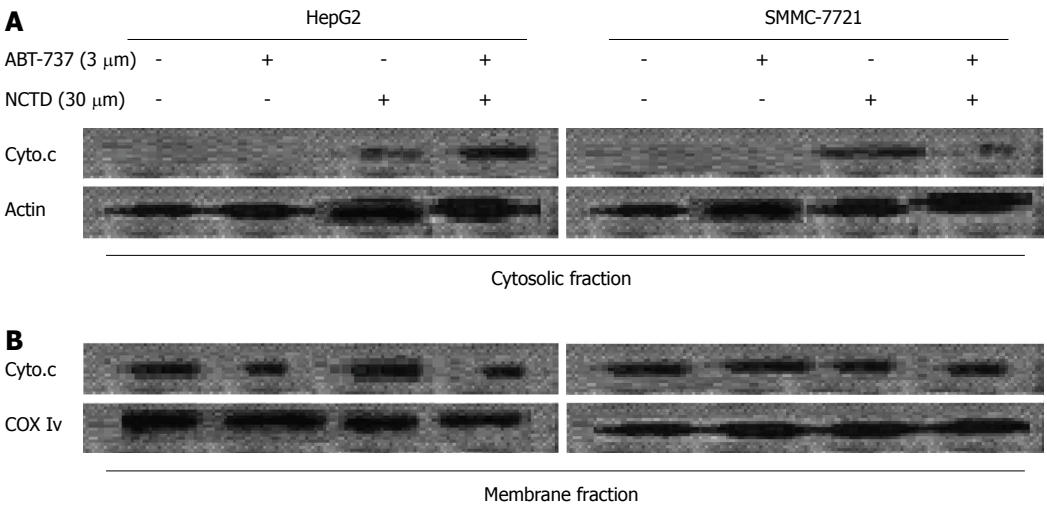

Figure 4 Expression of cytochrome C in HepG2 cells and SMMC-7721 cells after treatment with ABT-737 and norcantharidin detected by Western blotting. A: The expression of cytochrome C in HepG2 cells; B: The expression of cytochrome C in SMMC-7721 cells.

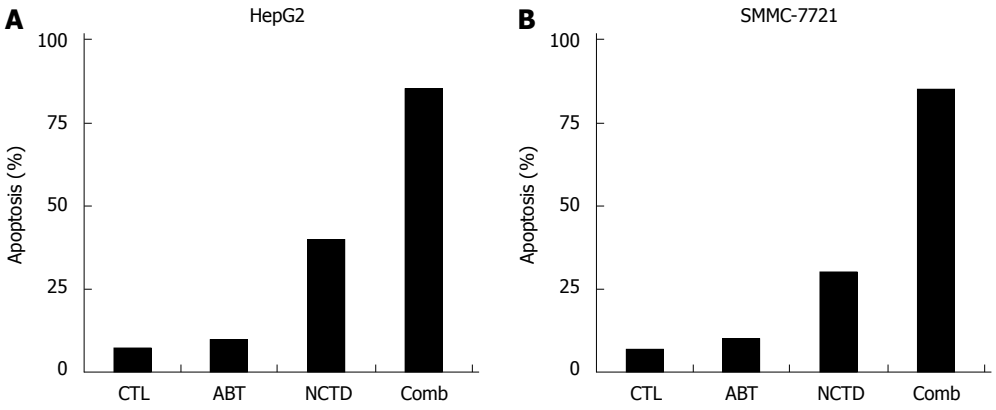

Figure 5 Apoptosis of HepG2 and SMMC-7721 cells detected by flow cytometry after treatment for 48 h. A: The apoptosis of HepG2 cells after treatment for 48 h; B: The apoptosis of SMMC-7721 cells after treatment for 48 h.

SMMC-7721 cells are treated for 48 h with different concentrations of NCTD, it is apparent that NCTD has a good inhibitory effect on cell proliferation; and comparing 1-3 μm of ABT-737 alone with ABT-737 combined with NCTD, results show that the ABT-737 combined with NCTD treatment has a stronger inhibition of cell proliferation compared with ABT-737 alone[29,30]. In order to verify this, we further detected its apoptotic effect by flow cytometry. The results showed that ABT-737 combined with NCTD treatment for 48 h applied to HepG2 and SMMC-7721 cells had a stronger apoptosis-inducing effect than ABT-737 and NCTD monotherapy; and thus, we confirm the rationality of these results. These results also show that NCTD enhances ABT-737 in inhibiting cell proliferation by inducing apoptosis.

Regarding detection of Mcl-1 expression in cells, results showed that after treatment of HepG2 cells and SMMC-7721 cells with NCTD 15 μm, the expression of Mcl-1 was partially inhibited, while the expression of Mcl-1 was almost undetectable when NCTD concentration was 30 and 60 μm. As expected for NCTD, there were better inhibitory effects on Mcl-1 expression in cells at higher doses. To study its mechanism, cytochrome C was further detected in the cytoplasm and the mitochondrial membrane.

In comparing HepG2 cells and SMMC-7721 cells treated in the control group, ABT-737 monotherapy group, NCTD monotherapy group, and ABT-737 combined with NCTD group, the following expressions of cytochrome C were found: in the control group and ABT-737 monotherapy group, the expression of cytochrome C in cells was not detected, while a low expression of cytochrome C was detected in cells in the NCTD monotherapy group; and cytochrome C showed a high expression in cells in the ABT-737 combined with NCTD group. This result prompts us to conclude that this two-drug combination can enhance the expression of cytochrome C, and it also proves that NCTD enhances the release of cytochrome C induced by ABT-737. These results are due to cytochrome C release from the mitochondria into the cytosol in cells, and this is an important symbol of the Bcl-2 family proteins in the regulation of apoptosis. Therefore, we can speculate that NCTD inhibits Mcl-1 enabling ABT-737 to release cytochrome C in cells.

Studies for ABT-737 drugs are promising. Although we have a number of significant results, there are still many issues that need to be explored in in-depth studies, such as: the inhibition by NCTD of the expression of Mcl-1 to enhance ABT-737 in the treatment of hepatocellular carcinoma drug resistance; the release of cytochrome C induced by ABT-737, and whether there is an impact on other factors; and to determine whether ABT-737 combined with other anti-cancer chemotherapy drugs will show improvements. In our study, these problems are not investigated, and the role of its mechanism needs to be further explored through in-depth studies.

In summary, NCTD and ABT-737 combined can solve the ABT-737 drug resistance problem for the treatment of liver cancer. NCTD can inhibit the expression of Mcl-1 to enhance the release of cytochrome C induced by ABT-737. NCTD has a role of inducing apoptosis to enhance ABT-737 in its inhibition of cell proliferation; thus, enhancing ABT-737 induces hepatocellular carcinoma cell apoptosis. Therefore, NCTD combined with ABT-737 has a positive impact on the treatment of hepatocellular carcinoma cells; clinical research in this field has great value, and it deserves further investigation.

COMMENTS

Background

Myeloid cell leukemia-1 (Mcl-1) is a special B-cell lymphoma 2 (Bcl-2) family protein. It cannot only control cell survival and death, but also plays an important role in regulating apoptosis signaling. Several studies have shown that Mcl-1 generally has high expression in hepatocellular carcinoma (HCC) and other malignant tumors; and this has become a cancer research focus of molecular targeted therapy. ABT-737 is a novel cancer therapeutic agent that has good prospects for clinical application. Norcantharidin (NCTD) is a derivative of the Chinese medicine cantharidin, which has good anti-tumor effects.

Research frontiers

The ABT-737-mediated apoptosis signal is limited when there is high expression of Mcl-1 in liver cancer and other solid tumors; and this has become a major obstacle point in clinical application. Research has shown that the treatment sensitivity of tumor cells to ABT-737 can be enhanced when it is in combination with other chemotherapy drugs. Studies have reported that the anti-tumor effect of NCTD may be related to the role of Bcl-2 family members, which can inhibit Mcl-l expression in HCC cells.

Innovations and breakthroughs

Research has shown the treatment sensitivity of tumor cells to ABT-737 can be enhanced by combination with other chemotherapy drugs. Therefore, this study aims to investigate the therapeutic effects of NCTD combined with ABT-737 on HCC cells, and to preliminarily analyze its mechanism of action for the future development of anticancer drugs and to provide theoretical guidance for clinical applications.

Applications

This study demonstrated that combining NCTD and ABT-737 can solve the ABT-737 drug resistance problem for the treatment of liver cancer. It shows that the effect on inducing apoptosis in HepG2 and SMMC-7721 cells with the treatment of ABT-737 combined with NCTD for 48 h was greater than that of ABT-737 or NCTD alone.

Terminology

HepG2 and SMMC-7721 hepatocellular carcinoma cell lines were tested.

Peer-review

This study demonstrated that NCTD can inhibit the expression of Mcl-1 to enhance the release of cytochrome C induced by ABT-737. NCTD has a role of inducing apoptosis to enhance ABT-737 for inhibiting cell proliferation; thus, enhancing ABT-737 induces hepatocellular carcinoma cell apoptosis. Therefore, NCTD combined with ABT-737 has a positive impact for the treatment of hepatocellular carcinoma cells; the clinical research has great value, and it deserves further investigation.

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ORIGINAL ARTICLE

Basic Study

Integrin-linked kinase overexpression promotes epithelial-mesenchymal transition *via* nuclear factor-κB signaling in colorectal cancer cells

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Author contributions: Shen H and Zeng S designed research; Shen H, Ma JL, Zhang Y, Deng GL, Qu YL, Wu XL, He JX, Zhang S and Zeng S performed research; Ma JL, Zhang Y, Deng GL and Qu YL contributed new reagents/analytic tools; Shen H and Zeng S analyzed data and wrote the paper.

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Data sharing statement: No additional data available.

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Abstract

AIM: To investigate the effect of integrin-linked kinase (ILK) on proliferation, metastasis, and invasion of the colorectal cancer cell line SW480.

METHODS: In this study, the colorectal cancer cell line SW480 was stably transfected with ILK plasmids, and small interfering RNA (siRNA) was used to knockdown expression of nuclear factor (NF)-κB/p65. Methylthiazolium tetrazolium (MTT) assay was performed to measure proliferation, and the wound healing migration assay and matrigel invasion assay were used to test the metastasis and invasion ability of SW480 cells. To explore the epithelial-mesenchymal transition (EMT) process, embryonic development, and the invasion and metastasis of tumors, the protein level of E-cadherin, vimentin, snail, and slug was detected by western blot. Immunofluorescence was also used to detect E-cadherin expression. Western blot was used to determine the level of phosphorylated-inhibitor of kappa B (IκB)a, inhibitor of gamma B (IγB)a, and nuclear factor kappa B (NF-κB) expressions and to

explore the ILK signaling pathway.

RESULTS: Western blot results revealed that ILK expression significantly increased when ILK was overexpressed in SW480 cells ($P < 0.05$). Proliferation, metastasis, and invasion ability were improved in the vector-ILK group compared to the vector group ($P < 0.05$). Immunofluorescence results revealed that E-cadherin fluorescence intensity decreased after ILK was overexpressed ($P < 0.05$). Western blot results revealed that the protein expression of E-cadherin was reduced, while vimentin, snail, and slug were upregulated when ILK was overexpressed in SW480 cells ($P < 0.05$). In order to determine the role of the NF-κB signaling pathway in ILK overexpression promoted EMT occurrence, we overexpressed ILK in SW480 cells and found that levels of NF-κB/p65 and cytoplasmic phosphorylated-IκBa were increased and that cytoplasmic IκBa levels were decreased compared to the control group ($P < 0.05$). Furthermore, NF-κB/p65 knockout revealed that E-cadherin was increased in the overexpressed ILK group.

CONCLUSION: ILK overexpression improved the proliferation, metastasis, and invasion ability of SW480 cells, and this effect may be mediated by the NF-κB signaling pathway.

Key words: Colorectal cancer; Integrin-linked kinase; Epithelial-mesenchymal transition; Nuclear factor-κB; Overexpression

Core tip: In this study, the colorectal cancer cell line SW480 was stably transfected with integrin-linked kinase (ILK) plasmids, and the proliferation, metastasis, and invasion ability of the cells were tested. The results demonstrated that ILK overexpression improved the proliferation, metastasis, and invasion ability of cell line SW4802 and promoted the occurrence of the epithelial-mesenchymal transition in colorectal cancer cells. These effects may be mediated by the nuclear factor-κB signaling pathway.

Shen H, Ma JL, Zhang Y, Deng GL, Qu YL, Wu XL, He JX, Zhang S, Zeng S. Integrin-linked kinase overexpression promotes epithelial-mesenchymal transition *via* nuclear factor-κB signaling in colorectal cancer cells. *World J Gastroenterol* 2016; 22(15): 3969-3977 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/3969.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.3969

INTRODUCTION

Integrin-linked kinase (ILK) is a multifunctional receptor protein and a protein interaction integrin. Moreover, ILK can recruit other adapter molecules and regulate a variety of cellular processes *via* coupled signaling pathways, including cell growth, proliferation, apoptosis, survival, differentiation, migration, and invasion. Recent studies have shown that ILK is overexpressed and excessively activated in a number of human cancers[1,2]. It has been reported that the overexpression of ILK can enhance the rate of lung cancer cell migration, and it was shown that this enhancement was regulated by nuclear factor (NF)-κB-mediated matrix metalloproteinase (MMP)-9 expression[3,4]. Researchers have found that ILK was highly expressed in colorectal cancer tissues; and that ILK promoted tumor transfer and corrosion, which is mediated through the epithelial-mesenchymal transition (EMT) process[5,6]. However, the role and mechanism of ILK in colorectal cancer cells remains unclear. Some experts have reported that ILK overexpression can induce transcription factor snail and zinc finger E-box binding homeobox 1 (ZEB1) expression, resulting in the inhibition of E-cadherin expression[7-9]. The colorectal cancer cell line SW480 was used in this study, and ILK expression levels in this cell line were found to be relatively low. The present study aims to investigate the effect of ILK in colorectal cancer cell proliferation, invasion, and metastasis and to explore its underlying mechanism.

MATERIALS AND METHODS

Materials

Transfection reagent lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States), PVDF film (Millipore, Bedford, MA, United States), anti-ILA antibody (Cell Signaling Technology, Danvers, MA, United States), anti-E-cadherin antibody and anti-Vimentin antibody (Santa Cruz Biotechnology, Dallas, TX, United States), anti-Slug antibody (Abcam, Cambridge, MA, United States), and anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, United States).

Construction of ILK overexpressed SW480 cell line

The human colorectal cancer cell line SW480 was obtained from the Cell Bank, Chinese Academy of Medical Sciences (Shanghai, China) and cultured in Leibovitz L-15 medium (Gibco, Grand Island, NY, United States) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, United States) and antibodies. Cells were cultured in an incubator containing 5% CO_2 at 37 ℃ and were passaged for 2-3 d until 85% confluence was achieved.

Human ILK gene coding sequence was obtained by polymerase chain reaction (PCR) amplification and connected the target gene to the pcDNA3.1 vector. Sequencing detection revealed no mutation in the target gene. In a six-well plate, 2×10^5 cells were seeded into each well. After 1 d of culture, cells were transfected, and transfection reagent lipofectamine 2000 was applied to transfect 2 μg/mL of

overexpressed ILK plasmids (pcDNA3.1-ILK) or empty vector. After 48 h of transfection, cells were placed into a selective medium (G418, 800 mg/mL) for 3-4 wk. G418-resistant clones were filtered and amplified after reverse transcriptase (RT)-PCR and western blot confirmation.

Cell proliferation experiment

Cells were cultured in 96-well plates (2×10^3 cells/well) for 24, 48, and 72 h. Then, 20 μL of MTT was added into each well and cultured in an incubator for another 2 h. At the end of the culture period, the liquid in the well was discarded, 200 μL of dimethylsulfoxide (DMSO) was added, and optical density (OD) was measured with a microplate reader at 450 nm.

Wound healing assay

Cells were cultured in six-well plates, and the culture medium was discarded when it reached approximately 80% confluence. Then, the monolayer was scraped using the tip of a 200-μL pipette, cells were washed three times with phosphate buffered saline (PBS), and a photograph was taken under a microscope. Subsequently, cells were maintained in serum-free medium for another 6 h. Then, a photograph was taken and the migration rate was computed.

The culture medium was discarded when cells reached 60% confluence. Cells were washed three times with PBS and cultured in an incubator for another 24 h. After trypsinization, cells were collected in a tube, centrifuged, and resuspended in FBS free culture medium to a final concentration of 1×10^5 cells/mL. The transwell chamber was placed onto a 24-well plate containing 800 μL of culture medium with 20% FBS. Then, cell suspensions were injected into the devices through the inlet channels. The plate was incubated for 24 h in 5% CO_2 at 37 ℃. After incubation, the transwell chamber was taken out, washed with PBS, and the upper layer of the chamber was cleaned with a cotton swab. The chamber was fixed with formalin for 20 min, dyed with hematoxylin for 5 min, and washed with PBS. Then, the number of cells were observed and counted with a microscope.

siRNA interference

NF-κBp65 small interfering RNA (siRNA) 5′-CCUCCUU UCAGGAGAUGAATT-3′; scramble control 5′-UUCUCCGA ACGUGUCACGUTT-3′. After transfection with lipofectamine 2000 for 48 h, cells were obtained and analyzed.

Western blot

Cells were lysed for cytosol-nuclear isolation; and nuclear and cytoplasmic lysates were collected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and proteins were transferred onto a polyvinylidene (PVDF) membrane. Primary antibodies used for incubation were as follows: membrane anti-ILK antibody (diluted 1:1000), anti-E-cadherin antibody (diluted 1:100), anti-NF-KBp65 antibody (diluted 1:1000), anti-inhibitor of kappa B (IkB)a antibody (diluted 1:1000), anti-phosphorylated IkBa antibody (diluted 1:1000), anti-vimentin antibody (diluted 1:1000), anti-E-cadherin antibody (diluted 1:500), anti-snail antibody (diluted 1:500), anti-slug antibody (diluted 1:1000), and anti-β-actin antibody (diluted 1:5000). Then, membranes were treated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Specific protein bands were detected using an enhanced chemiluminescence assay kit (Santa Cruz Biotechnology).

Immunofluorescence

Cells were cultured on round coverslips and fixed with 4% paraformaldehyde for 30 min. Then, 0.1% Triton X-100 was used for permeabilization. Cells were incubated in fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibodies for 90 min, washed with secondary antibodies, and fixed by 4′,6-diamidino-2-phenylindole (DAPI). A laser scanning confocal microscope (FV1000S-SIM/IX81; Olympus, Tokyo, Japan) was used to observe staining.

Statistical analysis

All data are presented as mean ± SD. Using SPSS 13.0 software (SPSS, Chicago, IL), data were compared by one-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

RESULTS

Overexpression of ILK in SW480 cells increased ILK protein expressions

To verify the low expression levels of ILK in stably transfected SW480 cells, there were three transfection treatment groups: SW480, pcDNA3.1, and pcDNA3.1-ILK. After 24 h of transfection, ILK expression level was detected by western blot. Results revealed that ILK expression was significantly increased in the pcDNA3.1-ILK group compared with the vector group (Figure 1A). In addition, grayscale analysis revealed that ILK expression increased in the pcDNA3.1-ILK group, compared with the vector group (Figure 1B), and this difference was statistically significant ($P < 0.05$).

Overexpression of ILK enhances proliferation of SW480

MTT assay was used to detect cell proliferation and validate whether the increase in ILK expression affected the proliferation of SW480 cells. The results (Figure 2) revealed that there was no statistical difference between the vector and control groups. However, the proliferation rate was much higher at 48 and 72 h in the vector-ILK group than in the vector group; and the difference was statistically significant ($P < 0.05$).

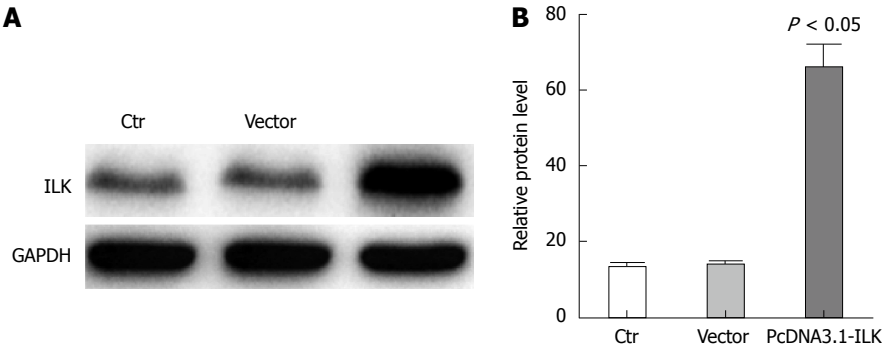


Figure 1 Overexpression of integrin-linked kinase in SW480 cells increased integrin-linked kinase protein expression. A: Integrin-linked kinase (ILK) expression in the control, vector, and PCDNA3.1-ILK groups were detected by western blot, with GAPDH as an internal control. This experiment was repeated three times; B: Statistical analysis revealed that ILK expressions significantly increased in the PcDNA3.1-ILK group compared with the vector group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

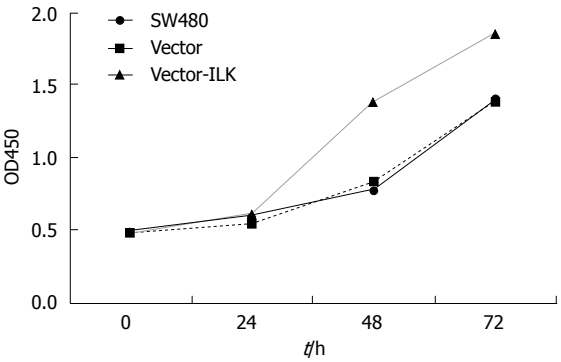


Figure 2 Proliferation rate of SW480 cells due to integrin-linked kinase overexpression.

Wound healing assay

Wound healing assay is an easily performed experiment that can detect migration ability, invasiveness, and metastasis of cells (Figures 3 and 4). Six and 12 h of culture after the scrape was made, the cells migrated much faster in the vector-ILK group than in the control group ($P < 0.05$).

Matrigel invasion assay

In the transwell method, cells can be induced to migrate from low nutrient culture medium to the high nutrient culture medium, allowing for the detection of the invasion ability of cells (Figures 5 and 6). The number of cells that migrated to the other side of the chamber was slightly higher in the vector group than in the control group, but the difference was not statistically significant ($P < 0.05$). More cells migrated to the other side of the chamber in the vector-ILK group ($P < 0.05$).

ILK can promote EMT occurrence

We over-expressed ILK in SW480 cells to investigate whether EMT occurrence and ILK expression in colorectal cancer are correlated. Using immunofluorescence staining, we found that E-cadherin fluorescence intensity was significantly reduced in the overexpressed ILK group (Figure 7B) compared with the no-load group (Figure 7A, $P < 0.05$). As explained, mutual adhesion between cells decreased, and EMT may have occurred. Therefore, EMT occurrence in colorectal cancer may be mediated by ILK. The transcription factors vimentin, snail, and slug have regulatory roles in cell EMT occurrence. To further validate our results, we overexpressed ILK in SW480 cells and used western blot for protein detection. We found that the expression of vimentin, snail, and slug was increased, while expression of E-cadherin was decreased (Figure 7C). In grayscale analysis, vimentin, snail, slug, and E-cadherin expression differences were statistically significant ($P < 0.05$, Figure 7D). Experimental results revealed that EMT occurrence in SW480 cells was promoted by ILK.

NF-κB signaling pathway mediated the ILK-induced EMT occurrence

In order to investigate whether ILK overexpression-induced EMT occurrence was directly or indirectly regulated *via* the NF-κB signaling pathway, we overexpressed ILK in SW480 cells and found using western blot that NF-κBp65 and cytoplasmic phosphorylated p-IκBa expression levels were significantly higher and that cytoplasmic IγBa expression was reduced in the overexpressed ILK group compared with the control group ($P < 0.05$, Figure 8A and B). In addition, we carried out siRNAp65 interference treatments on SW480 cells and simultaneously overexpressed ILK. By western blot, we found that E-cadherin expression in the vector-ILK cell line increased in the siRNAp65 treated group compared with that in the ILK overexpression group and the empty vector transfection group; and the difference was statistically significant ($P < 0.05$, Figure 8C and D). Taken together, these results demonstrate that the NF-κB signaling pathway mediated ILK-induced EMT occurrence.

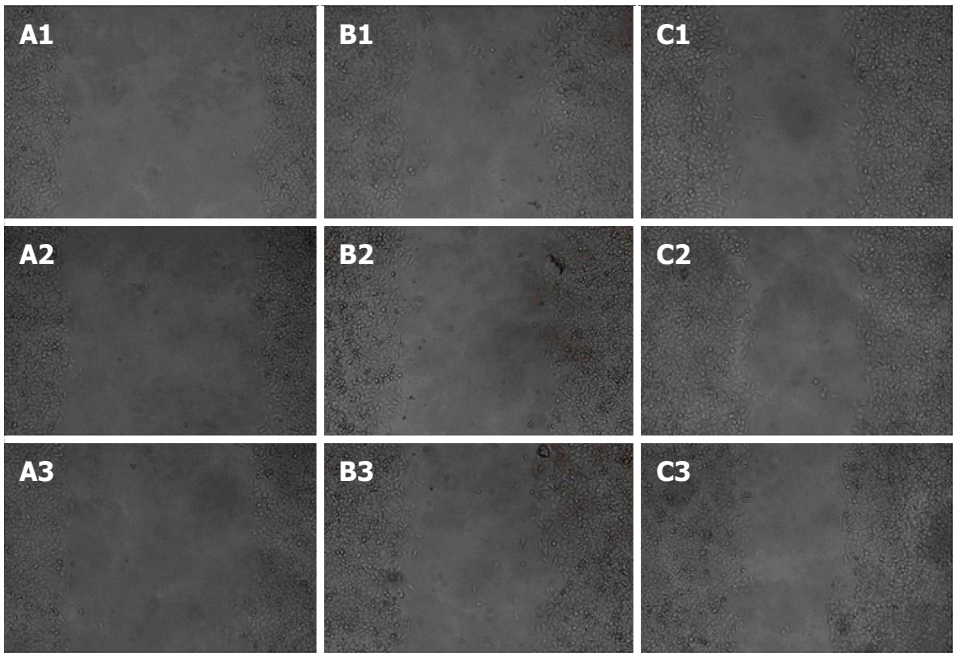


Figure 3 Wound healing assay. A1, A2, and A3 show the migration condition of cells in the control group after the scrape was made at 0, 6, and 12 h, in which only a small amount of cells migrated after 12 h; B1, B2, and B3 show the migration condition of the vector group, in which only a small amount of cells migrated after 12 h. C1, C2, and C3 show the migration condition of the vector-integrin-linked kinase group, in which a number of cells migrated into the center.

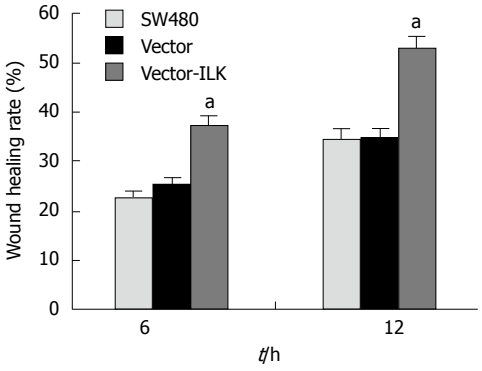


Figure 4 Wound healing rate in the three groups. $^{a}P < 0.05$ *vs* the vector group.

DISCUSSION

ILK is a vinculin serine/threonine kinase that is highly expressed in malignant tumors[10]. Some researchers have found that the role of ILK in different tumor types is not the same. In progressive pediatric tumors, breast cancer, and rhabdomyosarcoma tumors, ILK has been linked with tumor suppression[3,11,12], whereas in colon cancer, pancreatic cancer, melanoma, prostate cancer, and glioblastoma, ILK plays a role in the promotion of tumor metastasis and erosion[13-15]. Currently, many experts have reported that ILK has carcinogenic effects in colorectal cancer. Furthermore, pathological results have shown that high ILK expression levels are related to colorectal cancer staging, lymph node metastasis, and survival of patients[16,17]. For example, Li *et al*[6] reported that patients with colorectal cancer who have high ILK expression levels have a shorter survival time compared to patients with colorectal cancer who have low ILK expression levels. However, how ILK influences the migration of colorectal cancer cells and its mechanism remains unclear[6,18,19]. In this study, ILK was overexpressed in the cell line SW480 to detect the proliferation, migration, and invasion ability of cells. Then, immunofluorescence was used to detect E-cadherin levels (a biomarker of EMT) and to explore the possible mechanisms underlying the effects of ILK.

Tumor metastasis and recurrence caused by tumor cell invasion or metastasis are the leading causes of death among cancer patients[20,21]. A metastatic tumor manifests when cancer cells leave the tumor lesion, invade the adjacent tissue, grow, and proliferate in that area[22,23]. A portion of the tumor cells invades the lymphatic system, blood vessels, and body cavity and enters tissues far from the tumor lesion; resulting in the formation of a secondary tumor. In this study, we found that enhancing ILK expression levels in SW480 enhanced the proliferation ability of cells. Wound healing and matrigel invasion assays revealed that enhanced ILK expression levels improved metastasis and invasion of colorectal cancer cells. These results were consistent with some clinical reports in which patients with colorectal cancer who had high ILK expression levels were with poor prognosis.

Many theories can be used to explain the invasion and metastasis of tumors. Among these theories, EMT is a very classical theory[24,25]. EMT is a molecular program in which an epithelial cell loses its intercellular

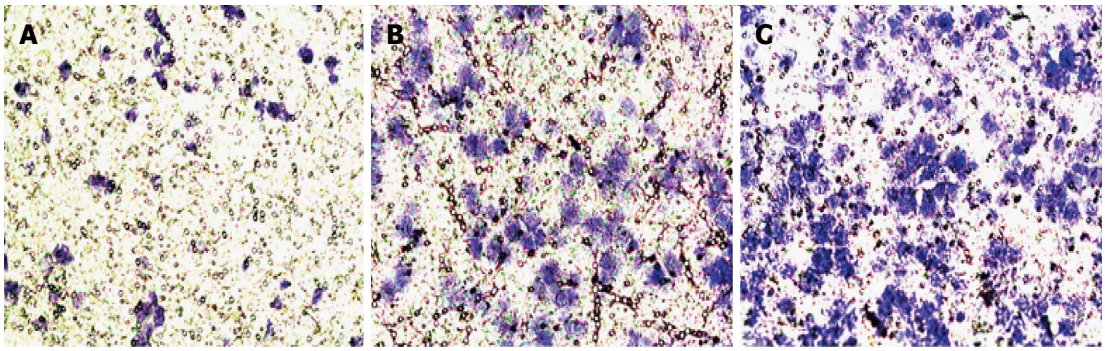


Figure 5 Matrigel invasion assay. A: The control group, only a small amount of cells migrated to the other side of the chamber; B: The vector group, only a small amount of cells migrated to the other side of the chamber; C: The vector-ILK group, a number of cells migrated to the other side of the chamber.

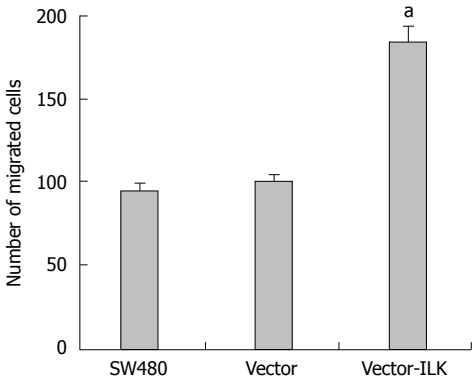


Figure 6 Number of cells that migrated to the other side of the chamber. $^{a}P < 0.05$ *vs* the vector group.

adhesion and acquires a migratory mesenchymal phenotype. E-cadherin expression is affected by this change, and the shape of cells are also converted from an epithelial state to a mesenchyma state; changing the motility of cells[26,27]. EMT plays an important role in the early stage of embryonic development, the invasion and metastasis of advanced tumors, and fibrosis after chronic inflammation[28]. Studies have found that cancer cells overexpress EMT-related genes and that these cells also have an initial cancer metastasis function. After EMT is induced in cells, epithelial cells lose their polarity. At the same time, cell adhesion ability also declines, cells relatively disperse, and migration ability is enhanced[29]. These changes become the main form of local tumor cell invasion and distant metastasis. In colorectal cancer experiments *in vitro*, ILK was overexpressed in SW480 cells. By western blot, we found that E-cadherin expression was significantly reduced, while vimentin expression was significantly increased; and by immunofluorescence staining, we found that E-cadherin in the overexpressed ILK group was significantly reduced ($P < 0.05$). It was further demonstrated in colorectal cancer that the increase in ILK expression levels and decrease in E-cadherin expression were linked. Our results showed that ILK can decrease the expression of E-cadherin; thereby promoting EMT, which leads to tumor metastasis and invasion. However, the expression of snail, slug, and other E-cadherin inhibitors was increased. Our results also confirmed that ILK can promote the EMT process in colorectal cancer *via* upregulation of the expression of snail and slug.

This study found that activation of the NF-κB signaling pathway may directly or indirectly regulate target proteins. Furthermore, activation of NF-κB is related to an aggressive phenotype, and its target proteins include snail and slug. Moreover, the NF-κB pathway plays a crucial role in EMT. Some experts have reported that ILK regulated melanoma angiogenesis *via* the NF-κB/interleukin (IL)-6 pathway[21]. Our results revealed that ILK overexpression activated the NF-κB signaling pathway, increased NF-κB/p65 levels, increased cytoplasmic levels of phosphorylated p-IκBa, and reduced cytosolic IγBa ($P < 0.05$). In colorectal cancer, we confirmed that the NF-κB signaling pathway participated in the overexpression of ILK *in vitro*, which induced EMT occurrence. In addition, we used siRNAp65 knockout experiments to successfully demonstrate that ILK inhibited the expression of E-cadherin partly by activation of the NF-κB signaling pathway. In conclusion, our results confirmed that the overexpression of ILK induces EMT occurrence, which promotes the invasion and metastasis of colorectal cancer *in vitro*. Moreover, this was partly mediated by the NF-κB signaling pathway; which also shows that the NF-κB pathway plays an important role in EMT in colorectal cancer.

In conclusion, this *in vitro* colorectal cancer study confirmed that overexpression of ILK can promote EMT occurrence in colorectal cancer cells, which was partly regulated *via* the NF-κB signaling pathway. As previously described, ILK plays an important role in promoting EMT occurrence in colorectal cancer cells and provides a new therapeutic target for the treatment of colorectal cancer. However, the mechanism of EMT regulatory factor expressions remains unclear and further research is needed.

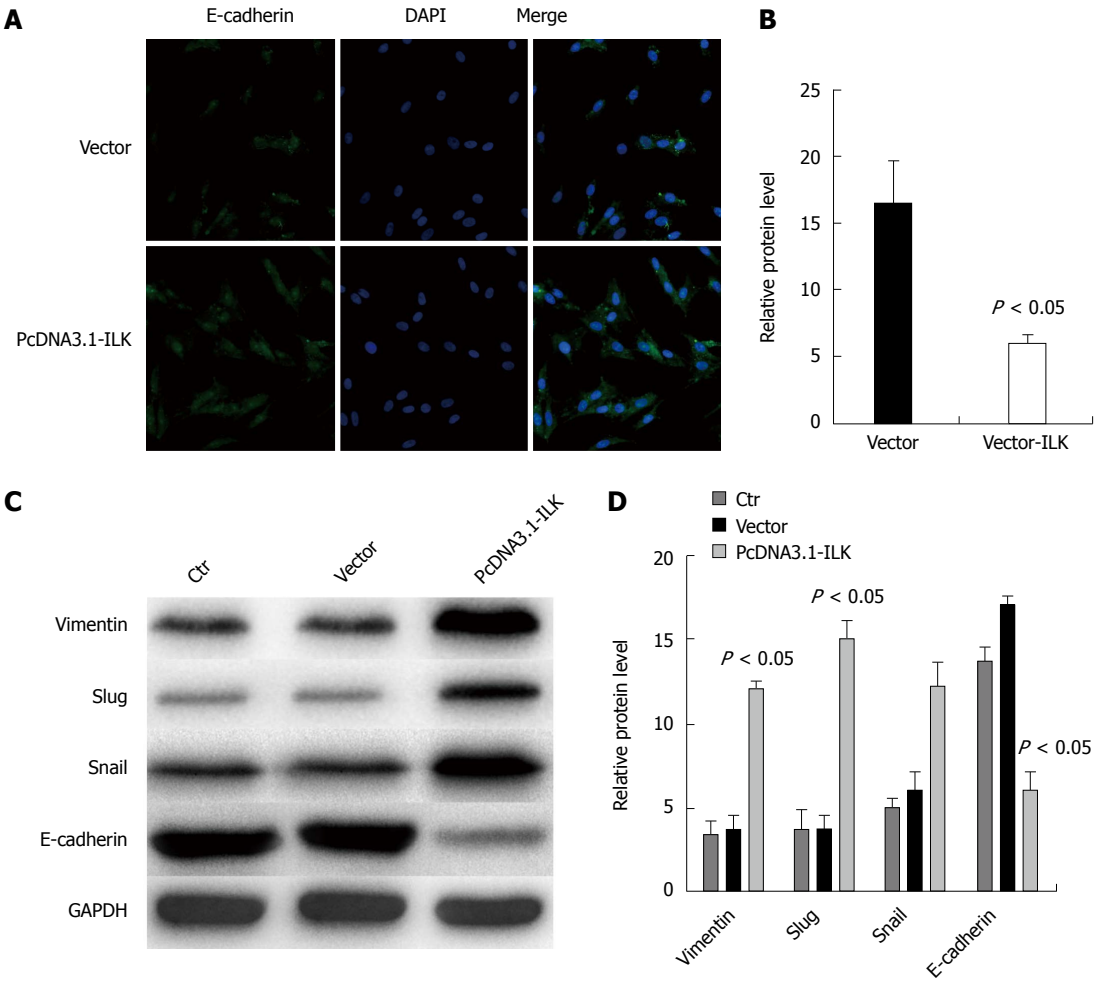
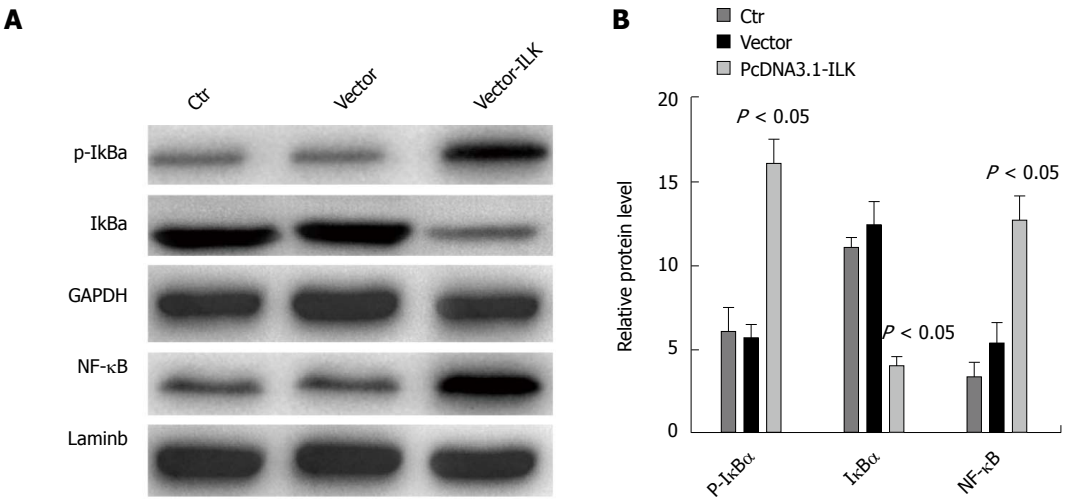


Figure 7 Integrin-linked kinase can promote epithelial-mesenchymal transition occurrence. A: Immunofluorescence revealed that E-cadherin fluorescence intensity significantly decreased in the pcDNA3.1-ILK group compared with the vector group; B: The difference between the vector group and pcDNA3.1-ILK group was statistically significant; C: Western blot revealed that vimentin, slug, and snail expression was increased, while E-cadherin expressions was reduced in the pcDNA3.1-ILK group; compared with the vector group. This experiment was repeated three times; D: The difference between the vector group and pcDNA3.1-ILK group was statistically significant. ILK: Integrin-linked kinase.



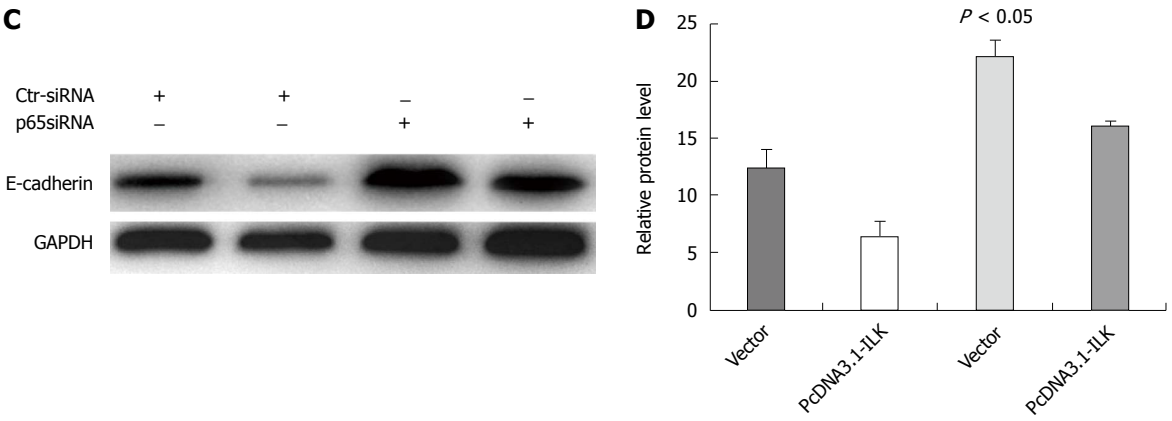


Figure 8 Nuclear factor-κB signaling pathway mediated the integrin-linked kinase-induced epithelial-mesenchymal transition occurrence. A: Western blot detection of p-IκBa, IγBa, and NF-κB expression; B: Statistical analysis, in which the difference between the vector group and vector-ILK group was statistically significant; C: After treatment with siRNA, E-cadherin expression was detected in cells; D: Statistical analysis: the difference between the vector-ILK cell line in the siRNAp65 treated group and control siRNA group was statistically significant. This experiment was repeated three times. ILK: Integrin-linked kinase; NF-κB: Nuclear factor-κB; siRNA, small interfering RNA.

COMMENTS

Background

Integrin-linked kinase (ILK) is a multifunctional receptor protein and a protein interaction integrin. Moreover, ILK can recruit other adapter molecules and regulate a variety of cellular processes *via* coupled signaling pathways, including cell growth, proliferation, apoptosis, survival, differentiation, migration, and invasion. Recent studies have shown that ILK is overexpressed and excessively activated in a number of human cancers.

Research frontiers

Some researchers have found that the role of ILK in different tumors is not the same. In progressive pediatric tumors, breast cancer, and rhabdomyosarcoma tumors, ILK can play a role in tumor suppression. However, in colon cancer, pancreatic cancer, melanoma, prostate cancer, and glioblastoma, ILK plays a role in promoting tumor metastasis and erosion. Currently, many experts have reported that ILK has carcinogenic effects in colorectal cancer. Furthermore, pathological results have shown that high ILK expression levels are related to colorectal cancer staging, lymph node metastasis, and survival of patients.

Innovations and breakthroughs

Overexpression of ILK promotes epithelial-mesenchymal transition (EMT) occurrence in colorectal cancer cells, which was partly regulated *via* the nuclear factor (NF)-κB signaling pathway.

Applications

ILK plays an important factor in the process of promoting EMT occurrence in colorectal cancer cells and provides a new therapeutic target for the treatment of colorectal cancer.

Peer-review

This *in vitro* colorectal cancer experiment confirms that overexpression of ILK can promote EMT occurrence in colorectal cancer cells, which was partly regulated *via* the NF-κB signaling pathway. ILK plays an important factor in the process of promoting EMT occurrence in colorectal cancer cells and may provide a new therapeutic target for the treatment of colorectal cancer.

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ORIGINAL ARTICLE

Basic Study

Mir-30d increases intracellular survival of *Helicobacter pylori* through inhibition of autophagy pathway

Xiao-Jun Yang, Ruo-Huang Si, Yu-He Liang, Bing-Qiang Ma, Ze-Bin Jiang, Bin Wang, Peng Gao

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Author contributions: Yang XJ, Si RH and Liang YH contributed equally to this work; Yang XJ and Liang YH carried out cell culture, transfection and the molecular genetic studies, drafted the manuscript; Si RH carried out the RT-PCR; Ma BQ and Jiang ZB participated in the GFP-LC3 Plasmid Transfection and Flow cytometry; Wang B performed the statistical analysis; and Gao P conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

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Abstract

AIM: To determine if mir-30d inhibits the autophagy response to *Helicobacter pylori* (*H. pylori*) invasion and increases *H. pylori* intracellular survival.

METHODS: The expression of mir-30d was detected by quantitative polymerase chain reaction (PCR), and autophagy level was examined by transmission electron microscopy, western blot, and GFP-LC3 puncta assay in human AGS cells and GES-1 cells. Luciferase reporter assay was applied to confirm the specificity of mir-30d regulation on the expression of several core molecules involved in autophagy pathway. The expression of multiple core proteins were analyzed at both the mRNA and protein level, and the intracellular survival of *H. pylori* after different treatments was detected by gentamicin protection assay.

RESULTS: Autophagy level was increased in AGS and GES-1 cells in response to *H. pylori* infection, which was accompanied by upregulation of mir-30d expression ($P < 0.05$, *vs* no *H. pylori* infection). In the two gastric epithelial cell lines, mimic mir-30d was found to repress the autophagy process, whereas mir-30d inhibitor increased autophagy response

to *H. pylori* invasion. mir-30d mimic decreased the luciferase activity of wild type reporter plasmids carrying the 3′ untranslated region (UTR) of all five tested genes (*ATG2B*, *ATG5*, *ATG12*, *BECN1*, and *BNIP3L*), whereas it had no effect on the mutant reporter plasmids. These five genes are core genes of autophagy pathway, and their expression was reduced significantly after mir-30d mimic transfection ($P < 0.05$, *vs* control cells without mir-30d mimic treatment). Mir-30d mimic transfection and direct inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

CONCLUSION: Mir-30d increases intracellular survival of *H. pylori* in gastric epithelial cells through inhibition of multiple core proteins in the autophagy pathway.

Key words: mir-30d; *Helicobacter pylori*; Autophagy; Gene expression; Gastric cancer

Core tip: In this study, we tested a hypothesis that mir-30d could repress autophagy in response to *Helicobacter pylori* (*H. pylori*) invasion by directly targeting multiple core genes of the autophagy pathway, including ATG2B, ATG5, ATG12, BECN1 and BNIP3L in gastric epithelial cells. Inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells, and the repression of autophagy by mir-30d may help the intracellular *H. pylori* to evade autophagic clearance. These findings provide a novel mechanism for elucidating persistent *H. pylori* infection and provide a promising target for gastric cancer prevention.

Yang XJ, Si RH, Liang YH, Ma BQ, Jiang ZB, Wang B, Gao P. Mir-30d increases intracellular survival of *Helicobacter pylori* through inhibition of autophagy pathway. *World J Gastroenterol* 2016; 22(15): 3978-3991 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/3978.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.3978

INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death in the world, and almost two-thirds of the cases occur in Asian countries, especially China and Japan[1,2]. The prognosis of gastric cancer is generally rather poor, and, therefore, prevention is a better choice than cure for patients with gastric cancer.

