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EDITORIAL

Acute appendicitis: What is the gold standard of treatment?

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Abstract

McBurney's procedure represented the gold-standard for acute appendicitis until 1981, but nowadays the number of laparoscopic appendectomies has progressively increased since it has been demonstrated to be a safe procedure, with excellent cosmetic results and it also allows a shorter hospitalization, a quicker and less painful postoperative recovery. The aim of this editorial was to perform a review of the literature in order to address controversial issues in the treatment of acute appendicitis.

Core tip: There are still controversial issues in the treatment of acute appendicitis such as comparison between laparoscopic and open appendectomy and the correct approach in special categories of patients. The aim of this editorial was to perform a review of the literature in order to address controversial issues in the treatment of acute appendicitis.

INTRODUCTION

In 1894, McBurney[1] described a new technique for the management of acute appendicitis: this method is still used when an open approach is required.

McBurney's procedure represented the gold-standard for acute appendicitis until 1981, when Semm[2] performed the first laparoscopic appendectomy in Germany, a "culture shock" in general surgery since a revolutionary method was discovered by a gynecologist[3]. But a real "laparoscopic revolution" took place only in 1985 with the first laparoscopic cholecystectomy performed by Erich Muhe, using Semm's technique and instruments. Laparoscopy was not easily accepted since it was not considered a safe procedure; nowadays laparoscopic surgery is gaining a primary role in many surgical settings.

The number of laparoscopic appendectomies (LA) has progressively increased since it has been demonstrated to be a safe procedure, with excellent cosmetic results; furthermore, LA allows a shorter hospitalization, a quicker and less painful postoperative recovery.

But is laparoscopic surgery the best choice for appendectomy? Which are the correct surgical indications? What are the results from the comparison between LA *vs* classic open appendectomy (OA)? Are there selected groups of patients in which one of these approaches should be preferred? The aim of this editorial was to perform a review of the literature in order to address these

controversial issues.

OPEN *VS* LAPAROSCOPIC APPENDECTOMY

Many comparative studies have already demonstrated the advantages of LA over OA in terms of length of hospital stay, use of postoperative analgesics and earlier return to work[4]. The most controversial issues of these studies have been taken into consideration.

Surgical-site infection

Surgical-site infection (SSI) rate was significantly lower in the LA than in the OA group (1.6% *vs* 3.2% respectively) and this gap between the two groups increased in severe forms of appendicitis, such as gangrenous and perforated. Some authors estimated that one wound infection could be prevented for every 23.7 patients treated with LA, instead of OA[5]: this can be explained with the use of the extraction bag (endo-bag) in LA, which prevents the direct contact between the infected appendix, the wound edges and the inflamed tissues around the appendix during its removal[5,6].

Other studies found a higher SSI rate in OA, but also a significantly higher intraabdominal abscess (ⅡA) rate in LA. The difference in the postoperative complications according to the surgical technique were remarkable when inflammation of the appendix was more severe: in fact, when a periappendiceal abscess was present, there were more cases of paralytic ileus (PI) in the LA group and more cases of SSI in the OA group. This result can be due to the leakage of infected substances, the appendiceal stump not being inverted and the resection side being exposed in the intraabdominal cavity during the removal of the appendix in LA[7]. Some authors suggest that the use of an Endo-GIA stapler could help minimize these adverse effects[8]. Finally, these differences are not statistically significant in case of gangrenous or/and perforated appendicitis[7].

Intraabdominal abscess

In an interesting study that considered 2464 patients, 52 experienced postoperative abscesses. The patients with a diagnosis of complicated appendicitis had a significant correlation with a higher incidence of intraabdominal abscess development (67% in complicated appendicitis *vs* 25% in uncomplicated appendicitis, $P = 0.01$). The majority of abscesses developed in the pelvis (41%), especially in those patients who had complicated rather than uncomplicated appendicitis (63% *vs* 18% respectively, $P = 0.01$). It is interesting to notice how the formation of an ⅡA in patients with a diagnosis of complicated appendicitis did not differ significantly between those who underwent LA and those who underwent OA (5.9% *vs* 4.1% respectively, $P = 0.44$). Moreover, in patients with complicated appendicitis there was no significant increase in presenting symptoms or in the severity of the case history, quite independently from the surgical approach.