Helicobacter pylori (*H. pylori*) is a class I carcinogen, appointed by the International Agency for Research on Cancer in 1994 due to its strong correlation with gastric cancer in humans[3]. One reason for *H.pylori*'s high resistance to biomedical therapy may be its residence inside host cells[4,5]. Although regarded generally as an extracellular pathogen, the intracellular survival of *H. pylori* in both gastric epithelial cells and immunocytes allows it to escape from the host immune response and resist destruction from membrane-impermeable antibiotics[6], leading to persistence in the stomach. Up to now, the detailed molecular mechanisms by which *H. pylori* escape host cell machineries for intracellular survival are remains obscure.

Autophagy is present in mammalian cells at a low basal level. As an evolutionarily conserved cellular activity, it delivers organelles and cellular materials to the lysosome for degradation within double-membraned vacuoles, called autophagosomes[7,8]. Autophagy is considered one of the innate immune effectors against intracellular bacterial infection (*e.g.*, *Streptococcus pyogenes*)[9,10]. Autophagic proteins act as cytosolic sensors to rapidly launch the autophagic pathway when the innate defense system recognizes invasive bacterial pathogens[11]. However, some intracellular pathogens use highly evolved machinery to deceive autophagic recognition, manipulate the autophagic pathway, and reconstruct the autophagosomal compartment for their own survival[12]. Over the last decade, many studies have reported that *H. pylori* infection can induce macroautophagy and that *H. pylori* may evade the autophagic machinery through downregulating the expression of autophagic proteins[6,13-15].

Recently, interest in the study of mir-30 has been growing. The mir-30 microRNA family is extensively expressed in multiple tissues and cell types[16,17]. It has been shown to be involved in a wide range of physiological activities in normal tissues and cancer tissues, including cell differentiation, development, proliferation, apoptosis, senescence, and cancer metastasis[18-22]. mir-30 expression is amplified in more than 30% of human epithelial tumors, including gastric cancer[15,23,24]. There is increasing evidence that mir-30 is a novel oncomir and understanding the mechanism underlying mir-30 function in tumorigenesis would be helpful for developing targeted cancer therapy against this miRNA family. Previously, we demonstrated that mir-30d regulated cellular autophagy by directly targeting multiple genes in the autophagy pathway[25]. Consistent with our finding, another mir-30 family member, mir-30a was found to regulate autophagy *via* repressing BECN1 expression in tumor cells[26,27]. In addition, compromised autophagy by mir-30b upregulation might benefit the intracellular survival of *H. pylori*[15]. These results shed light on the potential role of miRNAs on autophagy regulation during gastric tumorigenesis.

Here, we continue our investigation on mir-30d and *H.pylori* and suggest that mir-30d downregulated the expression of key autophagy genes, including ATG2B, ATG5, ATG12, BECN1 and BNIP3L, and inhibited the autophagy response to *H.pylori* invasion of

gastric epithelial cells, resulting in increased *H. pylori* intracellular survival.

MATERIALS AND METHODS

Plasmids

The green fluorescent protein (GFP)-LC3 and psiCHECK-2 vectors were purchased from Addgene (Cambridge, MA, United States) and Promega (Madison, WI, United States), respectively.

Antibodies and reagents

Antibodies against light chain 3 B (LC3B), autophagy related (ATG)2B, ATG5, ATG12, beclin 1 (BECN1), and BNip3-like protein (BNIP3L) were obtained from Cell Signaling Technology (CST, Beverly, MA, United States). 3-methyladenine (3-MA, M9281) and rapamycin (Rapa, R8781) were purchased from Sigma (St. Louis, MO, United States).

Cell lines and H. pylori strains

AGS cells (a human gastric adenocarcinoma cell-line) were obtained from American Type Culture Collection (Manassas, VA, United States) and cultured in F12 media (Gibco, Carlsbad, CA, United States). Human gastric mucosal epithelial cell line GES-1 (Purchased from Cell bank of Xiangya Medical School, Central South University, Hunan, China) was cultured in Roswell Park Memorial Institute (RPMI)1640 (Cellgro, Manassas, VA, United States) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, United States), and 100 U/mL penicillin/streptomycin (Gibco, 15140-122) in a humidified incubator at 37 ℃ with 5% CO_2. For autophagy induction, cells were either treated with 200 nM rapamycin (Sigma) supplemented in complete medium or serum starved with Hank's buffer (Stemcell Technologies, Vancouver, Canada), both at 37 ℃ for 4 h. The wild-type *H. pylori* strain 26695(700392) was obtained from American Type Culture Collection and cultured as previously described.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed using a high capacity RNA-cDNA kit (Applied Biosystems, Carlsbad, CA, United States). cDNA was quantified on an ABI Prism 7900 sequence detection system (Applied Biosystems). Polymerase chain reaction was performed using Power SYBR Green polymerase chain reaction (PCR) master mix (Applied Biosystems).

Western blotting

Cells were lysed in mammalian protein extraction reagent (Pierce, Rockford, IL, United States) with protease inhibitor cocktail (Sigma). After centrifugation at 5000 g for 15 min at 4 ℃, the protein concentration was measured with bicinchoninic acid (BCA) protein assay kit (Pierce, 23227). Fifteen micrograms of total protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, United States). Membranes were blocked in 5% non-fat milk (Bio-Rad, Hercules, CA, United States) and then incubated with the following primary antibodies: anti-ATG2B, anti-ATG5, anti-ATG12, anti-BECN1, anti-BNIP3L, and anti-LC3B. After incubation with a secondary antibody conjugated with horseradish peroxidase (HRP) (Amersham Biosciences, Chalfont St. Giles, United Kingdom) together with an HRP-conjugated primary antibody for b-actin (Sigma), immunoreactive proteins were visualized using the LumiGLO chemiluminescent substrate (Cell Signaling). Densitometric analyses were performed using Scion Image software.

GFP-LC3 plasmid transfection

Cells were seeded onto six-well plates and transfected with a GFP-LC3 expression plasmid at approximately 45%-55% confluence using the Lipofectamine RNAiMAX transfection reagent (Invitrogen). After 24 h, the cells were infected with or without *H. pylori* for 24 h. For observation, cells were fixed with 4% formaldehyde for 15 min and then washed twice in cold phosphate-buffered saline (PBS). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Transfection of mir-30d mimic and inhibitor oligonucleotides

Pre-mir miRNA precursor and control oligos were purchased from Ambion (Foster City, CA, United States), and miRCURY LNA miRNA inhibitors and control oligos were purchased from Exiqon (Vedbaek, Denmark). Transfections were performed using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) and then cells were incubated in the medium containing the transfection mixture for 24-48 h.

Luciferase reporter assay

Cells were plated on a 24-well plate 24 h before transfection at 50% confluence. miRNA mimics (30 nmol/L, Ambion) were transfected using Lipofectamine RNAiMAX. Twenty-four hours post-transfection, 0.125 μg of reporter vector was transfected using FuGENE6 transfection reagent (Roche, Basel, Switzerland). Forty-eight hours after reporter vector transfection, cells were harvested, and reporter assays were performed using a dual luciferase reporter assay system (Promega).

Transmission electron microscopy

AGS and GES-1 cells were digested with 0.25% trypsinase and rinsed twice with PBS. They were then collected, fixed in 2% paraformaldehyde, 0.1%

glutaraldehyde in 0.1 mol/L sodium cacodylate for 2 h, postfixed with 1% OsO_4 for 1.5 h, washed, and stained for 1 h in 3% aqueous uranyl acetate. The samples were then washed again, dehydrated with graded alcohol, embedded in Epon-Araldite resin (Canemco, Quebec, Canada), and then cut into 0.05 μm thick sections on an ultramicrotome. The cells were observed under JEM-1230 (Jeol Ltd., Tokyo, Japan) electron microscopy.

Gentamicin protection assay

After bacteria infection, the GES-1 and bacterium co-culture was washed three times with 1 mL of warm PBS per well to remove nonadherent bacteria. To determine the colony-forming unit (CFU) count corresponding to intracellular bacteria, the GES-1 cell monolayers were treated with gentamicin (100 mg/mL; Sigma, G1272) at 37 ℃ in 5% CO_2 for 1 h, washed three times with warm PBS, and then incubated with 1 mL of 0.5% saponin (Sigma, 47036) in PBS at 37 ℃ for 15 min. The treated monolayers were resuspended thoroughly, diluted, and plated on serum agar. To determine the total CFU corresponding to host associated bacteria, the infected monolayers were incubated with 1 mL of 0.5% saponin in PBS at 37 ℃ for 15 min without prior treatment with gentamicin. The resulting suspensions were diluted and plated as described above. Both the CFU of intracellular bacteria and the total CFU of cell-associated bacteria were given as CFU per well of GES-1 cells.

Bioinformatic analysis

miRNA and mRNA expression microarray data were retrieved from a public accessible database, Cell Miner. http://discover.nci.nih.gov/cellminer/. Gene set enrichment analysis (GSEA) algorithm was used to identify the pathways that were significantly enriched between mir-30d low and high tumor cells. http://www. broadinstitute.org/gsea/index.jsp. TargetScan algorithm was used to predict mir-30d targets. http://www.targetscan.org.

RESULTS

H. pylori infection increased autophagy increased and upregulated mir-30d in AGS and GES-1 cell lines in response to

To measure autophagy induction during *H. pylori* infection, a GFP-LC3 fusion protein expression reporter was used in the assay. Upon autophagy induction, LC3-I, one form of the microtubule-associated protein light chain 3 (LC3), converts to another form LC3-Ⅱ. LC3-Ⅱ is accumulated in the autophagosomal membranes, and its amount is correlates to the number of autophagosomes and may serve as a marker for autophagosome formation. Autophagy induction was evaluated by measuring the quantity of GFP-LC3 puncta formed in the tested cell. AGS and GES-1 cells were transfected with GFP-LC3 vector and infected with or without *H. pylori* for autophagy analysis. Under fluorescence microscopy observation (Figure 1A), the GFP-LC3 puncta was significantly increased in *H. pylori* infected AGS and GES-1 cells (compared to the control cells without infection) after 24 h infection. This finding indicated that *H. pylori* infection may induce LC3-Ⅱ production and autophagosome formation.

Meanwhile, a typical autophagosome, double-limiting membrane, was detectable in the autophagosome (black arrowheads) and autophagolysosome (white arrowheads) examined by transmission electron microscopy (TEM) (Figure 1B). Ultrastructural image analysis showed the presence of double-membrane autophagic vesicles containing *H. pylori* in the cytoplasm of AGS cells. The number of autophagic vacuoles (AV), including autophagosomes and autophagolysosomes in *H. pylori* infected AGS cells, was increased (Figure 1B). Similar results were obtained from GES-1 cells (Figure 1B).

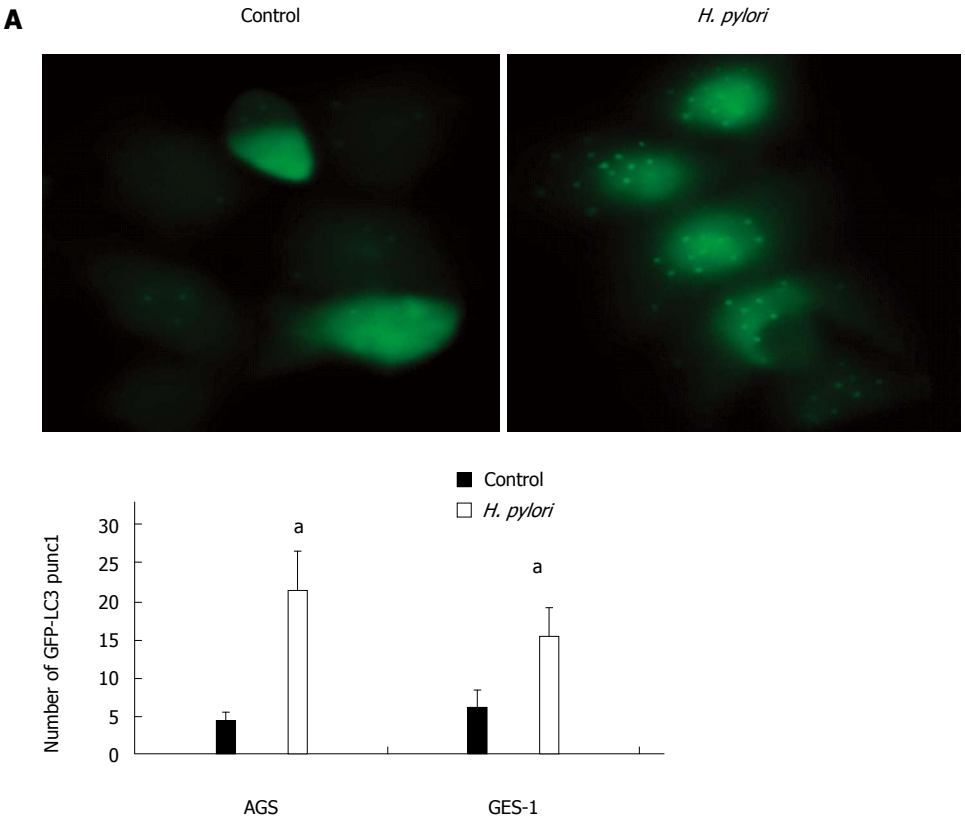
To further confirm this finding, western blots for LC3B protein were applied to analyze the conversion of LC3B-I to LC3B-Ⅱ. At 12 h and 24 h after *H. pylori* infection, LC3B-Ⅱ protein level was significantly increased in AGS cells when compared with non-infected cells. A similar pattern was observed in GES-1 cells as well (Figure 1C). The expression of mir-30d in *H. pylori* infected cells was analyzed by quantitative real-time PCR, and the results showed that the expression of mir-30d was obviously increased at 12 h and 24 h after being infected with *H. pylori* in both AGS and GES-1 cell lines ($P < 0.05$, *H. pylori* infected cells versus without *H. pylori* infected cells) (Figure 1D).

Taken together, these data demonstrate that *H.pylori* infection increased the conversion of LC3B-I to LC3B-Ⅱ (hence higher autophagosome formation), introduced a complete autophagic response, and upregulated mir-30d expression in AGS and GES-1 cell lines.

Transfection of mir-30d mimic in AGS and GES-1 cell lines downregulates autophagy after H. pylori *infection*

To determine whether mir-30d has a role in the negative regulation of autophagy during *H. pylori* infection, a mir-30d mimic was transfected into AGS and GES-1 cell lines for 24 h and then infected with *H. pylori*. The effect of the mir-30d mimic on autophagy was examined in both cell lines by western blotting, GFP-LC3 puncta assay, and TEM at 24 h post-*H. pylori* infection. Figure 2A showed that the expression of mir-30d was significantly increased in AGS cell lines either with or without *H. pylori* infection at 48 h after mir-30d mimic transfection ($P < 0.05$, mimics *vs* control). The same results were also found in GES-1 cells ($P < 0.05$ mimic transfected cells *vs* no mimic transfected control cells).

The results of western blotting revealed that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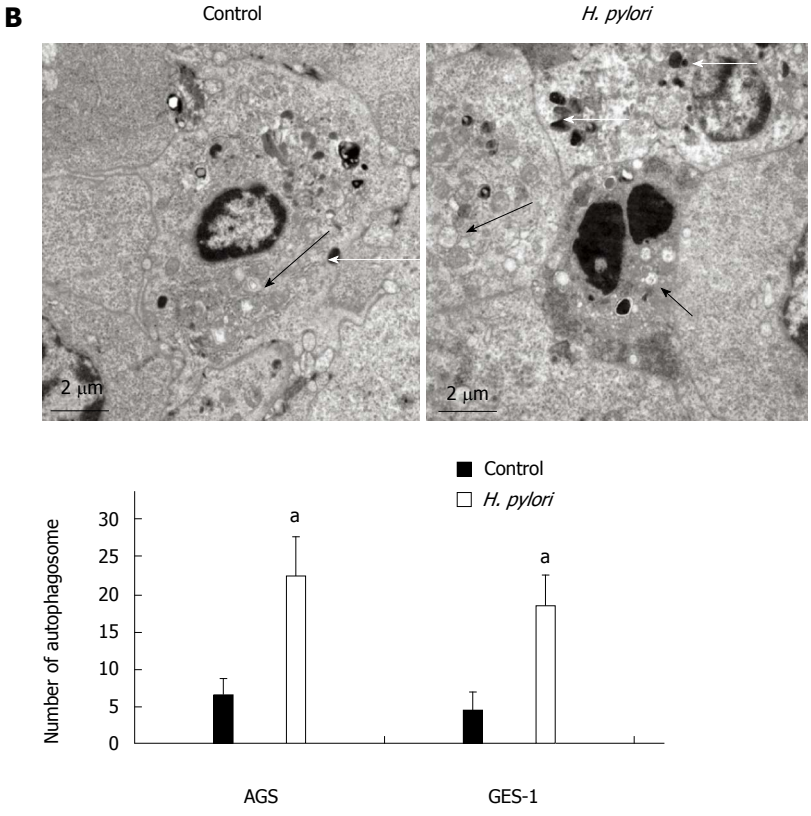
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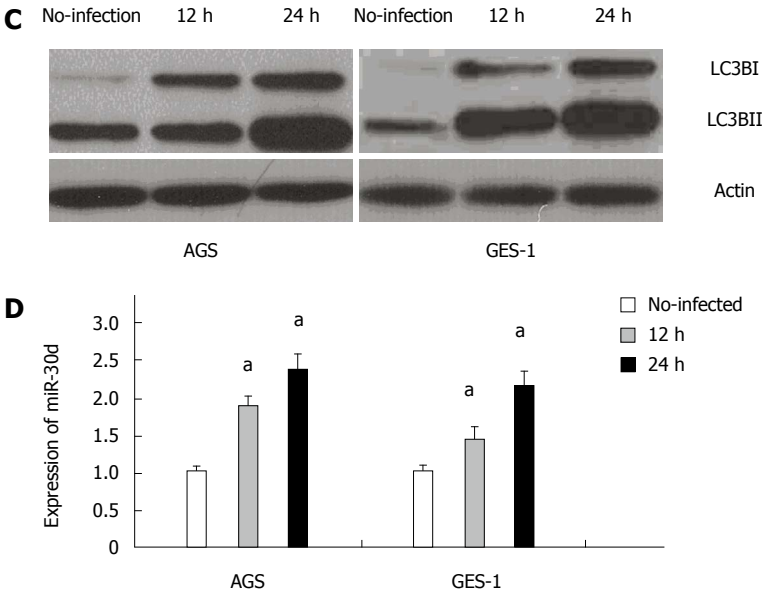


Figure 1 Autophagy and mir-30d are upregulated in AGS and GES-1 cell lines in response to *Helicobacter pylori* infection. A: GFP-LC3 puncta were observed in AGS cells with or without 24 h *Helicobacter pylori* (*H. pylori*) infection. Quantification of the number of GFP-LC3 puncta in AGS and GES-1 cells presented as mean ± SD, $^{a}P < 0.05$ control *vs H. pylori* infection; B: The autophagosomes and autophagolysosomes at 24 h after *H. pylori* infection assayed by transmission emission microscopy (TEM), shown are a typical autophagosome (black arrowheads) and autophagolysosome (white arrowheads). Quantification of GFP-LC3 puncta in AGS and GES-1 cells shown as mean ± SD, $^{a}P < 0.05$ *vs* control; C: The protein levels of light chain (LC)3B-I and LC3B-Ⅱ at 12 h and 24 h after infection with *H. pylori* analyzed by western blot; D: Analysis of the expression of mir-30d at 12 h and 24 h after infection with *H. pylori* in both cell lines by quantitative polymerase chain reaction (q-PCR). Results shown as mean ± SD, $^{a}P < 0.05$ *vs* control.

autophagy was enhanced, as evidenced by increased LC3B-Ⅱ expression, in both two cell lines during *H. pylori* infection, but transfection of mir-30d mimic significantly downregulated autophagy activity *(i.e.*, attenuated LC3B-Ⅱ conversion, Figure 2B).

In the GFP-LC3 puncta assay, GFP-LC3 plasmid and mir-30d mimic/control mimic were co-transfected into AGS and GES-1 cells using the lipofectamine RNAiMAX transfection reagent for 24 h and then infected with *H. pylori*. The treated cells were imaged under confocal laser-scanning microscope 24 h after *H. pylori* infection. The results showed that GFP-LC3 puncta were significantly increased in AGS and GES-1 cells infected with *H. pylori* compared to non- infected cells at 24 h after *H. pylori* infection, but transfection of mir-30d mimic significantly decreased GFP-LC3 positive puncta in AGS and GES-1 cells ($P < 0.05$, mimics *vs* control; Figure 2C).

Meanwhile, autophagosome and autophagolysosome were examined by TEM. The number of autophagic vacuoles (AV), including autophagosomes and autophagolysosomes, was increased in *H. pylori* infected AGS and GES-1 cells compared to non-infected cells at 24 h after *H. pylori* infection. However, transfection of mir-30d mimic significantly decreased autophagic vacuoles in both cell types ($P < 0.05$, mimics *vs* control; Figure 2D).

Transfection of mir-30d inhibitor in AGS and GES-1 cell lines upregulates autophagy after H. pylori *infection*

For loss of function experiments, endogenous mir-30d expression was blocked by mir-30d inhibitor in both cell lines and then the cells were infected with *H. pylori*. The effects of blocked mir-30d expression on autophagy in both cells lines were examined by western blotting, GFP-LC3 puncta assay, and TEM at 24 h after *H. pylori* infection.

In Figure 3A, the expression of mir-30d was decreased obviously in AGS cell lines with or without *H. pylori* infection at 48 h after mir-30d inhibitor transfection ($P < 0.05$, *vs* control oligos transfected cells). A similar result was found in GES-1 cells ($P < 0.05$, oligos transfected cells *vs* control cells). Western blotting showed that autophagy was enhanced (increased LC3B-Ⅱ expression) in both cell lines during *H. pylori* infection. After transfection of mir-30d inhibitor into both cell lines, this process was further increased significantly (increased LC3B-Ⅱ conversion; Figure 3B).

Co-transfection of GFP-LC3 plasmid and mir-30d inhibitor/control oligos into AGS and GES-1 cells was done using the lipofectamine RNAi MAX transfection reagent for 24 h and then the cells were infected with *H. pylori*. Cells were imaged under confocal laser-scanning microscope 24 h later. The results showed

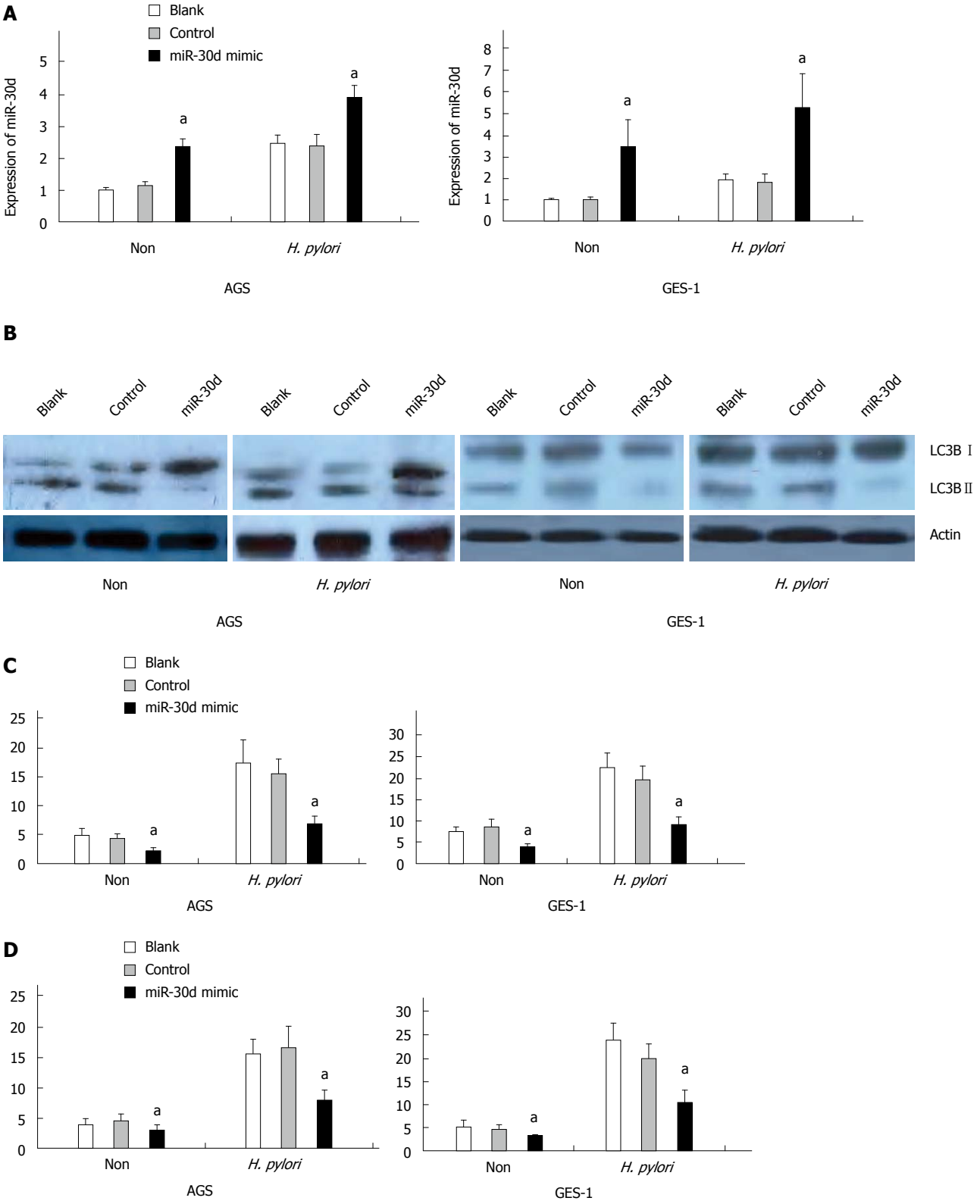


Figure 2 Mir-30d mimic represses autophagy in response to *Helicobacter pylori* infection in AGS and GES-1 cell lines. A: mir-30d expression in AGS and GES-1 cells with or without *H. pylori* infection at 48 h after mir-30d mimic transfection. Results shown as mean ± SD, $^{a}P < 0.05$ *vs* control; B: The protein levels of LC3B-I and LC3B-Ⅱ in mir-30d mimic transfected AGS and GES-1 cells with or without 24 h *H. pylori* infection; C: GFP-LC3 puncta in mir-30d transfected AGS and GES-1 cells with or without *H. pylori* infection (Results shown as mean ± SD, $^{a}P < 0.05$ *vs* control mimic transfected cells); D: Quantification of autophagosome and autophagolysosome in mir-30d mimic transfected AGS and GES-1 cells with or without 24 h *H. pylori* infection (results shown as mean ± SD, $^{a}P < 0.05$ *vs* control mimic transfected cells). *H. pylori*: *Helicobacter pylori*.

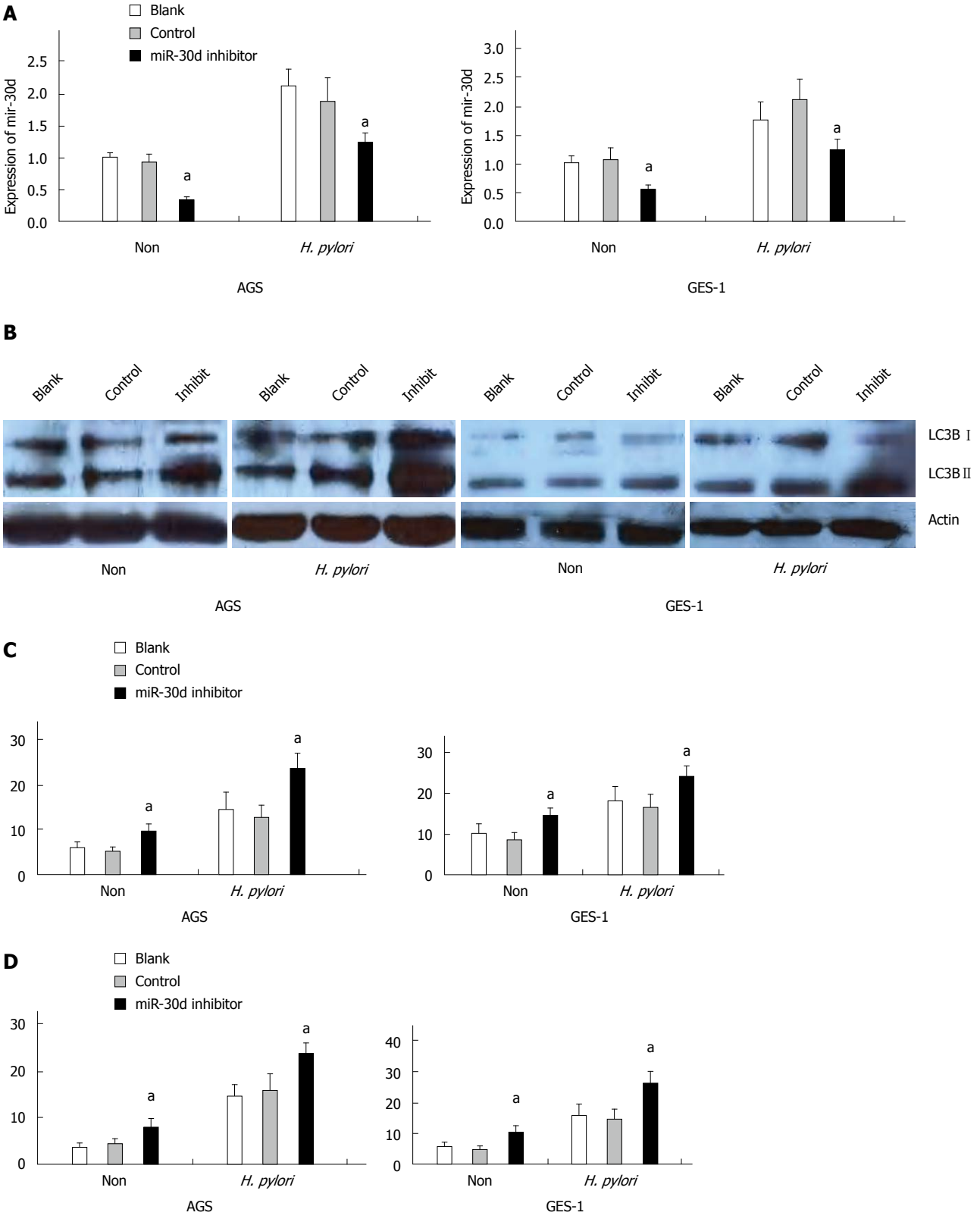


Figure 3 Mir-30d inhibitor upregulates autophagy in response to *Helicobacter pylori* infection in AGS and GES-1 cell lines. A: mir-30d expression in AGS and GES-1 cells with or without *H. pylori* infection at 48 h after mir-30d inhibitor transfection. $^{a}P < 0.05$, with mir-30d inhibitor *vs* without mir-30d inhibitor; B: LC3B-I and LC3B-Ⅱ protein levels in AGS and GES-1 cells with or without 24 h *H. pylori* infection; C: GFP-LC3 puncta in mir-30d transfected AGS and GES-1 cells with or without *H. pylori* infection (results shown as mean ± SD, $^{a}P < 0.05$ *vs* control oligos transfected cells); D: Autophagosome and autophagolysosome in mir-30d transfected AGS and GES-1 cells with or without *H. pylori* infection (results shown as mean ± SD, $^{a}P < 0.05$ *vs* control oligos transfected cells). *H. pylori*: *Helicobacter pylori*.

that GFP-LC3 puncta significantly increased in AGS and GES-1 cells infected with *H. pylori* as compared with non-infected cells. Nevertheless, mir-30d inhibitor significantly increased GFP-LC3 positive puncta in both cell types ($P < 0.05$, oligos transfected cells *vs* control cells, Figure 3C).

TEM assay also showed that there was an increased number of autophagic vacuoles (AV) in *H. pylori* infected AGS and GES-1 cells in contrast to non-infected cells at 24 h after *H. pylori* infection. After transfection of mir-30d inhibitor into the above cells, autophagic vacuoles were increased in both cell types ($P < 0.05$, oligos transfected cells *vs* control; Figure 3D).

Mir-30d suppresses the expression of multiple core autophagy proteins in gastric epithelial cells

Previously, it was found that mir-30d inhibited the autophagy process in ovarian cancer and breast cancer cell lines by directly targeting multiple genes of the autophagy pathway, including BECN1, BNIP3L, ATG12, ATG5 and ATG2[25]. To test the effect of mir-30d inhibition on gastric epithelial cells infected with *H. pylori*, we prepared reporter plasmids containing wild type or mutant mir-30d binding sites from 3′ untranslated region (UTR) of target genes (ATG2B, ATG5, ATG12, BECN1 and BNIP3L) for luciferase activity assay. Co-transfection of luciferase reporter plasmids and mir-30d mimic or control oligos showed that mir-30d potently decreased the luciferase activity of wild type reporter plasmids that represented all five target genes examined (ATG2B, ATG5, ATG12, BECN1, and BNIP3L), whereas it had no effect on the mutant reporter plasmids (Figure 4A). Perhaps mir-30d suppressed autophagy pathway gene expression by binding to its binding site within the 3′UTR of the target genes in a sequence-specific manner.

To further validate the repression of mir-30d on targeted autophagic genes in the autophagy pathway, mir-30d mimics were transfected in AGS and GES-1 cells, and target gene expression was analyzed with qRT-PCR. The mRNA levels of ATG2B, ATG5, ATG12, BECN1, and BNIP3L were remarkably suppressed by mir-30d mimic transfection compared with mimic control transfected in both AGS and GES-1 cells (Figure 4B). Similar results were obtained using western blots to detect the protein levels of these mir-30d potential targets in the above mir-30d mimic or control mimic treated cells. The protein levels for ATG2B, ATG5, ATG12, BECN1, and BNIP3L were reduced by mir-30d mimic transfection (Figure 4C). These results suggested that mir-30d regulated these autophagic genes at both the mRNA and protein level.

A loss of function experiment was applied with the mir-30d inhibitor. When endogenous mir-30d expression was blocked in the above cell lines (AGS and GES-1), the mRNA levels of ATG2B, ATG5, ATG12, BECN1, and BNIP3L were detected through qRT-PCR. In both cell lines (Figure 4D), the mRNA levels of the above genes were significantly increased by mir-30d inhibitor compared to control oligos.

Mir-30d increases intracellular survival of H. pylori *in AGS cells through inhibition of autophagy*

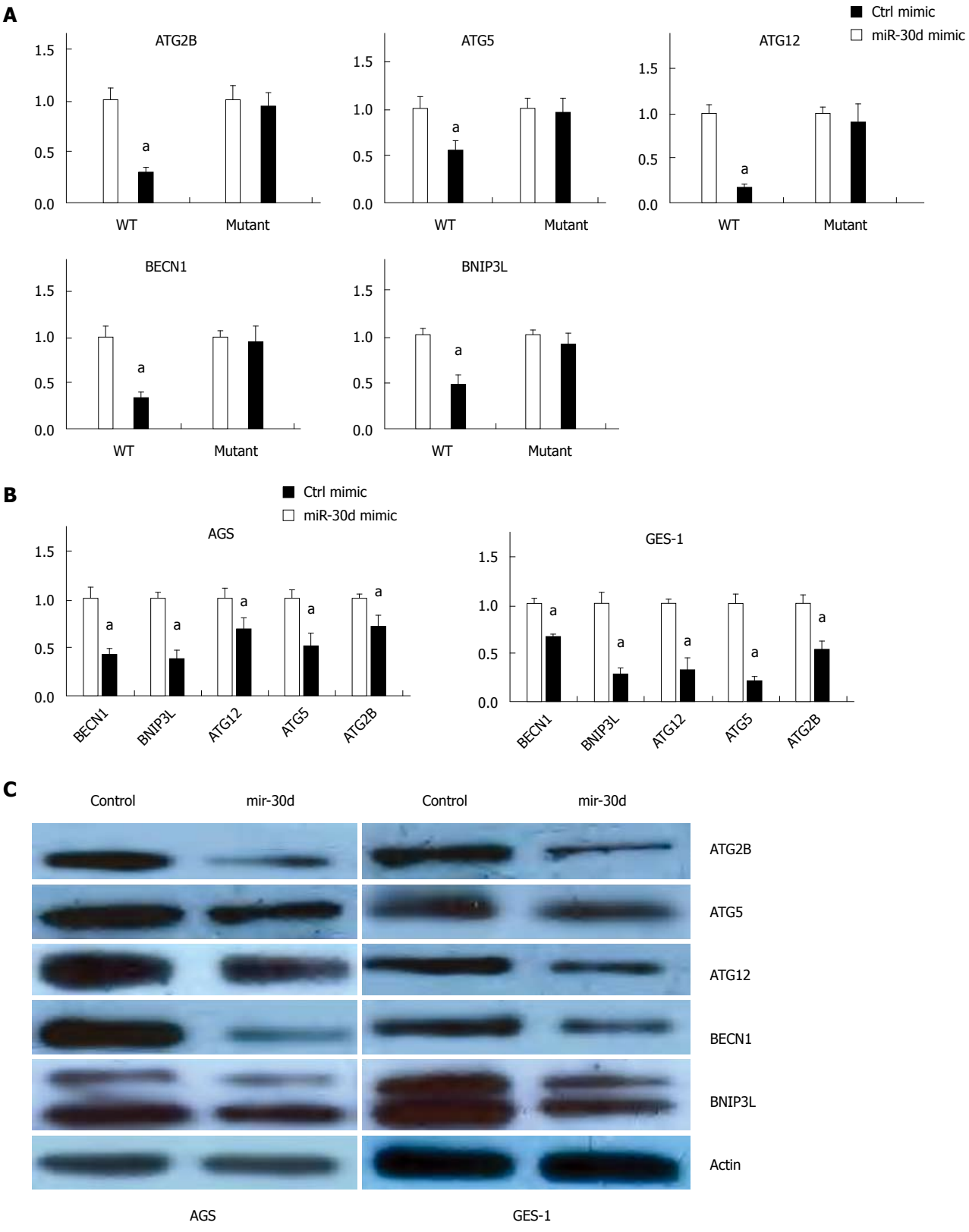
H. pylori invasion of gastric epithelial cells has been reported previously[24,26-28]. To evaluate the number of live internalized *H. pylori* cells, AGS cells were pretreated with PBS (as control), DMSO, autophagy inhibitor (3-MA), autophagy activators (starvation or rapamycin), mir-30d mimic, and mir-30d inhibitor, respectively, and then, a gentamicin protection assay was performed. The number of *H. pylori* CFU was increased approximately 10-fold 24 h after infection compared with 3 h of infection in all examined groups, indicating that internalized *H. pylori* underwent replication. Subsequently, the number of CFU decreased after 24 h in all groups (Figure 5A). However, compared with control (PBS) and DMSO groups, the number of CFU was obviously higher in mir-30d mimic and autophagy inhibitor 3-MA groups and lower in mir-30d inhibitor and autophagy activator (starvation or rapamycin) groups at all time points during a 60 h experiment (Figure 5A). The results from different treatments 24 h after infection with *H. pylori* are plotted in Figure 5B. These findings suggest that inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

DISCUSSION

H. pylori is a common phenomenon worldwide, reaching nearly one-half of the world's population. Chronic *H. pylori* infection is etiologically linked to gastric adenocarcinoma, especially non-cardia type (63% of all stomach cancer or 25% of cancers are associated with infectious etiology)[29]. These discoveries highlight the importance of basic research and clinical research on *H. pylori* infection and treatment. As resistance to the current proton pump inhibitor-based triple regimens or second-line therapies for the eradication of *H. pylori* continue to grow, so will the need to search for novel approaches.

It is known that varied autophagy is related to persistent *H. pylori* infection. Chu and colleagues[6] found that rapamycin, an inducer of autophagy, increased the clearance of *H. pylori*. However, they also found that many coccoid forms of *H. pylori* occurred on the membrane of the infected AGS cells. Autophagic vesicles were induced and their maturation was arrested with rapamycin[7], but it was not clear if *H. pylori* strains were killed inside the autophagic vesicles, as Amano *et al*[30] had indicated previously.

The role of autophagy in cancer development is important. Autophagy may be tumor-suppressing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AGS
GES-1

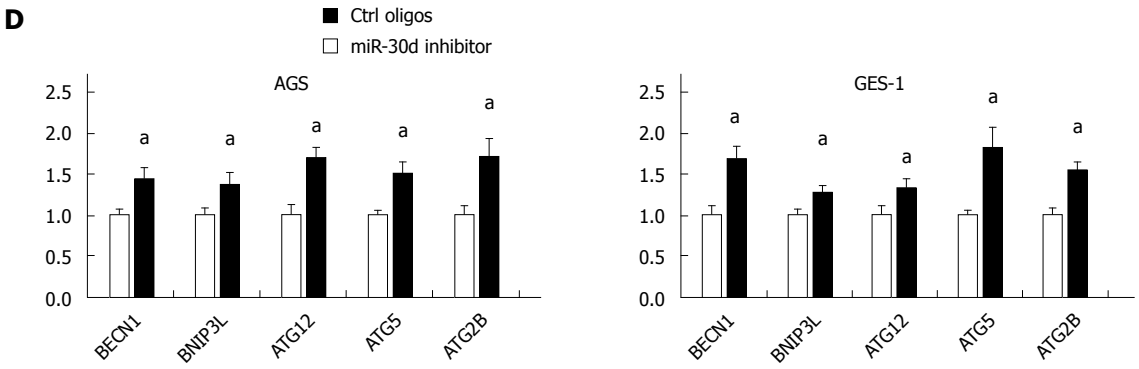

Figure 4 Multiple core proteins in the autophagy pathway are direct targets of mir-30d in gastric epithelial cells. A: Luciferase reporter assay with plasmids bearing wild type or mutant 3'UTR binding sites of mir-30d in AGS cells with mir-30d mimic or control oligos. Luciferase activity of mir-30d mimic transfected cells was normalized to control mimic transfected cells. Results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control; B: mRNA levels of autophagy related (ATG)2B, ATG5, ATG12, beclin 1 (BECN1), and BNip3-like protein (BNIP3L) in both cell lines with mir-30d mimic or control mimic transfection. Results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control; C: ATG2B, ATG5, ATG12, BECN1, and BNIP3L protein levels in both cell lines with mir-30d mimic or control mimic transfection; D: The mRNA levels of ATG2B, ATG5, ATG12, BECN1, and BNIP3L in two cell lines with or without mir-30d inhibitor transfection. Results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control.

during the early stages of tumorigenesis, as reduced expression of autophagy proteins was shown to contribute to the development or progression of human breast and other cancers[31-33]. Sometimes, however, autophagy promotes cancer development[25]. In this case, downregulation of autophagy may benefit the intracellular survival of *H. pylori*, and induction of autophagy may be beneficial indirectly for cancer prevention.

Recently, miRNAs were demonstrated to play a crucial role in autophagy regulation, such as mir-30a, mir-30b, mir-17/20/93/106, mir-204, and mir-10b[15,26,34-36]. Tang *et al*[15] found that compromised autophagy by mir-30b led to a failure to clear intracellular *H. pylori*, resulting in persistent *H. pylori* infection and proliferation in the host cells. Kobayashi *et al*[37] suggested that mir-30d is a prognostic maker for prostate cancer. Our previous study showed that mir-30d regulated the autophagy process by directly targeting multiple autophagic genes in the autophagy pathway[25].

In this study, we confirmed a hypothesis that mir-30d could inhibit autophagy in gastric epithelial cells induced by *H. pylori* invasion by down-regulating autophagy-related gene expression, resulting in increased *H. pylori* intracellular survival. The results obtained from GFP-LC3 puncta assay, TEM, and western blot demonstrated that autophagy could be induced in AGS and GES-1 cells in response to *H. pylori* infection. The expression of mir-30d was upregulated in both cells after *H. pylori* infection in our experiments. This event appeared to be unique to *H. pylori* infection, but it must be repeated with other pathogens to demonstrate its specificity. Study on mir-30b found that infection with other pathogens (*E. coli* DH5α and O157:H7) or autophagy modulators (*e.g.*, rapamycin and 3-methyladenine) had no effect on mir-30b expression[15]. We found that autophagy was upregulated in both cell lines after *H. pylori* infection, but upregulation of mir-30d significantly inhibited this process. In contrast, when mir-30d expression was blocked by mir-30d inhibitor, autophagy was obviously increased by downregulation of mir-30d. Mir-30d also repressed the autophagy process by directly targeting multiple core genes (ATG2B, ATG5, ATG12, BECN1, and BNIP3L). A gentamicin protection assay indicated that inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

Although overexpression of mir-30d could decrease autophagy by inhibiting the expression of multiple core genes of the autophagy pathway in gastric epithelial cells, the regulation event might have happened after *H. pylori*-induced autophagy. Moreover, downregulation of autophagy by mir-30d may not be sufficient to block the autophagy induced by *H. pylori*. In assayed AGS and GES-1 cells with exogenously added mir-30d mimic, autophagy in *H. pylori* infection was more than that in uninfected cells (Figure 2B, C and D). Given the complexity of *H. pylori* infection *in vivo*, other factors may also contribute to autophagy inhibition. As one of the factors, overexpression of mir-30d may slightly continue to inhibit autophagy pathway for a long time, leading to subversion of host autophagic responses for their survival or growth.

Based on the above results, we concluded that repression of autophagy by mir-30d may help intracellular *H. pylori* evade autophagic clearance through targeting ATG2B, ATG5, ATG12, BECN1, and BNIP3L. These findings provide a novel molecular mechanism for persistent *H. pylori* occupancy. Although much remains to be studied on the regulation of autophagy in gastric cancer, the current study provides a promising target for gastric cancer prevention. We suggest that enhanced autophagy by mir-30d inhibition may be protective against *H. pylori*-related gastric cancer.

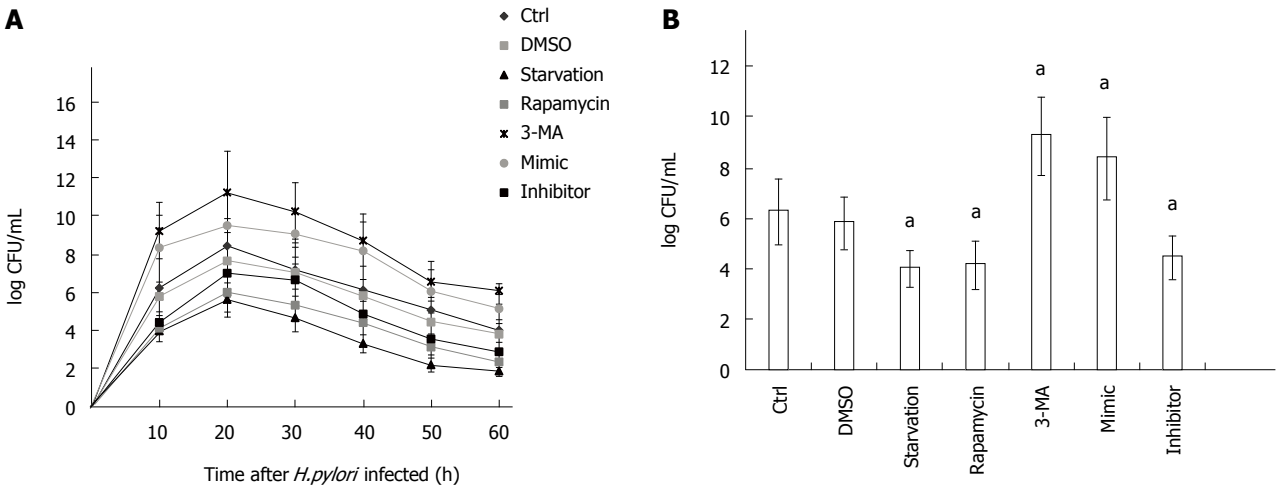

Figure 5 Mir-30d increases intracellular survival of *Helicobacter pylori* in AGS cells. A: Gentamicin protection assay for the number of colony forming units (CFU) of *H. pylori* during a 60 h *H. pylori* infection with different treatment; B: The results from 24 h *H. pylori* infection, results are shown as mean ± SD, $^{a}P < 0.05$ *vs* control. *H. pylori*: *Helicobacter pylori*.

Preventive measures for gastric cancer must include tertiary prevention and effective treatment of *H. pylori* infections. The long-term decline in gastric cancer mortality in developed countries has resulted, in part, from interrupting *H. pylori* transmission through provision of improved basic sanitation, housing, and socioeconomic status. However, secondary prevention may be attempted where simple diagnostic tests, follow-up treatment (urea breath test), and effective, short-term eradication treatment are available to mitigate individual risk[38].

In summary, our report indicates a novel molecular mechanism for the inhibition of autophagy by mir-30d by increasing the intracellular survival of *H. pylori*. Although a detailed mechanism for *H. pylori* persistence remains to be elucidated, the present study establishes a basis that will be helpful for future evaluation of mir-30d in *H. pylori* infections.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) was designated as a class I carcinogen by the International Agency for Research on Cancer in 1994 due to its strong correlation with gastric cancer in humans. One possible hypothesis for the relatively high resistance to therapy may be the ability of *H. pylori* to reside inside host cells.

Research frontiers

Over the last decade, several research groups have independently reported that infection by *H. pylori* can induce macroautophagy. However, *H. pylori* has been reported to evade the autophagic machinery by downregulating the expression of autophagic proteins.

Innovations and breakthroughs

In this study, the authors confirmed that mir-30d could represses autophagy in response to *H. pylori* invasion by directly targeting multiple core genes of the autophagy pathway in gastric epithelial cells, including *ATG2B*, *ATG5*, *ATG12*, *BECN1* and *BNIP3L*. Inhibition of autophagy increased the intracellular survival of *H. pylori* in AGS cells.

Applications

These findings may provide a novel mechanism for elucidating persistent *H. pylori* infection, and it appears to provide a promising target for gastric cancer prevention. Although the mechanism of *H. pylori* infection persistence remains to be fully determined, the present study provides the basis for future evaluations of mir-30d in *H. pylori* infections.

Terminology

Autophagy, which is present in cells at a low level basally, is an evolutionarily conserved process for delivering cellular materials and organelles to lysosome for degradation within double-membraned vacuoles, called autophagosomes. Autophagy is also regarded as one of the innate immunity effectors against intracellular bacterial infection.

Peer-review

This is a very good study on mir-30d and *H. pylori* in gastric epithelial cells. Mir-30d was shown to inhibit multiple core proteins in the autophagy pathway. The link between mir-30d in gastric cancer and *H. pylori* is novel, as no other study has indicated previously this relationship in the literature.

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ORIGINAL ARTICLE

Basic Study

CacyBP/SIP nuclear translocation regulates p27Kip1 stability in gastric cancer cells

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Author contributions: Niu YL, Hu JG and Zhai HH conducted laboratory studies, prepared figures and tables and drafted the manuscript; Li YJ and Wang JB conducted Western blot and co-IP; Liu ZX conducted cell cycle analysis; Lu YY and Feng SS constructed truncated fusion protein and transfected cells; all authors read and approved the manuscript.

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Data sharing statement: Technical appendix, statistical code and dataset available from the corresponding author at zhaihuihong@263.net. Participants have informed consent for data sharing.

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Abstract

AIM: To investigate the mechanism of calcyclin binding protein/Siah-1 interacting protein (CacyBP/SIP) nuclear translocation in promoting the proliferation of gastric cancer (GC) cells.

METHODS: The effect of CacyBP/SIP nuclear translocation on cell cycle was investigated by cell cycle analysis. Western blot analysis was used to assess the change in expression of cell cycle regulatory proteins and proteasome-mediated degradation of p27Kip1. Co-immunoprecipitation (co-IP) analysis was performed to examine the binding of CacyBP/SIP with Skp1. A CacyBP/SIP truncation mutant which lacked the Skp1 binding site was constructed and fused to a fluorescent protein. Subsequently, the effect on Skp1 binding with the fusion protein was examined by co-IP, while localization of fluorescent fusion protein observed by confocal laser microscopy, and change in p27Kip1

protein expression assessed by Western blot analysis.

RESULTS: CacyBP/SIP nuclear translocation induced by gastrin promoted progression of GC cells from G1 phase. However, while CacyBP/SIP nuclear translocation was inhibited using siRNA to suppress CacyBP/SIP expression, cell cycle was clearly inhibited. CacyBP/SIP nuclear translocation significantly decreased the level of cell cycle inhibitor p27Kip1, increased Cyclin E protein expression whereas the levels of Skp1, Skp2, and CDK2 were not affected. Upon inhibition of CacyBP/SIP nuclear translocation, there were no changes in protein levels of p27Kip1 and Cyclin E, while p27Kip1 decrease could be prevented by the proteasome inhibitor MG132. Moreover, CacyBP/SIP was found to bind to Skp1 by immunoprecipitation, an event that was abolished by mutant CacyBP/SIP, which also failed to stimulate p27Kip1 degradation, even though the mutant could still translocate into the nucleus.

CONCLUSION: CacyBP/SIP nuclear translocation contributes to the proliferation of GC cells, and CacyBP/SIP exerts this effect, at least in part, by stimulating ubiquitin-mediated degradation of p27Kip1.

Key words: Calcyclin binding protein/Siah-1 interacting protein; Gastric cancer; Cell cycle; P27kip1; Ubiquitin

Core tip: Calcyclin binding protein/Siah-1 interacting protein (CacyBP/SIP) is a component of the ubiquitination pathway. Our study showed that defects in G1 arrest led to an increase in embryonic fibroblast growth rate in SIP$^{-/-}$ mice, and CacyBP/SIP could promote G1/S transition of pancreatic cancer cells and regulate the glucose limitation-induced p27 degradation. In gastric cancer (GC) tissue, CacyBP/SIP was identified to be expressed in the nuclei and could translocate into the nucleus after induction with gastrin and promote cell proliferation; however, the mechanism remains unclear. In the present investigation, the mechanism of CacyBP/SIP nuclear translocation in promoting the growth of GC cells was studied.

Niu YL, Li YJ, Wang JB, Lu YY, Liu ZX, Feng SS, Hu JG, Zhai HH. CacyBP/SIP nuclear translocation regulates p27Kip1 stability in gastric cancer cells. *World J Gastroenterol* 2016; 22(15): 3992-4001 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/3992.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.3992

INTRODUCTION

Calcyclin binding protein (CacyBP) was originally discovered in Ehrlich ascites tumor cells and mouse brain as a calcyclin (S100A6) target protein[1], and later identified as a Siah-1 interacting protein (SIP)[2]. SIP, a component of ubiquitin-mediated proteolysis, binds the Skp1-Cul1-F box protein complex, thus called CacyBP/SIP.

Further investigations showed that CacyBP/SIP could bind other S100 proteins, including S100A1, S100A12, S100B, and S100P[3], which are the members of EF-hand Ca^{2+}-binding proteins. Meanwhile, CacyBP/SIP can also bind other target proteins including Skp1[2], tubulin[4] and ERK1/2[5]. Functionally, some reports have suggested the role of CacyBP/SIP in cellular processes such as ubiquitination, proliferation, differentiation, tumorigenesis, cytoskeletal rearrangement, and regulation of transcription. Most of all, CacyBP/SIP was found to be closely associated with the malignant phenotypes of gastric cancer (GC)[6,7], renal cancer[8], pancreatic cancer[9], as well as breast cancer[10]. However, the exact function of CacyBP/SIP has not been clarified.

Matsuzawa *et al*[2] were the first to report that CacyBP/SIP is a component of the ubiquitin pathway. P53 induces β-catenin degradation through the Siah-1-CacyBP/SIP-SCF (Skp1/Cullin1/F-box) complex pathway, in which CacyBP/SIP exerts its function by associating with Skp1. An additional study showed that defects in G1 arrest led to an increase in embryonic fibroblast growth rate in SIP$^{-/-}$ mice[11]. Recently, it was shown that CacyBP/SIP could regulate the glucose limitation-induced p27 degradation[12].

Our laboratory was the first to show that CacyBP/SIP was involved in the multidrug resistance capacity of GC cells[13,14]. In our previous studies, CacyBP/SIP expression profile in a broad range of normal and malignant human tissues was analyzed by immunohistochemistry (IHC) analysis using an anti-CacyBP/SIP monoclonal antibody first produced in our laboratory[15]. Weak staining was observed in various normal tissues. However, CacyBP/SIP was ubiquitously detected in most tumor tissues, especially in GC tissues[16]. A subsequent study showed that CacyBP/SIP could translocate into the nucleus after induction with gastrin[17]. However, the functional role of CacyBP/SIP nuclear translocation in GC cells remains unclear.

In the present investigation, the role of CacyBP/SIP nuclear translocation in GC cells was studied. Our results indicate that CacyBP/SIP nuclear translocation promotes G1 phase progression by stimulating ubiquitin-mediated degradation of p27Kip1.

MATERIALS AND METHODS

Cell culture, reagents, and treatment of cells

The human GC cell line SGC7901 (derived from poorly differentiated adenocarcinoma) was obtained from China Cell Resource Center of Academy of Life Sciences (Shanghai). Cells were cultured in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% FBS (Sijiqing, China), penicillin (100 units/mL) and streptomycin (100 μg/mL). Stably transfected SGC7901-CacyBP/SIPsi

cells were cultured in RPMI 1640 medium containing 10% FBS and 200 μg/ML G418 (Invitrogen, Carlsbad, CA). Nocodazole (Alexis Corporation, Switzerland) was added to the culture medium at 0.2 μg/mL to freeze cell cultures in mitosis. Gastrin (Sigma, St. Louis, MO) was dissolved in RPMI 1640 and used for cell treatment.

Cell cycle analysis

Cells were plated at a density of 50000 cells/well in 2 mL of complete RPMI1640 media in 6-well plates and allowed to grow for 24 h until 60%-70% confluence. To achieve synchronization, cells were starved in serum-free medium for 24 h. Upon return to regular growth medium, cells were treated or untreated with 10^{-8} mol/L gastrin. After 48 h of culture, cells were fixed overnight in 70% ethanol at 4 ℃, and then re-suspended in a buffer containing propidium iodide (PI). After 30 min of incubation, cells were subjected to DNA content analysis by flow cytometry (Coulter EPICS XL) using CellQuest software. This cell cycle analysis was performed in three independent experiments.

Western blot analysis

Treated and untreated cells were lysed in 300 μL of freshly prepared extraction buffer [1% SDS, 1 mmol/L Na3VO4, 150 mmol/L NaCl, 0.1 mol/L Tris (pH 7.4)]. To distinguish cytosolic from nuclear CacyBP/SIP, cell fractions were extracted using the NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL). Proteins were resolved at 40 μg/lane on 12% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 20-50 min at 20 V. Membranes were incubated at 4 ℃ overnight with one of the following primary antibodies: monoclonal antibodies against CDK2 (1:100), Cyclin E (1:100), Skp1 (1:100) and p27Kip1 (1:200) (all from NeoMarkers, United States); polyclonal antibodies against Skp2 (1:1000) and PARP (1:1000) (all from Cell Signaling Technology, Boston, MA); and monoclonal antibodies against CacyBP/SIP (1:1000) and β-actin (1:2000) (Sigma Chemical, St. Louis, MO). Membranes were then incubated with anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) and were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL). For each Western blot result, at least three independent experiments were conducted, and representative images are shown.

Immunoprecipitation assays

For immunoprecipitation, lysates were obtained from untreated or treated GC cells. When the cells had reached approximately 80% confluence, the cell layer was washed with ice-cold phosphate-buffered saline (PBS). Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction kit. Then salts were removed from the nuclear extract using the Pierce Slide-A-Lyzer MINI Dialysis Unit (Pierce Biotechnology, Rockford, IL). The supernatant was collected and total protein (300 μg) was used for immunoprecipitation. The supernatant was incubated at 4 ℃ for 2 h with 1 μg of monoclonal anti-CacyBP/SIP or anti-GFP (Sigma Chemical, St. Louis, MO) and 40 μL of 25% protein A/G agarose slurry. The protein A/G agarose was recovered by centrifugation at 5000 rpm and washed four times with ice-cold lysis buffer. Proteins were eluted with 20 μL of SDS loading buffer by boiling for 5 min and subjected to immunoblot analysis with anti-Skp1 antibodies.

Construction and transfection

The cDNA coding for full-length human CacyBP/SIP was generated from the plasmid pET28-CacyBP/SIP constructed and described previously[14]. The cDNA was generated by PCR amplification using the following primers: a forward primer containing a Pst I site, 5′-aactgcagtcATGGCTTCAGAAGAGCTACAGA-3′, and a reverse primer with a BamH I site, 5′-cgggatcc-TCAAAATTCCGTGTCTCCTTTG-3′. The PCR product was sub-cloned into the pEGFP/C1 vector (Invitrogen, Carlsbad, CA) using Pst I and BamH I, producing a fusion protein pEGFP/C1-CacyBP/SIP.

The cDNA encoding a truncated form of CacyBP/SIP (1-72) (*i.e.*, the N-terminal fragment of CacyBP/SIP containing residues 1-72) was derived from the full-length human CacyBP/SIP gene in plasmid pET28-CacyBP/SIP. This cDNA was generated by PCR amplification with the following primers: a forward primer with a Pst I site, 5′-aactgcagtcATGGCTTCAGAAGAGCTACAGA-3′; and a reverse primer with a BamH I site, 5′-cgggatcctca-CGTATAGCCCGTTGTAATGGG-3′. The PCR product was sub-cloned into the pEGFP/C1 vector using Pst I and BamH I, producing a fusion protein pEGFP/C1-CacyBP/SIP (1-72).

Stably-transfected GC cells SGC7901-CacyBP/SIP-siRNA were described previously and used in later experiments[17]. Cell transfection was performed with Lipofectamine^{2000} (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. For transient transfection of pEGFP/C1, pEGFP/C1-CacyBP/SIP or pEGFP/C1-CacyBP/SIP (1-72), cells were harvested for further experiments after 48 h of transfection. Cells were harvested at different time points (0, 4, 8, 12, and 24 h) for Western blot, cell cycle analysis, co-immunoprecipitation (co-IP), and confocal laser microscopy (Bio-Rad Laboratories, United States).

Statistical analysis

Bands from Western blots were quantified with Quantity One software (Bio-Rad). Relative protein levels were calculated by comparing absolute protein levels to the amount of β-actin. Numerical data are

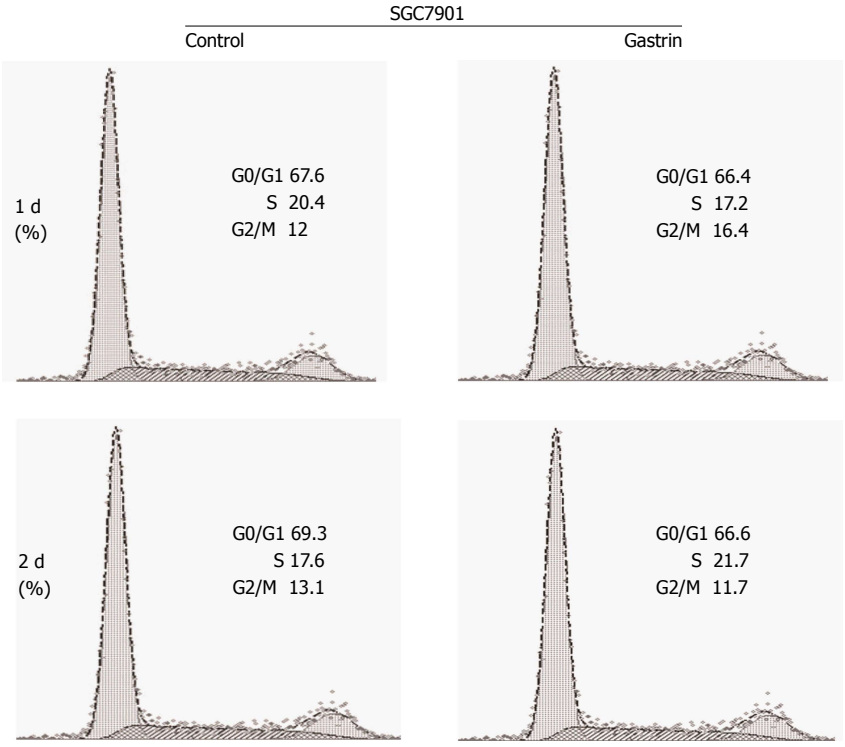

Figure 1 Gastrin-stimulated translocation of calcyclin binding protein/Siah-1 interacting protein into nucleus decreases the number of SGC7901 gastric cancer cell in the G0-G1 phases of the cell cycle. Cells were treated with gastrin (10^{-8} mol/L) for the indicated times and cell cycle variables were investigated by flow cytometry after propidium iodide (PI) staining. Data are presented as mean ± SD (n = 3), and graphs shown are representative of the three experiments.

presented as mean ± SD. Calculation of the difference between means was performed by ANOVA and then a post-hoc test. All statistical analyses were performed using SPSS software (version 11.0; Chicago, IL, United States). Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of CacyBP/SIP nuclear translocation on cell cycle in GC cells

The effect of CacyBP/SIP nuclear translocation on cell cycle phase distribution was investigated in SGC7901 cells with or without 2-d exposure to gastrin (10^{-8} mol/L). After 2 d of culture, 69.70% ± 0.46% of untreated and 65.80% ± 0.60% of gastrin-treated SGC7901 cells were observed in the G1 peak. The analysis showed that the G1 phase of gastrin-treated cells was shorter than that of untreated cells (P = 0.008; Figure 1).

Cells stably transfected with SGC7901-CacyBP/SIPsi1 which inhibited CacyBP/SIP expression to reduce the nuclear translocation of CacyBP/SIP were chosen for cell cycle assay. After 2 d of treatment, 71.09% ± 0.16% of untreated and 70.86% ± 0.25% of gastrin-treated SGC7901-CacyBP/SIPsi1 cells were observed in the G1 peak. Cell cycle analyses showed that no change was evident in the percentage of cells in G0-G1 phase in either cell line, whether untreated or treated with gastrin (P = 0.101; Figure 2).

Effects of CacyBP/SIP nuclear translocation on cell cycle regulatory proteins

To correlate the effect of CacyBP/SIP on cell cycle progression with some molecular effectors of the restriction point, SGC7901 cells were treated with nocodazole for 15 h to synchronize cells in G2-M phase. After nocodazole was washed away, cells were incubated in fresh serum-free media in the presence or absence of gastrin. From 4 to 24 h, gastrin treatment (10^{-8} mol/L for 0, 4, 8, 12, or 24 h) induced an increase in the amount of Cyclin E protein, whereas the levels of Skp1, Skp2, and CDK2 were not affected (Figure 3). Conversely, a significant decrease in the level of p27Kip1 protein was detected during the first 8 h of treatment.

Furthermore, SGC7901-CacyBP/SIPsi1 cells were also synchronized in G2-M phase with nocodazole. After stimulation by gastrin, no change in protein levels of p27Kip1 or Cyclin E was observed in SGC7901-CacyBP/SIPsi1 (Figure 4).

Proteasome-mediated degradation of p27Kip1 in GC cells

Our results shown in Figure 3 prompted us to investigate the mechanism of the decrease in p27Kip1 in more detail. It is well known that low p27Kip1 levels are attributed to increased rates of proteasome-mediated degradation. With this in mind, we studied the effect of CacyBP/SIP on p27Kip1 in the presence of the 26S proteasome inhibitor MG132. As shown in

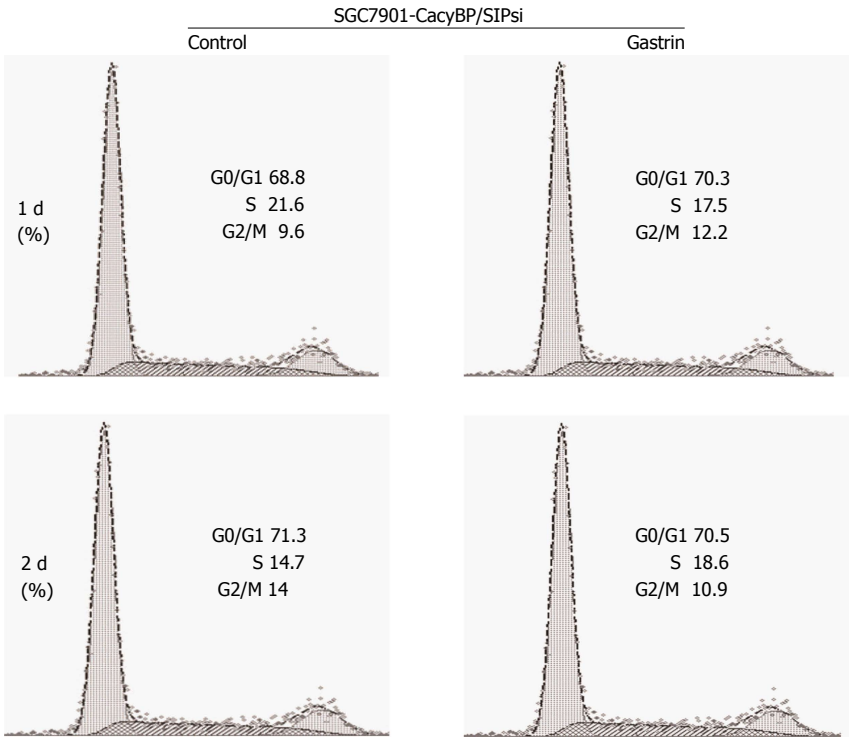

Figure 2 Treatment with gastrin increases the number of SGC7901-calcyclin binding protein/Siah-1si1 cells in the G0-G1 phases of the cell cycle. Cells were treated with gastrin (10^{-8} mol/L) for the indicated times and cell cycle variables were investigated by flow cytometry. Data are presented as mean ± SD (n = 3), and graphs shown are representative of the three experiments.

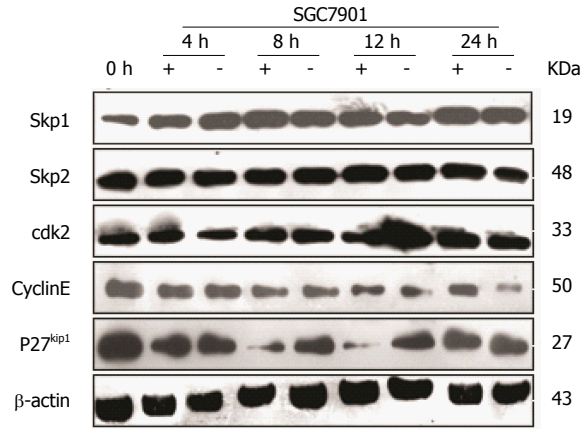

Figure 3 Effects of calcyclin binding protein/Siah-1 on cell cycle regulatory proteins. Cells were synchronized in G2-M phase with 0.2 μg/mL nocodazole for 15 h and nocodazole was removed by washing; cells were then incubated in fresh medium with (+) or without (-) gastrin for the indicated times. After treatment, cellular lysates were prepared and loaded per lane. Different blots with the same samples were detected with the indicated antibodies: Cyclin E, CDK2, p27Kip1, Skp1, Skp2, and β-actin as an internal control. Gastrin treatment induced an increase in the amount of Cyclin E protein and a decrease in the level of p27Kip1 protein during the first 8 h of treatment, whereas the levels of Skp1, Skp2, and CDK2 were not affected.

Figure 5, CacyBP/SIP nuclear translocation markedly decreased the stability of p27Kip1. Pre-incubation of growing cells with MG132 prevented p27Kip1 removal, which confirmed that the degradation of p27Kip1 in GC cells requires proteasome-mediated systems.

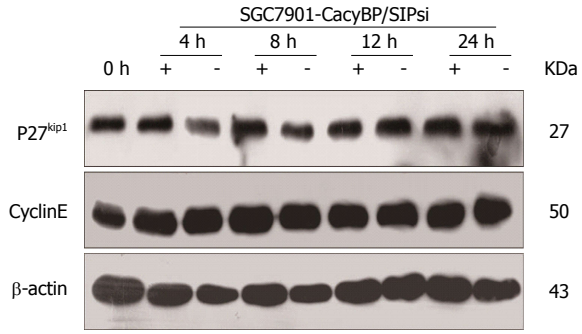

Figure 4 No change in protein levels of p27Kip1 and Cyclin E was observed in SGC7901-calcyclin binding protein/Siah-1si1 cells in which expression of calcyclin binding protein/Siah-1 was silenced. Cells were synchronized in G2-M phase with nocodazole for 15 h and nocodazole was removed by washing; and then cells were incubated in fresh medium with (+) or without (-) gastrin (10^{-8} mol/L). Different blots with the same samples were detected with the indicated antibodies: Cyclin E, p27Kip1, and β-actin as an internal control.

CacyBP/SIP promotes proteasome-dependent degradation of p27Kip1 in GC cells through interaction with the SCF component Skp1

Additionally, we examined whether degradation of p27Kip1 is also associated with changes in ubiquitination. P27Kip1 is the primary target of the SCF complex, which is involved in the proteolysis of core components of the cell cycle machinery. Recently, based on a domain mapping study, the central region of CacyBP/SIP was confirmed to interact with Skp1,

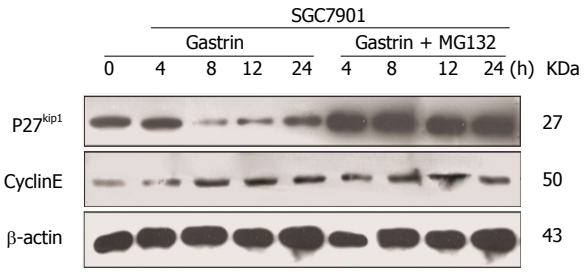

Figure 5 Proteasome-mediated degradation of p27Kip1 in gastric cancer cells. Western blot analysis of p27Kip1, Cyclin E and β-actin in cells 4, 8, 12, and 24 h after treatment with 10^{-8} mol/L gastrin in the absence or presence of MG132 (5 mmol/L, 15 min pre-treatment and co-treatment with gastrin).

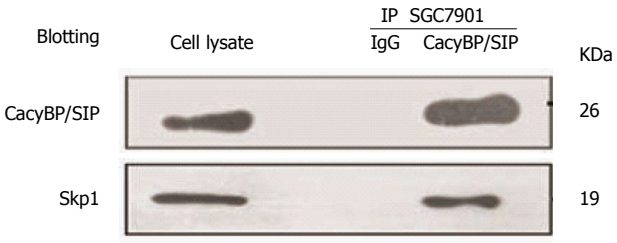

Figure 6 Physical interactions between Skp1 and calcyclin binding protein/Siah-1 in SGC7901 cells. Nuclear protein extracts from gastric cancer cells treated with 10^{-8} mol/L gastrin were immunoprecipitated with calcyclin binding protein/Siah-1 (CacyBP/SIP) MAb or control IgG. Immune complexes were analyzed by immunoblotting using an anti-Skp1 antibody with ECL-based detection.

the adaptor protein of the SCF complex[2]. Therefore, we explored whether CacyBP/SIP could bind Skp1 to trigger the degradation of p27Kip1. The nuclear fractions of cell lysates were immunoprecipitated with anti-CacyBP/SIP, and the immunoprecipitated complexes were analyzed by anti-Skp1 immunoblotting. As shown in Figure 6, CacyBP/SIP bound to Skp1 after exposure to gastrin in SGC7901 cells, in agreement with the cyclical proteasomal degradation of p27Kip1.

To explore the possibility that CacyBP/SIP increased the degradation of p27Kip1 through interaction with Skp1, a truncated form of CacyBP/SIP was constructed. CacyBP/SIP (1-72), the N-terminal fragment of CacyBP/SIP which deleted the binding site with Skp1, was constructed and fused into pEGFP/C1. To exclude any interference from internal CacyBP/SIP in GC cells, SGC7901-CacyBP/SIPsi cells in which the expression of CacyBP/SIP was suppressed were used for the latter experiment. In SGC7901-CacyBP/SIPsi cells, transiently transfected pEGFP/C1-CacyBP/SIP (1-72), the truncated protein was capable of translocating into the nucleus after gastrin induction (Figure 7). However, CacyBP/SIP (1-72) abolished interactions with Skp1 as assessed by co-IP assays (Figure 8). In contrast, no change was detected in the protein level of p27Kip1 after gastrin induction (Figure 9). The diagram containing the interaction and mechanism of gastrin, CacyBP/SIP, P27kip1 and Skp1 in gastric cancer is shown in Figure 10.

DISCUSSION

CacyBP/SIP nuclear translocation has been suggested as a promoter of GC, but the mechanism remains unknown[17]. In this study, we examined the interaction of CacyBP/SIP nuclear translocation and p27Kip1 in GC cells. Our results suggested that CacyBP/SIP nuclear translocation promotes GC cell growth through enhancing the ubiquitin-mediated degradation of p27Kip1.

Our previous studies showed that CacyBP/SIP could translocate to the nucleus in colon cancer cells upon stimulation with KCl, gastrin, epidermal growth factor, prostaglandin E2, and hypoxia[18-21]. A previous study also found that CacyBP/SIP could translocate into the nucleus after induction with gastrin and promote the proliferation of GC CELLS[17]. However, the mechanism underlying this observation is unclear.

The study by Fukushima *et al*[11] was the first to identify the potential function of CacyBP/SIP in thymocyte development and G1 checkpoint. The authors observed that $SIP^{-/-}$ embryonic fibroblasts showed a growth-rate increase resulting from defects in G1 arrest. Another study determined that CacyBP/SIP could promote proliferation and G1/S transition of human pancreatic cancer cells[9]. In our study, cell cycle analysis showed an increase in the number of cells in S phase at the expense of those in G1 phase. This result is consistent with previous findings[17].

In eukaryotic cells, progression of the cell cycle is controlled by a series of cyclin-dependent kinases (CDKs). The transition from G1 to S is regulated mainly by the G1-specific kinases, consisting of CDK2 and Cyclin E, which are controlled by a regulatory molecule, p27Kip1, the CDK inhibitor. P27Kip1 normally partners with Cyclin E/CDK2 and inhibits CDK2 activity. When p27Kip1 is phosphorylated at Thr187 and targeted for ubiquitin-dependent proteolysis by SCF^{Skp2}, CDK2/Cyclin E is released, allowing the transition of cells from G1 to S phase[22]. In the present study, we found that CacyBP/SIP nuclear translocation induced by gastrin was accompanied by decreased p27Kip1 and elevated Cyclin E protein levels. However, the level of cell cycle regulator p27Kip1 did not change when CacyBP/SIP nuclear translocation was reduced. These results indicate that the decreased level of p27Kip1, which led to the progression from G1 to S phase in GC cells, is the result of CacyBP/SIP nuclear translocation.

Recently, it was shown that CacyBP/SIP could regulate the glucose limitation-induced p27Kip1 degradation[12]. We supposed that CacyBP/SIP nuclear translocation affected the degradation of p27Kip1. It is widely accepted that the deregulation of p27Kip1 expression in human tumors is often due to post-transcriptional mechanisms[23]. Phosphorylation and ubiquitination are the two major types of post-translational protein modifications at work in the cell

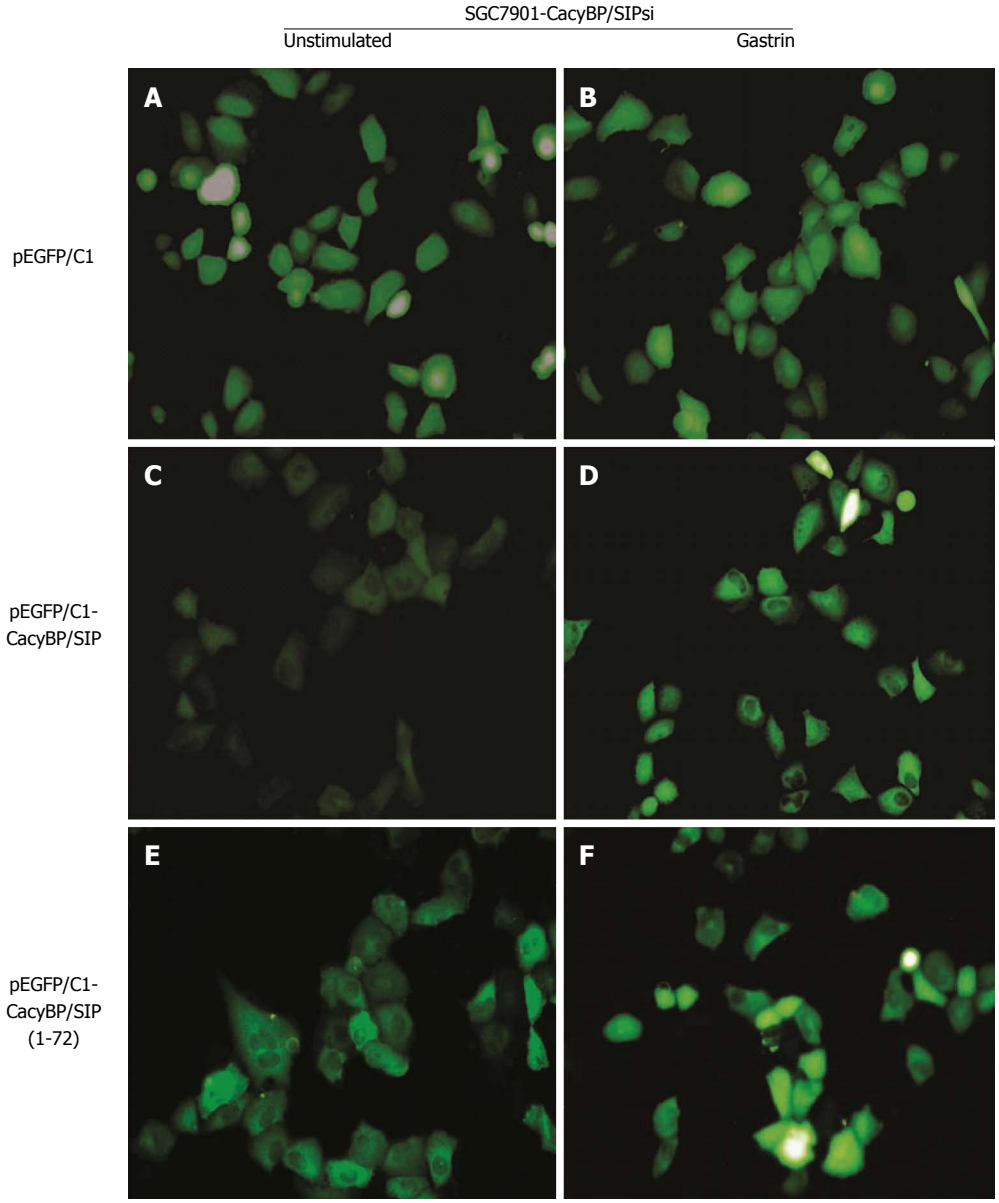

Figure 7 Immunofluorescent localization of calcyclin binding protein/Siah-1 (1-72) in SGC7901-calcyclin binding protein/Siah-1si cells. SGC7901-CacyBP/SIPsi cells were transfected with pEGFP/C1 vector, producing either GFP-tagged CacyBP/SIP or CacyBP/SIP (1-72) lacking the 155 central and C-terminal amino acids. Cells transfected with GFP-tagged pEGFP/C1 lacking a cDNA insert served as controls. Transfectants were treated or untreated with gastrin (10^{-8} mol/L), and EGFP fluorescence was analyzed under a confocal laser microscope. A, C, E: Unstimulated cells; B, D, F: Cells 8 h after gastrin stimulation.

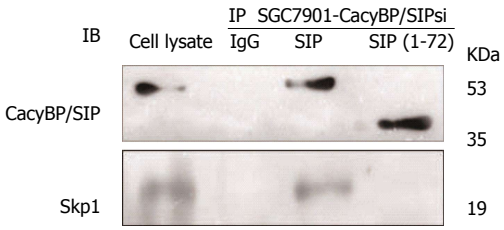

Figure 8 Interaction between Skp1 and calcyclin binding protein/Siah-1 (1-72) in SGC7901-calcyclin binding protein/Siah-1si transfected with pEGFP/C1-calcyclin binding protein/Siah-1 (1-72). Nuclear protein extracts from cells transfected with pEGFP/C1-CacyBP/SIP or pEGFP/C1- CacyBP/SIP (1-72) after treatment with 10-8 mol/L gastrin were immunoprecipitated with anti-GFP antibody or control IgG. Immune complexes were analyzed by immunoblotting using an anti-Skp1/GFP antibody with ECL-based detection. CacyBP/SIP: Calcyclin binding protein/Siah-1 interacting protein.

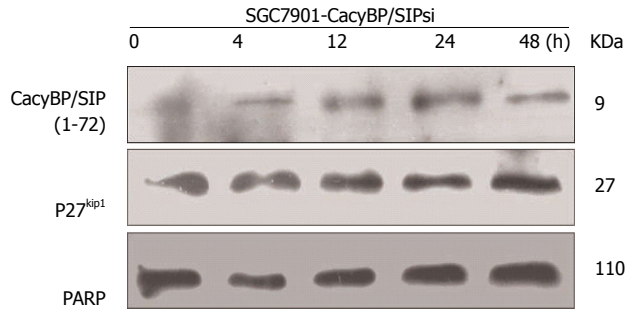

Figure 9 Lysates of the transfected cells were harvested for Western blot analysis 0, 4, 12, 24, and 48 h after treatment with gastrin (10^{-8} mol/L). Equal amounts of cellular protein (40 μg) were subjected to SDS-PAGE, followed by Western blot analysis for p27Kip1. PARP was used as a nuclear protein loading standard.

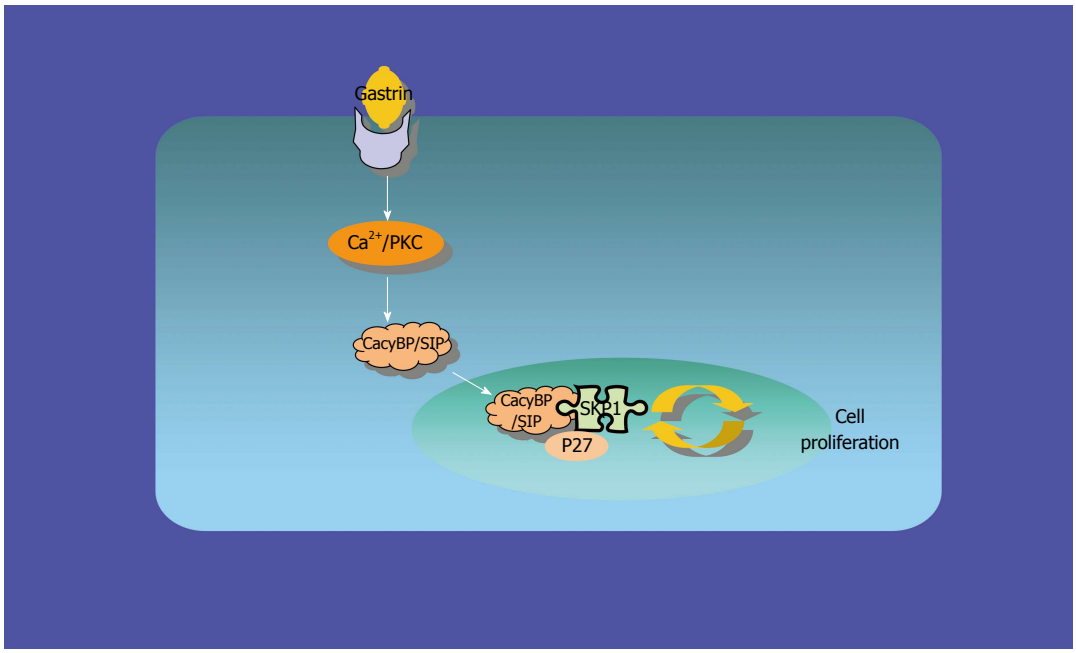

Figure 10 Interaction of gastrin, CacyBP/SIP, P27kip1 and Skp1 in gastric cancer.

cycle. We know that low p27Kip1 levels in tumors are attributed to increased rates of proteasome-mediated degradation[24]. Our study showed that treatment with proteasome inhibitors such as MG132 restored p27Kip1 expression levels. Therefore we posit that decrease in p27Kip1 in GC cells is the result of proteasome-mediated degradation.

Studies have shown that p27Kip1 is the target of the SCF complex of E3 ubiquitin ligases. The Skp1-Cul1-F-box protein (SCF) complex is responsible for the specific ubiquitination of many regulators, and plays an integral role in regulating the G1/S phase transition in mammalian cells[25]. The SCF complex targets p27Kip1 for degradation through the F-box protein and Skp2[26,27]. Our results showed that the protein levels of Skp1 and Skp2 were unchanged when the level of p27Kip1 decreased. These results indicate that the decrease in p27Kip1 is not associated with the elevated activity of SCF.

CacyBP/SIP has been reported to bind Skp1, which led us to speculate that CacyBP/SIP was induced by gastrin to translocate to the nucleus, where it binds to Skp1 in order to promote p27Kip1 degradation. Our further experiments confirmed this hypothesis. Using Co-IP assays, we found that CacyBP/SIP could interact with Skp1, which is consistent with reports from other studies[2]. The central and the C-terminal truncated CacyBP/SIP mutant, in which the Skp1 binding site was deleted, was unable to induce any change in p27Kip1 levels, although it still translocated to the nucleus upon gastrin stimulation. Therefore, our results suggest that CacyBP/SIP nuclear translocation promotes proliferation of GC cells, at least in part, due to enhanced ubiquitin-mediated degradation of p27Kip1.

In conclusion, the present study provides evidence that CacyBP/SIP nuclear translocation stimulates the progression of cell cycle in human GC cells, an effect that is at least partially mediated by enhancing the ubiquitin-mediated degradation of p27Kip1.

ACKNOWLEDGMENTS

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COMMENTS

Background

Calcyclin binding protein (CacyBP) was originally discovered as a calcyclin (S100A6) target protein, and later as a Siah-1 interacting protein (SIP), a component of ubiquitin-mediated proteolysis, thus named CacyBP/SIP. It has been reported that P53 induces β-catenin degradation through the Siah-1-CacyBP/SIP-SCF (Skp1/Cullin1/F-box) complex pathway, in which CacyBP/SIP exerts its function by associating with Skp1. A key study showed that defects in G1 arrest led to an increase in embryonic fibroblast growth rate in $SIP^{-/-}$ mice. It was also found that CacyBP/SIP could promote G1/S transition of pancreatic cancer cells. Recently, it was found that CacyBP/SIP could regulate the glucose limitation-induced P27 kip1 degradation. The authors' previous study showed that CacyBP/SIP could translocate to the nucleus in colon cancer cells upon stimulation with KCl, gastrin, epidermal growth factor, prostaglandin E2, and hypoxia. In gastric cancer (gc) tissue, CacyBP/SIP was highly expressed in the nuclei. Further studies showed that CacyBP/SIP translocated into nucleus induced by gastrin and promote the proliferation of gc cells. However, the mechanisms underlying these observations have been unclear. In the present investigation, the role of CacyBP/SIP nuclear translocation in gc cells was studied. These results indicate that CacyBP/SIP nuclear translocation promotes G1 phase progression by enhancing the degradation of p27Kip1.

Research frontiers

CacyBP/SIP nuclear translocation decreased the number of gc cells in the

G0-G1 phases of the cell cycle through increasing Cyclin E protein and decreasing p27Kip1 protein expression, whereas the levels of Skp1, Skp2 and CDK2 were unaffected. Inhibiting CacyBP/SIP nuclear translocation increased the number of cells in the G0-G1 phases, while no change in protein levels of p27Kip1 and Cyclin E was observed. Next, the authors studied the effect of CacyBP/SIP on p27Kip1 in the presence of the 26S proteasome inhibitor MG132. Pre-incubation with MG132 prevented p27Kip1 removal. The authors explored whether CacyBP/SIP could bind Skp1 to trigger the degradation of p27Kip1. Those results showed that CacyBP/SIP indeed bound Skp1. The authors also constructed CacyBP/SIP (1-72), the N-terminal fragment of CacyBP/SIP which deleted the binding site with Skp1 and fused it into pEGFP/C1. Transiently transfected pEGFP/C1-CacyBP/SIP (1-72) truncated protein was still capable of translocating into the nucleus. However, CacyBP/SIP (1-72) abolished interactions with Skp1 in co-immunoprecipitation assays. In contrast, no change was detected in the protein level of p27Kip1 after gastrin induction.

Innovations and breakthroughs

To explore the role of CacyBP/SIP nuclear translocation in gc cells, effect of CacyBP/SIP nuclear translocation on cell cycle of gc cells was studied. These studies showed that CacyBP/SIP nuclear translocation can shorten G1 phase. Using Western blot to explore the change of cell cycle regulatory proteins, the authors observed a decrease in p27Kip1 protein. Pre-incubation with proteasome inhibitor MG132 determined that the degradation of p27Kip1 was mediated by proteasome in gc cells. Using co-IP, the authors found that CacyBP/SIP promoted proteasome-dependent degradation of p27Kip1 in gc cells through interaction with the SCF component Skp1.

Applications

The study provides evidence that CacyBP/SIP nuclear translocation promotes the proteasome-mediated degradation of p27Kip1 through interaction with the SCF component Skp1. CacyBP/SIP may be a potential therapeutic target in gc.

Peer-review

In this study, the authors report the role of CacyBP/SIP nuclear translocation in GC. The study is technical sound and the results are properly analyzed. This study was based on the expression of cell cycle proteins such as CDK2, Cyclin E, Skp1, Skp2 and CacyBP/SIP detected by Western blot with or without gastrin. CacyBP/SIP was found to bind Skp1 which can trigger the degradation of P27kip1.

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ORIGINAL ARTICLE

Case Control Study

Socio-economic status and lifestyle factors are associated with achalasia risk: A population-based case-control study

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Abstract

AIM: To evaluate the association between various lifestyle factors and achalasia risk.

METHODS: A population-based case-control study was conducted in Northern Ireland, including *n* = 151 achalasia cases and *n* = 117 age- and sex-matched controls. Lifestyle factors were assessed *via* a face-to-face structured interview. The association between achalasia and lifestyle factors was assessed by unconditional logistic regression, to produce odds ratios (OR) and 95% confidence interval (CI).

RESULTS: Individuals who had low-class occupations were at the highest risk of achalasia (OR = 1.88, 95%CI: 1.02-3.45), inferring that high-class occupation holders have a reduced risk of achalasia. A history of foreign travel, a lifestyle factor linked to upper socio-economic class, was also associated with a reduced risk of achalasia (OR = 0.59, 95%CI: 0.35-0.99). Smoking and alcohol consumption carried significantly reduced risks of achalasia, even after adjustment for socio-economic status. The presence of pets in the house was associated with a two-fold increased risk of achalasia (OR = 2.00, 95%CI: 1.17-3.42). No

childhood household factors were associated with achalasia risk.

CONCLUSION: Achalasia is a disease of inequality, and individuals from low socio-economic backgrounds are at highest risk. This does not appear to be due to corresponding alcohol and smoking behaviours. An observed positive association between pet ownership and achalasia risk suggests an interaction between endotoxin and viral infection exposure in achalasia aetiology.

Key words: Achalasia; Risk factors; Epidemiology; Lifestyle; Socio-economic status

Core tip: Little is known about achalasia aetiology, with roles suggested for genetic conditions, auto-immune diseases and infectious agents. This population-based case-control study investigated lifestyle and household factors in adulthood and childhood in relation to achalasia risk, for the first time. Results indicate that achalasia is a disease of inequality, and individuals from low socio-economic backgrounds are at highest risk. The burden of achalasia in lower socio-economic groups cannot be explained by smoking or alcohol intake. Pet ownership was associated with a two-fold increased risk of achalasia. Further studies of environmental factors and achalasia risk are warranted.