The only remarkable difference was that the patients who underwent OA presented earlier symptoms and received a more timely diagnosis of ⅡA than the patients who underwent LA (6 d in OA group *vs* 11 d in LA group)[9].

A multivariate analysis has shown that development of abscesses has a higher correlation with the initial diagnosis than with the type of surgical approach. The evaluation of selected patients demonstrated a 30% increase of the risk of ⅡA for every decade of life. This could be clinically relevant because it suggests the need for careful monitoring of elderly patients who initially presented complicated appendicitis, since they are at higher risk for postoperative ⅡA[9]. Finally an explanation for the formation of ⅡA could be found in the surgical technique itself: currently, surgeons performing LA tend to apply irrigation more freely; therefore, contaminating the entire peritoneal cavity[10]; although irrigation as a cause of ⅡA is yet controversial.

Incisional hernia

The incidence of incisional hernia is low in both techniques (0.7% in OA group *vs* 1% in LA): the development of post incisional hernias is higher with McBurney's incision, whereas in LA there are incisional hernias only in those patients who undergo conversion[11].

Small bowel obstruction

Finally, as far as long-term complications are concerned, some studies assessed that small bowel obstruction can present many years after surgery, especially for open appendectomy. The prevalence of bowel obstruction after appendectomy increased from 0.63% after 1 year, to 0.97% after 10 years, to 1.30% after 30 years of follow up[11]. In a randomized study, a second look laparoscopy was performed on 40 patients who had histological confirmation of acute appendicitis, 3 mo after the first operation: there were adhesions in the 80% of patients that underwent OA, but only in 10% of LA group[5]. Therefore, LA seems to be associated with an easier second-look procedure and a minor infertility rate due to less adhesions[12].

Among long-term complications, small bowel obstruction has a very low incidence, between 0.33% and 1.51% in OA. It is known that the risk is higher with negative appendectomy or appendectomy through a midline laparotomic incision. Then, the choice of LA in suspected appendicitis is correct because it avoids unnecessary appendectomy if the appendix is normal and it prevents unnecessary wide incisions[13].

SUSPECTED APPENDICITIS

The differential diagnosis of most of the surgical abdominal emergencies is based on clinical grounds, laboratory data and diagnostic imaging. The problem, however, is to obtain a correct diagnosis of the exact localization of the lesion to determine surgical indications and to decide the best surgical approach. Laparoscopy is a valuable instru-

ment in the case of suspected appendicitis allowing the surgeon to correctly evaluate the intraperitoneal condition of practically every single patient[14].

At first, considering its exploratory nature and its diagnostic accuracy, besides the advantage of a shorter time of hospitalization and reduction of pain on day 1[15], LA can be considered the first choice in suspected appendicitis, especially in particular categories, such as premenopausal women. In fact, in these patients, in the presence of right lower quadrant pain, differential diagnosis between acute appendicitis, ectopic pregnancy and pelvic inflammatory disease (PID), is necessary. A laparoscopic exploration of the abdominal cavity allows a rapid and safe diagnosis; for the former two affections laparoscopy also represents a therapeutic option, while in the latter one, samples for culture may also be taken, with the advantage of avoiding "negative" appendectomies, with a high diagnostic accuracy (96% in women and 100% in men)[16].

Morino *et al*[17] evaluated, in a prospective, randomized, single-institution trial, the role of early laparoscopy in the management of nonspecific abdominal pain (NSAP) in young women. NSAP was defined as an abdominal pain in right iliac or hypogastric area lasting more than 6 h and less than 8 d, without fever, leukocytosis, or obvious peritoneal signs and uncertain diagnosis after physical examination and baseline investigations including abdominal sonography. Patients were randomly assigned to early (< 12 from admission) laparoscopic group or to clinical observation group. Compared with active clinical observation, early laparoscopy did not show a clear benefit in women with NSAP. A higher number of diagnosis and a shorter hospital stay in the laparoscopic group did not lead to a significant reduction in symptoms recurrences at 1 year.

LA may be performed safely in pregnant patients with appendicitis according to the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) guidelines[18].