Coleman HG, Gray RT, Lau KW, McCaughey C, Coyle PV, Murray LJ, Johnston BT. Socio-economic status and lifestyle factors are associated with achalasia risk: A population-based case-control study. *World J Gastroenterol* 2016; 22(15): 4002-4008 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4002.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4002

INTRODUCTION

Oesophageal achalasia is one of the most poorly understood diseases of the digestive tract. Achalasia is a neurodegenerative motility disorder that results in loss of normal lower oesophageal sphincter function and aperistalsis[1]. Oesophageal manometry is regarded as the ultimate diagnostic investigation for this condition[1]. Relatively little attention has been given to understanding the underlying aetiology of this disease, and more efforts are needed to ultimately achieve prevention of achalasia.

Although achalasia remains a rare condition, a recent review by our working group suggests that there has been an approximate two-fold increase in incidence since the mid-1980s up to the mid-2000s[2]. Reports from Canada[3] and Italy[4] estimate that achalasia incidence is now approximately 1.6 per 100000 population. Such a rise in incidence could well reflect changes in diagnostic criteria and increased awareness of achalasia amongst clinicians, however it could also point to a role for changing environmental risk factors over this timeframe.

Previously suggested risk factors for achalasia include genetic and autoimmune conditions[5,6], and infections such as the Herpes Simplex Virus (HSV-1)[7,8]. To our knowledge, no lifestyle factors have been investigated in relation to achalasia development. Associations and biologically plausible mechanisms have been reported for lifestyle factors in the role of other neurodegenerative disorders, such as Alzheimer's disease, motor neuron disease and multiple sclerosis[9-11].

Further, the timing of exposure to environmental risk factors may be important in disease aetiology. Childhood factors have been associated with risk of other oesophageal conditions in later life, including oesophagitis, Barrett's oesophagus and oesophageal adenocarcinoma in a series of Danish population-based studies[12-14]. Exposure to infectious diseases during childhood has also been speculated to contribute to the neurodegenerative Parkinson's disease risk in adulthood[15]. Given the broad age at diagnosis observed in achalasia cases[16,17], it would be interesting to study the potential role of childhood factors in achalasia development.

The aim of this novel population-based case-control study was to evaluate the association between exposure to environmental factors throughout the lifespan and risk of oesophageal achalasia.

MATERIALS AND METHODS

Subject recruitment

Patients were identified by records of all individuals undergoing oesophageal manometry performed in the Gastrointestinal Physiology Unit, Royal Victoria Hospital, Belfast, Northern Ireland, United Kingdom between 1989-2006. This was the regional centre for oesophageal manometry in Northern Ireland and diagnosed n = 304 primary achalasia patients, aged 16 years or older, during that timeframe. Of these, n = 202 cases were invited to participate in the study and n = 151 cases were successfully recruited (response rate = 74.8%). Population-based controls were identified *via* General Practitioner practices throughout Northern Ireland, and n = 117 controls took part in the study from the total n = 247 controls invited (response rate: 47.4%). Controls were frequency-matched within groups defined by age (< 50, 50-69, ⩾ 70 years) and sex to their corresponding cases, therefore similarities in age and sex distribution reflect this study design. This study was ethically approved by the Office for Research Ethics Committees Northern Ireland

Table 1 Descriptive characteristics of achalasia cases and matched controls *n* (%)

Characteristics	Achalasia cases	Controls	
	n = 151	*n* = 117	*P* value
Age, yr (mean ± SD)	55.9 ± 17.1	55.8 (16.0)	0.97
Age at diagnosis (mean ± SD)	47.6 ± 17.8	/	/
Sex			
Male	76 (50.3)	55 (47.0)	
Female	75 (49.7)	62 (53.0)	0.59
Previous medical history			
Ischaemic heart disease	12 (8.0)	7 (6.0)	0.53
Diabetes Mellitus	4 (2.7)	3 (2.6)	0.97
Hypertension	27 (17.9)	17 (14.5)	0.46
Hypercholesterolaemia	16 (10.6)	12 (10.3)	0.93
Asthma/COPD	11 (7.3)	4 (3.4)	0.17
Gastritis/Peptic ulcer	19 (12.6)	8 (6.8)	0.12
Autoimmune/Connective Tissue Disorders	1 (0.7)	1 (0.9)	0.86
Family history of achalasia	5 (3.3)	0 (0.0)	0.05
Number of years in education (mean, SD)	12.5 (3.3)	12.8 (3.1)	0.34
Occupation class			
High	39 (25.8)	45 (38.5)	
Medium	47 (31.1)	32 (27.4)	
Low	55 (36.4)	34 (29.1)	
Not classified	10 (6.6)	6 (5.1)	0.17

COPD: Chronic obstructive pulmonary disorder.

(ORECNI). Written informed consent was obtained from all study participants.

Assessment of clinical and demographic factors

Information on demographic data, lifestyle factors, past medical history, family history, childhood factors, medications and previous achalasia treatment, were collected by an interviewed questionnaire, administered by one of two trained interviewers who were not blinded to the case-control status of individuals. Socio-economic status was derived from occupation data, according to National Statistics Socio-Economic Classification as used by the Office for National Statistics[18]. Briefly in this classification system, professional, employer or manager occupations are considered to be high class; intermediate or junior non-manual occupations are categorised as medium class; skilled, semi-skilled or unskilled manual occupations are considered to be low class; students or not employed are considered as unclassified[18].

Statistical analysis

Statistical analysis comparing continuous or categorical variables between achalasia cases and controls was conducted using an independent *t*-test or chi-squared test, respectively. Odds ratios (OR) and corresponding 95% confidence interval (CI) were generated using unconditional logistic regression models to assess achalasia risk according to childhood and adult socio-demographic and lifestyle factors. Both unadjusted and adjusted regression models were performed, with the latter adjusting for age, sex, and socio-economic status (for adulthood factors) as potential confounders. Interaction between socio-economic status, smoking and alcohol status to influence achalasia risk was assessed using the likelihood ratio test. All statistical analysis was performed using Stata Version 11.2 (StataCorp, College Station, TX, United States).

RESULTS

Comparison of characteristics between achalasia cases and controls is shown in Table 1. Mean age at interview was 55.9 years for achalasia cases, of whom 50% were male. Cases were, on average, recruited 8.3 years after their incident diagnosis of achalasia. No significant differences in education, occupation or previous medical history were detected between cases and controls (Table 1), with exception of a family history of achalasia which was more prevalent in achalasia cases.

Table 2 shows the association between childhood household factors and achalasia risk. No significant associations were detected for number of rooms, household density or toilet location in the childhood home, and risk of achalasia. A non-significant inverse association was observed between the presence of smokers in the childhood home, and achalasia risk (OR = 0.85, 95%CI: 0.48-1.50). Non-significant increased risks of achalasia were also noted for childhood homes in which a pet was present (OR = 1.17, 95%CI: 0.67-2.04), and for low compared with high socio-economic households, as determined by occupation of head of household (OR = 1.64, 95%CI: 0.78-3.67). Having been breastfed did not seem to influence achalasia risk. Further adjustment for age and sex had little impact on observed associations.

The association between achalasia risk and various adult socio-demographic and lifestyle factors is shown in Table 3. The presence of pets in the house was associated with an almost two-fold increased risk of achalasia (OR = 1.92, 95%CI: 1.12-3.31). Years of education completed were unrelated to achalasia risk. However, individuals who had low-class occupations were at the highest risk of achalasia (OR = 1.88, 95%CI: 1.02-3.45), inferring that high-class occupation holders have a reduced risk of achalasia. A history of foreign travel, a lifestyle factor linked upper socio-economic class, was also associated with a reduced risk of achalasia (OR = 0.59, 95%CI: 0.35-0.99).

Smoking and alcohol consumption carried significant reduced risks of achalasia, even after adjustment for socio-economic status (Table 3). The potential interaction between alcohol, smoking and socio-economic status to influence achalasia risk was further explored in stratified analysis (data not shown). Reduced statistical power resulted in a lack of statistically significant findings. However, the reduced risk of achalasia for alcohol consumers and

Table 2 Early life and childhood household factors and achalasia risk *n* (%)

Early life risk factors	Achalasia cases *n* = 151	Controls *n* = 117	Unadjusted OR (95%CI)	Adjusted[2] OR (95%CI)
Number of rooms				
< 6	71 (47.0)	54 (46.2)	1	1
⩾ 6	80 (53.0)	63 (53.8)	0.97 (0.60-1.57)	0.96 (0.59-1.57)
Number of bedrooms				
< 3	35 (23.2)	29 (24.8)	1	1
⩾ 3	116 (76.8)	88 (75.2)	1.09 (0.62-1.92)	1.09 (0.61-1.96)
Household density				
< 2	55 (36.4)	44 (37.6)	1	1
⩾ 2	96 (63.6)	73 (62.4)	1.05 (0.64-1.73)	1.05 (0.64-1.73)
Toilet location				
Indoors	85 (56.3)	73 (62.9)	1	1
Outdoor	66 (43.7)	43 (37.1)	1.32 (0.80-2.16)	1.55 (0.85-2.82)
Presence of smokers				
No	38 (25.2)	26 (22.2)	1	1
Yes	113 (74.8)	91 (77.8)	0.85 (0.48-1.50)	0.85 (0.48-1.52)
Presence of any pets in the house[1]				
No	36 (23.8)	31 (26.5)	1	1
Yes	115 (76.2)	86 (73.5)	1.15 (0.66-2.31)	1.17 (0.67-2.04)
Occupation of head of household				
High	15 (9.9)	18 (15.4)	1	1
Medium	56 (37.1)	40 (34.2)	1.68 (0.76-3.73)	1.70 (0.77-3.79)
Low	78 (51.7)	57 (48.7)	1.64 (0.76-3.53)	1.69 (0.78-3.67)
Unclassified	2 (1.3)	2 (1.7)	1.20 (0.15-9.57)	1.25 (0.15-10.23)
Breastfed				
No	63 (41.7)	48 (41.0)	1	1
Yes	61 (40.4)	51 (43.6)	0.91 (0.54-1.55)	0.89 (0.49-1.59)
Unknown	27 (17.9)	18 (15.4)	1.14 (0.56-2.31)	1.12 (0.54-2.31)

[1]Compared with no pets present in the childhood house; [2]Adjusted logistic regression model includes age (at interview) and sex.

Table 3 Early life and childhood household factors and achalasia risk *n* (%)

Risk factors	Achalasia cases *n* = 151	Controls *n* = 117	Unadjusted OR (95%CI)	Adjusted OR[1] (95%CI)
Occupation class				
High	39 (25.8)	45 (38.5)	1	1
Medium	47 (31.1)	32 (27.4)	1.69 (0.91-3.15)	1.75 (0.93-3.29)
Low	55 (36.4)	34 (29.1)	1.87 (1.02-3.42)	1.88 (1.02-3.45)
Unclassified	10 (6.6)	6 (5.1)	1.92 (0.64-5.77)	1.90 (0.59-6.14)
Years in education				
< 13 yr	91 (60.3)	64 (54.7)	1	1
⩾ 13 yr	60 (39.7)	53 (45.3)	0.80 (0.49-1.30)	0.92 (0.52-1.61)
Smoking status				
Non-smoker	91 (60.3)	61 (52.1)	1	1
Former smoker	36 (23.8)	28 (23.9)	0.86 (0.48-1.56)	0.82 (0.44-1.54)
Current smoker	24 (15.9)	28 (23.9)	0.57 (0.30-1.08)	0.47 (0.24-0.92)
Alcohol consumer				
No	58 (38.4)	31 (26.5)	1	1
Yes	93 (61.6)	86 (73.5)	0.58 (0.34-0.98)	0.55 (0.32-0.95)
Combined alcohol/smoking status				
Non-drinker and Non-smoker	46 (30.5)	20 (17.1)	1	1
Drinks alcohol or ever smoker	57 (37.8)	52 (44.4)	0.48 (0.25-0.91)	0.48 (0.25-0.93)
Drinks alcohol and ever smoker	48 (31.8)	45 (38.5)	0.46 (0.24-0.90)	0.41 (0.21-0.83)
History of foreign travel outside Europe				
No	96 (63.6)	58 (49.6)	1	1
Yes	55 (36.4)	59 (50.4)	0.56 (0.34-0.92)	0.59 (0.35-0.99)
Presence of any pets in the house				
No	37 (24.5)	44 (37.6)	1	1
Yes	114 (75.5)	73 (62.4)	1.86 (1.10-3.14)	1.92 (1.12-3.31)

[1]Adjusted logistic regression model includes age (at interview), sex and socioeconomic status.

ever smokers remained evident across the three socio-economic groupings (OR = 0.39, 0.61 and 0.44). The reduced risk appeared to be somewhat driven by smoking in low-class occupation holders, and alcohol consumption in high-class occupation holders, however formal tests for interaction were not statistically significant.

DISCUSSION

The results from this novel population-based study suggest that achalasia disproportionately affects individuals from lower socio-economic backgrounds. Smoking and alcohol intake do not explain this inequality in achalasia risk. Pet ownership in adulthood was associated with an increased risk of achalasia, and raises interesting hypotheses about potential explanatory biological mechanisms for achalasia. None of the childhood factors evaluated were associated with achalasia risk, suggesting that early life exposures do not have a role in achalasia development.

This is the first study to assess the relationship between socio-economic status and achalasia. Our findings indicate an increased risk of developing achalasia in individuals with lower socio-economic status. Our results also demonstrate that this is not explained by the "usual" factors associated with lower socio-economic status, namely smoking and alcohol. Instead these factors carry a reduced risk. There is little evidence of biologically plausible mechanisms to link smoking and alcohol to a reduced risk of achalasia - in contrast, nicotine exposure is known to induce loss of lower oesophageal sphincter function[19]. The findings for smoking and alcohol are highly likely to reflect reverse causation bias, since the majority of achalasia cases in this study were prevalent cases who may have avoided these lifestyle factors to alleviate symptoms. However, such bias is unlikely to have occurred to the extent whereby it is masking an increased risk of achalasia, and recall bias is unlikely to influence the other characteristics enquired about in this study. There are several other plausible associations for the link with lower socio-economic status which merit further exploration.

Firstly, lower socio-economic status is associated with increased gastro-intestinal infection risk in this region[20]. One hypothesis for the aetiology of achalasia is of a neurotropic virus showing predilection for the squamous mucosa of the oesophagus and targeting the myenteric plexus. There has been some evidence supporting this link in the herpes virus family[8,21] and a large Spanish study has recently demonstrated increased herpes zoster prevalence/incidence in subjects with lower socio-economic status[22]. Secondly, autoimmunity has been suggested as a factor in the development of achalasia. Although our study found no significant increase in auto-immune diseases among achalasia patients, this has been demonstrated previously[6] and there is strong evidence linking auto-immune disease and lower socio-economic status[23]. Thirdly, the direct association with occupation (but not education) as a reflection of socio-economic status may reflect exposure to occupational hazards that play a role in achalasia aetiology, for example metal exposure has been linked with Parkinson's disease[15]. Finally, a hypothesis that has not previously been suggested relates to perinatal factors. Low birth weight has been associated with other oesophageal diseases[12-14] and is linked with lower socio-economic status[24]. Recent epigenetic studies have demonstrated methylation changes in the perinatal period, linked to lower socio-economic status and low birth weight babies[25]. The authors suggest that this is a key element in the development of subsequent disease in adulthood[25].

We were unable to assess birth weight and other perinatal factors in this study. However, we were able to evaluate other early childhood factors in relation to achalasia risk. No associations were identified between household density, toilet location or history of having been breastfed and achalasia risk. This contrasts with hypotheses that household crowding and resultant earlier/more frequent exposure to infections and antigens could protect against immune-related diseases, as has been noted for Type 1 diabetes[26,27]. The lack of association suggests that, even if a role for infectious agents does exist for achalasia, the timing of exposure in early childhood may be irrelevant. Three other childhood factors investigated also showed non-significant associations with achalasia, but the direction of associations parallel those seen for adult lifestyle factors. This includes the presence of smokers in the childhood home, which was non-significantly inversely associated with risk, while lower socio-economic occupations held by the head of the childhood household and owning a pet in childhood both carried a non-significant increased risk of achalasia.

Pet ownership in adulthood was associated with an increased risk of achalasia in this study. Evidence to suggest an association between pet ownership and the incidence of other immune-related conditions, such as rheumatoid arthritis and multiple sclerosis (MS), is conflicting[28-32]. Pet ownership may increase exposure to parasitic infections[29] , and while secondary achalasia is due to parasitic infection with *Trypanosoma cruzi*[33], there is no evidence to suggest a direct parasitic cause in primary achalasia. Finally, households with resident pets have higher levels of pro-inflammatory endotoxin in the house dust[34]. Endotoxin is speculated to be hypoallergenic and thereby protect against atopic conditions[34]. However, as part of a separate mechanistic pathway, endotoxins may interact with viral infection to induce an inflammatory response[35]. For example, lipopolysaccharide can increase expression of a survival protein (BAG3) that regulates the replication of HSV-1 and Varicella-Zoster virus[36], and so may act to exacerbate the impact of such viruses on achalasia

development. The finding of an increased risk of achalasia with pet ownership in the current study may also be due to chance, but is unlikely to be due to recall bias. Our observed inverse association between a history of foreign travel outside of Europe, even after adjustment for socio-economic status, may also reflect exposure to an unknown infectious agent that is actually protective against achalasia development.

One of the strengths of the study is that a large number of patients with primary achalasia were recruited into the study using a population-based approach. The response rate among cases was high (75%), suggesting excellent generalisability to the wider population of patients with primary achalasia. Also, to our knowledge, this is the first case-control study investigating potential environmental risk factors in primary achalasia, providing novel insight into mechanisms and potential prevention strategies for this incurable disease.

Certain potential limitations of this study must be acknowledged. Firstly, self-reported risk factors were relied upon in this study, and may be subject to recall and socially-desirable respondent bias. This is likely to explain the significant inverse association for smoking, alcohol and achalasia risk to some extent, but is unlikely to be masking a converse positive association for these lifestyle factors and achalasia risk. Certain factors such as family history were not verified with general practitioners or other medical records. The low response rate of controls (47%) may have introduced bias if the characteristics and exposures of the non-responders were different. Unfortunately, there was no access to medical records of non-responders to compare with responders to allow evaluation of this. The case-control nature of the study design also presents an opportunity for reverse causation to be skewing some of the observed associations, whereby achalasia cases may have altered their habits relating to certain risk factors, due to their disease and symptom experience. However, these limitations affect all epidemiological case-control studies, and we still believe that our analysis provides a useful and novel insight into potential modifiable risk factors for achalasia. Further case-control and cohort studies verifying our results are required.

In conclusion, achalasia appears to be a disease of inequality that disproportionately affects individuals from low socio-economic backgrounds. This does not appear to be due to corresponding alcohol and smoking behaviours. An observed positive association between pet ownership and achalasia risk may lend support to a role for interaction between endotoxin and viral infection exposure in achalasia aetiology. Further studies of environmental factors and achalasia risk are warranted.

COMMENTS

Background

Oesophageal achalasia is one of the most poorly understood diseases of the digestive tract. Achalasia is a neurodegenerative motility disorder that results in loss of normal lower oesophageal sphincter function and aperistalsis. Relatively little attention has been given to understanding the underlying aetiology of this disease, and more efforts are needed to ultimately achieve prevention of achalasia.

Research frontiers

To our knowledge, no lifestyle factors have been investigated in relation to achalasia development. Associations and biologically plausible mechanisms have been reported for lifestyle factors in the role of other neurodegenerative disorders. The aim of this novel population-based case-control study was to evaluate the association between exposure to environmental factors throughout the lifespan and risk of oesophageal achalasia.

Innovations and breakthroughs

Achalasia disproportionately affects individuals from low socio-economic backgrounds. This does not appear to be due to corresponding alcohol and smoking behaviours. An observed positive association between pet ownership and achalasia risk may lend support to a role for interaction between endotoxin and viral infection exposure in achalasia aetiology.

Applications

This is the first study to evaluate environmental risk factors for achalasia, and raises interesting hypotheses about potential explanatory biological mechanisms for achalasia. Further studies of environmental factors and achalasia risk are warranted.

Peer-review

Coleman *et al* present a questionnaire based populational enquiry about risk factors for achalasia. The topic is interesting and some data is original. The authors discussed their findings well.

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ORIGINAL ARTICLE

Retrospective Study

Follow-up of patients with functional bowel symptoms treated with a low FODMAP diet

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Author contributions: Maagaard L and Munkholm P designed the research; all authors contributed to the set-up and content of the questionnaires; Maagaard L performed the research; Maagaard L and Végh Z analysed the data; Maagaard L wrote the paper and all the co-authors made critical revisions of its content; all authors approved the final manuscript.

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Institutional review board statement: Not needed for a simple questionnaire analysis according to rules of the Ethics Committee of Science, Denmark.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent before study enrolment.

Conflict-of-interest statement: None to declare.

Data sharing statement: Questionnaires and data set are available from the corresponding author at louisemaagaard3@gmail.com. The Danish Data Protection Agency approved the study design. Participants gave informed written consent for data sharing and furthermore, the presented data are anonymised and risk of identification is low.

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Abstract

AIM: To investigate patient-reported outcomes from, and adherence to, a low FODMAP diet among patients suffering from irritable bowel syndrome and inflammatory bowel disease.

METHODS: Consecutive patients with irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD) and co-existing IBS fulfilling the ROME Ⅲ criteria, who previously attended an outpatient clinic for low FODMAP diet (LFD) dietary management and assessment by a gastroenterologist, were invited to participate in a retrospective questionnaire analysis. The questionnaires were sent and returned by regular mail and gathered information on recall of dietary

treatment, efficacy, symptoms, adherence, satisfaction, change in disease course and stool type, and quality of life. Before study enrolment all patients had to sign an informed written consent.

RESULTS: One hundred and eighty patients were included, 131 (73%) IBS and 49 (27%) IBD patients. Median age was 43 years (range: 18-85) and 147 (82%) were females. Median follow-up time was 16 mo (range: 2-80). Eighty-six percent reported either partial (54%) or full (32%) efficacy with greatest improvement of bloating (82%) and abdominal pain (71%). The proportion of patients with full efficacy tended to be greater in the IBD group than in the IBS group (42% *vs* 29%, $P = 0.08$). There was a significant reduction in patients with a chronic continuous disease course in both the IBS group (25%, $P < 0.001$) and IBD group (23%, $P = 0.002$) along with a significant increase in patients with a mild indolent disease course of 37% ($P < 0.001$) and 23% ($P = 0.002$), respectively. The proportion of patients having normal stools increased with 41% in the IBS group ($P < 0.001$) and 66% in the IBD group ($P < 0.001$). One-third of patients adhered to the diet and high adherence was associated with longer duration of dietary course ($P < 0.001$). Satisfaction with dietary management was seen in 83 (70%) IBS patients and 24 (55%) IBD patients. Eighty-four percent of patients lived on a modified LFD, where some foods rich in FODMAPs were reintroduced, and 16% followed the LFD by the book without deviations. Wheat, dairy products, and onions were the foods most often not reintroduced by patients.

CONCLUSION: These data suggest that a diet low in FODMAPs is an efficacious treatment solution in the management of functional bowel symptoms for IBS and IBD patients.

Key words: Low FODMAP; Irritable bowel syndrome; Inflammatory bowel disease; Adherence; Disease course

Core tip: This is a retrospective study based on patient-reported questionnaires to evaluate the low FODMAP diet (LFD) dietary course of patients with irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD). Effect was reported by 86% of patients with greatest relief of abdominal pain and bloating. Long-term IBS disease course and stool type improved significantly after dietary intervention. One-third of patients were adherent and the majority was satisfied with the treatment. These are the first data on changes of long-term IBS disease course following LFD treatment and the longest FU to date of IBS and IBD patients treated with the LFD with a median FU of 16 mo.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a highly prevalent functional gastrointestinal disorder characterised by abdominal pain or discomfort in association with altered bowel habits and no organic disease. IBS symptoms are prevalent in about 10%-20% of the general population, with a female gender predilection and a high proportion of undiagnosed sufferers, making IBS a major health issue[1-3]. Furthermore, IBS-like symptoms are common in patients with inflammatory bowel disease (IBD) and are present in between 30%-40% of patients in clinical remission[4-6].

Treatment of IBS is a challenging task for primary care physicians and gastroenterologists due to the heterogeneity of the disorder, a lack of reliable outcome measures, and high placebo response rates. In the last decades, a diet excluding foods high in short-chained carbohydrates, termed FODMAPs (fermentable oligo-, di-, and monosaccharides and polyols), has proven effective in the treatment of functional gastrointestinal symptoms[7]. FODMAPs are poorly absorbed in the small intestine and are passed on to the colon, where they exert an osmotic effect, drawing fluid into the lumen and, furthermore, causing an increase in gas production (mainly hydrogen and methane) due to the excess delivery of fermentable substrates to the colonic microflora[8]. These mechanisms can lead to abdominal pain, bloating, flatulence, and diarrhoea in susceptible subjects[9,10]. There is strong evidence supporting the low FODMAP diet (LFD) as an effective therapeutic tool in the management of IBS with an overall response rate of 75[7,9,11-13]. The diet also seems to reduce functional bowel symptoms in patients with inflammatory bowel disease[14,15] and patients without a colon[8].

Adherence to the diet is key to its success and according to previous studies, adherence can be expected in up to 75% of patients[12,14,16]. However, many struggle with implementing the LFD in daily life due to its complexity. Currently, studies of long-term efficacy of the LFD are lacking, and no one has yet investigated the dietary impact on IBS disease course.

The present retrospective study aimed to examine patient-reported long-term effects of the LFD, dietary adherence, and dietary impact on disease course in patients with IBS and patients with IBD and co-existing IBS.

MATERIALS AND METHODS

Study design

A retrospective, cross-sectional study was conducted

Table 1 Dietary adherence at follow-up, estimated by the FODMAP adherence report scale *n* (%)

FARS questions	All patients	IBD	IBS
"I change the content of a LFD meal despite the recommended content"	57 (35)	12 (28)	45 (37)
"I follow a modified LFD compared to the recommended LFD"	61 (37)	15 (34)	46 (38)
"I replace a LFD meal with a regular meal containing FODMAPs"	90 (55)	27 (63)	63 (53)
"I forget to follow the LFD"	111 (68)	33 (75)	78 (65)
"I stop taking the LFD for a period of time"	107 (65)	34 (77)	73 (61)

Each of the FARS questions had five possible responses, scoring 1-5 points. A score of ⩾ 4 points was regarded as adherence to the LFD. FARS: FODMAP adherence report scale; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; LFD: Low FODMAP diet; FODMAPs: Fermentable oligo-, di- and monosaccharides and polyols.

to investigate long-term adherence and effect on disease course in IBS and IBD patients treated with the LFD.

Study population

Consecutive patients with IBS or IBD and co-existing IBS fulfilling the Rome Ⅲ criteria[17] for IBS and having received LFD education followed by a dietary course of varying duration in the period 2009-2013, were invited to participate in the study. All patients had initially been treated by their general practitioner and subsequently been referred to Herlev University Hospital (HUH) for dietary management of IBS with clinical dieticians.

All study participants, or their legal guardian, provided informed written consent prior to study enrolment. Participants gave written informed consent for data sharing.

Prior to dietary consultation, gastroenterologists had assessed all patients and the majority of patients presented normal colonoscopy results, and were tested for lactose intolerance and celiac disease, among other relevant tests.

Patients were excluded from the study if they had significant gastrointestinal co-morbidities such as abdominal cancer or ileo-/colostomy. IBD patients were not tested for level of disease activity at follow-up.

eHealth: A web-program for IBS and IBD patients

Some of the recruited patients (103, 57%) had earlier been engaged in other LFD studies at HUH involving eHealth web-program monitoring and a dietary course of six weeks with follow-up evaluation[15,17,18]. The program has been described in previous papers[19]. The majority of IBD patients (40, 82%) included here participated in these eHealth studies, while patients with moderate to severe disease activity (assessed by HBI or SCCAI) were excluded.

Data collection

Patients eligible for the study received a letter containing an invitation, an informed consent form, and a questionnaire regarding the dietary treatment, adherence to diet, disease severity and course, stool pattern, and quality of life. If the patients did not reply to the invitation, reminders were sent by regular mail. Before accessing electronic patient files for extraction of additional demographic data, an informed written consent for data sharing had to be signed by the patients in accordance with the Danish health authority regulations.

Dietary advice

Four experts in clinical nutrition and the LFD performed the dietary consultations. One dietician was FODMAP-certified at King's College, London, United Kingdom[20]. The initial consultation lasted 60-90 min and included IBS education, dietary history, LFD education, and individualisation of LFD advice in order to facilitate implementation of the diet. Patients were to stay on the diet for 6-8 wk and then review the treatment response with support from the dietary expert. If the response was considered satisfactory, patients continued the restriction of FODMAPs, but with reintroduction of small amounts of foods high in FODMAPs in order to determine individual tolerance level and ensure variety in diet. The patients were offered follow-up consultations either in clinic or by telephone and were also able to email the experts.

Questionnaires

At follow-up, all patients were asked to complete a questionnaire analysis including four self-developed questionnaires and three or four internationally validated questionnaires.

The first questionnaire, developed in cooperation with clinical dieticians, consisted of 23 questions with limited answering options or visual analogue scales (VAS) addressing efficacy of diet, dietary management, and compliance. The FODMAP Adherence Report Scale (FARS) was constructed to evaluate dietary adherence and was inspired by the validated Medication Adherence Report Scale by Byrne *et al*[21]. The scale consists of five questions (see Table 1), each question offering five possible answers (always, often, sometimes, rare, and never) scoring from one to five points with a maximum score of 25 points. A total score of at least 20 points (⩾ 80%) was considered as adherence to the diet. Furthermore, a questionnaire previously applied in a study by Pedersen *et al*[17] at HUH was included in order to assess satisfaction with the dietary treatment. Its six questions were accompanied by VAS scales, with a scoring range of 0-100 points (*i.e.*, 1 cm = 10 points), and covered the following items: dietary consultations, distributed written material, flavour of diet, compliance/adherence to diet, and availability of appropriate foods in supermarkets. The maximum score was 600 points and a total score of a minimum of 360 points (⩾

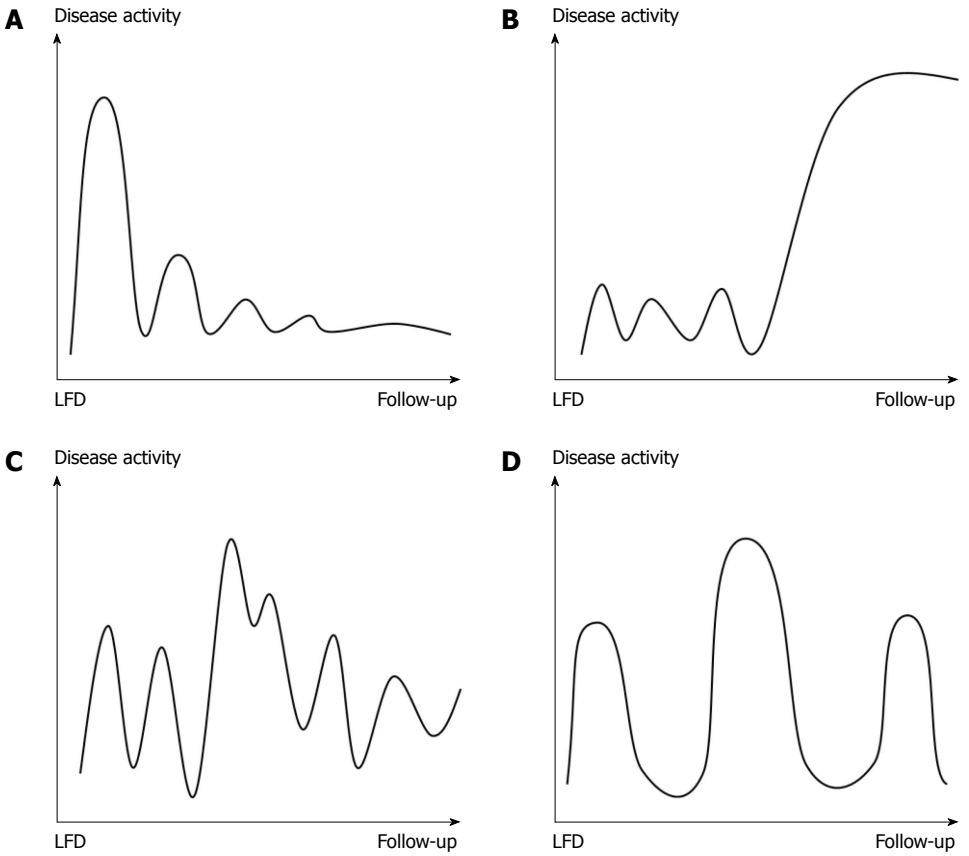


Figure 1 Copenhagen irritable bowel syndrome disease courses. The four figures each depict a different type of IBS disease course with varying disease activity over time. The time span is from introduction of the LFD until follow-up. The same figures were used from time of diagnosis to introduction of the LFD (not shown). A: Mild IBS with indolent course: The disease activity fades over time; B: Mild IBS with aggressive course: The disease activity increases over time; C: Chronic IBS with continuous course: There is constant disease activity without remission periods; D: Chronic IBS with intermittent course: The disease activity appears in relapses with remission periods in between. LFD: Low FODMAP diet; IBS: Irritable bowel syndrome.

60%) was considered as satisfaction with the dietary treatment.

The last questionnaire developed addressed changes in IBS disease course prior to, and after, dietary intervention and consisted of four figures depicting different types of disease courses, the Copenhagen IBS disease courses (see Figure 1). The figures were constructed based on several years of clinical experience in IBS management and studies of pattern recognition of IBD disease courses[22,23]. The mild indolent disease course was considered the preferred type, as disease activity decreased over time. The patients had to choose one figure representing their disease course before and after dietary management.

The Bristol Stool Chart (BSC)[24,25] illustrates seven different stool types representing constipation (type 1-2), normal stools (type 3-4), and diarrhoea (type 5-7), and was used retrospectively to assess stool type prior to and after dietary treatment. The IBS Severity Scoring System (IBS-SSS)[26] was applied to measure IBS disease severity at follow-up and consists of five questions combined with VAS-scales, with the maximum score being 500 points. Remission/mild disease is classified as a score less than 175 points, moderate disease as a score between 175 and 300 points, and above 300 points the disease is severe. Quality of life was evaluated at follow-up using the IBS Quality of Life questionnaire (IBS-QoL)[27] that contains 34 questions, each with five possible answers and a scoring range of 34-170 points. Due to the absence of an official cut-off for good quality of life, we arbitrarily set the bar at $\leqslant$ 102 points (50%).

Finally, the Short IBD Questionnaire (SIBDQ)[28] is composed of 10 questions and was used to measure quality of life at follow-up for IBD patients only. A total score of 50 points or more was considered to indicate a good quality of life[29].

Statistical analysis

Statistical analysis was performed using the SAS v. 9.3 (NC, United States) and SPSS v. 20 (IL, United States) statistical software packages. Standard descriptive statistics were carried out including frequency distributions for categorical data and calculation of medians and ranges for continuous variables. Fisher's exact and χ^2 tests were used to investigate whether or not the differences in descriptive data between groups were significant. The Wilcoxon ranked test was applied to determine if there had been significant changes in disease course and stool type from baseline to follow-

Table 2 Demographic data at follow-up *n* (%)

	IBS	IBD
Patients	131 (73)	49 (27)
Participation in eHealth studies	63 (48)	40 (82)
IBD type		
UC		32 (65)
CD		12 (25)
IBDU		5 (10)
Female	107 (82)	40 (82)
Age, median (range)	43 (18-85)	44 (19-70)
Height, median (range)	168 (133-189)	171 (160-189)
Weight, median (range)	65 (43-115)	75 (49-146)
BMI, median (range)	23 (16-45)	25 (18-53)
Smokers	15 (14)	5 (12)
IBS subtypes		
IBS-D	47 (40)	28 (67)
IBS-C	44 (37)	6 (14)
IBS-M	17 (14)	3 (7)
IBS-U	10 (9)	5 (12)
IBS disease severity		
Mild	41 (32)	23 (53)
Moderate	56 (43)	14 (33)
Severe	32 (25)	6 (14)
Lactose intolerance[1]	18 (25)	1 (6)
Gluten intolerance[2]	2 (2)	1 (8)
Food allergy[3]	5 (24)	5 (42)
Dietary consultations		
None	4 (4)	0 (0)
1	30 (28)	14 (33)
2	33 (30)	22 (53)
3 or more	41 (38)	6 (14)
Follow-up time, median (range)	15 (2-80)	17 (5-32)

[1]Diagnosed by lactose intolerance test or genetic test of lactase; [2]Diagnosed by serologic test for immunoglobulin A tissue transaminase; [3]Diagnosed by serologic tests for specific immunoglobulins. IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; IBDU: Undetermined inflammatory bowel disease; BMI: Body mass index; IBS-D: IBS with diarrhoea; IBS-C: IBS with constipation; IBS-M: Mixed IBS; IBS-U: Unsubtyped IBS.

up. Fisher's exact test and multiple logistic regression were performed to examine the relationship between responses and explanatory variables. All reported *P*-values are two-sided and tests were performed with a 5% level of significance. The statistical methods of this study were reviewed by Henrik Wachmann, Larix A/S.

RESULTS

Demographic data

Four hundred and three (294 IBS, 109 IBD) patients eligible for the study were identified. Fifteen were excluded due to co-morbidity (eight), migration (four), or uncertain IBS diagnosis (three). Forty patients rejected the invitation. Of the remaining 348 patients, a total of 180 patients (52%), 131 IBS and 49 IBD, answered one or more questionnaires and were included in the study. Demographic characteristics are presented in Table 2. Twenty (11%) patients did not consent to extraction of data from their electronic patient files; therefore, only data from questionnaires

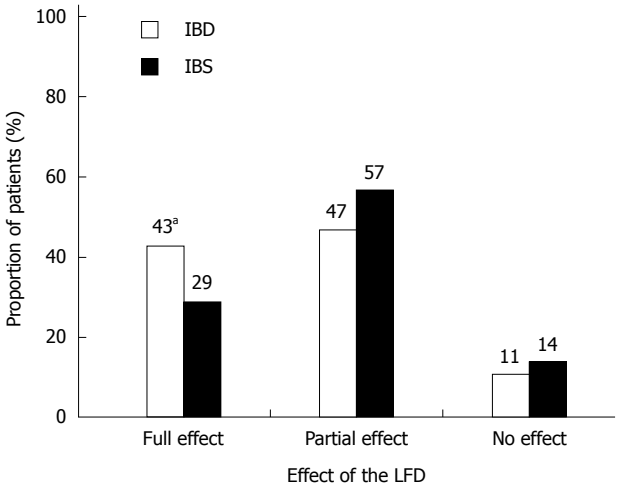


Figure 2 Patient-reported effectiveness of the low FODMAP diet in inflammatory bowel disease and irritable bowel syndrome patients at follow-up. Effectiveness was categorised as full, partial, or no effect. There were more IBD patients with full effect than IBS patients ([a]*P* = 0.08). IBD: *n* = 47; IBS: *n* = 126. LFD: Low FODMAP diet; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome.

were available. There were significant differences in demographic data between the two groups regarding IBS subtypes and IBS severity at follow-up. The IBS-D subtype and IBS-C subtype were more frequent in the IBD ($P < 0.01$) and IBS ($P < 0.01$) group, respectively, and the proportion of IBD patients with mild IBS at follow-up was significantly greater when compared to the IBS patients ($P = 0.01$). The median duration of follow-up from the initial dietary consultation to the completion of the questionnaire analysis was 16 (range: 2-80) months overall, with 15 (range: 2-80) months for the IBS group, and 17 (range: 5-32) mo for the IBD group.

Efficacy and symptoms

One hundred and fifty patients (86%) reported either partial (94, 54%) or full (56, 32%) effectiveness of dietary treatment (Figure 2). The proportion of patients experiencing full effectiveness was greater in the IBD group than in the IBS group (42% *vs* 29%, $P = 0.08$). The diet showed greatest effect on bloating (82%) and abdominal pain (71%) (Figure 3). Furthermore, 46 (37%) IBS patients and 21 (24%) IBD patients became asymptomatic while following the diet.

Disease course and stool type

Figure 4 illustrates changes in IBS disease course related to the LFD. After dietary treatment, the number of patients with a chronic continuous course was significantly reduced in both patient groups (IBS: -25%, $P < 0.001$; IBD: -23%, $P = 0.002$), while the mild indolent course became the predominant type (IBS: +37%, $P < 0.001$; IBD: +23%, $P = 0.002$). The mild indolent disease course following LFD intervention was associated with good quality of life and normal stool pattern ($P < 0.0001$). Furthermore, mild indolent

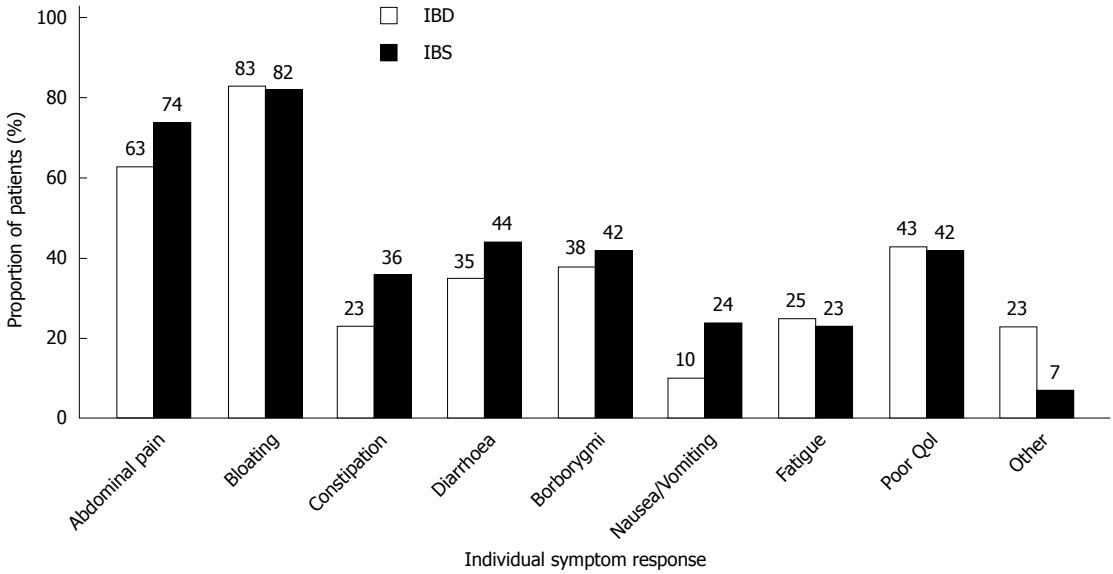


Figure 3 Patient-reported symptom relief for individual symptoms. Patients were able to select as many symptoms as they felt appropriate according to subjective symptom improvement following LFD treatment. The majority experienced relief of abdominal pain and bloating. IBD: *n* = 40; IBS: *n* =113. QoL: Quality of life; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome.

disease course prior to LFD was a strong predictor of a disease course persisting beyond the LFD (84%, $P < 0.001$). Patients starting on one of the three other less favourable disease courses had a probability of about 40% (range: 39%-46%) of transitioning to the mild indolent course after dietary treatment. The baseline variables showed no influence on the probability of changing from one of the three less favourable disease course types to the mild indolent course.

There was a significant improvement of stool pattern in both patient groups (Figure 5). After dietary intervention, the proportion of patients producing normal stools increased, with 41% in the IBS group ($P < 0.001$) and 66% in the IBD group ($P < 0.001$).

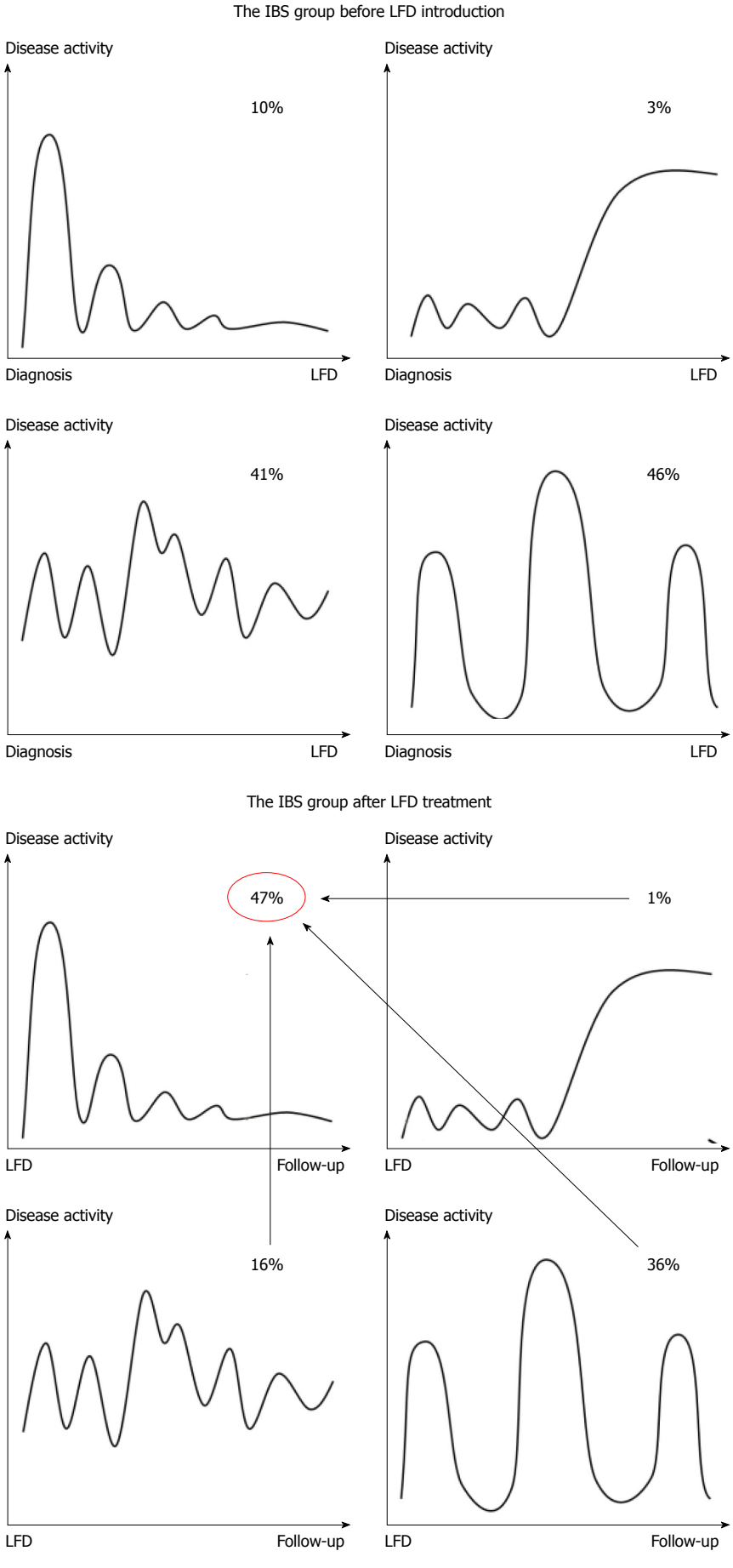
Adherence and satisfaction

In both patient groups, approximately one-third were adherent to the diet according to the FARS. Adherence was highest with regards to remembering to follow the diet and not taking breaks, and poorest when asked if patients followed the diet "by the book" and without making their own modifications (Table 2). Increased adherence was associated with longer duration of dietary treatment ($P < 0.001$), but otherwise no associations were found. Thirty-two percent of the IBS group and 37% of the IBD group were on the diet for less than three months, while 47% and 50%, respectively, stayed on the diet until follow-up. Fifty-four percent used the LFD on and off depending on symptom severity, while the rest were continuously on the diet. Eighty-four percent lived on a modified LFD, where some foods rich in FODMAPs were reintroduced in varying amounts according to individual tolerance level, whereas the remaining patients followed the LFD by the book without deviations. Wheat, dairy products, and onions were the foods most often not reintroduced by patients. Weight loss occurred in 29% of patients, while 7% gained weight during the dietary course. Thirty-three (26%) IBS patients and 8 (20%) IBD patients answered that they quit the diet before completion of the standard dietary period. The most common reasons for quitting were that the diet was too complicated to follow (50%), too expensive (23%), or bland in taste (15%), and other reasons (53%) were co-morbidities and detrimental effect on functional gastrointestinal symptoms. Satisfaction with dietary management was seen in 83 (70%) IBS patients and 24 (55%) IBD patients.

Disease severity and quality of life

The overall median IBS-SSS score at follow-up was 211 (range: 16-487). Mild IBS was associated with mild indolent disease course prior to LFD ($P < 0.01$) and, furthermore, was trending towards a normal stool type after LFD ($P = 0.068$). IBD patients were more likely to have less severe IBS at follow-up than were IBS patients ($P < 0.05$).

At follow-up, the median IBS-QoL score was 75 (range: 37-145) for the IBS group and 63 (range: 36-126) for the IBD group corresponding to 70 (range: 15-98) and 79 (range: 32-99) on the transformed scale, respectively. For the IBD patients only, the median SIBDQ score was 55 (range: 23-69) and 75 (range: 22-98) on the transformed scale. Good quality of life was associated with normal stool type after dietary therapy ($P < 0.001$) and duration of dietary course ($P < 0.01$). A tendency to report good quality of life at follow-up was observed in non-smokers and patients with normal stool pattern before introduction of LFD ($P = 0.068$, $P = 0.060$). IBD patients were observed to be more content with life as compared to IBS patients (89% *vs* 73%, $P < 0.05$).


The IBS group before LFD introduction
Disease activity
10%
Diagnosis
LFD
Disease activity
3%
Diagnosis
LFD
Disease activity
41%
Diagnosis
LFD
Disease activity
46%
Diagnosis
LFD
The IBS group after LFD treatment
Disease activity
47%
LFD
Follow-up
Disease activity
1%
LFD
Follow-up
Disease activity
16%
LFD
Follow-up
Disease activity
36%
LFD
Follow-up

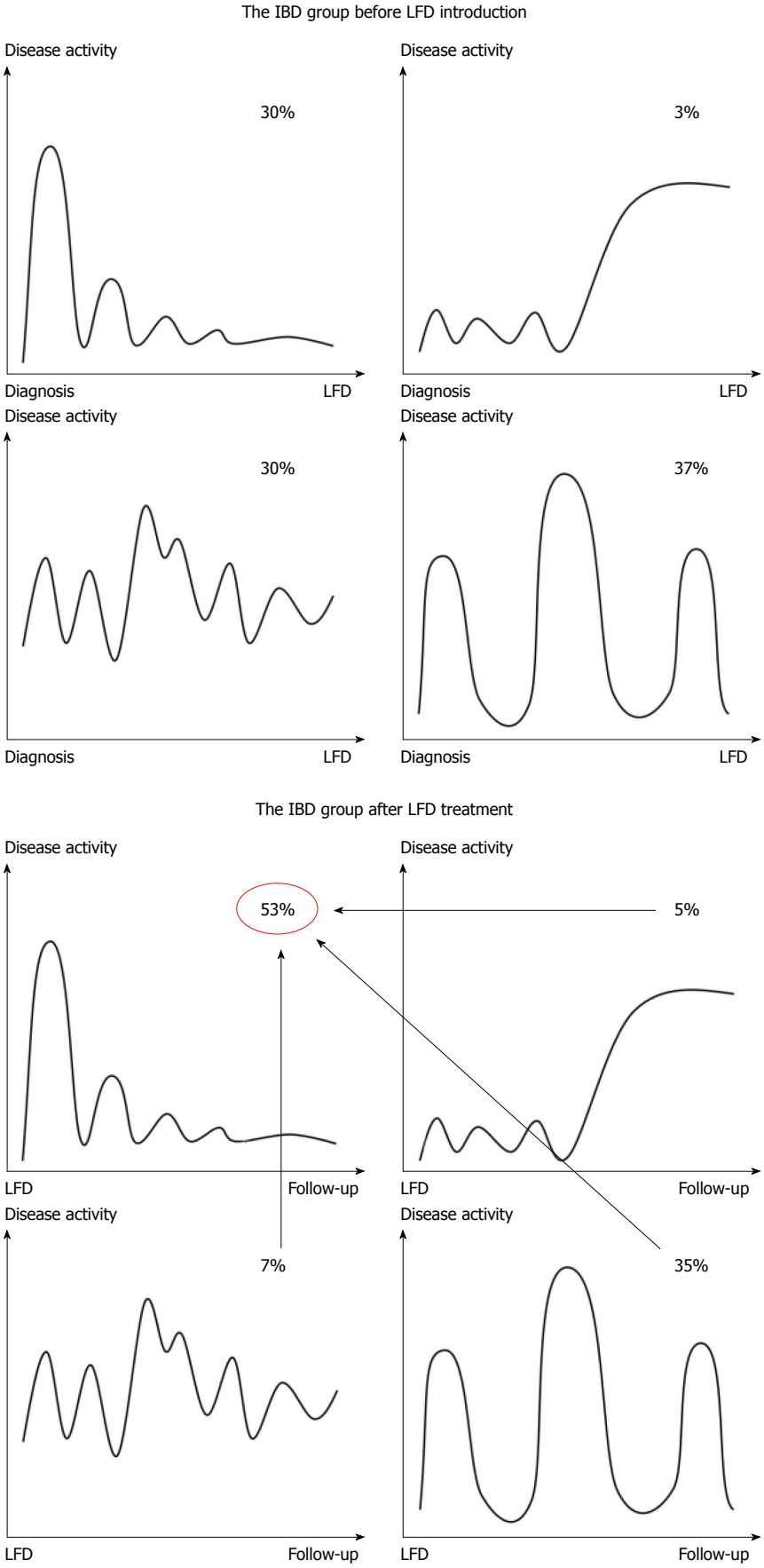


Figure 4 Changes in Copenhagen IBS disease courses after low FODMAP diet treatment. The four different disease courses are depicted before and after the LFD intervention for the IBD and IBS group, separately. Above each figure, the prevalence is denoted as a percentage. The mild indolent course increased significantly following LFD introduction in both patient groups ($P < 0.001$), while the chronic continuous course and the intermittent course were less common at follow-up. IBD: $n = 43$; IBS: $n = 120$. LFD: Low FODMAP diet; IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

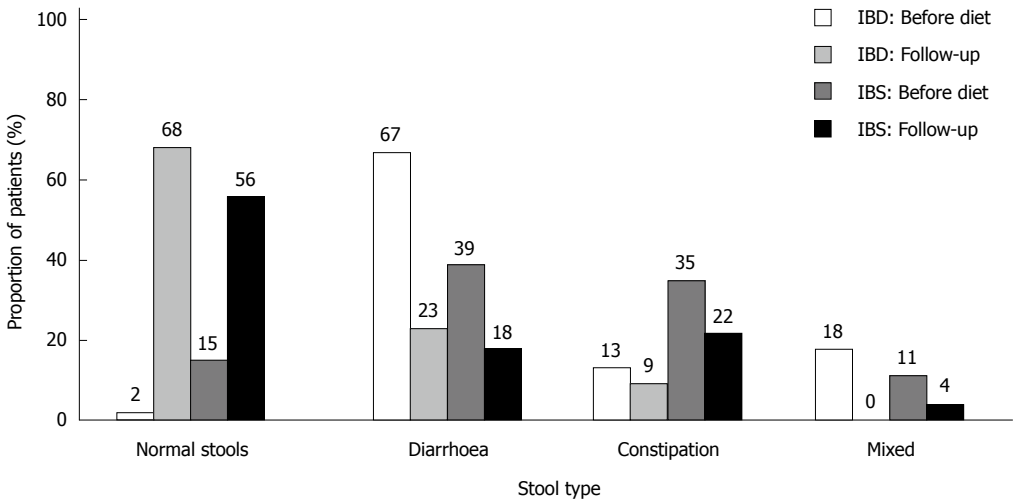


Figure 5 Changes in stool type after low FODMAP diet treatment. The stool types (Bristol stool chart) was categorised as normal stools, diarrhoea, constipation, and mixed. After the LFD, there was a significant increase in the prevalence of normal stools in both IBD and IBS patients ($P < 0.001$) with decreases in the remaining three categories of stool type, in particular the diarrhoea group ($P < 0.001$). IBD: $n = 43$; IBS: $n = 121$. LFD: Low FODMAP diet; IBS: Irritable bowel syndrome; IBD: Inflammatory bowel disease.

DISCUSSION

This study focuses on long-term outcomes of LFD treatment and hopes to increase global awareness of FODMAP restriction. It is the first survey ever to assess the impact of LFD on long-term IBS disease course and, furthermore, the longest follow-up study on IBS and IBD patients treated with the LFD.

Long-term IBS disease courses improved significantly after the LFD period. Mild indolent disease course at baseline was a reliable predictor of remaining on this course after treatment. However, patients starting with one of the less favourable disease courses had a probability of about two in five of transitioning to a mild indolent course. This suggests that patients with more severe chronic courses might be able to improve their disease course with LFD management. Having said that, these data are the first on IBS disease course in relation to LFD and prospective cohort studies are needed for further investigation of whether or not LFD can improve disease course of IBS patients.

The prevalence of a normal stool type increased significantly after dietary therapy. Improvement of stool pattern occurred across all three groups of stool types, particularly in the diarrhoea group. An improved outcome among patients with diarrhoea was also found in a recent prospective study comparing LFD to a normal diet[13]. The enhanced efficacy of LFD in patients with diarrhoea could be explained by the osmotic nature of pooled FODMAPs in a regular diet which contributes to increased water output[8].

Previous studies found adherence to be of paramount importance to the diet's success[12,14,16]. In this study, only one-third of the patients were adherent to the diet. High adherence was associated with longer duration of dietary treatment. No more than half of patients were still on the diet at follow-up; nevertheless, the majority was satisfied with the dietary treatment and did not quit the dietary course before planned. The probability of patients discontinuing dietary management increases with duration of dietary course, as motivation tends to dwindle. Gearry *et al*[14] found that use of resources (*e.g.*, cookbooks), a higher educational level, and working fewer than thirty-five hours a week were significantly related to better adherence.

At follow-up, most patients reported good quality of life and had only mild or moderate disease severity. Good quality of life was associated with normal stool type after diet and longer duration of dietary treatment, while mild disease severity was related to having a mild indolent disease course before LFD introduction. Two recent prospective studies by Pedersen *et al*[15,17] investigated the efficacy of LFD as compared to a normal diet in IBS and IBD patients and demonstrated a significant reduction in IBS disease severity and IBD disease activity, along with a significant increase in quality of life among IBD patients and IBS-D patients during the six weeks of LFD intervention.

A retrospective study is not the ideal way to assess efficacy of therapy and is accompanied by some limitations. The high rate of patients (48%) not replying to the invitation to join the study could have led to selection bias which, together with possible recall bias, might have resulted in type I errors.

In the last decade it has been suggested that FODMAPs increase endothelial barrier permeability and, together with other factors, cause immune activation and low-grade inflammation, which could play a crucial role in the pathogenesis of IBS[30,31]. FODMAPs have been shown to influence on the colonic microbiota, and Halmos *et al*[32] recently found that diets differing in FODMAP content significantly affected the gut microflora composition and that a low FODMAP intake

was associated with reduced absolute abundance of bacteria. As a LFD might lead to detrimental changes in gut microbiota, caution should be taken when recommending the LFD for long-term treatment.

Although there are limitations of this study, the retrospective follow-up provided data supporting the use of LFD in both IBS and IBD with co-existing IBS. A majority of patients reported beneficial effects and satisfaction with the dietary treatment and, furthermore, long-term disease courses and stool pattern improved significantly. Long-term, prospective studies are needed to further investigate those characteristics of patients responding to the diet, dietary impact on IBS disease course, and the safety of long-term FODMAP restriction.

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COMMENTS

Background

Treatment of irritable bowel syndrome (IBS) is challenging due to the heterogeneity of the disorder, a lack of reliable outcome measures, and high placebo response rates. The low FODMAP diet excludes foods high in short-chained carbohydrates termed FODMAPs (fermentable oligo-, di-, and monosaccharides and polyols) and has been demonstrated to be effective in the treatment of functional gastrointestinal symptoms. FODMAPs are poorly absorbed in the small intestine and are passed on to the colon, where they exert an osmotic effect, drawing fluid into the lumen and causing an increase in gas production (mainly hydrogen and methane) due to the excess delivery of fermentable substrates to the colonic microflora. These mechanisms can lead to abdominal pain, bloating, flatulence, and diarrhoea in susceptible subjects. In this long-term follow-up study, we evaluated the patient-reported outcomes among those with IBS and inflammatory bowel disease (IBD) after low FODMAP diet (LFD) treatment.

Research frontiers

Currently, studies of long-term efficacy of the LFD are lacking, and no one has yet investigated the dietary impact on IBS disease courses. The results of this study provide long-term patient-reported outcomes of LFD treatment and details on implementing the LFD.

Innovations and breakthroughs

The majority of IBS and IBD patients reported beneficial effects and satisfaction with the dietary treatment, although only one third was adherent. Furthermore, long-term IBS disease courses and stool patterns improved significantly. Eighty-four percent of patients lived on a modified LFD, where some foods rich in FODMAPs were reintroduced, and 16% followed the LFD by the book without deviations. Wheat, dairy products, and onions were the foods most often not reintroduced by patients.

Applications

This study suggests that the LFD is effective in the management of functional gastrointestinal (GI) symptoms in IBS patients and IBD patients in remission.

Peer-review

This study is of relevance and importance given the widespread use of the low FODMAP diet in the management of patients with IBS, and those with functional GI symptoms in IBD. Although there are clearly limitations with such a retrospective study based on patient self-report questionnaires, the study provides some interesting long-term data.

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ORIGINAL ARTICLE

Retrospective Study

Risk of lymph node metastasis in mixed-type early gastric cancer determined by the extent of the poorly differentiated component

Chung-Su Hwang, Sangjeong Ahn, Bong-Eun Lee, So-Jeong Lee, Ahrong Kim, Chang In Choi, Dae Hwan Kim, Tae-Yong Jeon, Gwang Ha Kim, Geum Am Song, Do Youn Park

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Author contributions: Hwang CS and Ahn S contributed equally to this work; Park DY designed the research; Lee BE, Lee SJ, Kim A, Choi CI, Kim DH, Jeon TY, Kim GH and Song GA performed the research; Park DY and Hwang CS analyzed the data and wrote the paper; Ahn S made critical revisions; Park DY and Ahn S approved the final version of the article.

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Institutional review board statement: This study was reviewed and approved by the institutional Review Board of the Pusan National University Hospital (E-2014130).

Informed consent statement: This retrospective study was performed under IRB approval, all data is de-identified for statistical analysis. Therefore, we think that a waiver of informed consent may be justifiable under this situation. The bio-specimens for this study were provided by the Pusan National University Hospital, a member of the National Biobank of South Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. All samples derived from the National Biobank of South Korea were obtained with informed consent under institutional review board-approved protocols.

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Abstract

AIM: To predict the rate of lymph node (LN) metastasis in diffuse- and mixed-type early gastric cancers (EGC) for guidelines of the treatment.

METHODS: We reviewed 550 cases of EGC with

diffuse- and mixed-type histology. We investigated the clinicopathological factors and histopathological components that influence the probability of LN metastasis, including sex, age, site, gross type, presence of ulceration, tumour size, depth of invasion, perineural invasion, lymphovascular invasion, and LN metastasis status. We reviewed all slides and estimated the proportions of each tumour component; pure diffuse type, mixed-predominantly diffuse type (diffuse > intestinal type), mixed-predominantly intestinal type (intestinal > diffuse type), and mixed diffuse = intestinal type. We calculated the extents of the respective components.

RESULTS: LN metastasis was observed in 12.9% (71/550) of early gastric cancers cases [15/288 mucosal EGCs (5.2%) and 56/262 submucosal EGCs (21.4%)]. Of 550 cases, 302 were diffuse-type and 248 were mixed-type EGCs. Of 248 mixed-type EGCs, 163 were mixed-predominantly diffuse type, 82 were mixed-predominantly intestinal type, and 3 were mixed diffuse = intestinal type. Mixed-type cases with predominantly diffuse type histology showed a higher frequency of LN metastasis (20.2%) than cases of pure diffuse type (9.3%) and predominantly intestinal type (12.2%) histology. We measured the dimensions of each component (intestinal and diffuse type) to determine the association of the extent of each component with LN metastasis in mixed-type gastric carcinoma. The total tumour size and the extent of poorly differentiated components was associated with LN metastasis, while that of signet ring cell components was not.

CONCLUSION: We recommend careful identification and quantitative evaluation of mixed-type early gastric cancer components after endoscopic resection to determine the intensity of the treatment.

Key words: Lymph nodes; Metastasis; Gastric cancer; Histology; Endoscopic gastrointestinal surgery

Core tip: This study consolidates correlations between clinicopathological characteristics of early gastric cancer and the risk of lymph node (LN) metastasis, which is important to select patients that will benefit from endoscopic submucosal dissection. This paper also shows that the amount of poorly differentiated tumor cells within mixed type early gastric cancer is correlated with the highest risk of LN metastasis, thus highlighting the need for diligent histological characterization of patient specimens.

Hwang CS, Ahn S, Lee BE, Lee SJ, Kim A, Choi CI, Kim DH, Jeon TY, Kim GH, Song GA, Park DY. Risk of lymph node metastasis in mixed-type early gastric cancer determined by the extent of the poorly differentiated component. *World J Gastroenterol* 2016; 22(15): 4020-4026 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4020.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4020

INTRODUCTION

Endoscopic submucosal dissection (ESD), a recently developed technique, is a widely accepted treatment modality for early gastric cancer (EGC), including for diffuse (undifferentiated) type EGC within the expanded criteria (mucosal cancer measuring less than 2 cm without ulceration and lymphovascular tumour emboli). Large datasets indicate almost no risk of lymph node (LN) metastasis in diffuse type EGCs[1-3]. A considerable proportion of gastric adenocarcinomas show a mixed composition with both intestinal and diffuse histological components. No consensus has been established regarding the management of mixed-type histology, *i.e.*, varying proportions of diffuse and intestinal components in the tumour.

Generally, gastric adenocarcinoma can be subclassified into intestinal (differentiated) and diffuse (undifferentiated) types based on gland formation[4,5]. According to Japanese guidelines, diffuse components are further subdivided into signet ring cell carcinoma and poorly differentiated carcinoma[6]. These guidelines refer to mixed-type EGCs by their predominant histological component, whether intestinal or diffuse. There are several reports on the management and outcomes of mixed-type EGCs[7-11]. Takizawa *et al*[7] reported that mixed-predominantly diffuse type showed significant LN metastasis and suggested that their treatment be similar to that of diffuse-type EGCs. Hanaoka *et al*[8] reported that the predominantly undifferentiated mixed-type tumours showed considerably higher LN metastasis than other types of submucosal invasive EGCs. Furthermore, Min *et al*[9] reported that mixed intestinal- and diffuse-type EGCs could be managed with ESD under the expanded criteria.

We investigated 550 cases of diffuse- and mixed-type EGCs to determine which components of the diffuse subtypes (signet ring cell or poorly differentiated carcinoma) are more closely associated with LN metastasis, providing evidence to establish a consensus for the management of diffuse- and mixed-type EGCs with ESD.

MATERIALS AND METHODS

Patient selection and clinicopathological evaluation

We retrieved early gastric cancer cases that involved gastrectomies with LN dissection at the Pusan National University Hospital treated between 2008 and 2013, and selected 550 cases of EGC with diffuse- and mixed-type histology based on the Japanese classification of gastric cancer[6]. Cases involving

Table 1 Clinicopathological factors and lymph node metastasis in early gastric cancers *n* (%)

	Total	Lymph node status		*P* value
		Negative (*n* = 479)	Positive (*n* = 71)	
Sex				0.950
Male	304	265 (87.2)	39 (12.8)	
Female	246	214 (87.0)	32 (13.0)	
Age (yr)	550	54.5 ± 0.7	55.5 ± 2.5	0.686
Site[1]				0.059
Upper	129	120 (93.0)	9 (7.0)	
Middle	252	217 (86.1)	35 (13.9)	
Lower	169	142 (84.0)	27 (16.0)	
Gross type				0.030
Elevated	59	45 (76.3)	14 (23.7)	
Flat	51	46 (90.2)	6 (9.8)	
Depressed	440	388 (88.2)	52 (11.8)	
Ulceration				0.640
Absent	445	389 (87.4)	56 (12.6)	
Present	105	90 (85.7)	15 (14.3)	
Tumour size (cm)[2]				< 0.0001
≤ 1	40	39 (97.5)	1 (2.5)	
≤ 2	126	120 (95.2)	6 (4.8)	
≤ 3	141	125 (88.7)	16 (11.3)	
≤ 3	243	195 (80.2)	48 (19.8)	
Depth of invasion[3]				< 0.0001
Mucosa	288	273 (94.8)	15 (5.2)	
M2	50	49 (98.0)	1 (2.0)	
M3	238	224 (94.1)	14 (5.9)	
Submucosa	262	206 (78.6)	56 (21.4)	
SM1	33	30 (90.9)	3 (9.1)	
SM2	67	55 (82.1)	12 (17.9)	
SM3	162	121 (74.7)	41 (25.3)	
Depth (μm)	262	1754 ± 89.4	2017 ± 130.3	0.153
Perineural invasion				< 0.0001
Absent	495	442 (89.3)	53 (10.7)	
Present	55	37 (67.3)	18 (32.7)	
LV invasion				< 0.0001
Absent	518	464 (89.6)	54 (10.4)	
Present	32	15 (46.9)	17 (53.1)	

[1]Between upper *vs* middle+lower; [2]Between ≤ 2 cm *vs* > 2 cm; [3]Between M *vs* SM. LV: Lymphovascular; M2: Lamina propria invasion; M3: Muscularis mucosae invasion without penetration; SM1: Upper third; SM2: Middle third; SM3: Lower third.

preoperative chemotherapy or radiotherapy or multiple gastric cancers were excluded. Pathological reports and electronic medical records were reviewed, for attributes, including sex, age, site, gross type, presence of ulceration, tumour size, depth of invasion, perineural invasion, lymphovascular invasion, and LN metastasis status. Lesions were classified into the following three groups according to gross type: elevated type (Ⅰ, Ⅱa, Ⅱa + Ⅱb, and Ⅱa + Ⅱc), flat type (Ⅱb, Ⅱb + Ⅱa, and Ⅱb + Ⅱc), and depressed type (IIc, Ⅱc + Ⅱb, Ⅱc + Ⅲ, Ⅱc + Ⅱa, and Ⅲ). Ulceration was defined as a deformity of the muscularis propria or fibrosis in the submucosal layer. Intramucosal carcinoma was categorized as M2 (lamina propria invasion) or M3 (muscularis mucosae invasion without penetration). Submucosal invasion was graded as SM1 (upper third), SM2 (middle third), or SM3 (lower third). The depth of submucosal invasion was measured directly using a micrometre and was defined as the distance from the lowest point of the muscularis mucosa (or surface of ulceration) to the point of deepest tumour penetration. The Institutional Review Board at the Hospital approved this study.

Histopathologic evaluation with regard to histological subtypes

We reviewed all slides and estimated the proportions of each tumour component. Diffuse components (undifferentiated) were further subdivided into signet ring cell (sig) or poorly differentiated (por) types. Subclassification was according to the most prevalent component, applying the 50% criterion, as follows: pure diffuse type, mixed-predominantly diffuse type (diffuse > intestinal type), mixed-predominantly intestinal type (intestinal type > diffuse), and mixed diffuse = intestinal type[7]. We calculated the extents of the respective components using the following formula: extent of respective component (cm) = dimension of tumour (cm) × estimated percentage of the respective component/100.

Statistical analysis

Statistical calculations were performed using SPSS for Windows (version 12K, Chicago, IL, United States). To identify the predictive risk factors for LN metastasis, the data were statistically analysed using the chi-squared test and an unpaired Student's *t* test. The independent factors for LN metastasis in diffuse- and mixed-type EGCs were analysed by binary logistic regression analysis (backward, stepwise). The ability of histological component extent to predict LN metastasis was assessed using receiver operating characteristic (ROC) curve analysis. The total areas under the ROC curves ranged from 0.5 to 1.0, with 1.0 indicating perfect predictive value and 0.5 indicating random chance. A pairwise comparison of the areas under the ROC curve was subsequently performed to assess the predictive value of each histological component. A $P < 0.05$ was deemed statistically significant.

RESULTS

Clinicopathological characteristics relevant to LN metastasis

Clinicopathological characteristics as a status of LN metastasis are listed in Table 1. Of 550 cases, 71 (12.9%) showed LN metastasis [15/288 mucosal EGCs (5.2%) and 56/262 submucosal EGCs (21.4%)]. Increased tumour size, gross type (elevated), depth of invasion, and perineural and lymphovascular invasion were associated with LN metastasis. Furthermore, we investigated the independent predictive risk factors for LN metastasis using binary logistic regression analysis (Supplemental Table 1). Tumour size ($P = 0.001$), depth of invasion ($P < 0.0001$), and lymphovascular invasion ($P = 0.001$) were independent predictive risk

Table 2 Lymph node metastasis according to depth of invasion, ulceration, and tumour size *n* (%)

	Mucosal carcinoma				Submucosal carcinoma			
	No ulcer		Ulcer		SM1		SM2	SM3
	≤ 2 cm	> 2 cm	≤ 3 cm	> 3 cm	≤ 3 cm	> 3 cm	Any size	
Diffuse (n = 302)	1/61 (1.6)	5/86 (5.8)	2/22 (9.0)	1/19 (5.3)	0/12 (0)	2/7 (28.6)	6/32 (18.8)	11/63 (17.5)
Mixed (n = 248)	0/19 (0)	5/64 (7.8)	0/4 (0)	1/10 (10)	0/6 (0)	1/8 (12.5)	6/35 (17.1)	30/99 (30.3)
Total (n = 550)	1/80 (1.3)	10/150 (6.7)	2/26 (7.7)	2/29 (6.9)	0/18 (0)	3/15 (20.0)	12/67 (17.9)	41/162 (25.3)

SM1: Upper third; SM2: Middle third; SM3: Lower third.

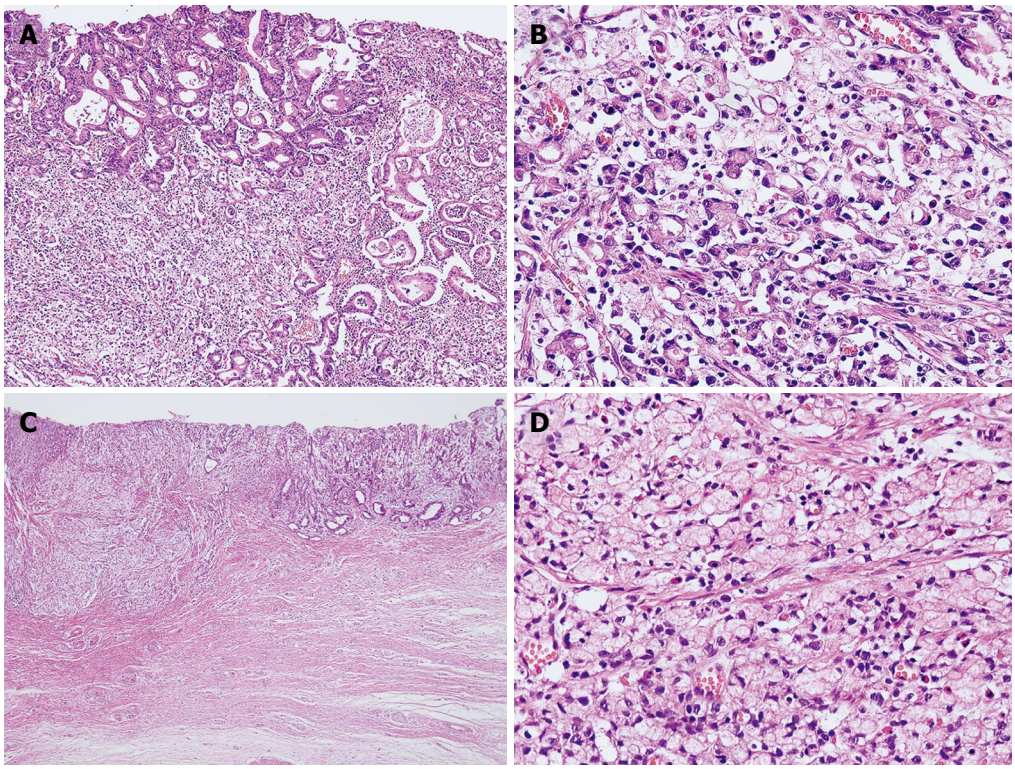

Figure 1 Pathology of mixed-type gastric carcinoma. A: Mixed-type gastric carcinoma showing moderately differentiated tubular adenocarcinoma, intestinal type (upper left and right) and poorly differentiated adenocarcinoma (lower left); B: High magnification view of poorly differentiated lesion (× 400); C: Mixed-type gastric carcinoma showing moderately differentiated tubular adenocarcinoma, intestinal type (right) and signet ring cell carcinoma (left); D: High magnification view of signet ring cell carcinoma lesion (× 400).

factors for LN metastasis in diffuse- and mixed-type EGCs. In our dataset, there were 80 cases of mucosal EGCs less than 2.0 cm in size and without ulceration; among them, only 1 case showed LN metastasis (Table 2). Hence, only 1 case within ESD eligibility criteria showed LN metastasis in this study.

Histological subtypes and LN metastasis

Of 550 cases, 302 were diffuse-type and 248 were mixed-type EGCs (Figure 1; Supplemental Tables 2 and 3). Furthermore, 28 of the 302 diffuse-type (9.3%) and 43 of the 248 mixed-type EGCs (21.0%) showed LN metastasis. The clinicopathological features associated with LN metastasis in each type were similar to those associated with LN metastasis when diffuse and mixed types were considered together, *e.g.*, tumour size, depth of invasion, and perineural and lymphovascular tumour invasion. Depth of submucosal invasion was significantly associated with LN metastasis in mixed-type (P = 0.044), but not in diffuse-type EGCs (P = 0.905)

Of 248 mixed-type EGCs, 163 were mixed-predominantly diffuse type, 82 were mixed-predominantly intestinal type, and 3 were mixed diffuse = intestinal type (Table 3). Interestingly, LN metastasis was more frequent in the mixed-predominantly diffuse type (33/163, 20.2%) than in either the pure diffuse type (28/302, 9.3%) or the mixed-predominantly intestinal histological type (10/82, 12.2%; P = 0.003).

Quantitative impact of histopathological subtypes on LN metastasis

We measured the extent of the individual components to determine the quantitative power of each to predict LN metastasis. The total tumour size and the

Table 3 Histopathological subtypes and lymph node metastasis *n* (%)

	Total	Status of lymph node metastasis	
		Negative	Positive
Pure diffuse type	302	274 (90.7)	28 (9.3)[a]
Sig	233	210 (90.1)	23 (9.9)
Por	69	64 (92.8)	5 (7.2)
Mixed type	248	205 (83.7)	43 (16.3)
Predominantly diffuse	163	130 (79.8)	33 (20.2)[a]
Sig predominant	107	86 (80.4)	21 (19.6)
Por predominant	56	44 (78.6)	12 (21.4)
Predominantly intestinal	82	72 (87.8)	10 (12.2)[a]
Diffuse = intestinal	3	3 (100.0)	0 (0.0)

[a]*P* = 0.003 (between pure diffuse, predominantly diffuse, and predominantly intestinal). Sig: Signet ring cell; Por: Poorly differentiated.

Table 4 Relationship between the extent of various histopathological components and lymph node metastasis in 550 diffuse- and mixed-type early gastric cancers

	Lymph node status		*P* value
	Negative (*n* = 479)	Positive (*n* = 71)	
Total (cm)	3.30 ± 0.09	4.46 ± 0.29	< 0.001
Diffuse type (cm)	2.73 ± 0.11	3.61 ± 0.24	0.005
Signet ring cell	2.07 ± 0.12	2.54 ± 0.29	0.161
Poorly differentiated	0.66 ± 0.05	1.07 ± 0.20	0.012
Intestinal type (cm)	0.65 ± 0.05	0.87 ± 0.17	0.172

extent of the poorly differentiated component were significantly larger in the metastatic LN group (Table 4). Interestingly, the extent of the poorly differentiated component was associated with LN metastasis (*P* = 0.012), whereas that of the signet ring cell component was not (*P* = 0.161).

In the 248 mixed-type EGCs, we assessed the ability of the histological components to predict LN metastasis using ROC curve analysis (Figure 2). The areas under the ROC curve for each component were as follows: diffuse component (0.687, 95%CI: 0.604-0.701, *P* < 0.0001), total size (0.610, 95%CI: 0.523-0.698, *P* = 0.023), poorly differentiated component (0.598, 95%CI: 0.505-0.691, *P* = 0.043), and signet ring cell component (0.556, 95%CI: 0.450-0.661, *P* = 0.249). These results confirm that poorly differentiated component was significantly associated with LN metastasis (*P* = 0.043) while signet ring cell component was not (*P* = 0.249).

DISCUSSION

With the development of ESD, the criteria for diffuse-type EGCs eligible for endoscopic resection expanded to include mucosal lesions measuring less than 2.0 cm without ulceration or lymphovascular tumour emboli; this was based on the risk of LN metastasis[1-3]. Hirasawa *et al*[12] reported that 310 diffuse-type EGCs within the expanded criteria showed no LN metastasis,

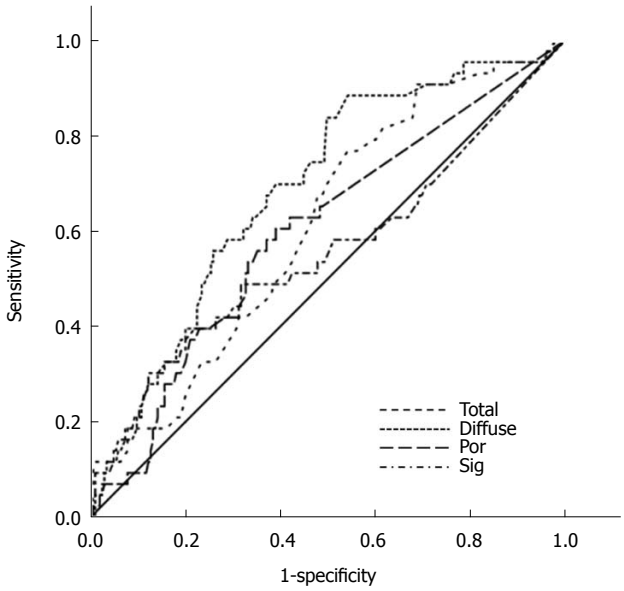

Figure 2 Receiver operating characteristics curve analysis of scores for predicting lymph node metastasis in mixed-type early gastric cancers yielded an area under the curve of 0.687 (95%CI: 0.604-0.701, *P* < 0.0001) for the diffuse component, 0.610 (95%CI: 0.523-0.698, *P* = 0.023) for total size, 0.598 (95%CI: 0.505-0.691, *P* = 0.043) for the poorly differentiated component, and 0.556 (95%CI: 0.450-0.661, *P* = 0.249) for the signet ring cell component.

in contrast to an earlier report by Gotoda *et al*[13]. Shim and Lee summarized previously published datasets describing the risks of LN metastasis for diffuse-type EGCs that meet the proposed criteria[3], concluding that these expanded criteria for ESD of diffuse type EGC are feasible with regard to efficacy and safety.

Consistent with other reports that reported low or no LN metastasis rates, we noted only one such metastasis among cases within the established expanded ESD criteria (1/80, 1.3%). We also identified various clinicopathological factors associated with LN metastasis, including increased size, gross type (elevated), depth of invasion, and perineural and lymphovascular invasion; this was consistent with previously published data[14,15]. Taken together, the expanded criteria for diffuse-type EGC can be used to determine the primary option for treatment. Mixed- predominantly diffuse EGCs showed a higher frequency of LN metastasis (20.2%) than either the pure diffuse type (9.3%) or the mixed-predominantly intestinal type (12.2%), in accordance with previous reports[7,8], suggesting that mixed-predominantly diffuse EGC should be more stringently managed than pure diffuse-type EGC.

We further sub-categorized diffuse histological components into signet ring cell and poorly differentiated carcinoma, and evaluated the impact of each component on LN metastasis. Interestingly, we identified an increase in the extent of the total diffuse component - especially the poorly differentiated

component - was associated with LN metastasis. There was no such association between the extents of signet ring cell components or intestinal components with LN metastasis. Furthermore, ROC curve analysis of mixed-type EGCs showed that the total tumour size along with the extents of the diffuse and poorly differentiated components affected LN metastasis with statistical significance, while the extent of the signet ring cell component did not (Figure 2).

Many studies reported favourable outcomes of early signet ring cell carcinoma and recommended endoscopic resection as the primary treatment option, which is not the case for poorly differentiated carcinoma[16-18]. Ha *et al*[17] demonstrated that signet ring cell carcinoma within the expanded criteria showed no LN metastasis and favourable outcomes compared to undifferentiated carcinoma. Kim *et al*[18] reported that signet ring cell carcinoma had a lower rate of LN metastasis compared to poorly differentiated or tubular adenocarcinoma of the submucosal type. However, none of aforementioned studies demonstrated the association of signet ring cell carcinoma in mixed-type EGCs with LN metastasis. This study demonstrates that a larger, poorly differentiated histological component was associated with LN metastasis, whereas a larger signet ring cell component was not.

There are many limitations and possible errors in our method of measuring the exact size of the lesions. We measured the extent of each component by multiplying the percentage of each component with the largest diameter of the lesion. Although this method is not perfect, we believed that it would be sufficiently indicative of the role of each component.

In conclusion, our results support the use of the extended criteria for the therapeutic use of ESD in cases of diffuse-type EGCs, and we recommend careful identification and quantitative evaluation of each tumour component in mixed-type EGC specimens obtained by endoscopic resection.

ACKNOWLEDGMENTS

This study was presented in part at the 2015 USCAP meeting (Boston, MA, United States).

COMMENTS

Background

A considerable proportion of gastric adenocarcinomas show a mixed composition with both intestinal and diffuse histological components. No consensus has been established regarding the management of mixed-type histology.

Research frontiers

The authors investigated which components of the diffuse subtypes (signet ring cell or poorly differentiated carcinoma) are more closely associated with lymph node (LN) metastasis, providing evidence to establish a consensus for the management of diffuse- and mixed-type early gastric cancers (EGCs) with endoscopic submucosal dissection (ESD).

Innovations and breakthrough

This study consolidates correlations between clinicopathological characteristics of early gastric cancer and the risk of LN metastasis, which is important to select patients that will benefit from endoscopic submucosal dissection. This paper also shows that the amount of poorly differentiated tumor cells within mixed type early gastric cancer is correlated with the highest risk of LN metastasis.

Applications

The results of the our present study support the use of the extended criteria for the therapeutic use of ESD in cases of diffuse-type EGCs, recommending careful identification and quantitative evaluation of each tumour component in mixed-type EGC specimens obtained by endoscopic resection.

Terminology

Gastric adenocarcinoma can be subclassified into intestinal (differentiated) and diffuse (undifferentiated) types based on gland formation. Diffuse components are further subdivided into signet ring cell carcinoma and poorly differentiated carcinoma. In practice, mixed-type EGCs, consisted of intestinal and diffuse type, are frequently encountered. The expanded criteria for ESD include diffuse (undifferentiated) mucosal EGC measuring less than 2 cm without ulceration and lymphovascular tumour emboli.

Peer-review

This paper shows that the amount of poorly differentiated tumor cells within mixed type early gastric cancer is correlated with the risk of LN metastasis. They emphasize the need for diligent histological characterization of patient specimens as conclusion. Their findings are interesting and useful for the future treatment of early gastric cancer.

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ORIGINAL ARTICLE

Retrospective Study

Intestinal histoplasmosis in immunocompetent adults

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Author contributions: Yang JL and Wang YP designed the research; Zhu LL, Wang J and Wang ZJ performed the research and collected the clinical data; Zhu LL and Wang J analyzed the data; Zhu LL performed the follow-up and wrote the manuscript; all authors have read and approved the final version to be published.

Institutional review board statement: This research was reviewed and approved by the Ethics Committee of the West China Hospital of Sichuan University.

Informed consent statement: This is a retrospective study using routinely collected data, results did not have any impact on participants. Patients were not required to give informed consent for the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Intestinal histoplasmosis in immunocompetent adults".

Data sharing statement: No additional unpublished data are available.

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Abstract

AIM: To present a retrospective analysis of clinical and endoscopic features of 4 cases of immunocompetent hosts with intestinal histoplasmosis (IH).

METHODS: Four immunocompetent adults were diagnosed with IH between October 2005 and March 2015 at West China Hospital of Sichuan University. Clinical and endoscopic characteristics were summarized and analyzed retrospectively. GMS (Gomori methenamine silver), PAS (periodic acid-Schiff) and Giemsa staining technique were used to confirm *Histoplasma capsulatum*(*H. capsulatum*). The symptoms, signs, endoscopic presentations, radiographic imaging, pathological stain results and follow-up are presented as tables and illustrations.

RESULTS: The cases were male patients, ranging from 33 to 61 years old, and primarily presented with nonspecific symptoms such as irregular fever, weight loss, abdominal pain and distention. Hepatosplenomegaly and lymphadenopathy were the most common signs. Endoscopic manifestations were localized or diffuse congestion, edema, ulcers, and polypoid nodules with central erosion involving the terminal ileum, ascending

colon, transverse colon, descending colon, sigmoid colon and rectum, similar to intestinal tuberculosis, tumor, and inflammatory bowel disease. Numerous yeast-like pathogens testing positive for PAS and GMS stains but negative for Giemsa were detected in the cytoplasm of the histiocytes, which were highly suggestive of *H. capsulatum*.

CONCLUSION: Immunocompetent individuals suffering from histoplasmosis are rarely reported. It is necessary that gastroenterologists and endoscopists consider histoplasmosis as a differential diagnosis, even in immunocompetent patients.

Key words: Intestinal histoplasmosis; Disseminated histoplasmosis; Immunocompetence; Endoscopic characteristics; Differential diagnosis

Core tip: Intestinal histoplasmosis (IH) is an uncommon disease. It is more likely to be encountered in immunocompromised patients. No case series of IH in immunocompetent patients has been published so far. This retrospective study included 4 cases of immunocompetent adults with intestinal histoplasmosis and focused on presenting the endoscopic characteristics. It is necessary that gastroenterologists and endoscopists consider histoplasmosis as a differential diagnosis, even in immunocompetent patients.

Zhu LL, Wang J, Wang ZJ, Wang YP, Yang JL. Intestinal histoplasmosis in immunocompetent adults. *World J Gastroenterol* 2016; 22(15): 4027-4033 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4027.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4027

INTRODUCTION

Disseminated histoplasmosis (DH) is more likely to be encountered in patients whose $CD4^+$ cell counts are < 200 cells/mm^3[1,2], a condition mostly found in acquired immune deficiency syndrome (AIDS) patients, and extremely rarely in patients with human T-lymphotropic virus 1 (HTLV-1) infection. Gastrointestinal (GI) involvement in disseminated histoplasmosis can occur at any site along the GI tract, and particularly in the terminal ileum due to its abundance of lymphoid tissue. Intestinal involvement is also primarily found in immunocompromised patients, whereas cases of intestinal histoplasmosis (IH) in immunocompetent hosts have rarely been reported. Although more than 20000 colonoscopies are performed in our department every year, we found only one patient who presented with intestinal multiple ulcers as an initial manifestation in 2012. Therefore, we searched all patients with intestinal histoplasmosis and summarized the results. Here, we present a retrospective analysis of clinical and endoscopic features of 4 cases of immunocompetent hosts at our hospital between 2005 and 2015. Among them, 3 cases were DH; the other case was localized ascending colon IH. Diagnoses were confirmed by bone marrow or GI tissue culture. Few of the physicians and endoscopists considered this disease as a differential diagnosis, and colonoscopies and fungal cultures were rarely performed. Based on the collection of clinical and endoscopic manifestations, gastroenterologists and endoscopists should consider histoplasmosis as a differential diagnosis, even in immunocompetent patients.

MATERIALS AND METHODS

We reviewed the medical records of 4 patients at our hospital in whom intestinal histoplasmosis was diagnosed from 2005 to 2015. The diagnosis of IH was made if lesions of the small bowel, colon or rectum were identified on endoscopy results in conjunction with any of the following: positive blood, bone marrow, or GI tissue culture for *Histoplasma capsulatum* (*H. capsulatum*); histopathologic observation of microorganisms morphologically consistent with *H. capsulatum* in tissue specimens[3]. The baseline characteristics of these patients are shown in Table 1. All these immunocompetent patients were male. Two cases were diagnosed in 2008, one in 2012 and the last in 2014. None of the patients were infected with the human immunodeficiency virus or treated with immunosuppressive drugs.

RESULTS

Signs, symptoms and sites of involvement

The most common presenting symptoms were irregular fever (75%) and weight loss of different degrees (75%). The highest body temperature was 40.3 ℃, accompanied or not by night sweats and chills. Abdominal pain and distention (75%) and anorexia (75%) were the main manifestations of the GI system. However, empirical antibiotic treatment was ineffective in improving symptoms. On physical examination, 3 of the patients showed hepatosplenomegaly and 2 showed peripheral lymphadenectasis involving the submentum or the left armpit, with only one patient having abdominal tenderness and rebound pain while he experienced peritonitis.

Among the 4 IH patients, 3 had multiple colon lesions, though only one lesion was localized at the ascending colon (Case A). Multiple intestinal lesions involving the terminal ileum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum were in continuous or multifocal distribution. DH lesions were mainly distributed in the bone marrow

Table 1 Baseline characteristics of 4 patients

	Case A	Case B	Case C	Case D
Age (yr)	61	33	59	28
Sex	Male	Male	Male	Male
Underlying diseases	Arthritis[1]	No	AP, HBV[2]	Onychomycosis[3]
Risk factor				
HIV	Negative	Negative	Negative	Negative
Injection drug use	Deny	Deny	Deny	Deny
Glucocorticoids	Not used	Not used	Not used	Not used
Other-immunosuppressive agents	Not used	Not used	Not used	Not used
CD4 counts (/mm^3)				
Before treatment	Not reported	159	145	86
After treatment	Not reported	221	229	Not reported

[1]Arthritis of the left shoulder with amyotrophy; [2]Acute pancreatitis 30 years previously, healed; hepatic B virus (HBV) infection 9 years previously, but HBsAg (-), anti-HBs(-), HBeAg (-), anti-HBe (+), anti-HBc (+) on admission; [3]Onychomycosis in multiple fingernails. HIV: Human immunodeficiency virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis E antigen.

Table 2 Symptoms, signs and sites of involvement

	Case A	Case B	Case C	Case D
Symptoms				
Fever	No	Yes	Yes	Yes
Temperature (℃)		35.3-40.3	35.8-38.7	35-40.3
Night sweat	No	Yes	Yes	Not reported
Chills	No	No	No	Yes
Antibiotic[1]	Moxifloxacin[2] Metronidazole[2]	Levofloxacin[3]	Anqi[4]	Penicillin[5], Amikacin[5] Azithromycin[5] Levofloxacin[6] Ceftriaxone[5], Xianshu[7]
Abdominal pain	Right abdomen	No	Yes	Whole abdomen
Abdominal distention	Yes	No	Yes	Upper abdomen
Diarrhea	No	No	No	Yes[8]
Anorexia	Yes	Yes	Yes	Not reported
Pharyngalgia	No	Yes	No	Not reported
Cough	No	Yes	No	No
Expectoration	No	Yes	No	No
Weight loss	Not apparent	15 kg	4 kg	10 kg
Signs				
Lymphadenectasis	No	Yes[9]	No	Yes
Abdominal tension	No	No	No	Yes
Hepatomegaly	No	Not palpable	Yes[10]	Yes
Splenomegaly	No	Yes[11]	Yes[12]	Yes
(Rebound) tenderness	No	No	No	Yes
Shifting dullness	Negative	Negative	Negative	Positive
Involvement				
Bone marrow		√	√	√
Pulmonary				
Colon	√	√	√	√
Terminal ileum		√	√	√

[1]Empirically used, but invalid; [2]For 5 d, dosage unknown; [3]0.4 g iv daily for 26 d; [4]Amoxicillin and potassium clavulanate tablets 0.3125 g tid po for 1 wk; [5]Dosage unknown; [6]0.4 g iv daily for 28 d in our hospital; dosage unknown in other hospital; [7]Cefoperazone sulbactam sodium 2.0 g iv bid for 8 d then 2.0 g iv tid for 7 d; [8]Black watery stool with amount unknown, 7-10 times per day; [9]1 × 1 cm soft lymphatic node without adhesion in the left armpit; [10]3 cm below the right rib border and 5 cm below the xiphoid; [11]5 cm below the left rib border; [12]3 cm below the left rib border.

and in the lung (Table 2).