COMPLICATED APPENDICITIS

Excellent results are mentioned in several studies about the use of LA in complicated appendicitis, though a higher incidence of intraabdominal abscesses has been noticed. Some studies have demonstrated that LA is almost totally comparable to OA as far as operating time, hospital stay and postoperative complications are concerned. The rate of postoperative ⅡA was significantly higher in LA when compared with OA (respectively, 14% *vs* 0%), while wound infection and pulmonary complication rate were significantly lower (respectively 2.3% *vs* 8.2% in OA group and 0% *vs* 4.9% in LA group)[19].

The incidence rate of ⅡA increases considerably when a periappendiceal abscess or a postoperative ileus are present. Particularly, the incidence of ⅡA in complicated appendicitis increases remarkably (67% in complicated *vs* 25% in uncomplicated appendicitis): in these patients, there are no significant differences in the postoperative outcome or in the development of the ab-

scess according to the surgical technique; therefore in the presence of an initial diagnosis of complicated appendicitis with a severe clinical background there is a higher probability of developing an abscess regardless of the adopted surgical approach[9].

In another 5-year non randomized study considering 1133 patients of which 244 had a complicated appendicitis (and among them, 175 underwent LA and 69 OA), LA patients had a shorter operative time (55 min *vs* 70 min), reduced length of stay (5 d *vs* 6 d) and a lower incidence of SSI (0.6% *vs* 10%)[10]. In the case of complicated appendicitis (gangrenous or perforated), the laparoscopic approach also reduced postoperative pain[20].

SPECIAL CATEGORIES OF PATIENTS

There are clinical settings in which laparoscopy may be the preferred approach: obese patients, immunocompromised patients and elderly patients.

In obese patients, in fact, laparoscopy is undeniably useful[21], considering at first the difficult exposure of the right lower quadrant during OA, which may require large, morbidity-prone incisions that are at risk of infections and of wound complications[5,22]. It is known that BMI is a risk factor for SSI[23]. Furthermore, obese patients have a higher risk of incisional hernias: laparoscopic approach reduces the risk of incisional hernia[24].

Immunocompromised patients include heart transplanted patients and those who received immunosuppressive therapy for autoimmune diseases, cancer and AIDS; the risk of infections is higher and the immunity response could be partial and ineffective due to immunodepression. Therefore, these patients may not exhibit the typical signs and symptoms of appendicitis and may only have a barely positive examination[25]. In these patients laparoscopic approach represents the best option: compared with OA, LA is characterized by a lower rate of postoperative complications (10.36% in LA group *vs* 22.56% in OA group), a shorter hospitalization (2.9 d *vs* 4.9 d) and a lower mortality (0.16% *vs* 0.61%). These results can be observed in both uncomplicated and complicated appendicitis, with a considerably lower incidence of complications (27.52% in LA group *vs* 57.50% in OA group) and a shorter hospital stay (5.92 d in LA group *vs* 9.67 d in OA group)[26].

Finally, elderly patients might significantly benefit from a laparoscopic approach[24]; in these patients it is quite difficult to collect anamnestic data, in addition to a mild abdominal examination and to laboratory and radiological tests which might not be so diriment. Laparoscopy can clarify the diagnosis and also represent a good therapeutical strategy[27].

INFLAMMED APPENDICEAL STUMP

Stump appendicitis is the acute inflammation of the residual portion of the appendix and is a rare complication of incomplete appendectomy[28].

Due to the relevant recurrence rate, a second appen-

dectomy 3 mo after the outbreak of inflammation, could be necessary. In a histopathological study Gahukamble demonstrated that 13 of the 14 removed appendices had a pervious lumen with a higher risk of recurrent appendicitis. More recently authors focused the problem of a very long stump also on patients undergoing LA; in fact, the presence of an excessively long appendiceal stump could be at risk of recurrence also in these patients. Pain in the lower right abdominal quadrant in a patient that has undergone LA does not rule out a second episode of acute appendicitis[29]. The possibility of a recurring appendiceal stump abscess as a complication of LA is high. When performing LA, the appendiceal stump should be as short as possible and its ligation should not determine ischemia of the stump[30].