Endoscopic and radiographic imaging

The details of endoscopic and radiographic analyses are available in Tables 3 and 4. Abnormal findings of radiographic imaging included bowel wall thickening, retroperitoneal or intraperitoneal lymphadenopathy, ascites and hepatosplenomegaly. Colonoscopy showed the following: an isolated mucosal nodular bulging lesion approximately 2 cm in diameter with its central erosion at the ascending colon near the hepatic flexure (Figure 1A); manifestation as edematous mucosa, diffuse nodular changes accompanied by aphthoid ulcers or erosion measuring 0.5-1.0 cm, with colon marsupium disappearing and intestinal strictures (Figure 1B); a 1.5 × 1 cm area of isolated swollen

Table 3 Endoscopic findings

	Case A (cm)	Case B (cm)	Case C (cm)	Case D (cm)
Lesions				
Protrusions with erosion	√			
Flat polypoid uplift				√
Nodular deposits		√	√	
Edematous mucosa		√	√	√
Multiple ulcers		√	√	
Stricture		√		
Location and size				
Ascending colon	2.0	0.5-1.0[1]	0.3 × 0.3	0.5
Transverse colon		0.5-1.0[1]	1 × 0.8	0.3-0.6
Descending colon		0.5-1.0[1]		0.3-0.6
Sigmoid colon		0.5-1.0[1]		0.3-0.6
Terminal ileum			1.5 × 1.0	0.5
Rectum		0.5-1.0[1]		0.3-0.6
Biopsy texture	Soft	Fragile hemorrhage	Soft	Soft

[1]Lesion distribution in all segments from rectum to descending colon until the colonoscope could not continue.

rough mucosa in the terminal ileum and 0.3 cm × 0.3 cm, 0.8 cm × 1.0 cm ulcers covered with white fur located in the ascending and transverse colon, with the surrounding mucosa hyperemic and swollen (Figure 1D); diffuse flat polypoid mucosa measuring 0.3-0.5 cm located in the rectum, sigmoid colon, descending colon, transverse colon, ascending colon, and terminal ileum (Figure 1C).

Histology

All of the biopsy specimens of the ascending colon, transverse colon, sigmoid colon, rectum, and descending colon showed increased accumulation of histiocytes, with formations of multinucleated giant cells in the mucosa; numerous yeast-like pathogens testing positive for PAS (periodic acid-Schiff) and GMS (Gomori methenamine silver) stains but negative for Giemsa were detected in the cytoplasm of the histiocytes (Cases B, C;). No specific staining was observed for Case D. The ascending colon specimen of Case A showed inflamed granulated tissue, with a large number of interstitial foam cells; PAS, GMS, and Giemsa stains were positive, whereas acid-fast staining was negative, indicating mycotic granulomatous inflammation consistent with histoplasmosis. Bone marrow smears showed multiple oval or round organisms with amaranth nuclei and surrounding capsule-like unstained halos, which within the context of phagocytes are highly suggestive of *H. capsulatum* (Cases B, D; Figure 2A-D). Bone marrow biopsy was also positive for PAS and GMS staining, compatible with a diagnosis of histoplasmosis (Case C).

Treatment and follow-up

Three of the DH patients were started on intravenous amphotericin B deoxycholate at an initial dose of 1 mg/d for a total of 1.47-2.79 g followed by oral itraconazole 200 mg bid for 6 mo. There was a dramatic response with a rapid change in temperature. Follow-up endoscopic examinations involving second and third colonoscopies after 42 d to 1 year (Cases B, C) were normal. Case D was lost during follow-up. It is worth noting that the localized ascending colon histoplasmosis patient (Case A) had fully recovered without any anti-fungal drugs by his second colonoscopy after 35 d.

DISCUSSION

H. capsulatum is a 2-4 μm yeast which enters the body primarily through the lungs via inhalation, and largely causes a self-limited respiratory infection in healthy individuals[4], mainly observed at endemic levels in areas of the upper Mississippi and Ohio river valleys[5]. As it is difficult for macrophages to eliminate these organisms in immunodeficient patients due to the absence of cellular immunity, the infection can disseminate to other organs such as the skin, bone marrow, spleen, liver, lymphoid node, adrenal gland, renal tract, central nervous system, and even the GI tract, which presents as disseminated histoplasmosis. Reported immunodeficiency conditions include AIDS[1], HTLV-1 infection[6], hepatitis C infection[4], renal failure, and the recent use of a glucocorticosteroid or biological agents such as infliximab or etanercept[7]. However, less than 0.05% of patients have no obvious immunosuppressive risk factors[8], which is difficult to reconcile with DH. Regarding our cases, because the $CD4^+$ count was initially 86-159/mm^3 but increased to 221-229/mm^3 after the infection was treated, we considered this to be an outcome of the infection, though we did not find any evidence that *H. capsulatum* impairs the immune function of T-cells[9]. GI involvement occurs in 70%-90% of cases of DH[10], whereas the colon may be involved in 59.6%. Symptomatic GI histoplasmosis (GIH) is uncommon (3%-12% of patients)[11], primarily manifesting as abdominal pain, diarrhea, anorexia, nausea, bilious vomiting, constipation, tenesmus, and abdominal tenderness[10]. There are also reports of GI complications such as bleeding[12], bowel obstruction and perforation[1], and even protein loss due to enteropathy and hypogammaglobulinemia[11]. Missed diagnoses can occur because healthcare providers often do not arrange GI endoscopy examinations due a lack of GI symptoms.

Four distinct pathological forms have now been recognized: (1) subclinical, with microscopic clusters of macrophages in the lamina propria; (2) plaques and pseudopolyps caused by fungus-containing macrophages; (3) tissue necrosis and ulceration leading to abdominal pain, diarrhea and bleeding; and (4) localized thickening with inflammation of the bowel that can mimic malignancy or Crohn's disease[13,14].

Table 4 Radiographic findings

	Case A	Case B	Case C	Case D
Abdominal				
Lesions	Bowel-wall thickening	Hepatosplenomegaly	Hepatic cyst	Hepatosplenomegaly Splenic infarction Lymphadenopathy Ascites
Location	Ascending colon Ileocecal			Intraperitoneal Retroperitoneal
Chest				
Lesions	Small nodular	Nodular shadows[1]	Pleural-effusion Atelectasis	Pleural-effusion
Location	right middle lobe left inferior lobe	right lower lobe	right lower lobe	

[1]1.7 cm nodular shadow in the dorsal segment of the right lower lobe, with shrinkage after treatment.

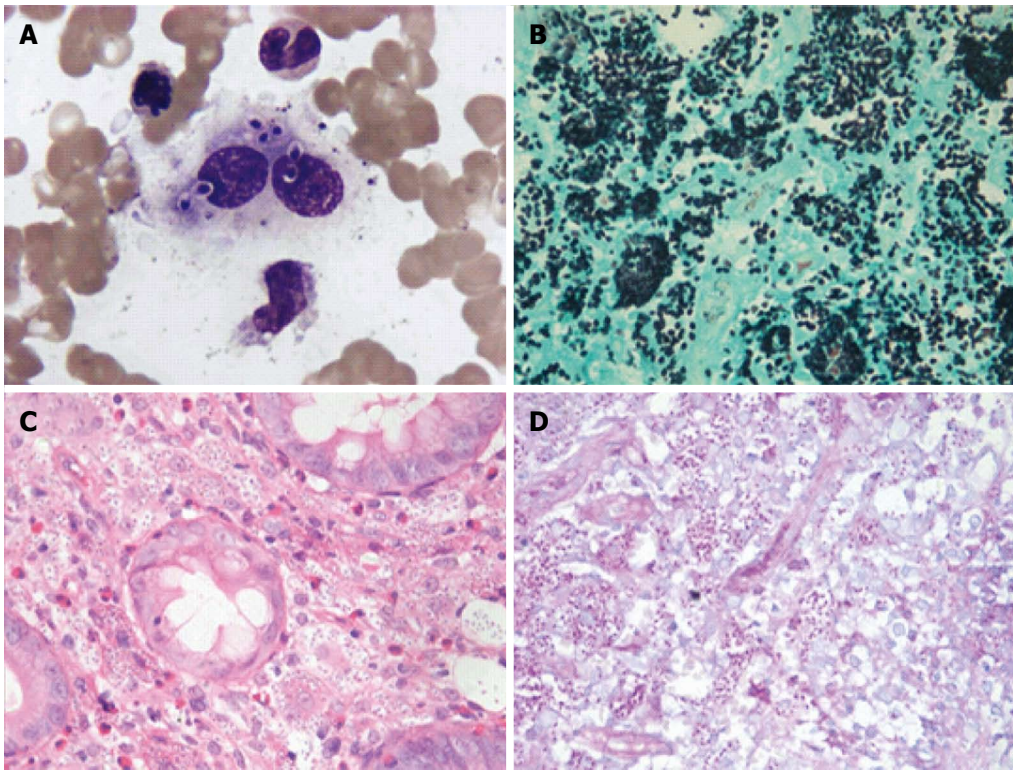

Figure 1 Pathological stain results of *Histoplasma capsulatum* in bone marrow. A: Wright-Giemsa stained bone marrow aspirate. There were numerous round or oval *H. capsulatum* of relatively uniform size in phagocyte and cytoplasm. It is round at one end and pointed at the other. Karyon was stained fuchsia, surrounded by peri-nuclear halos and the shape was capsule-like; B-D: Numerous uniform oval-shaped yeasts suggesting *H. capsulatum* were found in the amina propria stroma in the descending colon biopsy. [B: Gomori methenamine silver stain (magnification × 100); C: Hematoxylin and eosin stain (magnification × 40); D: Periodic acid-Schiff stain (magnification × 40)]. *H. capsulatum*: *Histoplasma capsulatum*.

Lesions found during endoscopies, laparotomies, or autopsies include single or continuous superficial mucosal ulcerations, deep bleeding ulcers with or without frank perforations, friable and mass-containing areas of necrosis, and obstructions due to circumferential exophytic thickening[10]. Diffuse ulcerations were detected in 85.7% (12/14) of AIDS GIH patients, with 10/14 (71.4%) involving only the colon or cecum[3]. Despite the fact that these manifestations mimic many other GI diseases, such as Crohn's disease, tuberculosis, carcinomas, and lymphomas, the cause is often not considered to be histoplasmosis in the differential diagnosis, leading to inappropriate therapies and unnecessary surgical interventions[10].

A variety of diagnostic exams, including direct microscopic examination, cultures, antigen detection, and serological tests for antibodies have been described, and a tissue biopsy should be performed as soon as possible. Samples can be obtained from the blood, bone marrow, liver, skin lesions, or any other site of infection. Cultures are positive in approximately 85% of cases, though multiple specimens must be cultured to achieve the highest yield[10,15]. Overall,

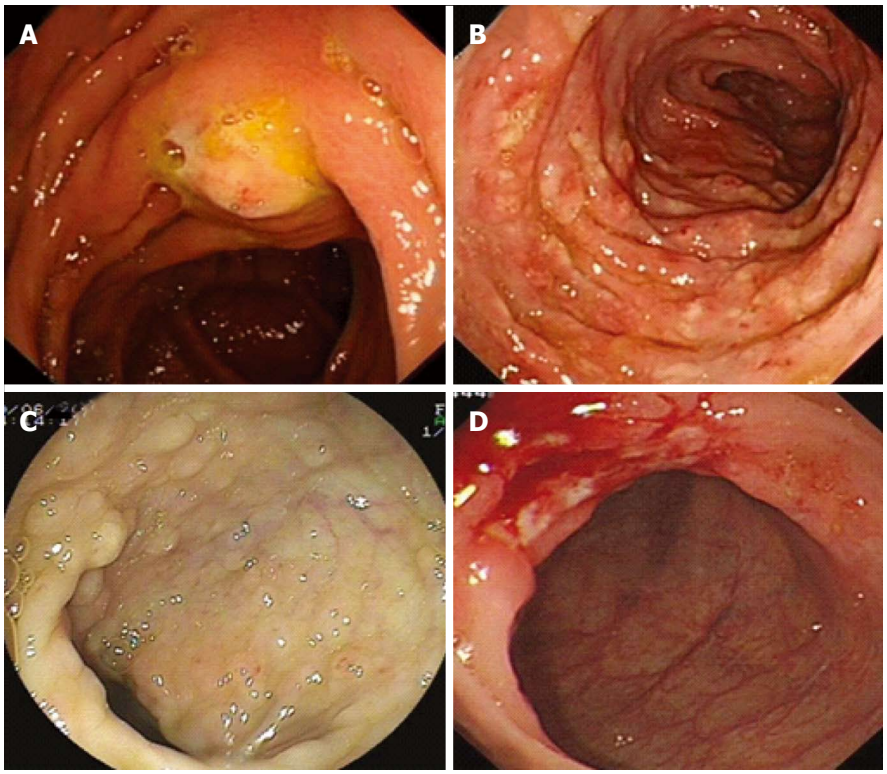

Figure 2 Endoscopic manifestations of *Histoplasma capsulatum* infection. A: Isolated mucosal nodular bulging lesion about 2 cm with central erosion; B: Diffuse nodular changes accompanied by aphthoid ulcers or erosion measuring 0.5-1.0 cm; C: Diffuse flat polypoid mucosa measuring 0.3-0.5 cm; D: 0.8 cm × 1.0 cm ulcer covered with white fur.

52.9% of patients exhibited positive culture results for blood, bronchoalveolar lavage, lymph node, liver, and spleen specimens, whereas 90.9% of GI specimens were positive[1]. Thus, in addition to pathoscopy, specimens should be submitted for microscopic examination, and fungi from mucosal lesions of the GI tract identified at endoscopy should be cultured[10]. GIH has excellent long-term survival with aggressive therapy, such as anti-mycete therapies. However, untreated disseminated histoplasmosis is almost always fatal.

In summary, it is important to arrange GI endoscopies for DH cases, even with a lack of GI tract symptoms or in patients with normal immune function. Endoscopists and GI physicians should be aware of histoplasmosis in the GI tract, especially when it manifests as ulceration, and a sample should be sent for culture as soon as possible.

COMMENTS

Background

Intestinal histoplasmosis (IH) is an uncommon disease. The majority of IH cases are found in disseminated histoplasmosis (DH), which is more likely to be encountered in immunodeficient patients, such as those with AIDS. Conversely, immunocompetent individuals suffering from histoplasmosis are rarely reported. To our knowledge, no case series of IH in immunocompetent patients has been published. This retrospective study concluded 4 cases of immunocompetent adults with intestinal histoplasmosis, and focused on presenting the endoscopic characteristics.

Research frontiers

IH in immunocompetent adults is an uncommon disease. Few reports have included this disease so far. It is necessary that gastroenterologists and endoscopists consider it as a differential diagnosis.

Innovations and breakthroughs

To improve the clinical recognition of intestinal histoplasmosis, this study presents the symptoms, signs, endoscopic presentations, radiographic imaging, pathological staining, and follow-up of 4 IH patients.

Applications

Endoscopic manifestations of IH are somewhat similar to intestinal tuberculosis, tumor, and inflammatory bowel disease. Based on collection of clinical and endoscopic manifestations, gastroenterologists and endoscopists should consider histoplasmosis as a differential diagnosis, even in immunocompetent patients.

Terminology

DH: is an AIDS defining illness which usually involves the gastrointestinal tract.

Peer-review

This study summarized the characteristics of 4 cases of immunocompetent adults with intestinal histoplasmosis. Although the cases are few, they are interesting.

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ORIGINAL ARTICLE

Retrospective Study

Sorafenib after resection improves the outcome of BCLC stage C hepatocellular carcinoma

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Author contributions: Li J and Hou Y contributed equally to this work; Li J and Hou Y designed the study and analyzed the data; Li J, Hou Y, and Cai XB acquired data and drafted the manuscript; Li J, Hou Y, and Liu B defined the intellectual content; Cai XB researched literature; Liu B provided administrative, technical, and material support and critically revised the manuscript; all authors guarantee the integrity of the entire study and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the First Affiliated Hospital of Kunming Medical University Institutional Review Board.

Informed consent statement: This is a retrospective study, informed consent is not required.

Conflict-of-interest statement: None declared.

Data sharing statement: No additional data are available.

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Abstract

AIM: To evaluate whether sorafenib use after resection impacts tumor relapse and survival in Barcelona Clinic Liver Cancer (BCLC) stage C hepatocellular carcinoma (HCC).

METHODS: This retrospective study enrolled 36 male BCLC stage C HCC patients with portal vein thrombus and Child-Pugh class A liver function. Twenty-four patients received only surgical resection (SR), and 12 patients received oral sorafenib within 30 d after surgery. The primary outcomes were time to progression (TTP) (the time from surgical resection until HCC recurrence or extrahepatic metastases) and overall survival (OS). The secondary outcome was the rate of postoperative recurrence or metastasis. TTP and OS were analyzed using Kaplan Meier curves.

RESULTS: There were no significant differences between the two groups in the serum levels of alpha-fetoprotein, copies of hepatitis B virus-DNA, preoperative laboratory results, degree of hepatic fibrosis, types of portal vein tumor thrombus, number of satellite lesions, tumor diameter, pathological results, volume of blood loss, volume of blood transfusion, or surgery time (all $P > 0.05$). Patients in the SR + sorafenib group had a significantly longer TTP (29 mo *vs* 22 mo, $P = 0.041$) and a significantly longer median

OS (37 mo *vs* 30 mo, *P* = 0.01) compared to patients in the SR group. The SR group had 18 cases (75%) of recurrence/metastasis while the SR + sorafenib group had six cases (50%) of recurrence/metastasis. A total of 19 patients died after surgery (five in the SR + sorafenib group and 14 in the SR group). The most common sorafenib-related adverse events were skin reactions, diarrhea, and hypertension, all of which were resolved with treatment.

CONCLUSION: Sorafenib after SR was well-tolerated. Patients who received sorafenib after SR had better outcomes compared to patients who received only SR.

Key words: Hepatocellular carcinoma; Survival; Hepatic resection; Sorafenib; Recurrence

Core tip: Barcelona Clinic Liver Cancer stage C patients with portal vein thrombus and Child-Pugh class A liver function who received sorafenib after surgical resection had significantly longer overall survival (37 mo *vs* 20 mo, *P* = 0.01) and significantly longer time to progression compared to patients who received only resection (29 mo *vs* 22 mo, *P* = 0.041). Our data suggested that better outcomes can be achieved with sorafenib after surgical resection, rather than sorafenib monotherapy.

Li J, Hou Y, Cai XB, Liu B. Sorafenib after resection improves the outcome of BCLC stage C hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(15): 4034-4040 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4034.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4034

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer-related deaths worldwide[1]. The most important risk factors for HCC include hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, and the presence of liver cirrhosis[2-4]. The Barcelona Clinic Liver Cancer (BCLC) classification system recommended a standard classification system for the treatment of HCC that has been accepted by the American Association for the Study of Liver Disease (AASLD) and the European Association for the Study of Liver[5]. Based on these guidelines, patients diagnosed with HCC at an early stage (BCLC Stage 0, A) currently undergo surgical resection (SR), liver transplantation, or percutaneous ablation and have a survival rate of 60%-70%[3,6,7]. However, HCC recurrence after these therapies is still common. Patients at an intermediate stage (BCLC Stage B) undergo transarterial chemoembolization (TACE)[8,9], where abnormal neovascularization is identified and injected with an emulsion of chemotherapeutic drug and lipidol and then embolized with gelfoam to induce tumor necrosis[10]. Although repeated treatment with TACE was associated with improved survival in patients with intermediate HCC[9], its use is limited to patients with well-compensated cirrhosis[11]. SR was shown to result in high hepatic functional reserve in HCC patients with portal vein tumor thrombus (PVTT), while postoperative TACE delayed recurrence and prolonged overall survival (OS) in patients who could tolerate the treatment[12]. Interestingly, TACE has been shown to induce neoangiogenesis and upregulation of vascular endothelial growth factor (VEGF), which is an independent negative predictor of survival[13].

Patients with advanced HCC have a poor prognosis[14]. The multi-kinase inhibitor sorafenib, which is the only approved agent recommended by the AASLD for HCC BCLC Stage C, is currently recommended as the first-line therapy in these patients[14-16]. Two randomized controlled clinical trials recently showed that sorafenib prolonged OS and delayed the time to progression (TTP) in patients with advanced HCC[17,18], likely by inhibiting a number of growth factor pathways, including VEGFR-1,-2,-3, platelet derived growth factor receptor (PDGFR)-β, Raf, rearranged during transfection (RET), and FMS-like tyrosine kinase (FLT)-3[19]. However, certain BCLC stage C patients with Child-Pugh class A liver function have been shown to have better outcomes with SR than with sorafenib monotherapy[20,21]. Additionally, recent reports, which showed that (1) advanced HCC patients at BCLC stage C had favorable outcomes with SR, and (2) the presence of multinodular tumors, macrovascular invasion, and portal hypertension were not contraindications for SR, suggested that the guidelines for the use of sorafenib monotherapy for advanced HCC should be re-evaluated[22,23].

The main purpose of this retrospective study was to evaluate whether sorafenib use after liver resection had an impact on tumor relapse and OS in BCLC stage C HCC patients. We also evaluated the safety and tolerability of oral sorafenib after surgical resection in these patients.

MATERIALS AND METHODS

This retrospective study evaluated the medical records of 36 male HCC patients who underwent surgical resection and were treated at the First Affiliated Hospital of Kunming Medical University between January 2009 and December 2013. All patients were HBV positive and had cirrhosis.

Inclusion criteria were: (1) age 18-70 years old; (2) newly diagnosed liver cancer with no treatment received prior to surgical resection; (3) BCLC stage C [tumor thrombus in left/right branches or main portal vein; Eastern Cooperative Oncology Group (ECOG) performance status (PS) ⩽ 2], Child-Pugh liver function class A; (4) tumor confined to left/right

lobe (single or multiple); maximum tumor diameter ⩾ 5 cm; all patients underwent anatomic left/right lobe resection + thrombus dissection, with negative surgery margin (R0). All operative procedures were classified according to the Brisbane terminology[24]. Anatomic left/right lobe resection was defined as resection of the tumor along with the related portal vein branches and corresponding hepatic artery territory; (5) patients treated with oral sorafenib received a dose of 200-800 mg/d within 30 d after surgery. The dose was reduced to 200 mg twice daily in the event of drug-related adverse effects; and (6) time of follow up ⩾ 6 mo (before June 2014); patients underwent at least once B-type ultrasound or computed tomography (CT)/magnetic resonance (MR) + chest X-ray every 2 mo during the follow-up period (if B-type ultrasound or X-ray found a new lesion, a confirmatory CT/MR scan was performed).

Exclusion criteria were: (1) presence of acute or chronic lesions in other organs outside the liver; (2) presence of metastases or suspected metastatic lesions outside the liver; (3) presence of other tumors at the time of diagnosis or during the follow-up period; (4) patient received any treatments (including TACE or RF) other than sorafenib after SR and before tumor recurrence or metastasis; and (5) tumor recurrence or metastasis within 3 mo after surgical resection or unnatural death during follow-up.

Outcome measures

The primary outcome was TTP (the time from SR until HCC recurrence or extrahepatic metastases discovered by CT/MR) and OS. The secondary outcome was the rate of postoperative recurrence or metastasis.

Statistical analysis

The demographic data and clinical characteristics of the patients were summaized as mean ± SD for continuous data, *n* (%) for categorical data, and median (range: min to max) for time-related data. Differences between groups were compared using two-sample *t*-test for continuous data, Pearson χ^2 test or Fisher exact test for categorical data, and log-rank test for time-related data. Additionally, a Mann-Whitney *U* test was considered for continuous data if data did not follow normal distribution. Time-related data (TTP and OS times) are represented using Kaplan-Meier curves, and differences between groups were analyzed by the log-rank test. The TTP and OS times were also summarized as median with 95%CI for both groups, separately. All statistical assessments were two-tailed, and $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Sciences 17.0 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

Of a total of 36 study patients, 12 patients received oral sorafenib after SR (SR + sorafenib group) and 24 received only SR (SR group). None of the patients exhibited any serious complications, including liver dysfunction, bleeding, or infection, within 30 d of surgical resection.

Demographics and baseline clinical characteristics

Table 1 summarizes the demographics and baseline clinical characteristics in the SR + sorafenib and the SR groups. All study patients were male. The average ages of patients in the SR + sorafenib and the SR groups were 49.8 ± 6.5 years and 52.8 ± 6.9 years, respectively ($P = 0.226$). There were no significant differences in the serum levels of alpha fetoprotein (AFP), copies of HBV-DNA, preoperative laboratory results, degree of hepatic fibrosis, types of PVTT, number of satellite lesions, tumor diameter, pathological results, volume of blood loss, volume of blood transfusion, or surgery time between the two groups (all $P > 0.05$).

Post-operative recurrence rate, TTP, and OS

The median follow-up time after SR for all patients was 23 mo (range of 9-54 mo). During the follow-up period, a total of 19 patients experienced residual liver relapse ($n = 5$ in the SR + sorafenib group, and $n = 14$ in the SR group), three patients developed lung metastasis ($n = 1$ in the SR + sorafenib group, and $n = 2$ in the SR group), one patient developed right adrenal gland metastasis ($n = 1$ in the SR group), and one patient developed thoracic vertebral metastasis ($n = 1$ in the SR group) (data not shown). The rate of patients with at least once recurrence (including relapse or metastasis) was, therefore, derived as 50% (6/12) for the SR + sorafenib group and 75% (18/24) for the SR group ($P = 0.157$) (Table 1).

Patients in the SR + sorafenib and SR groups had a median TTP of 29 mo (95%CI: 26.5-31.5 mo) and 22 mo (95%CI: 18.0-26.0 mo), respectively. The log-rank test showed that this difference was significant ($P = 0.041$), suggesting that sorafenib therapy after SR might prolong the time until recurrence compared to SR alone (Figure 1).

A total of 19 patients died after surgery (five in the SR + sorafenib group and 14 in the SR group) (Table 1). The SR + sorafenib and SR groups had median OS times of 37 mo (95%CI: 34.8-39.2 mo) and 30 mo (95%CI: 24.1-36.0 mo), respectively. The log-rank test showed that this difference was significant ($P = 0.010$), suggesting that patients who received sorafenib therapy after SR had longer survival times compared to patients who received only SR (Figure 2)

Adverse events

The most common sorafenib-related adverse events during the follow-up period included hand-foot-skin reaction (11 patients; 91.67%), diarrhea (10 patients; 83.3%), and hypertension (10 patients; 83.3%). All the sorafenib-related adverse events were resolved with treatment, and no treatment-related deaths

Table 1 Patients' demographics and clinical characteristics by group *n* (%)

Variables	SR + sorafenib (*n* = 12)	SR (*n* = 24)	*P* value
Age[1], yr	49.8 ± 6.5	52.8 ± 6.9	0.226
ECOG PS score[2]			1.000
0	10 (83.3)	19 (79.2)	
1	2 (16.7)	5 (20.8)	
Largest tumor diameters[1], cm	9.8 ± 2.1	11.2 ± 2.5	0.103
Pathologic results[3]			0.404
Well differentiated (grade 1)	3 (25.0)	2 (8.3)	
Moderately differentiated (grade 2)	7 (58.3)	18 (75)	
Poorly differentiated (grade 3)	2 (16.7)	4 (16.7)	
Satellite lesion[4], *n*	4 (33.3)	9 (37.5)	1.000
AFP			0.700
< 400 ng/mL	4 (33.3)	6 (25)	
⩾ 400 ng/mL	8 (66.7)	18 (75)	
HBV-DNA			0.664
< 1000 cps/mL	9 (75)	20 (83.3)	
⩾ 1000 cps/mL	3 (25)	4 (16.7)	
Degree of fibrosis[5]			1.000
0-2	2 (16.7)	3 (12.5)	
3-4	10 (83.3)	21 (87.5)	
Types of PVTT[6]			0.764
First-order branch (VP3)	10 (83.3)	19 (79.2)	
Main trunk (VP4)	2 (16.7)	5 (20.8)	
Preoperative laboratory results			
ALT[1], μmol/L	57.3 ± 19.9	50.8 ± 22.1	0.397
Albumin[1], g/L	39.5 ± 3.5	39.9 ± 4.5	0.781
Bilirubin[1], μmol/L	17.4 ± 4.5	19.8 ± 6.0	0.236
Hemoglobin[1], g/L	137.3 ± 10.9	132.0 ± 7.8	0.103
Platelet count[1], 10^9/L	185.4 ± 46.2	164.3 ± 48.6	0.220
Prothrombin time[1], s	12.1 ± 1.0	12.0 ± 1.0	0.785
INR[7]	1.4 ± 0.5	1.3 ± 0.4	0.436
Blood loss[7], mL	304.2 ± 151.4	343.8 ± 143.2	0.458
Blood transfusion[7], mL	37.5 ± 93.2	62.5 ± 124.5	0.679
Surgery time[7], min	218.3 ± 33.3	232.5 ± 47.8	0.265
TTP times[8], mo	26.5 (14-54)	21.5 (9-34)	0.041[9]
Patients with recurrence after SR[2]	6 (50)	18 (75)	0.157
Survival times[8], mo	32 (21-58)	25.5 (11-37)	0.010[9]
Patients died after SR[2]	5 (41.7)	14 (58.3)	0.483

[1]mean ± SD for continuous data, differences between groups were compared using two-sample *t*-test; [2]*n* (%) categorical data and differences between groups were compared using Pearson χ^2 test; [7]Mann-Whitney *U* test; [3]Median (range: min to max) for time-related data, and differences between groups were compared using Fisher exact test; [8]Log-rank test was used for time-related data; [4]Satellite lesions were defined as tumors ⩽ 2 cm in size that were located within a distance of 2 cm from the main tumor; [5]The degree of fibrosis of the hepatic parenchyma was graded according to the classification of Ishak *et al*[39] grade 0-2, no or minimal fibrosis; grade 3-4, incomplete bridging fibrosis; grade 5-6, complete fibrosis and nodules; [6]PVTT was typed according to the classification of primary liver cancer by the Liver Cancer Study Group of Japan[40]. Patients were defined as having a tumor thrombus in the first-order branch (VP3) and the main trunk (VP4) of the portal vein; [9]$P < 0.05$, indicates a significant difference between two groups. SR: Surgical resection; AFP: Alpha fetoprotein; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; PVTT: Portal vein tumor thrombus; TTP: Time to progression.

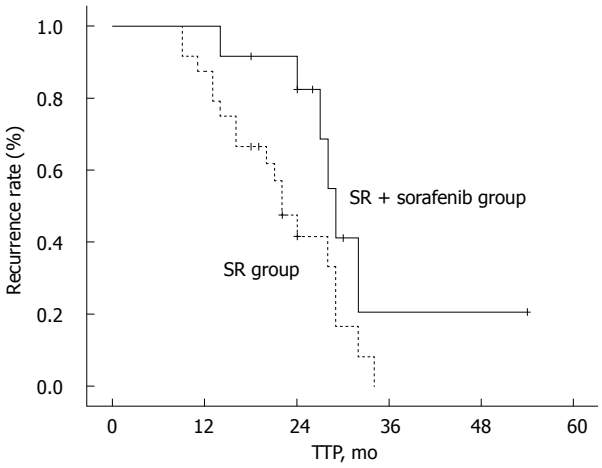

Figure 1 Kaplan-Meier curve of time-to-progression in the surgical resection + sorafenib and surgical resection groups. The median time to progression (TTP) was estimated from the patients with equivalent as 0.5 and derived as 29 mo (95%CI: 26.5-31.5 mo) and 22 mo (95%CI: 18.0-26.0 mo) for surgical resection (SR) + sorafenib and SR groups, respectively. + indicates censored patients. The log-rank test showed a significant difference in the TTP between groups ($P = 0.041$).

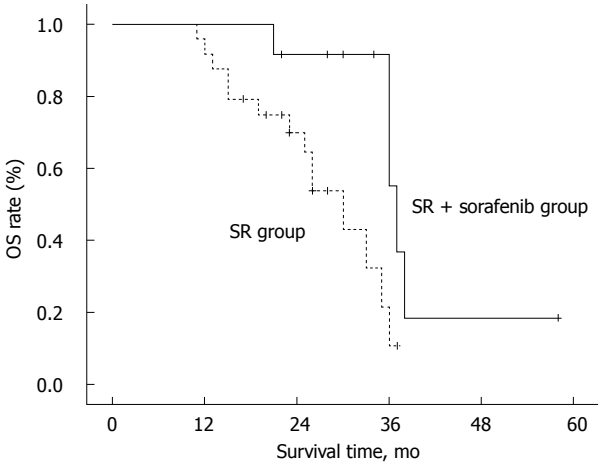

Figure 2 Kaplan-Meier curve of overall survival times in the surgical resection + sorafenib and surgical resection groups. The median overall survival (OS) time was estimated from the patients with equivalent as 0.5 and derived as 37 mo (95%CI: 34.8-39.2 mo) and 30 mo (95%CI: 24.1-36.0 mo) for the surgical resection (SR) + sorafenib and SR groups, respectively. + indicates censored patients. The log-rank test showed a significant difference in the OS times between groups ($P = 0.010$).

occurred (data not shown).

DISCUSSION

In this study, we showed that BCLC stage C patients who received sorafenib after SR had significantly longer OS and significantly longer TTP compared to patients who received only SR. There were fewer cases of recurrence/metastasis in the SR + sorafenib group

compared to the SR only group. Sorafenib after SR was generally safe and well-tolerated.

Based on the current standard of care, patients with advanced HCC (stage B or C) who cannot undergo radical resection, receive local palliative treatment, including TACE, hepatic arterial infusion chemotherapy, and systemic chemotherapy[10,25,26]. Patients with stage C (defined as portal vein aggressiveness, lymph node or distant metastasis, ECOG PS ⩽ 2, Child-Pugh liver function class A or B) are treated with sorafenib, which is an oral multi-kinase inhibitor with anti-tumor activity[27-29]. The multi-center European Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial, which evaluated the efficacy of sorafenib monotherapy (400 mg twice daily) in 602 HCC BCLC stage C patients with Child-Pugh class A liver function, showed a significantly lower OS in the placebo group compared to the sorafenib group (7.9 mo *vs* 10.7 mo, HR = 0.69; 95%CI: 0.55-0.87)[18]. These data were similar to a study in the Asia-Pacific region that demonstrated an improvement in OS from 4.2 mo in the placebo group to 6.5 mo in the sorafenib group (HR = 0.68; 95% CI: 0.50-0.93)[17]. The shorter OS in the latter trial compared to the SHARP study could be because of the higher number of cases with extrahepatic metastases, larger tumor diameters, higher ECOG PS scores, and higher AFP levels compared to the SHARP trial. Data from both trials formed the basis for the widespread recommendation of sorafenib for the treatment of advanced liver cancer. It was recently suggested that the relatively low response rates in sorafenib-treated patients could be because sorafenib induces tumor dormancy and a prolonged duration of stable disease (SD). Interestingly, patients with a longer duration of SD had a better OS compared to patients with a shorter duration of SD[30].

In contrast, data from two HCC clinical institutes in Asia showed that selected HCC patients at BCLC stage C had better outcomes with SR compared to other treatment modalities[20,21]. Similarly, a recent study that used the same inclusion criteria as the SHARP trial (BCLC stage C, Child-Pugh class A, ECOG PS ⩽ 2) showed that patients who received SR (n = 68), locoregional ablation therapy (n = 8); transarterial embolization (n = 140); systemic chemotherapy or radiotherapy (CT/RT, n = 96) and sorafenib (n = 11) had a median OS of 33.4 mo, 9.5 mo, 9.2 mo, 6.6 mo, and 15.7 mo, respectively[31]. These data suggested that some advanced HCC patients who had tumors in a single lobe without extrahepatic spread had a better prognosis with SR compared to other methods.

There are currently no studies that have investigated the use of sorafenib after SR for advanced HCC. In our present study, we used the same inclusion criteria as the SHARP study. We included only newly diagnosed, naïve HCC patients in order to avoid the confounding effects of previous therapy. Additionally, all our study patients who underwent SR received no other treatment except for sorafenib prior to tumor recurrence. We used a follow-up time period of > 6 mo because of the difficulty in differentiating early recurrence from a residual tumor. A longer term follow-up is necessary to evaluate therapeutic efficacy. In this study, all the patients in the SR + sorafenib group received sorafenib within 30 d after surgery. This was because (1) comparisons between the two groups would be more reliable if all the patients were at a similar stage of recovery after SR, and (2) sorafenib would inhibit any VEGF-mediated promotion of tumor growth in patients who had a sub-optimal response to SR. It is important to note that sorafenib treatment is usually initiated 2 wk after SR, since sorafenib is known to delay healing.

Our data showed that BCLC stage C HCC patients who received oral sorafenib treatment after SR had a significantly longer TTP and a significantly longer median OS than patients who received only SR. Patients in the SR + sorafenib group also had a lower rate of tumor recurrence and extrahepatic metastasis than the SR only group. Interestingly, our data showed that patients in the SR + sorafenib as well as the SR groups had a longer TTP than the OS of patients in the sorafenib group from the SHARP study (29 mo and 22 mo *vs* 11 mo). These data suggested that it may be advantageous to use sorafenib after SR, rather than sorafenib monotherapy, in order to have better outcomes. Our data also showed that sorafenib was safe and well-tolerated. All sorafenib-related adverse events were resolved with dose reduction treatment, as previously reported[32]. Our data were consistent with a recent study that reported that adjuvant sorafenib therapy after hepatic resection in HCC patients resulted in reduced mortality and prolonged OS, possibly *via* inhibition of tumor growth after tumor recurrence[33].

Sorafenib has been shown to have anti-angiogenic as well as antitumor activities, possibly mediated *via* a number of tyrosine kinases, including VEGF, PDGFRs, Raf, and the phosphatidylinositol 3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathways[29,34-37]. Since vascular invasion was shown to be a critical predictor of HCC recurrence[38], it will be important to define further the mechanism of action of sorafenib in order to optimize the clinical management of patients with advanced HCC.

Since the cost of sorafenib therapy in China is almost three times the cost of SR, a few patients with advanced HCC are sometimes recommended SR by experienced physicians after a careful case-by-case assessment. The criteria for allowing SR in this group of patients are very restricted, making it a challenge to study large sample sizes. Although it is important to investigate whether the two groups were comparable for characteristics, such as presence of satellite tumors, and etiology of cirrhosis with HBV/HCV, the small sample size of our study precluded such an analysis. Indeed, the major limitation of this study was

the small sample size. Other limitations were (1) its retrospective nature; and (2) factors such as surgeon preference and the socio-economic heterogeneity of our study population. The decision to be treated with sorafenib was largely based on the condition of the patients, and this could result in an overestimation of the effect of sorafenib after SR. Additionally, our study patients received different HCC treatment modalities (TACE, RAF, Chinese traditional medicines, or experimental immunotherapy) after tumor recurrence or metastasis. OS comparison may be impacted by these confounding factors.

In conclusion, our data showed that BCLC stage C HCC patients with Child-Pugh class A liver function who received oral sorafenib after SR had better postoperative TTP. It is important to validate our data *via* large multicenter randomized controlled trials.

COMMENTS

Background

Patients with advanced hepatocellular carcinoma (HCC) have a poor prognosis. The multi-kinase inhibitor sorafenib is the currently recommended first-line therapy for patients with HCC Barcelona Clinic Liver Cancer (BCLC) Stage C based on data from two recent randomized controlled clinical trials that showed that sorafenib prolonged overall survival (OS) and delayed time to progression (TTP). However, certain BCLC stage C patients with Child-Pugh class A liver function had better outcomes with surgical resection than with sorafenib monotherapy, suggesting a need to re-evaluate the guidelines for the use of sorafenib monotherapy for advanced HCC. This retrospective study evaluated whether sorafenib use after liver resection had an impact on tumor relapse and OS in BCLC stage C HCC patients.

Research frontiers

Although sorafenib is currently the standard of care for advanced HCC, certain patients have better outcomes with surgical resection rather than with sorafenib monotherapy. This study investigated the use of sorafenib after surgical resection in specific groups of patients with advanced HCC and contributes to our ability to improve the clinical management of these patients.

Innovations and breakthroughs

There are currently no studies that have investigated the use of sorafenib after surgical resection (SR) for advanced HCC. The authors' data indicated that sorafenib after SR was safe and well-tolerated. BCLC stage C patients who received sorafenib after SR had significantly longer OS, significantly longer TTP, and a lower rate of recurrence/metastasis compared to patients who received only SR.

Applications

Sorafenib therapy after surgical resection may result in better postoperative TTP in certain populations of HCC BCLC stage C patients with Child-Pugh class A liver function who may not benefit from sorafenib monotherapy. This study calls for re-evaluation of current guidelines for treating advanced HCC.

Peer-review

The manuscript is an interesting study showing the benefit of using adjuvant sorafenib in patients in BCLC C after liver resection. The article is well-organized, and the study objectives are clearly stated in the introduction, pointing out the relevance of this study. The study is built stepwise, and the description of the results is well-written.

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ORIGINAL ARTICLE

Observational Study

Worldwide practice in gastric cancer surgery

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Abstract

AIM: To evaluate the current status of gastric cancer surgery worldwide.

METHODS: An international cross-sectional survey on gastric cancer surgery was performed amongst international upper gastro-intestinal surgeons. All surgical members of the International Gastric Cancer Association were invited by e-mail to participate. An English web-based survey had to be filled in with regard to their surgical preferences. Questions asked included hospital volume, the use of neoadjuvant treatment, preferred surgical approach, extent of the lymphadenectomy and preferred anastomotic technique. The invitations were sent in September 2013 and the survey was closed in January 2014.

RESULTS: The corresponding specific response rate was 227/615 (37%). The majority of respondents: originated from Asia (54%), performed > 21 gastrectomies per year (79%) and used neoadjuvant chemotherapy (73%). An open surgical procedure was performed by the majority of surgeons for distal gastrectomy for advanced cancer (91%) and total gastrectomy for both early and advanced cancer (52% and 94%). A minimally invasive procedure was preferred for distal gastrectomy for early cancer (65%). In Asia surgeons preferred a minimally invasive procedure for total gastrectomy for early cancer also (63%). A D1+ lymphadenectomy was preferred in early gastric cancer (52% for distal, 54% for total gastrectomy) and a D2 lymphadenectomy was preferred in advanced gastric cancer (93% for distal, 92% for total gastrectomy)

CONCLUSION: Surgical preferences for gastric cancer surgery vary between surgeons worldwide. Although the majority of surgeons use neoadjuvant chemotherapy, minimally invasive techniques are still not widely adapted.

Key words: Gastric cancer; Gastrectomy; Laparoscopy;

Neoplasm; Minimally invasive surgery

Core tip: Since surgical techniques might differ over time and between countries, we aimed to evaluate international preferences in gastric cancer surgery by means of a cross-sectional survey. Surgical preferences for gastric cancer surgery vary between surgeons worldwide. Minimally invasive gastrectomy is still not widely adapted, but most popular in Asia to treat patients with early gastric cancer. Neo-adjuvant chemotherapy is used by the majority of surgeons worldwide. A D1+ lymphadenectomy is preferred for early gastric cancer and a D2 lymphadenectomy is preferred for advanced gastric cancer.

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INTRODUCTION

Gastric cancer is the fifth most common type of cancer worldwide[1]. Its treatment consists of (neo-) adjuvant chemotherapy and/or chemoradiation and surgical resection of the tumor and lymph nodes. The worldwide surgical practices may vary between surgeons, countries and continents. Current topics of debate in gastric cancer surgery are: (1) The influence of volume of hospitals and surgeons on the outcome after gastrectomy; (2) The technique of surgery: open or minimally invasive gastrectomy; (3) Reconstruction of the alimentary tract by means of a jejunal pouch. (4) The extent of lymph node dissection and need for omental resection and/or pancreaticosplenectomy; and (5) The type of (neo-)adjuvant treatment in patients with gastric cancer.

In this article the current practice of surgeons worldwide will be evaluated by means of a survey.

MATERIALS AND METHODS

An international cross-sectional survey about the surgical treatment of gastric cancer was performed amongst international gastric surgeons. All surgical members of the International Gastric Cancer Association (IGCA) were invited by email to participate after approval of the IGCA was obtained. An English web-based survey had to be filled in according to the surgeons' preferences. Questions asked included hospital volume, the use of neoadjuvant treatment, preferred surgical approach, extent of the lymphadenectomy and preferred anastomotic technique and are attached to the manuscript (Appendix 1). Definitions of the extent of lymph node dissection and gastric cancer classification were according to the Japanese gastric cancer classification system[2,3]. The invitations were sent in September 2013 and the survey was closed in January 2014. Statistical analysis was performed with the χ^2 test using the IBM SPSS Statistics (version 21; IBM Corporation, Armonk, NY, United States). Data were considered significant if $P < 0.05$.

RESULTS

Demographics

The survey was completed by 248 of 615 (40%) members of the IGCA. The 21 duplicate respondents were consequently excluded. The corresponding specific response rate was therefore 227/615 (37%). The respondents originated from Asia (54%), Europe (27%), South America (12%), North America (6%), Africa (0.4%), and Oceania (0.4%) (Figure 1).

Volume

The volume of the participating surgeons was ⩽ 10 gastrectomies per year in 16 (7%) respondents and > 21 resections in 180 (79%) respondents. In Asia, the majority of respondents (57%) performed > 61 resections (Figure 2). Medium and high volume surgeons worked in a university hospital (74%) more often than in a regional hospital (5%, $P = 0.048$).

Open vs minimally invasive gastrectomy

The current survey revealed that minimally invasive distal gastrectomy was preferred by 65% of surgeons in the treatment of early gastric cancer. The Asian respondents performed minimally invasive distal gastrectomy for early gastric cancer in 82% of the cases (Figure 3). In South America minimally invasive and open distal gastrectomy were equally performed. Minimally invasive distal gastrectomy for advanced gastric cancer was performed by only 9% of respondents. These results were comparable in all continents. For total gastrectomy, minimally invasive total techniques were favored by 49% for early gastric cancer and by 6% for advanced gastric cancer. However, in Asia the majority (64%) of respondents performed minimally invasive total gastrectomy for early gastric cancer, whereas other continents preferred the open procedure. For total gastrectomy for advanced cancer, there was no difference between continents.

Anastomoses

The preference of 83% of the participating surgeons in the survey was to construct a direct esophagojejunostomy without jejunal pouch reconstruction after total gastrectomy. In merely 17% of surgeons a pouch was the preferred method of reconstruction. This percentage was consistent between all continents.