The tactical modification of appendiceal stump closure, replacing the invaginating suture that nowadays has become the procedure of choice consists in a single endoligature. Alternatively, there are methods which make use of an endostapler, endoligature (endo-loop), metal clips, bipolar endocoagulation and polymeric clips. All the different techniques have advantages and disadvantages depending on the different stages of acute appendicitis; so, the right knowledge about the possible methods and the appropriate choice between them according to every single case allows a safe and efficient management of patients as well as a reduction in hospital costs[31].

Drainage placement, ultrasound and perhaps an exploratory-therapeutical laparoscopy could be very useful in the management of this complication[30]. Finally the use of CT imaging allows a precise definition of the surrounding anatomy, in particular of the length of the appendiceal stump[32]. Several authors identify the removal of the whole appendiceal stump as the major suggested mean to avoid recurrence of appendicitis[33].

CONSERVATIVE MANAGEMENT OF ACUTE APPENDICITIS

Acute appendicitis is one of the most frequent conditions seen in a surgical department; urgent appendectomy is considered the treatment of choice because of the low incidence of major complications and the relative rapidity of operation and hospital stay. Nevertheless surgical treatment exposes the patient to risks due to general anaesthesia and other complications such as surgical site infection, adhesions and intestinal obstruction, incisional hernia, infertility in female and pneumonia[34]; in this setting, the role of conservative treatment with antibiotics has been investigated in literature.

A recent Cochrane review assessed five low to moderate quality randomized controlled trials[35]; with the limit of the analyzed studies, surgical approach remains the gold standard treatment for acute uncomplicated appendicitis. Another large meta-analysis compared the two strategies in the scenario of complicated appendicitis, abscess or phlegmon[36]; in this case, radiologic-assisted drainage of appendiceal abscess could be another helpful conservative strategy. The analysis of seventeen studies revealed that conservative management, with or without interval appendectomy, was associated with less overall complication rates, less reoperations and similar hospital stay compared with urgent appendectomy.

In the absence of high quality studies, laparoscopic or traditional appendectomy is still the treatment of choice for acute appendicitis; some in-progress prospective studies[34,37] could be helpful in understanding the role of conservative management.

NORMAL APPENDIX: LAPAROSCOPIC MANAGEMENT

Negative or white appendectomy refers to the removal of non-inflamed appendix and is performed in about 15%-25% of patients undergoing surgery for suspected acute appendicitis[38]. White appendectomy rate is declining over time as cited by large studies, due to the availability of computed tomography and laparoscopy[39]; in open surgery, the appendix is generally always removed[40].

Thanks to the widespread use of laparoscopy, laparoscopic management of normal appendix represents a dilemma for the surgeon and no guidelines are available in this field[41]. When laparoscopy is performed for suspected appendicitis, exploration is negative in 8%-15% but in up to 27% another condition is diagnosed[40]. The risks of leaving in situ an apparent normal appendix are: later appendicitis, misdiagnosed subclinical or "endo"-appendicitis, missed appendiceal malignancy (carcinoid), risk of patient confusion and persisting symptoms[42]. At present, the laparoscopic strategy in front of a normal appendix remains controversial.

Conversions from laparoscopic to laparotomic appendectomy

In case of conversion, it is useful to perform an adequate laparotomic incision and an accurate and complete abdominal toilette. The conversion of perforated appendicitis is often burdened with a higher postoperative morbidity [60% in conversion appendectomy (CA), 22% in LA and 38% in OA][8].

A recent study in 2011, which included 745 patients that underwent LA or OA, asserts that conversion rate was about 8.6% and mentions that the first cause of conversion was the presence of a severe acute inflammatory process (38.7% of the factors which determine conversion to OA during operation). In this study, 77.42% of the patients that underwent CA had previous abdominal surgery and only 25.81% had a conversion due to adhesions.

Conversion was necessary especially in women over 65 years old (4.30% rather than 4.02% in the rest of patients)[43]. It is quite interesting that surgeons who performed at least 50 LA through their study period had a higher CA rate and this could reflect their will to attempt LA in the greatest part of patients, even in not strictly indicated cases. At the same time the number of conver-

sions decreases progressively throughout the career of a surgeon and his equipe[43].