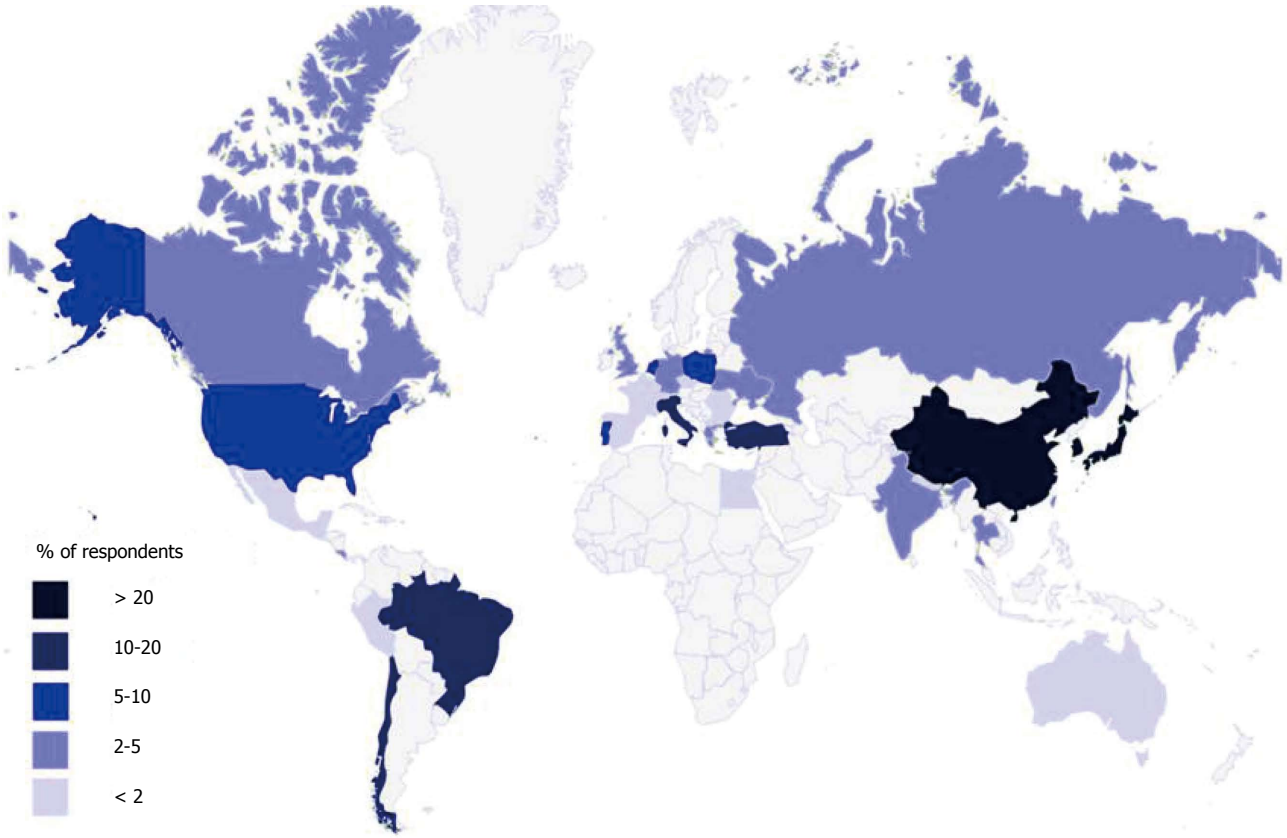

Figure 1 Contribution per country.

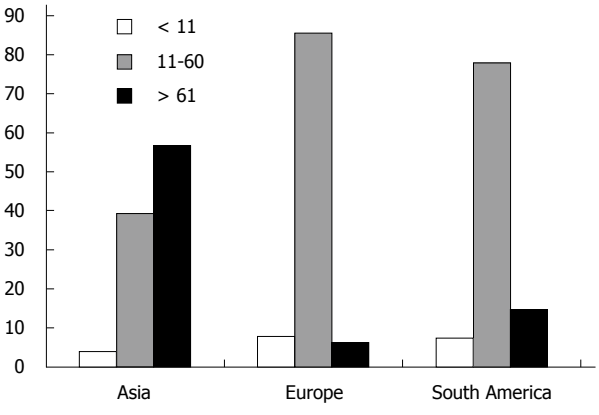

Figure 2 Annual number of gastrectomies per surgeon.

A pouch reconstruction was slightly more popular amongst surgeons from a university hospital than surgeons from a regional hospital (19% *vs* 11%, $P = 0.50$). The data from the survey reveal that anastomoses were preferably performed by means of a mechanical stapler by 92% of respondents, compared to 8% of surgeons who favored a hand-sewn anastomosis.

Extent of dissection

The current survey indicated that surgeons preferred a D1+ resection in 52% of distal gastrectomies and 54% of total gastrectomies for early cancers (Figure 4). In Asia and Northern America a D2 dissection was performed less frequently for early stage tumors compared to Europe and Southern America (Table 1). A D2 resection was favored by 93% of distal gastrectomies and 92% of total gastrectomies for advanced tumors. Resection of the spleen was preferably performed by 20% of all respondents: 33% of Asian respondents, 19% of South American respondents, and 15% of European respondents. The survey reveals that resection of the greater omentum was preferred by 89% of the participating surgeons.

(Neo)adjuvant therapy

The results of our survey on the use of neoadjuvant treatment for gastric cancer indicated that chemotherapy was preferred by 73% and chemoradiation was favored by 12% of respondents. Only 16% favored treatment without neoadjuvant treatment. These results did not differ significantly over continents (Table 2).

DISCUSSION

In this study the current worldwide trends in gastric surgery for cancer were evaluated by means of a survey amongst surgeons. It was found that the majority of surgeons have a high annual volume of gastrectomies. Open gastrectomy was still the preferred procedure in most procedures (68%)

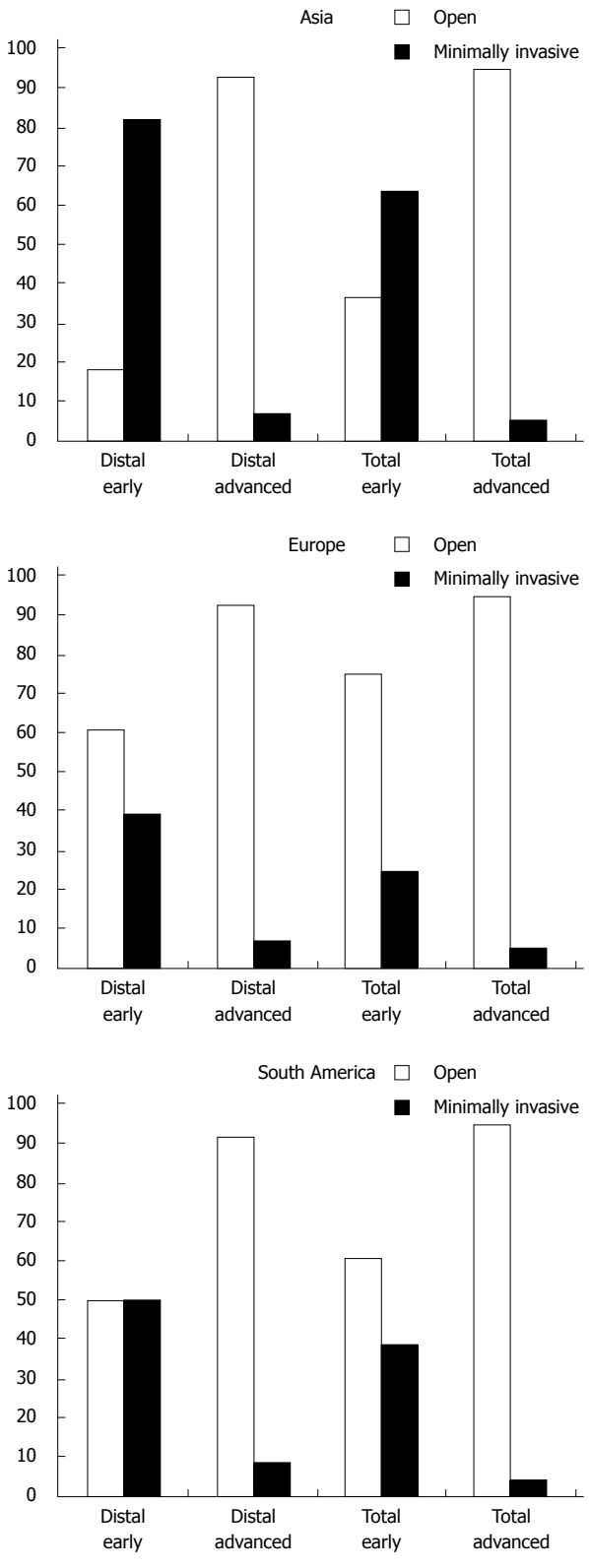

Figure 3 Open *vs* minimally invasive gastrectomy for early and advanced cancer.

combined with neoadjuvant chemotherapy in the majority of cases (73%). Differences in surgical approach and lymphadenectomy were found across continents.

In our survey, 79% of respondents performed

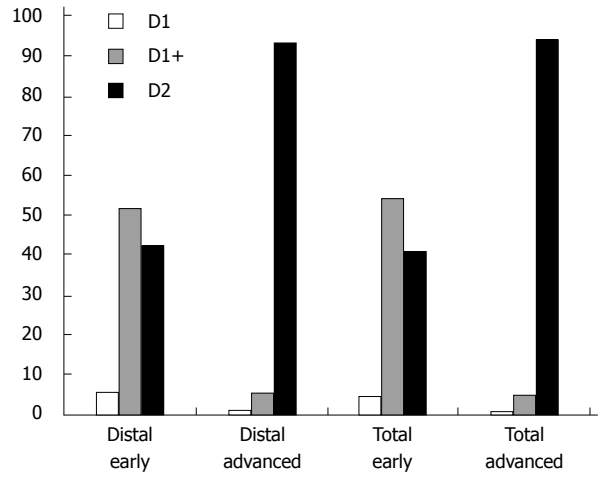

Figure 4 Lymph node dissection for distal and total gastrectomy.

Table 1 Percentage of D2-dissections for different tumor stages worldwide (%)

	Asia	Europe	South America	North America
Distal early	33.9	57.1	54.2	38.5
Total early	27.7	66.7	50.0	30.8
Distal advanced	98.2	85.7	91.7	92.3
Total advanced	95.5	93.0	95.8	92.3

Table 2 Percentage of different neo-adjuvant therapy regimens worldwide (%)

	Asia	Europe	South America	North America
Chemotherapy	69.6	76.8	68.2	84.6
Radiotherapy	0.0	1.8	0.0	0.0
Chemoradiation	12.5	12.5	9.1	7.7
None	17.9	8.9	22.7	7.7

> 20 gastrectomies per year which is considered a high volume in literature. With regard to annual volume of resection, both the volume of the individual surgeon and the volume of the hospital are related to mortality[4-8]. For example, one study showed that the 30-d mortality rate in centers performing > 21 resections per year was lower compared to centers performing ⩽ 10 gastrectomies per year (4.4% *vs* 6.7%, $P = 0.047$)[9]. Interestingly, more than half of the respondents from Asia perform > 60 gastrectomies.

The preferred method for gastric cancer surgery for most surgeons is an open gastrectomy. Only for early gastric cancer requiring a distal gastrectomy, the majority of surgeons preferably use a minimally invasive method. This is supported by recent short term results of the KLASS-01 trial, which found that the complication rate was significantly lower after laparoscopic distal gastrectomy compared to the open distal gastrectomy (13% *vs* 20%, $P = 0.001$)[10]. Evidence for all other types of gastric cancer are from

small randomized trials and retrospective studies only, but suggests minimally invasive gastrectomy to be safe[11-15]. The absence of long-term results might explain the lack of generalized usage of these minimally invasive techniques. Randomized studies in both Asian and Western populations are awaited before worldwide implementation can take place. In South Korea, the KLASS-02 trial and KLASS-03 (NCT01584336) trial are investigating the use of laparoscopy for distal gastrectomy for advanced cancer and total gastrectomy for early cancer respectively[16]. Recently, in Europe two randomized controlled trials (LOGICA-trial and STOMACH-trial) started comparing open with laparoscopic gastrectomy[17,18]. Regarding the current developments in minimally invasive surgery, one can expect the use of these techniques to increase. Since minimally invasive gastrectomy is associated with a considerable learning curve, expert training and proctoring is essential to provide for a safe implementation of this technique[19,20].

This survey showed that the majority of surgeons do not construct a jejunal pouch after total gastrectomy. Although literature remains scarce, studies have shown possible benefits of jejunal pouch reconstruction. Two studies demonstrated an improved quality of life in patients with a jejunal pouch, measured with the Gastro-Intestinal Quality of Life Index. In addition, they found no increase in postoperative morbidity after jejunal pouch reconstruction[21-24]. Several trials on the use of a jejunal pouch are currently running to further investigate this possibly beneficial technique. This study demonstrated the preferable technique for constructing the anastomosis was by means of a mechanical stapler. Studies support these results, since a mechanical anastomosis is constructed significantly quicker compared to the hand-sewn method (11.4 min *vs* 38.7 min, $P < 0.001$) with a comparable complication rate[25,26].

For early cancers the majority of surgeons perform a D1+ dissection worldwide. For advanced tumors, the majority of surgeons perform a D2 dissection. The minority of surgeons performs resection of the spleen. These findings are in compliance with literature. In Western countries, pancreas and spleen preserving D2 dissection has been the preferred resection technique since two large trials from the Netherlands and the United Kingdom[27,28]. These trials demonstrated D2 dissection with preserving the pancreas and spleen along with the associated lymph nodes (stations 10 and 11) to results in similar morbidity and mortality as D1 dissection and with better long-term results[27,29-31]. In addition, it was argued that the reason for resecting station 10 and 11 is questionable since metastasis in these lymph nodes confers a poor prognosis (11 year survival: positive station 10 = 8%; negative station 10 = 27%; positive station 11 = 11%; negative station 11 = 35%)[32]. In Asia, surgeons perform a more tailored lymph node dissection. This survey showed that for early gastric cancer, a D2-dissection is performed less frequently in Asia compared to Europe and South America. On the other hand, the Japanese Gastric Cancer Guidelines still advise considering complete clearance of lymph node stations 10 by splenectomy for potentially curable T2-T4 tumors invading the greater curvature of the upper stomach[3]. Some surgeons in Asia also perform a D3 dissection, since a Taiwanese trial showed an improved survival compared to D1 dissection[33]. However, a D3 dissection did not improve survival compared to a D2 dissection in a Japanese trial[34]. Evidence for a D1+ dissection is scarce. Only one small randomized trial demonstrated that D1+ dissection could be a safe alternative to D2 dissection for locally advanced non-junctional tumors. Therefore it seems justifiable that the majority of surgeons perform a D1+ dissection for early gastric cancer[35]. More studies are needed to clarify the role of D1+ dissection for all types of gastric tumors.

The survey reveals that resection of the greater omentum was preferred by 88.5% of the participating surgeons. The value of resection of the greater omentum is currently debated. Advocates of its resection underline the importance of dissection of possible tumor deposits, whereas opponents argue that it is a time consuming procedure associated with additional morbidity. Literature on this topic is scarce and international guidelines vary. A retrospective cohort study in an Asian patient population demonstrated that the 3- and 5-year survival rates were not significantly different between gastrectomy with and without resection of the greater omentum for advanced gastric cancer[36].

Lastly, the majority of surgeons (84.4%) report the application of neoadjuvant chemotherapy or chemoradiation prior to gastrectomy. Literature presents various possible treatment strategies before and after surgery for gastric cancer. In Western countries, perioperative chemotherapy has shown the most beneficial, with an increase in survival around 13%[37]. Interestingly, the percentage of surgeons in Europe using neo-adjuvant therapy in this study (2013-2014) is higher compared to the period of 2011-2012, where many patients did not receive neoadjuvant therapy[38]. In Asia, adjuvant chemotherapy alone demonstrated better results compared to surgery alone with an increase in 3-year survival of 5%-10%[39-41] adjuvant radiation in addition to perioperative chemotherapy can possibly increase survival and is currently investigated in Western countries[42,43]. In Asia however, adjuvant radiation in addition to adjuvant chemotherapy did not increase 7-year overall survival (75% *vs* 73%, $P = 0.484$)[39-41,44].

A limitation of this study is that it only evaluates expert opinions rather than objective measurements, which should be taken into account before generalizing these findings. However, its international design provides a unique insight in the current practice of gastric cancer surgeons. Its discussion in the light

of contemporary literature can be used for further improvement of gastric cancer surgery worldwide. Lastly, these results can be used for future evaluation of worldwide gastric cancer surgery.

In conclusion, this study is unique in its international design revealing the expert opinion on gastric cancer surgery. Minimally invasive gastrectomy is still not widely adapted and variations between continents are present. Minimally invasive gastrectomy is most popular in Asia to treat patients with early gastric cancer. Neoadjuvant chemotherapy is used by the majority of surgeons worldwide. A D1+ lymphadenectomy is preferred for early gastric cancer and a D2 lymphadenectomy is preferred for advanced gastric cancer.

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COMMENTS

Background

Gastric cancer is the fifth most common type of cancer worldwide. Its treatment consists of (neo)adjuvant chemotherapy and/or chemoradiation and surgical resection of the tumor and lymph nodes.

Research frontiers

The worldwide surgical practices may vary between surgeons, countries and continents. Current topics of debate in gastric cancer surgery are the use of (neo)adjuvant treatment, preferred surgical approach, extent of the lymphadenectomy and preferred anastomotic technique.

Innovations and breakthroughs

Since the introduction of minimally invasive surgery for gastric cancer in 1994, the number of surgeons performing minimally invasive gastrectomy is rising. Although the procedure is technically demanding, the evidence for advantages of laparoscopic total gastrectomy is increasing. It is therefore expected that the majority of surgeons will adopt this technique in the future. (Neo)adjuvant treatment has increased survival of patients with gastric cancer. The preferred treatment type and regimen differs amongst countries worldwide.

Applications

The majority of surgeons start performing minimally invasive gastrectomy for early gastric cancer. Early gastric cancer is predominantly seen in Asia. Western countries often see advanced gastric cancer. It is unknown if minimally invasive techniques result in at least comparable outcomes to open surgery for patients with advanced gastric cancer.

Peer-review

This is an interesting paper describing the current trend of surgical treatment of gastric cancer. The authors performed an international cross-sectional survey and concluded that surgical preferences for gastric cancer surgery vary between surgeons worldwide; and the results are informative.

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ORIGINAL ARTICLE

Prospective Study

Extensively drug-resistant bacteria are an independent predictive factor of mortality in 130 patients with spontaneous bacterial peritonitis or spontaneous bacteremia

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Abstract

AIM: To evaluate the epidemiology and outcomes of culture-positive spontaneous bacterial peritonitis (SBP) and spontaneous bacteremia (SB) in decompensated cirrhosis.

METHODS: We prospectively collected clinical, laboratory characteristics, type of administered antibiotic, susceptibility and resistance of bacteria to antibiotics in one hundred thirty cases (68.5% males) with positive ascitic fluid and/or blood cultures during the period from January 1, 2012 to May 30, 2014. All patients with SBP had polymorphonuclear cell count in ascitic fluid > 250/mm^3. In patients with SB a thorough study did not reveal any other cause of bacteremia. The patients were followed-up for a 30-d period

following diagnosis of the infection. The final outcome of the patients was recorded in the end of follow-up and comparison among 3 groups of patients according to the pattern of drug resistance was performed.

RESULTS: Gram-positive-cocci (GPC) were found in half of the cases. The most prevalent organisms in a descending order were *Escherichia coli* (33), *Enterococcus spp* (30), *Streptococcus spp* (25), *Klebsiella pneumonia* (16), *S. aureus* (8), *Pseudomanas aeruginosa* (5), other Gram-negative-bacteria (GNB) (11) and anaerobes (2). Overall, 20.8% of isolates were multidrug-resistant (MDR) and 10% extensively drug-resistant (XDR). Health-care-associated (HCA) and/or nosocomial infections were present in 100% of MDR/XDR and in 65.5% of non-DR cases. Meropenem was the empirically prescribed antibiotic in HCA/nosocomial infections showing a drug-resistance rate of 30.7% while third generation cephalosporins of 43.8%. Meropenem was ineffective on both XDR bacteria and *Enterococcus faecium* (*E. faecium*). All but one XDR were susceptible to colistin while all GPC (including *E. faecium*) and the 86% of GNB to tigecycline. Overall 30-d mortality was 37.7% (69.2% for XDR and 34.2% for the rest of the patients) (log rank, P = 0.015). In multivariate analysis, factors adversely affecting outcome included XDR infection (HR = 2.263, 95%CI: 1.005-5.095, P = 0.049), creatinine (HR = 1.125, 95%CI: 1.024-1.236, P = 0.015) and INR (HR =1.553, 95%CI: 1.106-2.180, P = 0.011).

CONCLUSION: XDR bacteria are an independent life-threatening factor in SBP/SB. Strategies aiming at restricting antibiotic overuse and rapid identification of the responsible bacteria could help improve survival.

Key words: Spontaneous bacterial peritonitis; Spontaneous bacteremia; Multidrug-resistant bacteria; Extensively drug-resistant bacteria; Susceptibility to antibiotics

Core tip: This is a prospective, observational, single Center study seeking to evaluate the epidemiology and outcomes of 130 patients with decompensated cirrhosis and culture-positive spontaneous bacterial peritonitis or spontaneous bacteremia. Both multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria were isolated in about one third of the cases. Patients with XDR demonstrated high mortality compared to the rest of the patients. All MDR/XDR associated infections were health-care associated and/or nosocomial. Independent factors adversely affected survival included XDR infection, renal dysfunction and coagulation disorder.

Alexopoulou A, Vasilieva L, Agiasotelli D, Siranidi K, Pouriki S, Tsiriga A, Toutouza M, Dourakis SP. Extensively drug-resistant bacteria are an independent predictive factor of mortality in 130 patients with spontaneous bacterial peritonitis or spontaneous bacteremia. *World J Gastroenterol* 2016; 22(15): 4049-4056 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4049.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4049

INTRODUCTION

Infections are the most common precipitating event in acute-on-chronic liver failure[1] and are associated with high mortality in patients with decompensated cirrhosis[2]. Pathological bacterial translocation (BT) in cirrhosis involves alterations in gut microbiota, deficiency in intestinal barrier and an impaired immune response by the gut associated lymphatic tissue[3,4]. The most typical clinical expressions of pathological BT are spontaneous bacterial peritonitis (SBP) and spontaneous bacteremia (SB) (positive blood culture with no cause of bacteremia)[5]. Prompt and appropriate treatment is important in patients with bacterial infections and decompensated cirrhosis, to cover the most commonly isolated bacteria and maximize the patient's chance of survival[6]. In recent years, a change in epidemiology of bacterial infections in cirrhosis has been observed worldwide characterized by an increasing rate of multi-drug resistant (MDR) bacteria and a decreased efficacy of antibiotics[7-12]. Risk factors associated with resistance to antibiotics are nosocomial or health care-associated acquisition, long-term norfloxacin prophylaxis, recent use of b-lactams and recent infection with MDR[5,7,12-14]. A position statement based on the EASL Special Conference 2013 recommended as empirical treatment for community-acquired infections either cefotaxime (or ceftriaxone) or amoxicillin/clavulanate and for nosocomial either piperacillin/tazobactam or meropenem ± glycopeptide[5].

MDR are difficult to treat bacteria including extended-spectrum beta-lactamases (ESBL)-producing *Enterobacteriaceae*, nonfermentable Gram-negative bacteria (GNB) *Pseudomanas aeruginosa* (*P. aeruginosa*) and methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA). Recently, extensively drug-resistant bacteria (XDR) such as Carbapenemase-producing (KPC) *Klebsiella pneumonia* (*K. pneumoniae*) and vancomycin-resistant enterococci (VRE) emerged in patients with cirrhosis[12,14,15]. We recently reported an increased prevalence in MDR in SBP cases in the period 2008-2011[14].

In this study we aimed at assessing the possible changes in microbial etiology of culture-positive SBP

and SB, the risk factors of acquisition of microorganisms resistant to third generation cephalosporins and quinolones and the difference in survival between patients with different patterns of drug-resistance and those with no drug-resistance.

MATERIALS AND METHODS

Study design

This prospective, observational study was conducted in the 2nd Department of Internal Medicine of our hospital from January 1, 2012 to May 30, 2014. We prospectively collected data on patients with decompensated cirrhosis and either spontaneous bacteremia without SBP or (ascitic fluid and/or blood) culture-positive SBP. Patients with human immunodeficiency virus infection, previous transplantation or any other type of immunodeficiency, multi-microbial infections, in peritoneal dialysis or those with secondary bacterial peritonitis were excluded. The study protocol was approved by the Hospital Ethical Committee. All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

The diagnosis of SBP was based on neutrophil count in ascitic fluid of > 250/mm^3 as determined by microscopy[6]. After SBP or SB diagnosis and in patients with clinical suspicion of infection, empirical treatment was administered intravenously and was maintained or replaced subsequently following a second paracentesis in two days or the *in vitro* susceptibility of isolated organisms from ascitic fluid or blood. In patients with SB thorough investigation did not reveal any specific cause of bacteremia. Infections were classified as HCA in patients with a hospitalization for at least 2 d in the previous 180 d or as nosocomial those which developed in more than 48 h after admission[16]. We did not include any patients with a previous hospitalization in intensive care unit (ICU). The remaining infections were considered community-acquired (CA) when they were present on admission or developed within the first 48 h after admission[16]. Clinical doctors and infectious diseases specialists decided about the empirical antibiotic regimens and assessed the clinical course and the resolution of the infection. The empirical antibiotic regimen according to local policies and recent guidelines[5] was ceftriaxone IV for the community-acquired and meropenem for HCA and/or nosocomial infections. SBP and SB considered as cured if polymorphonuclear cell count was < 250/mm^3 and negative blood cultures were obtained after antibiotic treatment, respectively. Only fifteen patients (11.5%) had been receiving prophylactic quinolone treatment as secondary prevention for SBP.

Bacterial cultures technique and multi-drug resistant bacteria definition

On admission, diagnostic paracentesis and inoculation of ascitic fluid into two blood culture bottles for aerobic and anaerobic bacteria was routinely performed at bedside. Separate and simultaneous blood cultures were collected. Aerobic, anaerobic and broth cultures were initiated in BACTEC 9240 (Becton, Dickinson). All the isolated organisms were identified by the VITEK2 system (Biomerieux, Marcy l'Etoile, France). Antibiotic susceptibility testing was performed by the Kirby-Bauer method according to Clinical and Laboratory Standards Institute guidelines. The minimum inhibitory concentrations were determined by the E-test system (AB Biodisk, Solna, Sweden) and by the VITEK2 system. Double disc synergy (DDST), ESBL E-test (AB) and VITEK2 ESBL card (Biomerieux) were used to detect ESBL frequency. The detection of KPC carbapenemase was based on a phenotypic screen for carbapenem resistance followed by the modified Hodge test as a confirmatory test. DDST and E-test-MBL were used to detect MBL carbapenemase frequency. Only monomicrobial infections were included. All patients with simultaneous fungi-positive culture were excluded from the study. When the same strain was isolated twice or more from the same patient, it was counted only once. Multi-drug resistant (MDR) bacteria are strains non susceptible to $\geqslant$ 1 agent in $\geqslant$ 3 antimicrobial categories[17]. Extensively drug-resistant bacteria (XDR) defined as non-susceptible to $\geqslant$ 1 agent in all but $\leqslant$ 2 antimicrobial categories[17]. *Enterococcus faecium* (*E. faecium*) was considered an MDR organism and was classified as such.

Collection of the data

Clinical and laboratory data were collected at the time of admission including age, gender, cause of admission, etiology of cirrhosis, full blood count, international normalized ratio of prothrombin time (INR), renal and hepatic function biochemical tests and ascitic fluid evaluation. Severity of liver disease was assessed by Model for End-Stage Liver Disease (MELD) score for each patient. Days of hospitalization within 6 months before infection were also recorded.

Statistical analysis

All data were analysed using the statistical package SPSS (version 21.0 SPSS Inc., Chicago, IL, United States). The characteristics of the patients were assessed using median (interquartile range) for continuous variables and count (percentage) for categorical variables. In order to test for differences in the univariate analysis among the different categories of patients with MDR, XDR and non-DR bacteria, we used Mann-Whitney test for continuous variables and chi-squared test for categorical variables. Survival rates were evaluated using the Kaplan-Meier estimator and were compared between groups by the log-rank test. The Cox proportional-hazards model was used to estimate the risk of death due to MDR or XDR. Factors associated with mortality with a *P* value of < 0.10 in the univariate analysis were entered in the multivariate

Table 1 Demographic characteristics of patients with bacterial infections classified according to the drug resistance of the organism

	Total	Non-DR	MDR	XDR	*P* vaule[1]	*P* vaule[2]
	n = 130	*n* = 90	*n* = 27	*n* = 13		
Age (yr)	62 (55-73)	62 (54-63)	62.5 (54.7-73.2)	62 (59-79)	0.928	0.379
Gender (Males)	89 (68.5)	57 (63.3)	23 (85.2)	9 (69.2)	0.768	0.035
Etiology of liver cirrhosis						
Alcoholic	54 (41.5)	35 (38.9)	13 (48.1)	6 (46.2)	0.476	0.718
Viral	47 (36.1)	32 (35.6)	10 (37)	5 (38.5)		
Other	29 (22.3)	23 (25.6)	4 (14.8)	2 (15.4)		
MELD	20 (15-25)	20 (15-25)	19.5 (16-23)	25 (18-36)	0.947	0.044
Hospitalization days over the preceding 6 mo	10 (5-19)	9 (4-16)	15 (6.75-28)	11 (7.5-22)	0.014	0.249
Nosocomial infections, *n* (%)	47 (36.1)	23 (25.5)	16 (59.2)	8 (61.5)	0.001	0.008
HCA infections, *n* (%)	75 (57.6)	48 (53.3)	18 (66.6)	9 (69.2)	0.220	0.281
HCA and/or nosocomial infections, *n* (%)	99 (76.1)	59 (65.5)	27 (100)	13 (100)	< 0.001	0.011

[1]Comparison between MDR and non-DR; [2]Comparison between XDR and Non-DR, values are expressed in median (interquartile range). HCA: Health care associated infections; MDR: Multi-drug resistant; XDR: Extensively drug resistant.

Table 2 Laboratory characteristics of patients with bacterial infections classified according to the drug resistance of the organism

	Total	Non-DR	MDR	XDR	*P* vaule[1]	*P* vaule[2]
	n = 130	*n* =90	*n* = 27	*n* = 13		
Leucocyte count × 10^9/L	7.38 (4.6-11.3)	7.34 (4.85-11.27)	7.2 (4.18-11.73)	7.51 (4.39-11.61)	0.797	0.967
Neutrophil/leucocyte%	81 (71-88)	79 (70-87)	84 (72-88)	83 (76-90)	0.608	0.163
Total Bilirubin (mg/dL)	3.8 (1.9-8.7)	4.2 (1.9-9.2)	2.6 (1.6-6.1)	4.2 (1.7-19.7)	0.305	0.792
Creatinine (mg/dL)	1.1 (0.8-1.7)	1.1 (0.8-1.5)	1.2 (0.8-1.7)	2 (0.8-5.0)	0.269	0.093
INR	1.6 (1.4-2.1)	1.6 (1.3-2.2)	1,5 (1.4-1.9)	2 (1.3-2.2)	0.958	0.423
AST (IU/L)	53 (30-105)	55 (30-121)	45 (32-74)	23 (18-78)	0.423	0.057
C-reactive protein (mg/L)	59 (23-108)	52 (22-102)	68 (30-138)	101 (18-120)	0.318	0.302
Fibrinogen (mg/dL)	239 (134-496)	344 (130-516)	170 (156-273)	247 (119-460)	0.393	0.628

[1]Comparison between MDR and Non-DR; [2]Comparison between XDR and non-DR; values are expressed in median (interquartile range). MDR: Multi-drug resistant; XDR: Extensively drug resistant.

model and non-significant factors were removed by a backward selection process. A two-tailed *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Clinical and laboratory characteristics in overall

We prospectively recorded 130 cases (68.5% males, median age 62 years) with culture positive-SBP (70 cases) or spontaneous bacteremia without SBP (60 cases) in patients with decompensated cirrhosis. In total, 58 (44.6%) cases had positive ascitic fluid culture, 64 (49.2%) positive blood culture and 8 (6.2%) both. Patients were classified into 3 groups according to the drug-resistance pattern: ninety (69.2%) patients had infections with non-DR, 27 (20.8%) with MDR and 13 (10%) with XDR bacteria. Etiology of cirrhosis was chronic viral hepatitis in 47 (36.1%), alcohol in 54 (41.5%) and other causes in 29 (22.3%). The median MELD score was 20 (15-25). Ninety nine patients (76.1%) had been hospitalized within the last six months (HCA) and/or developed nosocomial infections (23 were both HCA-associated and nosocomial) (Table 1). Only 31 (23.8%) infections were community-acquired. The laboratory characteristics of patients on admission are demonstrated in Table 2.

Comparison of 3 groups of patients according to different patterns of drug resistance

No difference in age, gender and etiology of cirrhosis was observed between patients with XDR or MDR and those with non-DR bacteria. More severe liver disease was observed in patients with XDR than in those with non-DR bacteria [median MELD score 25 (interquartile range 18-36) *vs* 20 (15-25), respectively *P* = 0.044] (Table 1). Nosocomial infections were more frequent in the XDR or MDR groups compared to the non-DR one. Community-acquired infections were evident only in the non-DR group (Table 1). No difference in laboratory characteristics was shown among the groups (Table 2).

Type of bacteria and drug resistance

Gram-positive cocci (GPC) were found in half of the cases (48.5%). The most prevalent organisms in a descending order were *Escherichia coli* (*E. coli*) (33), *Enterococcus spp* (30, including 17 *E. faecium*), *Streptococcus spp* (25), *K. pneumonia* (16), *S. aureus* (8), *P. aeruginosa* (5), other *GNB* (11) and anaerobes (2). Twenty seven (20.8%) of the isolated bacteria were MDR, including ESBL - GNB (9), *P. aeruginosa* (3) and *E. faecium* (15). Thirteen (10%) of the bacteria were XDR including KPC *K. pneumonia* (5), colistin-resistant KPC-producing *K. pneumonia* (1), *P.*

Table 3 Isolated bacteria and antibiotic susceptibility tests

Antibiotic	OX	AMC	MEM	CTX	FEP	TGC	VA	CT	CIP	SXT
Gram-positive bacteria (63)										
Resistance	34/63	22/63	25/63	27/63	26/63	0/63	2/63	51/63	38/63	24/63
S. aureus (8)	0/8	5/8	8/8	8/8	8/8	0/8	0/8	8/8	2/8	0/8
Streptococcus spp. (23)	3/22	0/23	0/23	0/23	0/23	0/23	0/23	11/23	11/23	0/23
S. pneumonia (2)	2/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	2/2	0/2
E. faecalis (13)	12/13	0/13	0/8	2/13	1/13	0/13	0/13	13/13	6/13	7/13
E. faecium (15)	15/15	15/15	15/15	15/15	15/15	0/15	0/15	15/15	15/15	15/15
E. faecium VRE (2)	2/2	2/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	2/2
Gram-negative bacteria (65)										
Resistance	65/65	34/65	15/65	28/65	20/65	9/65	65/65	1/65	24/65	33/65
E. coli (25)	25/25	3/25	0/25	1/25	0/25	0/25	25/25	0/25	5/25	5/25
ESBL-*E. coli* (7)	7/7	7/7	0/7	7/7	6/7	0/7	7/7	0/7	7/7	6/7
MBL-*E. coli* (1)	1/1	1/1	1/1	1/1	1/1	0/1	1/1	0/1	1/1	1/1
K. pneumonia (9)	9/9	1/9	0/9	0/9	0/9	0/9	9/9	0/9	1/9	7/9
KPC-*K. pneumonia* (5)	5/5	5/5	5/5	5/5	5/5	2/5	5/5	0/5	4/5	1/1
ESBL-*K. pneumonia* (1)	1/1	1/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1
K. pneumonia KPC+, col-R (1)	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
P. aeruginosa (5)	5/5	5/5	2/5	5/5	3/5	5/5	5/5	0/5	2/5	5/5
S. marcescens (2)	2/2	2/2	0/2	0/2	0/2	0/2	2/2	0/2	0/2	0/2
S. maltophilia (3)	3/3	2/3	3/3	3/3	1/3	0/3	3/3	0/3	0/3	0/3
A. baumannii (2)	2/2	2/2	2/2	2/2	2/2	1/2	2/2	0/2	2/2	2/2
ESBL P. mirabilis- (1)	1/1	1/1	0/1	1/1	1/1	0/1	1/1	0/1	1/1	1/1
E. cloacae (2)	2/2	2/2	1/2	0/2	0/2	0/2	2/2	0/2	0/2	2/2
E. aerogenes (1)	1/1	1/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1
Anaerobes (2)										
Resistance	2/2	1/2	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
Bacteroides spp (2)	2/2	1/2	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2

OX: Oxacillin; AMC: Amoxicillin/Clavulanic acid; MEM: Meropenem; CTX: Cefotaxime; FEP: Cefepime; TGC: Tigecycline; VA: Vancomycin; CT: Colistin; CIP: Ciprofloxacin; SXT: Cotrimoxazole; ESBL: Extended-spectrum-beta-lactamase-producing; KPC+, col-R: Carbapenemase-producing colistin-resistant *K. pneumonia*; MBL: Metallo-β-lactamase-producing; VRE: Vancomycin-resistant *E. Faecium*. *S. aureus*: *Staphylococcus aureus*; *S. pneumonia*: *Streptococcus pneumoniae*; *E. faecalis*: *Enterococcus faecalis*; *E. faecium*: *Enterococcus faecium*; *E. coli*: *Escherichia coli*; *K. pneumonia*: *Klebsiella pneumonia*; *P. aeruginosa*: *Pseudomanas aeruginosa*; *S. marcescens*: *Serratia marcescens*; *S. maltophilia*: *Stenotrophomonas maltophilia*; *A. baumannii*: *Acinetobacter baumannii*; *P. mirabilis*: *Proteus mirabilis*; *E. cloacae*: *Enterobacter cloacae*; *E. aerogenes*: *Enterobacter aerogenes*.

aeruginosa (2), *A. baumannii* (2), VRE *E. faecium* (2) and metallo-β-lactamase-producing (MBL) - *E. coli* (1).

Resistance to antimicrobial agents

Fifty seven (43.8%) of the isolated bacteria were third-generation cephalosporin-resistant. Resistance to quinolones was observed in sixty four (49.2%) microorganisms. Amoxycilline-clavulanate resistance was demonstrated in 57 (43.8%) cases (Table 3). The 50/57 (88%) third-generation cephalosporine-resistant bacteria showed cross-resistance to amoxicillin/clavulanate and the 42/57 (73.7%) were also resistant to quinolones (Table 3). Meropenem, the most frequent empirically prescribed antibiotic for HCA/nosocomial infections, showed a drug resistance rate of 30.7%. Meropenem was ineffective on both XDR bacteria and *E. faecium*. Ten out of 13 (77%) XDR were susceptible to colistin while all GPC including *E. faecium* and the 86% of GNB to tigecycline. Only 54% of the XDR were susceptible to tigecycline. All but one XDR bacteria were susceptible to a possible combination of colistin and tigecycline.

Survival analysis according to drug resistance pattern

Kaplan-Meier analysis showed that patients with XDR bacteria had a worse 30-d survival compared to those with MDR or non-DR bacteria (log rank, $P = 0.015$) (Figure 1). The outcome of patients with XDR was worse compared to MDR- and non-DR-infected patients separately (log rank $P = 0.012$ and $P = 0.008$, respectively). Consequently, patients with MDR had similar 30-d-survival rate to those with non-DR bacteria (log rank $P = 0.604$). In overall, 30-d-mortality was 37.7%. In particular, 30 d-mortality rate for patients with XDR *vs* the rest of the patients were 69.2% *vs* 34.2%, respectively.

In Cox univariate analysis, variables that had at least a trend ($P < 0.10$) for association with 30-d survival included age ($P = 0.089$), neutrophil-to-leucocyte ratio ($P = 0.072$), INR ($P = 0.001$), creatinine ($P = 0.036$), total bilirubin ($P = 0.001$) and XDR infection ($P = 0.007$). In multivariate Cox regression analysis, factors adversely affecting outcome were XDR infection (HR = 2.263, 95%CI: 1.005-5.095, $P = 0.049$), creatinine (HR = 1.125, 95%CI: 1.024-1.236, $P = 0.015$) and INR (HR = 1.553, 95%CI: 1.106-2.180, $P = 0.011$) (Table 4).

DISCUSSION

A worrisome increase in infections caused by MDR pathogens in decompensated cirrhosis has been

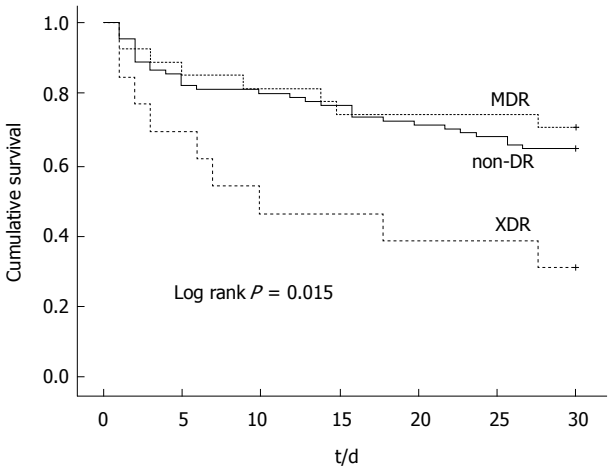

Figure 1 Comparison of survival among patients infected with extensively drug-resistant, multi-drug-resistant and non-drug-resistant bacteria. Non-DR: Non-drug-resistant bacteria; MDR: Multi-drug resistant; XDR: Extensively drug-resistant.

Table 4 Univariate and multivariate Cox regression analysis of factors predicting 30-d mortality in 130 patients with bacterial infections

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (per 1 yr)	1.017 (0.997-1.037)	0.089		
Gender	1.134 (0.833-1.542)	0.425		
Neutrophil-to-Leucocyte ratio	1.026 (0.998-1.055)	0.072		
C-reactive protein	1.004 (0.999-1.008)	0.129		
XDR infections	2.7 (1.310-5.565)	0.007	2.263 (1.005-5.095)	0.049
Total bilirubin	1.051 (1.020-1.082)	0.001		
Creatinine	1.097 (1.006-1.197)	0.036	1.125 (1.024-1.236)	0.015
INR	1.746 (1.271-2.399)	0.001	1.553 (1.106-2.180)	0.011
AST	1.000 (0.999-1.001)	0.442		

XDR: Extensively drug-resistant; INR: International normalized ratio.

emerged in many countries[7-12]. An increasing frequency in MDR in 47 cases with culture-positive-SBP was described in a previous study from our Center (2008-2011)[14]. The current collection of data from culture-positive infections of both blood stream and ascitic fluid over the period 2012-2014, showed similar rates of Gram-positive cocci, MDR pathogens and *E. faecium*, with those reported previously.

However, an increasing variety in XDR was currently recorded. MBL-producing *E. coli*, VR-*enterococci* and colistin-resistant KPC-producing *K. pneumonia* were isolated for the first time, while ESBL-producing *Enterobacteriaceae,* KPC-producing *K. pneumonia*, A. baumanni and multi-drug resistant *P. aeruginosa* were also observed in the previous cohort. Notably, the MDR/XDR pathogens were coming from regular hospital wards and not from the ICU. In addition, no previous hospitalizations in ICU were recorded. MDR/XDR bacteria were exclusively associated with HCA/nosocomial infections. On the other hand, non-DR bacteria were encountered only in patients with community-acquired infections. It is remarkable that community-acquired infections prevalence is reduced in current cohort compared to that reported in the literature[12].

E. faecium is a common pathogen in the current cohort comprising the 57% of the total *Enterococci*. *E. faecium* is considered by previous investigators as a multiresistant organism[5,18] because of resistance to ampicillin, third generation cephalosporins and quinolones[17]. Hence, we decided to include susceptible to vancomycin *E. faecium* in the MDR group. Eventually, we observed that all the *E. faecium*-associated infections were health-care-associated and/or nosocomial and patients with *E. faecium* had prolonged previous hospitalizations comparable to those recorded in MDR/XDR bacteria. The above findings advocated that this pathogen belongs to MDR bacteria.

Regarding microbial resistance, no major changes were recorded in amoxicillin-clavulanate, third generation cephalosporins and quinolone compared to the previous study. As reported before in the local nosocomial setting, the above antibiotics were ineffective to treat health care-associated and/or nosocomial infections[14]. In addition, a relatively high prevalence of ESBL-producing *Enterobacteriaceae* was previously described[14]. For all reasons reported above, clinical doctors and infection specialists decided to administer ceftriaxone to the community-acquired and meropenem to the health care-associated (HCA) and/or nosocomial infections during the whole period of current study. The widespread administration of carbapenems in the majority of the patients with HCA/nosocomial infections may have resulted in the emergence of new XDR strains and warns against the expanded use of these antibiotics. KPC-producing *K. pneumonia*, colistin-resistant or not, MBL-producing bacteria and multi-resistant *P. aeruginosa* may be the consequences of the aforementioned clinical approach which allows few available therapeutic options. As the clinicians usually approve the early antibiotic administration in cirrhotics, many non-infected patients may receive antibiotics, increasing the rate of colonization of enteric flora with resistant bacteria which eventually may become virulent.

The study illustrates two major changes in clinical practice with important clinical implications. Firstly, only few patients were receiving quinolone prophylaxis and as a result MDR/XDR infection development was not associated with the use of prophylaxis against SBP. The high prevalence of Gram positive cocci observed worldwide[12,19-22] was also confirmed in both 2008-2011[14] and 1998-2002[23] studies in culture-positive SBP cases from our Department. In addition, a remarkable number of MDR/XDR-associated infections were demonstrated

in 2008-2011 study. More specifically, GPC accounted for the half and MDR/XDR for the one third of the cases. Besides quinolone resistance accounted for almost half of the cases rendering quinolones ineffective for secondary prophylaxis. Similar high rates of quinolone resistance in isolated bacteria were reported in previous investigations[12,24-26]. The low rate of quinolone prophylaxis in the current cohort prevented us from considering quinolone as a risk factor for the development of multiresistant bacteria. Second, similar 30-d-survival rates were found between patients infected with non-DR and those with MDR pathogens. On the contrary, patients with XDR had significantly lower survival rate compared to both MDR and non-DR groups. This finding is not surprising since the impact of MDR on survival is blunted by the extended use of carbapenems, producing bacteriological clearance and achieving the best outcomes in the most MDR Enterobacteriaceae-associated infections. However, carbapenems are inefficient at treating both XDR bacteria and *E. faecium*. Colistin seemed to be the optimal choice for the former and glycopeptides for the later except for vancomycin-resistant strains where tigecycline is highly effective. As empirical treatment administered contained neither colistin nor glycopeptides, antibiotic failure is the cause of poor survival in patients infected with XDR bacteria. It is a routine in our Center to take both blood and ascitic fluid culture on admission and perform a second paracentesis after 48 h of start of treatment to check the effect of empirical antibiotic therapy. In case of XDR bacteria, first choice antibiotic treatment failure was frequent. Thus, the delay of *in vitro* susceptibility test, may also delay the modification of antibiotic treatment and have deleterious effects on outcome. XDR bacteria were a strong predictive factor of death in current study even in multivariate analysis. Hence, empirical antibiotic treatment requires the use of broad spectrum antibiotics adapted to the local epidemiological pattern. A recent investigation recommended a broad spectrum antibiotic combination including meropenem plus daptomycin as first line treatment of nosocomial SBP with favorable effect on survival compared to ceftazidime alone[27].

In conclusion, extensively drug-resistant bacteria are an independent life-threatening factor of outcome in cirrhotic patients with spontaneous bacterial peritonitis and spontaneous bacteremia. Even if our results could not be extrapolated to other institutions, it is useful to know local bacterial epidemiology of infections in cirrhosis in order to restrict overuse and make a more rational use of antibiotics. In addition, new microbiological methods aiming at rapid identification of the responsible bacteria could help improve survival. As monotherapy seems to be ineffective in a significant proportion of patients, a more complex approach including broad spectrum antibiotic combinations should be considered for empirical therapy of HCA/nosocomial infections.