Another study indicates the presence of a generalized purulent peritonitis as the only significant risk factor for conversion. Moreover, although patients with previous abdominal surgery are at higher risk of conversion, this is not significantly correlated with sex and age. Converted patients are at higher risk of relaparotomy and incisional hernia, independently of the duration of the operation[11].

Finally, for patients that underwent LA with complications requiring reintervention following laparoscopy, there is the possibility of a relaparoscopy for a second look: this has the advantage of maintaining the reduced morbidity allowed by the first operation. Relaparoscopy is very useful for abscess drainage, because it provides the accurate identification of the causes, for example in case of appendicular stump insufficiency[44].

LAPAROSCOPY *VS* LAPAROTOMY: WHICH FACTORS DETERMINE SURGEON'S DECISION?

It is known that laparoscopic approach is more expensive, as many studies have reported: an American study evaluated hospital cost behaviour in the years 2000-2005, including all patients undergoing both LA and OA. Costs for LA are 22% higher in uncomplicated and 9% higher in complicated appendicitis. They estimate that in 2005 exclusive use of open appendectomy would have saved 93 million dollars: this finding is particularly important because appendectomy is a common routine operation in all hospitals. The authors suggest OA as the gold standard for acute appendicitis, reserving LA only for special categories of patients[45].

Cothren *et al*[46] compared the costs for LA and OA, which were significantly higher for LA: the authors noted that the total costs for LA were higher although operative time and stay in hospital were not so different between the two methods. Higher costs for LA might be due to the use of specific disposable surgical material for laparoscopy.

Another important factor for the hospital costs is the severity of illness of the patients at the initial diagnosis[47]. Even if more expensive, throught the years LA has become more common because there are undeniable benefits in hospitalization time and in recovery time: this way, higher costs are balanced out by a more precocious return to work of working patients. Recently, one study found that predicted costs for LA were 1856$ lower than for OA while the postoperative complication rate did not differ significantly[47].

Another crucial factor which influences the choice between LA and OA is the training and experience of surgical equipe. An interesting study compares the experience in academic-affiliated and community hospitals. The rate of LA and OA in the two kinds of hospitals is quite the same, but in academic-affiliated ones the opera-

tive time is longer both for LA and for OA (47 min *vs* 38 min for LA and 49 min *vs* 44 min for OA): this could be explained considering the intrinsic didactic nature of academic hospitals which inevitably causes a little delay in the operations. Finally in both types of hospitals, hospitalization for LA was shortened by 1 d[48].

A parameter to assess the value of a surgical approach is long-term quality of life. A German study determined how a group of patients - including both LA and OA - perceived their quality of life 7 years after appendectomy, through the administration of a specific questionnaire. The most satisfied patients were those who underwent LA, both for the quick recovery and for the cosmetic result[49]. Another work obtained information about overall satisfaction by a telephone interview: the LA group had fewer complications and returned earlier to work (median 13 d for OA *vs* 8 d for LA)[13].

Laparoscopic appendectomy: Techniques

Recently several methods have been proposed to perform appendectomy in a laparoscopic fashion. In the most popular approach, 3 abdominal wall incisions are performed to insert instruments in the abdominal cavity. According to the patients' demand of scar-free surgery, new minimally invasive methods have been developed.

Traditional laparoscopic appendectomy [3 port(s) laparoscopic appendectomy]: In conventional laparoscopic appendectomy, 3 ports are used to place instruments in the abdomen (Figure 1). The laparoscope is inserted in the umbilicus and pneumoperitoneum is induced; the site of the other 2 trocars for operative instruments is variable, according to the surgeon's preference and ability. The most used locations for trocars are: the lower left quadrant and suprapubic or lower left quadrant and lower right quadrant or suprapubic and lower right quadrant or both trocars placed on the "bikini line" (suprapubic)[50]. Nevertheless, the trocars are inserted respecting the triangulation rule, with the appendix at the apex of a triangle. The umbilical port is 5-12 mm in diameter while the others are generally 5 mm large[51].

During surgical procedure, many methods are used to amputate and extract the appendix and to perform proper hemostasis; the routinely use of peritoneal irrigation and drainage placement is not recommended[52]. The number of trocars can be reduced to 2 using the "puppeteer technique"; in this variant, the appendix is suspended using transabdominal threads[53].

A laparoscopic surgeon must be skilled with the open approach; in fact, open appendectomy represent the first step in the training of an operator who desires to perform laparoscopic appendectomy. But when is the learning curve completed? It is generally accepted that it is completed after 20 operations[54].

To improve the cosmetic result, needlescopic appendectomy has been developed; this term refers to an evolution of conventional laparoscopy. The only difference between the two regards the instruments' diameter,
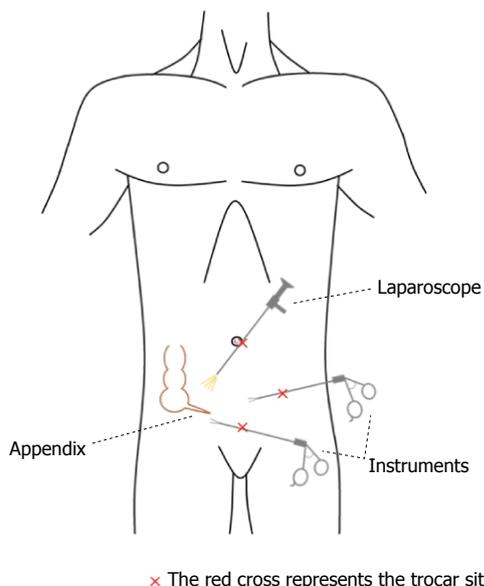
Figure 1 Traditional laparoscopic appendectomy: 3 ports are used to place instruments in the abdomen.

in fact in the needlescopic approach 3-mm or less trocars are used[55]. The first needlescopic appendectomy was performed in 1994. The use of smaller trocars potentially reduces postoperative pain and length of hospital stay due to minor abdominal wall incisions[56]; patients can quickly return to normal activity. On the other hand, this technique is more challenging for surgeons with a risk of longer duration of surgery and higher conversion rate[57]; these disadvantages will probably disappear after an appropriate learning curve and an increase of surgical skill. Needlescopic appendectomy is likely to be more expensive than the traditional approach due to equipment costs[58]. This fascinating laparoscopic evolution is not routinely recommended because of the lack of scientific evidence: large randomized controlled trials are necessary. It can, however, represents an option in selected patients, like young women.

Single-incision laparoscopic surgery: The continuous evolution of laparoscopic surgery and the ambition of better cosmetic results always tend to less invasive procedures. Single Incision Laparoscopic Surgery (SILS) for acute appendicitis in children began in 1992[59]. The development and diffusion of this technique was quite slow due to the lack of adequate instruments; healthcare engineering ideated multilumen ports, special laparoscopes and articulating instruments to facilitate the surgeon's work[60]. SILS is now diffused in many surgical specialties and skilled surgeons can perform several operations in this way, *i.e.*, adrenalectomy, Heller myotomy, large bowel surgery, splenectomy, bariatric surgery[61].

In SILS, a multi-luminal and single port device is placed transumbilically: through this device, laparoscope and instruments can reach the abdominal cavity. The proposed advantages of SILS are better cosmetic results, reduced wound infection, postoperative pain, bleeding,

visceral injury and port site hernia due to the presence of a unique abdominal wall incision: for this reason it is known as "scarless" surgery. In a recent randomized controlled trial, SILS was associated with higher post-operative pain and more intravenous analgesics requirement; better wound cosmesis and higher satisfaction scores were also observed[62]. On the other hand it also has some technical challenges, like loss of triangulation (the cornerstone of laparoscopy) and instrument crowding (sword fighting)[63]. Although it is a technical challenge, in skilled hands, it is considered a safe procedure; patients seem to appreciate when a SILS approach is performed because surgical incisions are hidden in the umbilicus. Recent studies compared SILS and conventional laparoscopic appendectomy: no significative differences in the operative time, length of hospital stay, post operative pain and complication were observed[64,65].