COMMENTS

Background

Spontaneous bacterial peritonitis (SBP) and spontaneous bacteremia (SB) (positive blood culture with no cause of bacteremia) are the most typical infections in patients with decompensated cirrhosis related to bacterial translocation. Early administration of the appropriate empirical antibiotics is important in order to cover the most commonly isolated bacteria and maximize the patient's chance of survival.

Research frontiers

Recently, a change in epidemiology of bacterial infections in cirrhosis has been observed worldwide characterized by an increasing prevalence of multi-drug resistant (MDR) bacteria and a decreased efficacy of antibiotics. In this study, there is an increased rate of MDR and extensively drug-resistant (XDR) bacteria even in the absence of quinolone prophylaxis particularly in health-care associated and/or nosocomial infections.

Innovations and breakthroughs

The literature suggests a high mortality of patients infected with MDR bacteria. The current study showed a similar mortality in patients with MDR and non-DR bacteria probably because of the wide use of meropenem in health-care associated and/or nosocomial infections in local area. However, a high mortality of XDR was observed.

Applications

This study highlights the relatively high prevalence of XDR pathogens in SBP and SB in decompensated cirrhosis in the local setting and the importance to modify regional epidemiological factors in order to improve outcome. In addition, rapid identification of the causative organism and empirical treatment with a combination of broad-spectrum antibiotics may help improving survival.

Terminology

Multidrug resistant bacteria: strains non susceptible to $\geqslant$ 1 agent in $\geqslant$ 3 antimicrobial categories. Extensively drug-resistant bacteria: strains non-susceptible to $\geqslant$ 1 agent in all but $\leqslant$ 2 antimicrobial categories.

Peer-review

This is a nicely written paper about an important and interesting area. In this paper, Alexopoulou *et al* evaluated the epidemiology and outcomes of culture-positive SBP and SB in decompensated cirrhosis patients and revealed the factors adversely affecting outcome included XDR infection, elevated creatinine and INR. The drug resistance of isolated bacteria to antibiotics also were investigated and authors provided the explanations of drug-resistance and the measures of preventing antibiotic-resistance. These findings are useful for clinicians to restrict overuse, make a more rational use of antibiotics, and eventually improve the survival of these patients.

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CASE REPORT

Nasogastric tube syndrome induced by an indwelling long intestinal tube

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Abstract

The nasogastric tube (NGT) has become a frequently used device to alleviate gastrointestinal symptoms. Nasogastric tube syndrome (NTS) is an uncommon but potentially life-threatening complication of an indwelling NGT. NTS is characterized by acute upper airway obstruction due to bilateral vocal cord paralysis. We report a case of a 76-year-old man with NTS, induced by an indwelling long intestinal tube. He was admitted to our hospital for treatment of sigmoid colon cancer. He underwent sigmoidectomy to release a bowel obstruction, and had a long intestinal tube inserted to decompress the intestinal tract. He presented acute dyspnea following prolonged intestinal intubation, and bronchoscopy showed bilateral vocal cord paralysis. The NGT was removed immediately, and tracheotomy was performed. The patient was finally discharged in a fully recovered state. NTS be considered in patients complaining of acute upper airway obstruction, not only with a NGT inserted but also with a long intestinal tube.

Key words: Nasogastric tube syndrome; Nasogastric tube; Long intestinal tube; Acute upper airway obstruction; Tracheotomy

Core tip: Nasogastric tube syndrome (NTS) is an uncommon but potentially life-threatening complication of an indwelling nasogastric tube (NGT). NTS is characterized by acute upper airway obstruction due to bilateral vocal cord paralysis. We report a

case of a 76-year-old man with NTS, induced by an indwelling long intestinal tube. He presented acute dyspnea following prolonged intestinal intubation, and bronchoscopy showed bilateral vocal cord paralysis. The NGT was removed immediately, and tracheotomy was performed to establish a safe airway. NTS be considered in patients complaining of acute upper airway obstruction, not only with a NGT inserted but also with a long intestinal tube inserted.

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INTRODUCTION

Nasogastric tube (NGT) is commonly used for a variety of indications since its first description by Hunter in 1790[1]. Despite extensive adaptation, serious complications have occasionally been reported. Iglauer and Molt first described damage to the larynx due to an indwelling enteric tube in a case series in 1939[2]. NTS was later formally defined by Sofferman *et al*[3] in 1990. This syndrome consists of throat pain, the presence of a NGT, and vocal cord abductor dysfunction[3]. It is encountered only rarely, but it is potentially life threatening.

To our knowledge, in all of the medical literature, a total of 20 case reports (Table 1) and 2 reviews (including the case report described) of NTS have been published through 2014[3-14]. Of these cases, only one case was induced by indwelling a long intestinal tube[6].

It is hypothesized that early and adequate investigation of throat pain and hoarseness in patients with an indwelling NGT or a long intestinal tube is critical to the diagnosis and timely management of NTS.

CASE REPORT

A 76-year-old man was admitted to the Tsukuba Medical Center Hospital for the treatment of sigmoid colon cancer. The physiological examination, abdominal X-ray (Figure 1A), and computed tomography (Figure 2) indicated colonic obstruction caused by a tumor in the sigmoid colon. Sigmoidectomy was performed urgently, and a long intestinal tube was placed into the jejunum during surgery for bowel decompression (Figure 1B). Six days after tube placement, the patient developed throat pain, acute inspiratory stridor, and oxygen failure. The long intestinal tube was removed immediately, and the patient was intubated to improve the oxygenation failure. When intubating, post-cricoid ulceration and arytenoid edema were observed. To improve the laryngeal injury, the patient was extubated two days later. Because wheezing persisted even after extubation, the patient was submitted bronchoscopy, which showed bilateral vocal cord dysfunction. Tracheotomy was performed for dyspnea. Four weeks following tracheotomy, bronchoscopy showed improving bilateral vocal cord paralysis. Finally, the patient recovered one month after the onset of NTS and was discharged with the tracheotomy closed.

DISCUSSION

NGT is one of the most commonly used devices in hospital patient care. Most often, NGT is placed for days to weeks before digestive symptoms become manifest. NTS is a rare but potentially fatal complication of nasogastric intubation, comprising acute upper airway obstruction due to bilateral vocal cord paralysis. However, due to ignorance of this disease, it is believed that there have been fewer cases reported than have actually occurred. Bilateral vocal cord paralysis is the principal sign that has brought this condition to clinical awareness. We experienced an extremely rare case of NTS induced by indwelling a long intestinal tube[6].

The term NTS was introduced by Sofferman in 1990 to describe the triad of throat pain, nasogastric intubation, and vocal cord paralysis man[3]. Brousseau *et al*[8] reported that 71% cases of NTS occurred in men, and 29% of cases occurred in women. Harmon *et al*[13] reported that this syndrome developed in both children and adults. Brousseau *et al*[8] also estimated a range of NTS onset from 12 h after intubation to 2 wk after extubation. Time to recovery from respiratory symptoms and vocal cords dysfunction has been reported at 1 d to 3 mo[3-6,9,10,12,13]. In our case, the symptoms were present 6 d after long intestinal intubation, and a month was required for complete recovery.

Throat pain is an important symptom. Most often, the discomfort is correctly attributed to the NGT, but its significance is usually minimized. Unless the pain is closely followed by stridor, otolaryngologic consultation can be delayed by several days to more than a week after the initial complaint. Odynophagia and referred otalgia are additional symptoms that coexist within the broad framework of this syndrome.

Diabetes mellitus and immunocompromised states have been suggested as risk factors for NTS[3]. Moreover, time to recovery have been reported to be longer in diabetic patients[3]. In our case, the patient did not have diabetes mellitus. Because the number of NTS case reports is very small, the risk factors for the disease are currently unclear. It is necessary to accumulate further case reports.

It is important first to diagnose NTS accurately. If it is not suspected at the time of intubation, bronchoscopy might be omitted and the correct

Table 1 Case reports of nasogastric tube syndrome

Author	Year	Age (yr), sex	Tube	Treatment	Outcome
Sofferman *et al*[3]	1990	28, male	NGT	Removal of NGT, tracheotomy	Full recover
Sofferman *et al*[3]	1990	42, male	NGT	Removal of NGT, tracheotomy	Full recover
Sofferman *et al*[3]	1990	36, male	NGT	Removal of NGT, tracheotomy	Death
Sofferman *et al*[3]	1990	45, female	NGT	Removal of NGT, tracheotomy	Full recover
Apostolakis *et al*[4]	2001	77, male	NGT	Removal of NGT, tracheotomy	Not recover
Apostolakis *et al*[4]	2001	73, male	NGT	Removal of NGT, tracheotomy	Full recover
Leclerc *et al*[14]	2002	71, female	NGT	Tracheotomy	Not recover (cricoid necrosis)
Nehru *et al*[5]	2003	60, male	NGT	Removal of NGT, tracheotomy	Full recover
Sanaka *et al*[6]	2004	85, male	Long intestinal tube	Removal of long intestinal tube, tracheotomy	Full recover
Isozaki *et al*[7]	2005	73, male	NGT	None	Death
Isozaki *et al*[7]	2005	77, female	NGT	Removal of NGT	Death
Isozaki *et al*[7]	2005	79, female	NGT	Undescribed	Undescribed
Isozaki *et al*[7]	2005	72, female	NGT	Undescribed	Undescribed
Marcus *et al*[9]	2006	72, male	NGT	Removal of NGT, tracheotomy	Full recover
Vielva del Campo *et al*[12]	2010	70, female	NGT	Removal of NGT, tracheotomy	Full recover
Ohshima *et al*[11]	2010	62, female	NGT	Removal of NGT	Full recover
Harmon *et al*[13]	2014	2 mo, male	NGT	Removal of NGT	Full recover
Harmon *et al*[13]	2014	3 mo, female	NGT	Removal of NGT	Full recover
Harmon *et al*[13]	2014	3 mo, male	NGT	Removal of NGT	Full recover
Our case	2015	76, male	Long intestinal tube	Removal of NGT, tracheotomy	Full recover

NGT: Nasogastric tube.

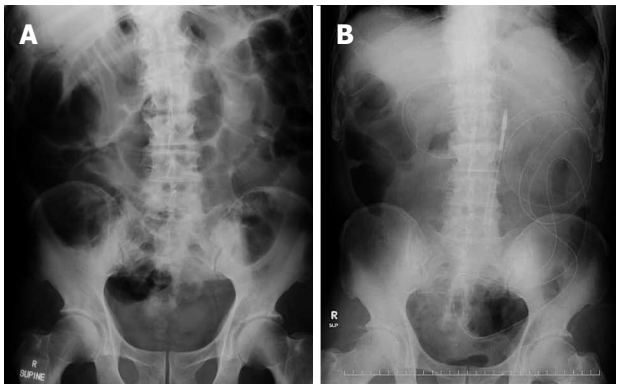

Figure 1 Abdominal X-ray. A: Intestinal obstruction at hospitalization; B: abdominal X-ray after long intestinal intubation.

diagnosis missed. The diagnosis of vocal cord paralysis and a post-cricoid ulcer can be excluded only by direct visualization of the entire post-cricoid area[4]. The most common findings, using the scope of the pharynx and the larynx in all of the published cases, have been bilateral vocal cord paralysis and ulceration of the post-cricoid region[8]. In our case, bronchoscopy showed only bilateral vocal cord paralysis induced by an indwelling long intestinal tube. The assessment of suspected cases of vocal cords paralysis in the setting of NGT or a long intestinal tube should include a full head and neck examination to exclude other causes and bronchoscopy to evaluate vocal cord function, ulceration, and the post-cricoid area, as well as ultrasound or computed tomography of the neck to exclude compressive pathologies.

The mechanisms of NTS can be explained in three parts[3,8,9,12]. First, the mobile laryngeal structures rub against the fixed NGT. Second, while the patient is supine, the cricoid bone compresses the tube against the supine. Finally, tonic contraction of the cricopharyngeus muscle pulls the tube against the delicate and thin post-cricoid mucosa. This combination generates local irritation, edema and eventual ulceration of the tissues, leading to impaired vocal cord function. It is believed that the upper airway is closed by the swelling that occurs due to the above mechanism.

The key treatment of NTS is removal of the NGT or long intestinal tube because it can lead to rapid resolution of respiratory distress. If a patient suffering from NTS requires prolonged bowel decompression, a percutaneous gastrostomy is an option. There have also been cases that improved only with the removal of the tube. However, when removal of the tube does not improve symptoms, tracheotomy will be required. Nehru *et al*[5] reported that 77% of all NTS cases required tracheotomy. Tracheotomy is preferable to prolonged endotracheal intubation because the latter can delay the recovery of vocal cord function for several months. With regard to other treatments, parenteral corticosteroids should be used to reduce inflammation, and antibiotics should be used to prevent the formation of retro-cricoid abscesses. Moreover, the patient should refrain from oral ingestion for several days, with daily inspection of the larynx to detect reduction in arytenoid edema. If necessary, intravenous fluids, hyperalimentation, or gastrostomy could be required during this interval. In our case, the long intestinal tube was removed immediately, and tracheotomy was performed.

To prevent the onset of NTS, adaptation of the NGT or long intestinal tube insertion should be carefully determined. Moreover, a more narrow tube diameter

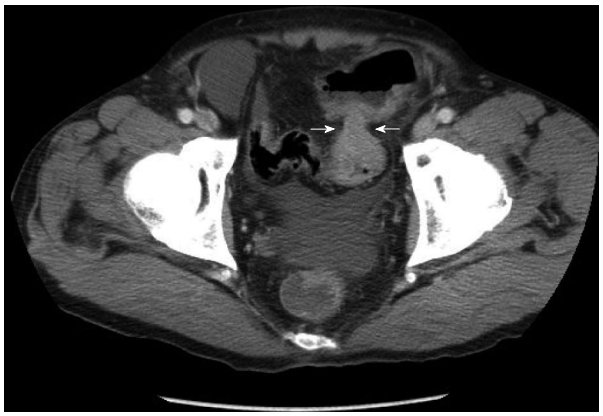

Figure 2 Computed tomography revealed sigmoid colon cancer. The white arrows indicated the site of stenosis due to the cancer.

should be chosen to reduce the pressure with which the tube presses against the organization. Friedman *et al*[15] reported that midline tube placement generated severe inflammation in the post-cricoid region more often than lateral tube placement. A long intestinal tube with a large diameter might be more easily placed in the midline than a NGT and lead to NTS.

In summary, we have reported a very rare but life-threatening case of the NTS induced by a long intestinal tube. We believe that this is only the second report of a case of NTS associated with a long intestinal tube for postoperative bowel decompression. NTS should be considered in all patients who present with throat pain, hoarseness, or shortness of breath after a nasogastric or a long intestinal intubation. It is hoped that careful attention to these conditions will prevent the full syndrome from becoming manifest. NTS requires prompt treatment, such as removal of the tube and tracheotomy, and close follow-up with bronchoscopy. If patients are properly diagnosed, almost all of them will eventually recover.

COMMENTS

Case characteristics

A 76-year-old man presented acute upper airway obstruction due to bilateral vocal cord paralysis.

Clinical diagnosis

The patients present acute upper airway obstruction following prolonged intestinal intubation, and bronchoscopy showed bilateral vocal cord paralysis.

Differential diagnosis

Diseases that cause acute upper airway obstruction such as the infection and foreign substances may be the main differential diagnosis.

Laboratory diagnosis

Except for desaturation, most laboratory values were within normal limits.

Imaging diagnosis

Bronchoscopy showed bilateral vocal cord paralysis.

Treatment

The key treatment is removal of the nasogastric tube or long intestinal tube. When removal of the tube does not improve symptoms, tracheotomy will be required.

Related reports

Very few cases of nasogastric tube syndrome have been reported in the literature. In most of the cases have been induced by an indwelling the nasogastric tube. The authors report a case induced by an indwelling long intestinal tube.

Term explanation

NTS is defined as nasogastric tube syndrome and NGT is defined as nasogastric tube.

Peer-review

The authors have described a case of nasogastric tube syndrome, which is rare and should, therefore, be reported. The patient was finally discharged in a fully recovered state by treatment with the appropriate diagnosis.

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CASE REPORT

Atypical onset of bicalutamide-induced liver injury

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Author contributions: All authors contributed to the acquisition of data, writing, and revision of this manuscript.

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Conflict-of-interest statement: All the authors have no conflicts of interests to declare.

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Abstract

Anti-androgen therapy is the leading treatment for advanced prostate cancer and is commonly used for neoadjuvant or adjuvant treatment. Bicalutamide is a non-steroidal anti-androgen, used during the initiation of androgen deprivation therapy along with a luteinizing hormone-releasing hormone agonist to reduce the symptoms of tumor-related flares in patients with advanced prostate cancer. As side effects, bicalutamide can cause fatigue, gynecomastia, and decreased libido through competitive androgen receptor blockade. Additionally, although not as common, drug-induced liver injury has also been reported. Herein, we report a case of hepatotoxicity secondary to bicalutamide use. Typically, bicalutamide-induced hepatotoxicity develops after a few days; however, in this case, hepatic injury occurred 5 mo after treatment initiation. Based on this rare case of delayed liver injury, we recommend careful monitoring of liver function throughout bicalutamide treatment for prostate cancer.

Key words: Bicalutamide; Drug-induced liver injury; Prostate neoplasm

Core tip: This case report describes a 62-year-old man with prostate cancer who experienced delayed

liver injury after bicalutamide therapy. In previous case reports on bicalutamide-induced liver injury, liver failure occurred shortly after bicalutamide therapy initiation. However, in this case, liver injury occurred 5 mo after bicalutamide treatment initiation. Therefore, our case emphasizes that liver function measurements should be monitored from baseline for at least the first 6 mo of therapy, and then periodically during the entire period of treatment with bicalutamide.

Yun GY, Kim SH, Kim SW, Joo JS, Kim JS, Lee ES, Lee BS, Kang SH, Moon HS, Sung JK, Lee HY, Kim KH. Atypical onset of bicalutamide-induced liver injury. *World J Gastroenterol* 2016; 22(15): 4062-4065 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4062.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4062

INTRODUCTION

Prostate neoplasms represent the second most common reason for male cancer-related mortality in the United States[1]. Mean age at diagnosis is 72 years; the condition is therefore called an "old man's" disease. The 5-year overall survival rates have been estimated to be 92%-95% for localized, 80%-83% for locally advanced, and 29% for metastatic disease. In metastatic prostate cancer, anti-androgen therapy is the chief treatment. More import role of anti-androgen therapy is the neoadjuvant or adjuvant therapy in the management of less advanced cancers[2].

Bicalutamide is a non-steroidal anti-androgen agent frequently administered during the initiation of androgen deprivation therapy along with a luteinizing hormone-releasing hormone agonist; it relieves the flare symptoms in patients with advanced prostate cancer. The frequent drug-induced toxicities caused by bicalutamide are hot flashes, gynecomastia, and breast pain[3]. Liver function test abnormalities, particularly in elevated transaminases, are also seen in bicalutamide use. To our knowledge, there are currently only four previous reports on bicalutamide-induced liver injury worldwide, with no previous case reported in Korea. In these previous cases, the liver function impairments were typically transient and occurred within a few days of bicalutamide use[2,4-6].

In this case report, we present an uncommon case of delayed liver injury after bicalutamide therapy, showing prolonged liver dysfunction maintained for approximately 2 mo. This is the first description of bicalutamide-induced liver injury in a Korean patient.

CASE REPORT

A 62-year-old South Korean man who was diagnosed with prostate cancer (T2N0M0; Gleason score 6; initial prostate-specific antigen, 6.75 ng/mL) presented with jaundice for a few days. He had been orally taking 100 mg bicalutamide daily for 19 wk as neoadjuvant chemotherapy prior to presentation. He did not admit to use of illegal drugs or alcohol. Physical examination revealed scleral icterus. Blood work revealed acute liver dysfunction with alanine aminotransferase, 677 U/L; aspartate aminotransferase, 440 U/L; and international normalized ratio, 1.17. The total bilirubin, gamma-glutamyl transpeptidase, and alkaline phosphatase levels were 1.62 mg/dL, 80 U/L, and 87 U/L, respectively. The international normalized ratio was in the normal range during the entire period. He had normal baseline laboratory results at the initiation of bicalutamide administration. The result for hepatitis A immunoglobulin M was negative. Hepatitis B surface antigen was negative. Hepatitis C RNA was undetectable. The results for hepatitis E immunoglobulin M and G were also negative. On the other hand, the hepatitis B surface antibody was positive. Other etiologies like autoimmune disease, drugs, common toxins, and copper or iron-induced insult were considered. However, the antibodies for anti-mitochondrial, antinuclear, and anti-smooth muscle were negative, and the serum copper, ceruloplasmin, and 24-h urine copper levels were in the normal ranges. The modified Roussel Uclaf Causality Assessment Method scale score was 8. These findings strongly suggested drug-induced liver injury. Abdominal CT showed non-specific findings, whereas liver biopsy revealed acute intrahepatic cholestasis in zone 3 and sinusoidal dilation with moderate lobular inflammation (Figure 1), suggesting liver injury caused by androgen, estrogen, or glucocorticoid administration.

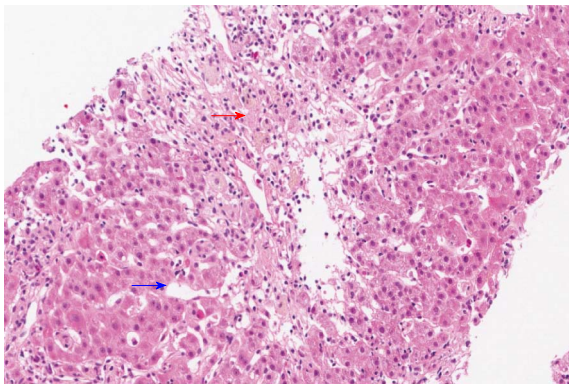

Figure 1 Liver biopsy showed acute intrahepatic cholestasis (red arrow) in zone 3 and sinusoidal dilation with moderate lobular inflammation (blue arrow) (hematoxylin and eosin, × 200).

As a result, bicalutamide was immediately withdrawn, and the patient was started on 75 mg/d biphenyl-dimethyl-dicarboxylate and 300 mg/d ursodeoxycholic acid. Laboratory abnormalities reduced with alanine aminotransferase and aspartate aminotransferase levels of 11 U/L and 21 U/L, respectively, after 12 wk. Consequently, the patient

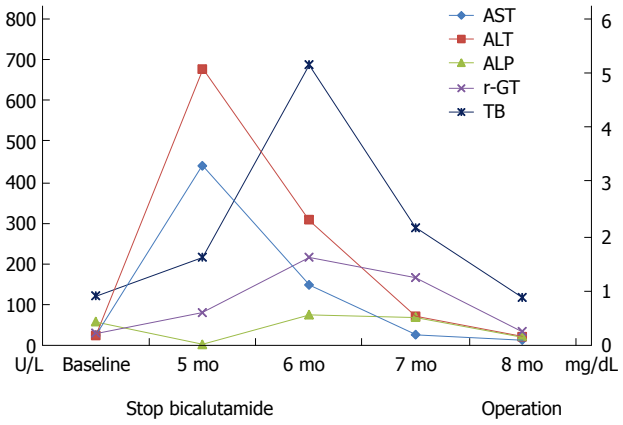

Figure 2 Courses of the laboratory findings from baseline (treatment initiation) to 8 mo later. The right axis (0-6) shows the values of TB (mg/dL). The left axis shows the values for AST, ALT, ALP, and r-GT (U/L). ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin, r-GT: Gamma-glutamyl transpeptidase.

underwent radical prostatectomy (Figure 2).

DISCUSSION

Several patterns of liver injury can occur secondary to many drugs, including cholestasis, hepatitis, and mixed-form injuries. Such drug-induced liver injury is usually divided into idiosyncratic and intrinsic reactions depend on the predictability and dose dependency. Intrinsic hepatotoxicity is dose dependent and can be predicted once a specific threshold amount has been absorbed. Conversely, idiosyncratic hepatotoxicity is dose independent and is subsequently unpredictable[2,7-10].

Liver biopsy is not routinely performed for evaluating drug-induced liver injury. However, it provides the opportunity to determine the form of injury, which may help confirm or exclude drug-induced liver injury, along with characterizing the distribution and severity of injury in the liver[11]. In this case, our patient underwent liver biopsy, which indicated drug-induced liver injury (*e.g.*, erythromycin, estrogen, androgen, diazepam, diphenylhydantoin, glucocorticoid, thioguanine, or azathioprine-induced injury). Owing to the rarity of bicalutamide-induced liver toxicity, no specific pathologic findings have been described, and our findings may hence provide a basis for the diagnosis of bicalutamide-induced liver injury.

Bicalutamide is an orally active non-steroidal anti-androgen. It competitively antagonizes the actions of androgens of both testicular and adrenal origin at the receptor level, thereby preventing the spread of prostate cancer[12]. Unlike steroidal anti-androgens (*e.g.*, cyproterone acetate), non-steroidal anti-androgens (*e.g.*, bicalutamide, nilutamide, and flutamide) do not suppress testosterone production and provides a better quality of life over castration. Among the non-steroidal anti-androgens, flutamide has been established to induce liver injury and cause mild aminotransferase elevation in 42%-62% of patients[13].

However, while an article search for case reports of non-steroidal anti-androgens revealed many cases of flutamide-induced liver injury, cases of bicalutamide toxicity were rare. In four previously reported cases of bicalutamide-induced liver injury, the injury occurred after receiving 50 mg/d orally for 2 d, 50 mg/d orally for 4 d, 50 mg/d orally for 3 mo, and 150 mg/d orally for 3 wk. Of these, two patients died as a result of fulminant hepatic failure while the other two patients showed clinical and serological improvement within days[2,4-6]. These previous reports suggest that the possible mechanism of bicalutamide-induced liver injury comprise direct hepatotoxicity and idiosyncratic reaction. Initially, our patient was first treated in the urology department where he received 100 mg bicalutamide daily. He developed liver injury after daily bicalutamide use for 19 wk, but slowly showed improved liver function 12 wk after ceasing medication use. The higher daily dose (100 mg), compared to that administered to patients described in the previous case reports (50 mg), and may be associated with a dose-response effect. On the other hand, the delayed liver injury may indicate an idiosyncratic reaction, because of the unpredictable latency. Irrespective of the mechanism, potentially life-threatening and clinically significant liver injury can result from the use of bicalutamide.

Therefore, immediate recognition and stopping bicalutamide is vital to avoid severe complications such as fulminant hepatitis. Liver function tests should be regularly conducted during and after bicalutamide administration.

Actually, the patient described herein was referred to our department from the urology department 5 mo after bicalutamide treatment initiation. The exact time at which bicalutamide-induced liver injury occurred may be unclear, because liver enzyme measurements were not followed at the urology department. This case emphasizes that liver function measurements should be checked from the baseline for at least the first 6 mo of treatment, and then regularly during the entire period of treatment with bicalutamide.

COMMENTS

Case characteristics

A 62-year-old South Korean man with prostate cancer (T2N0M0; Gleason score 6) presented with jaundice for a few days.

Clinical diagnosis

Physical examination revealed scleral icterus.

Differential diagnosis

Viral hepatitis, autoimmune hepatitis, and metastasis of prostate cancer to the liver are differential diagnoses.

Laboratory diagnosis

The aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, total bilirubin, and international normalized ratio levels were 440 U/L, 677 U/L, 87 U/L, 80 U/L, 1.62 mg/dL, and 1.17, respectively.

Imaging diagnosis

Abdominal CT showed non-specific findings.

Pathological diagnosis

Liver biopsy suggested liver injury caused by androgen, estrogen, or glucocorticoid administration.

Treatment

Bicalutamide was immediately discontinued.

Related reports

There are only four previous case reports on bicalutamide-induced liver injury. In these previous cases, hepatic failure occurred within a few days of bicalutamide use.

Term explanation

There are no unusual terms that require explanation.

Experiences and lessons

Although rare, clinically significant and potentially life-threatening liver injury can result from the use of bicalutamide. Prompt recognition and discontinuation of bicalutamide is necessary to avoid serious complications such as fulminant hepatitis. Liver function measurements should be monitored from baseline for at least the first 6 mo of therapy, and then periodically during the entire period of treatment with bicalutamide.

Peer-review

The authors describe a rare case of bicalutamide-induced hepatotoxicity, a 62 year-old Korean man with prostate cancer was administered with bicalutamide as a neoadjuvant chemotherapy and he experienced delayed liver injury. This is an interesting case study with valuable insights for the monitoring of liver enzymes in patients being treated with bicalutamide.

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CASE REPORT

Gastric inverted hyperplastic polyp: A rare cause of iron deficiency anemia

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Author contributions: Yun JT and Lee SW designed the report; Kim DP, Choi SH and Kim SH collected the patient's clinical data; Park JK, Jang SH, Park YJ and Sung YG contributed to revising the manuscript; Yun JT and Lee SW wrote the paper; all authors contributed to the manuscript.

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Institutional review board statement: The case has been discussed with the most senior member of staff in charge of the patient's care who has given consent for this, and consent was obtained for use of accompanying pathologic images from the consultant pathologist. The study was reviewed and approved by the Daejeon St. Mary`s Hospital of the Catholic University of Korea Institutional Review Board.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to enrollment.

Conflict-of-interest statement: We declare that we have no financial or personal relationships with other individuals or organizations that would inappropriately influence our work. There is no professional or other personal interest of any nature in any product or service.

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Abstract

Gastric inverted hyperplastic polyp (IHP) is a rare gastric polyp characterized by the downward growth of hyperplastic mucosal components into the submucosal layer. Macroscopically, a gastric IHP resembles a subepithelial tumor (SET); as a result, accurately diagnosing gastric IHP is difficult. This issue has clinical significance because gastric IHP can be misdiagnosed as SET or as malignant neoplasm In addition, adenocarcinoma can accompany benign gastric IHP. Although in most cases, gastric IHPs are asymptomatic and are found incidentally, these polyps may cause anemia secondary to chronic bleeding. Here, we report one case involving gastric IHP accompanied by chronic iron deficiency anemia that was successfully managed using endoscopic submucosal dissection.

Key words: Stomach; Hyperplasia; Polyp; Anemia

Core tip: Gastric inverted hyperplastic polyp (IHP) is a rare gastric polyp characterized by the downward growth of hyperplastic mucosal components into the submucosal layer. It is difficult to diagnose accurately without endoscopic resection and pathological investigation because of its inverted grown into the submucosal layer and the paucity of case reports. In most cases, gastric IHPs are asymptomatic and are found incidentally. Rarely, it may manifest as anemia secondary to chronic bleeding and can be ignored by inexperienced endoscopist. Importantly, it is reported to be related with dysplasia and adenocarcinoma. So, en bloc resection using endoscopic submucosal dissection was recommended for diagnosis and treatment.

Yun JT, Lee SW, Kim DP, Choi SH, Kim SH, Park JK, Jang SH, Park YJ, Sung YG, Sul HJ. Gastric inverted hyperplastic polyp: A rare cause of iron deficiency anemia. *World J Gastroenterol* 2016; 22(15): 4066-4070 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4066.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4066

INTRODUCTION

Gastric inverted hyperplastic polyp (IHP) is a rare gastric polyp characterized by the downward growth of hyperplastic mucosal components into the submucosal layer[1]. In 1993, Kamata *et al* first referred to this type of lesion as an IHP[2]. This polyp has also been called a hamartomatous polyp and a solitary polypoid hamartoma[3]. Macroscopically, a gastric IHP resembles a subepithelial tumor (SET); as a result, accurately diagnosing gastric IHP is difficult. This issue has clinical significance because gastric IHP can be misdiagnosed as SET or as malignant neoplasm. In addition, adenocarcinoma can accompany benign gastric IHP[1]. Although most gastric IHPs are symptomatic and are identified incidentally, we encountered a case of gastric IHP that had the primary manifestation of chronic iron deficiency anemia (IDA) and was successfully managed using endoscopic submucosal dissection (ESD).

CASE REPORT

A 64-year-old woman presented with several year history of dizziness and general weakness that had recently become aggravated. Her medical history was otherwise unremarkable. Approximately 7 years prior to the events described here, the patient had been diagnosed with gastric SET (Figure 1A). A biopsy indicated the presence of chronic gastritis. Although the patient had undergone endoscopic examinations nearly every year, no treatment for her gastric SET had been considered.

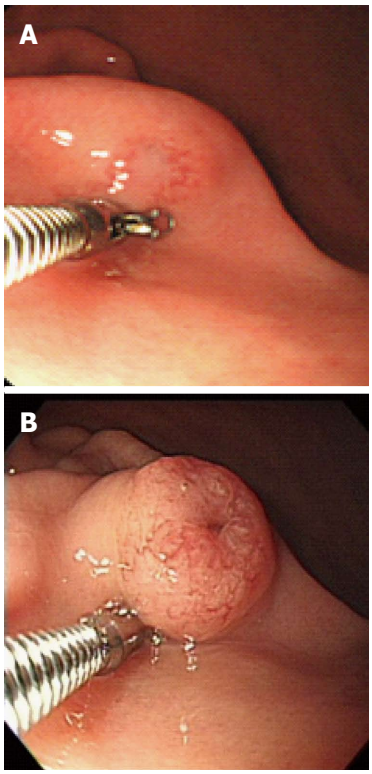

Figure 1 Gastric subepithelial tumor on the greater curvature side of the lower body. A: 7 years ago, gastric subepithelial tumor (SET) with hypervascular mucosal change was shown. When pushed the gastric SET by cold biopsy forcep, mucous secretion flowed from the lesion; B: At admission, gastric SET had shown the growth of size. Top of the gastric SET was shown slightly depressed, irregular and hypervascular change.

At admission, a physical examination produced unremarkable findings. Complete blood count results indicated a white blood cell count, hemoglobin level, and platelet count of 5900/mm^3, 6.5 g/dL, and 497000/mm^3, respectively. The patient's anemia was diagnosed as IDA and her stool was negative for occult blood. Blood chemistry results were unremarkable. An endoscopic examination revealed a 1.5 cm SET on the greater curvature (GC) side of the lower body. This tumor, which had increased in size since the previous examination, was covered with normal mucosa exhibited irregular, hypervascular changes and a central orifice at its surface (Figure 1B). Endoscopic forceps biopsy results indicated chronic gastritis with intestinal metaplasia. A colonoscopic examination produced unremarkable findings. Endoscopic ultrasonography (EUS) revealed a 13.2 mm × 11.2 mm heterogeneous hypoechoic tumor in the submucosal layer of the gastric wall (Figure 2A). Abdominal contrast-enhanced computed tomography (CT) indicated the presence of a 1.5 cm, oval-shaped enhancing mass in the stomach, on the GC side of the lower body (Figure 2B). Because this lesion could have been a cause of the patient's IDA and because we wished to obtain an appropriate diagnosis, we decided to respect the mass in question using ESD. Grossly, the resected specimen measured 5.0 cm × 3.0 cm

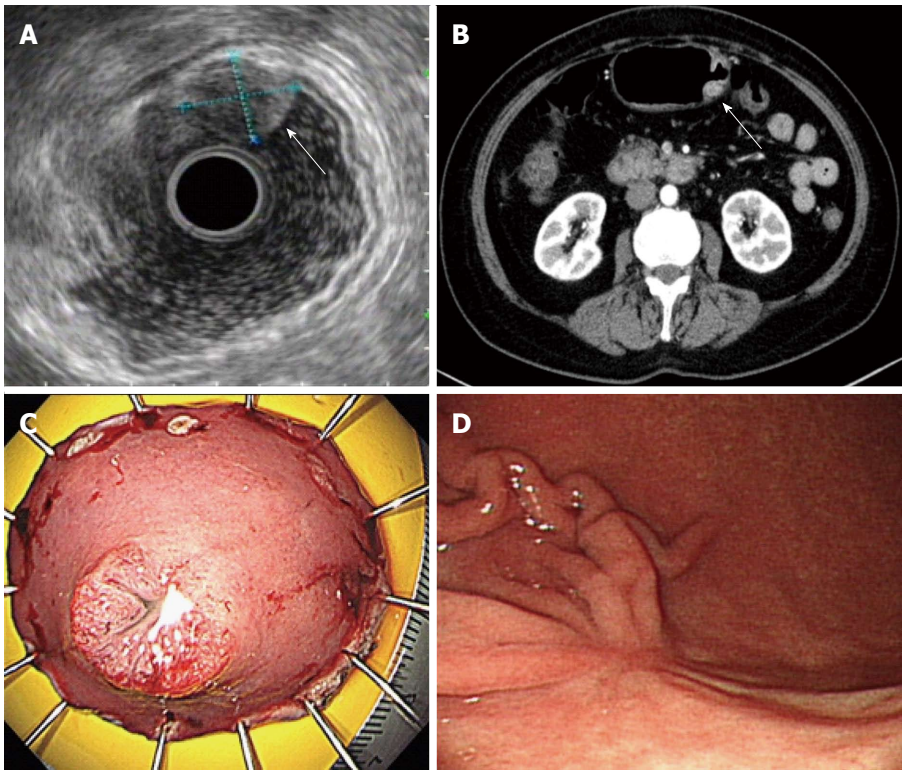

Figure 2 Colonoscopic examinations. A: Endoscopic ultrasonography (EUS) demonstrated a 13.2 mm × 11.2 mm-sized heterogeneous hypoechoic tumor (white arrow) in the submucosal layer of the gastric wall; B: Abdominal contrast-enhanced computed tomography (CT) demonstrated a 1.5 cm-sized, oval-shaped enhancing mass (white arrow) on the GC side of the lower body; C: ESD specimen measuring 5.0 cm × 3.0 cm showed a 1.5 cm-sized, well-circumscribed polypoid lesion; D: Follow-up endoscopic examination demonstrated a post ESD scar with converging fold on the GC side of the lower body.

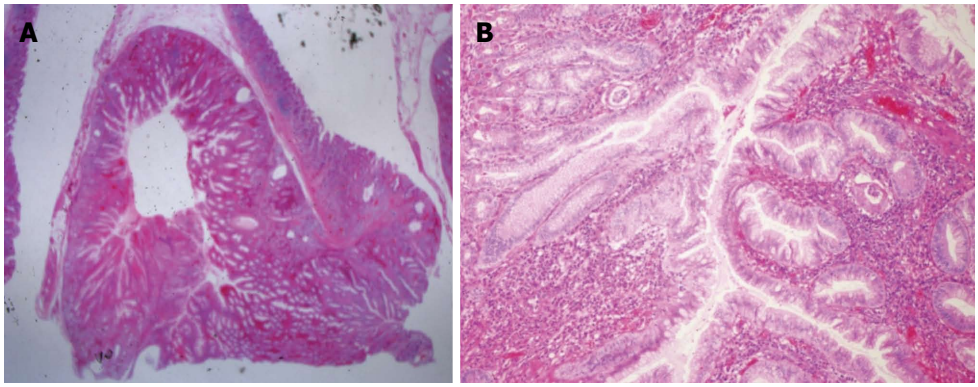

Figure 3 Microscopy of the endoscopic submucosal dissection specimen. A: Well-circumscribed inverted growth pattern into the submucosal layer was shown (HE staining, × 10); B: Endophytic proliferation of hyperplastic columnar cells and connected with inflamed surface epithelium was shown. No architectural or cytological atypia was found (HE staining, × 100).

and included a well-circumscribed 1.5 cm polypoid lesion (Figure 2C). Histologically, the lesion mainly consisted of inverted proliferating columnar cells and was primarily composed of hyperplastic foveolar-type glands; focal cystic dilatation, inflammatory cells, and smooth muscle bundles in the stroma were observed (Figure 3). The pathologic diagnosis was consistent with gastric IHP. No architectural or cytological atypia were detected.

The patient was discharged following endoscopic treatment of gastric IHP; 5 mo later, the patient's hemoglobin had normalized to 12.4 g/dL, and anemic symptoms such as general weakness and dizziness had disappeared. A follow-up endoscopic examination revealed a post-ESD scar with a converging fold on the GC side of the lower body (Figure 2D).

DISCUSSION

Gastric polyps can be classified by morphology as either protruding or inverted. Most gastric polyps are protruding, whereas inverted gastric polyps are rare. Gastric IHP is characterized by the marked endophytic proliferation of foveolar-, pyloric-, or fundic-

type glandular epithelium, which leads to a polypoid lesion[4]. Although the pathogenesis of IHP is not well understood, studies have suggested that inflammation and subsequent healing may promote epithelial displacement[4].

The connection between the inflamed overlying mucosa and hyperplastic components in combination with the presence of the slit-shaped cavity in the polyp's center suggested that repeated mucosal inflammation caused a break in the muscularis mucosae that allowed for the downward herniation and submucosal trapping of mucosal glands[4].

Our literature review discovered 19 reported cases of gastric IHP[1-11]. Most of this gastric IHP were asymptomatic and were found incidentally. Rarely, gastric IHP may manifest as anemia secondary to chronic bleeding. Among the 19 previously reported IHP cases, only 1 case presented with mild anemia. Thus, our IHP case is the first report of symptomatic, severe anemia as a symptom of gastric IHP.

Gastric IHP is difficult to diagnose accurately without endoscopic resection and pathological investigation because of the polyp's inverted growth into the submucosal layer and the existence of extremely few case reports. Gastric IHP is diagnosed based on the tumor's pathological characteristics, including fibroblast cells, smooth muscle proliferation, nerve components, vasoformative tissue, glandular hyperplasia, and cystic gland dilatation[1]. The pathological finding of inverted ectopic gastric pseudopyloric glands in the submucosal layer is critical for diagnosing gastric IHP[1].

Gastric IHP is reportedly related to dysplasia and adenocarcinoma[3]. Although gastric IHPs are benign tumors, dysplastic or cancerous areas are found within a large lesion in approximately 20% of gastric IHP cases[5]. Kono *et al*[6] suggested that p53 dysregulation may play an important role in the malignant transformation of gastric IHPs. In addition, multifocal adenocarcinomas may be present and randomly distributed across the superficial and deep regions of a gastric IHP. Therefore, a negative biopsy cannot reliably exclude the presence of dysplasia or adenocarcinoma. As a result, a patient with gastric IHP should be subjected to complete resection with a negative margin and a thorough histological examination should be performed[4]. Several methods of diagnosing and treating gastric IHP have been used, including conventional polypectomy, endoscopic mucosal resection with piecemeal resection, ESD, and surgical approaches such as laparoscopic wedge resection. Recently, en bloc resection using ESD was recommended for the diagnosis and treatment of gastric IHP.

In conclusion, gastric IHP can cause symptoms such as anemia and can be ignored by an inexperienced endoscopist. Therefore, it is important to recognize the clinical significance of these lesions and consider treatment. We have reported a case of gastric IHP accompanied by chronic IDA that was successfully managed using ESD.

COMMENTS

Case characteristics

A 64-year-old woman presented with several year history of dizziness and general weakness that had recently become aggravated.

Clinical diagnosis

An endoscopic examination revealed a 1.5 cm subepithelial tumor on the greater curvature (GC) side of the lower body.

Differential diagnosis

Carcinoid, Ectopic pancreas, Granular cell tumor, Leiomyoma, Lymphoma.

Laboratory diagnosis

Hemoglobin level was 6.5 g/dL and all other labs were within normal limits.

Imaging diagnosis

Abdominal CT indicated the presence of a 1.5 cm, oval-shaped enhancing mass in the stomach, on the GC side of the lower body and endoscopic ultrasonography revealed a 13.2 mm × 11.2 mm heterogeneous hypoechoic tumor in the submucosal layer of the gastric wall.

Pathological diagnosis

Gastric inverted hyperplastic polyp (IHP).

Treatment

Endoscopic submucosal dissection (ESD).

Related reports

Gastric IHP is a rare gastric polyp characterized by the downward growth of hyperplastic mucosal components into the submucosal layer. It is difficult to diagnose accurately without endoscopic resection and pathological investigation. Rarely, it may manifest as anemia secondary to chronic bleeding and is related with dysplasia and adenocarcinoma.

Term explanation

ESD is a well-established technique of endoscopic resection that allows for en bloc removal of GI epithelial lesions.

Experiences and lessons

Gastric IHP can cause symptoms such as anemia and can be ignored by an inexperienced endoscopist. Therefore, it is important to recognize the clinical significance of these lesions and consider treatment.

Peer-review

The manuscript is modern, well conceived and well written. The iconography is very comprehensive and discussion is exhaustive.

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Effects of daily telephone-based re-education before taking medicine on *Helicobacter pylori* eradication

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Author contributions: Demirci H designed research, performed research and wrote the paper; Ozturk K contributed new reagents or analytic tools; and Kurt O analyzed data.

Conflict-of-interest statement: No conflict of interest

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Abstract

We read the article "Effects of daily telephone-based re-education before taking medicine on *Helicobacter pylori* (*H. pylori*) eradication: A prospective single-center study from China" written by Wang *et al* with great interest. It is reported in American and European guidelines that there is no sufficient test for the diagnosis of *H. pylori* except culture and that using at least two different tests for diagnosis of *H. pylori* is recommended. Patients who used antibiotics or bismuth salts in the previous 2 wk were excluded from study. But patients who used probiotics and antioxidant vitamins such as vitamins C and E were not excluded.

Key words: *Helicobacter pylori*; Erdication rates; Telephone based education

Core tip: We read the article "Effects of daily telephone-based re-education before taking medicine on *Helicobacter pylori* (*H. pylori*) eradication: A prospective single-center study from China" written by Wang *et al*. It is reported in guidelines that there is no sufficient test for the diagnosis of *H. pylori* except culture and that using at least two different tests for diagnosis of *H. pylori* is recommended. Patients who used antibiotics or bismuth salts in the previous 2 wk were excluded from study.

Demirci H, Ozturk K, Kurt O. Effects of daily telephone-based re-education before taking medicine on *Helicobacter pylori* eradication. *World J Gastroenterol* 2016; 22(15): 4071-4072 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i15/4071.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i15.4071

TO THE EDITOR

We read the article "Effects of daily telephone-based re-education before taking medicine on *Helicobacter pylori* eradication: A prospective single-center study from China" written by Wang *et al*[1] with great interest. The authors investigated the effects of daily

telephone based re-education before taking medicine for the eradication of *Helicobacter pylori* (*H. pylori*) on the compliance and the eradication rate. We thank the authors for their contribution of well-presented study. We believe that these findings of current study will encourage further studies for *H. pylori* eradication.

H. pylori remains one of the most widespread worldwide human infections and is associated with upper gastrointestinal states, including peptic ulcer disease, chronic gastritis, and gastric malignancy[2]. The prevalence of *H. pylori* is closely related to socioeconomic status. This infection is more prevalent in developing countries than in Western countries. Wang *et al*[1] informed us that in their study that the daily TRE before taking medicine had no significant impact on the patients' compliance, satisfaction, or *H. pylori* eradication, but reduced the rate of adverse events. However, we believe that some points that should be emphasized in the study.

The authors reported in the present study that they had used diagnosis of *H. pylori* infection by at least one of the following methods: ^{13}C-urea breath test, histology, rapid urease test or bacterial culture. It is reported in American and European guidelines that there is no sufficient test for the diagnosis of *H. pylori* except culture and that using at least two different tests for diagnosis of *H. pylori* is recommended[2,3]. Hence, we consider that the number of *H. pylori* positive patients is different from that found in this present study. It is obviously seen that this distinction in number of *H. pylori* positive patients will impress the results of the study.

Additionally, patients who used antibiotics or bismuth salts in the previous 2 wk were excluded from study. But patients who used probiotics and antioxidant vitamins such as vitamins C and E were not excluded. It has been thought that vitamins C and E break the microenvironment created by *H. pylori* or directly inhibit bacteria. Vitamin C can inactivate the urease enzyme, which allows the endurance of *H. pylori* and the colonization of the gastric mucosa at a low pH. Thus, vitamin C may inhibit the spread, growth, and colonization of *H. pylori* in the early periods of infection[4]. Probiotics reduce the adverse effects of *H. pylori* eradication treatment, this could help increasing the adherence of patients to treatment and could increase the eradication rate. Probiotics may also inhibit the growth of *H. pylori*, stimulate an immunological response and reduce the inflammatory effects of infection[5]. It is clearly seen that this factors can change the results of the study.

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