The learning curve of single incision laparoscopic appendectomy is between 5 to 10 cases[66]. To reduce the need of special materials and the costs, SILS can be performed using nonarticulating instruments and conventional trocars: early data suggests that it can represent an economic and safe option, even if operative time is longer[67]. In this approach, an adequate follow-up to detect the risk of post-incisional hernia is needed because many trocars are inserted in a very small area. There are also original ideas to reduce costs, *i.e.*, the use of a surgical glove like a multi-lumen port where instruments pass via the cutting fingers[63]. However, it is very difficult to determine the costs of SILS[68].

Lacking of available evidence, no recommendations can be made on the effectiveness of SILS *vs* conventional multi-incision laparoscopic appendectomy[69].

Natural orifice transluminal endoscopic surgery: In 2004 Rao *et al*[70] described a new real "scarless" procedure performing a transgastric appendectomy. Natural Orifice Transluminal Endoscopic Surgery (NOTES) represents the forefront of laparoscopic surgery and the next worldwide focus on minimally invasive surgery[71]; using a multichannel endoscope, the access to the peritoneal cavity is obtained via natural orifices like vagina, rectum, stomach and bladder. This technique allows to perform many surgical operations without visible scars; avoiding abdominal-wall incisions, postoperative pain is minor and recovery is faster. SILS is considered a bridge between conventional multi-ports laparoscopy and NOTES.

Regarding acute appendicitis, in female patients a transvaginal approach can be used (TVA, TransVaginal Appendectomy); an incision performed in the posterior fornix of vagina permits the access to the peritoneal cavity (Figure 2).

A prospective study comparing TVA to traditional 3-port laparoscopic appendectomy showed significantly less post-operative analgesia demand (Patient Controlled Analgesia morphine utilization) and faster return to normal activity; compared with the conventional laparoscopic approach there were no differences in the length
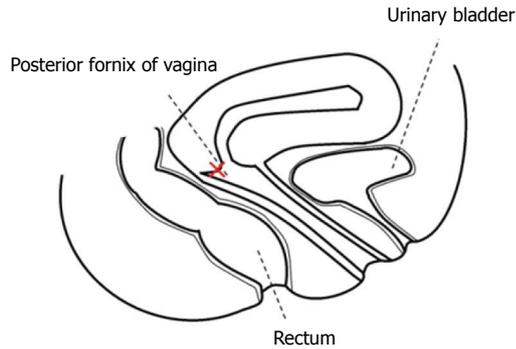
Figure 2 Notes procedure: Transvaginal approach is performed through the posterior fornix of the vagina.

of stay and operative time[72]. There were no differences in pre- and post-operatively sexual function; no post-operative dispareunia was noted and TVA *vs* conventional laparoscopy sexual outcome was comparable. Even though the authors of this prospective study concluded that TVA is a safe and feasible procedure in women with acute non-perforated appendicitis, the authors of this review believe that large randomized controlled trials are necessary before proposing this procedure to a young woman.

CONCLUSION

Patient selection is important in both LA and OA. LA is the preferred approach in immunocompromised, obese and elderly patients. LA presents longer operative time, but also a shortening of hospital stay, a better and earlier recovery and return to everyday occupations and to work and, last but not least, a better cosmetic result.

ACKNOWLEDGMENTS

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TOPIC HIGHLIGHT

Surveillance for early diagnosis of hepatocellular carcinoma: How best to do it?

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Abstract

Surveillance for hepatocellular carcinoma (HCC) is considered a standard of care for patients with chronic liver disease who are at risk of developing this malignancy. Several studies have shown that surveillance can improve the prognosis of patients diagnosed with HCC through an increased likelihood of application of curative or effective treatments. Repetition of liver ultrasonography (US) every 6 mo is the recommended surveillance program to detect early HCCs, and a positive US has to entrain a well-defined recall policy based on contrast-enhanced, dynamic radiological imaging or biopsy for the diagnosis of HCC. Although HCC fulfills the accepted criteria regarding cost-effective cancer screening and surveillance, the implementation of surveillance in clinical practice is defective and this has a negative impact on the cost-effectiveness of the procedure. Education of both physicians and patients is of paramount importance in order to improve the surveillance application and its benefits in patients at risk of HCC. The promotion of specific educational programs for practitioners, clinicians and patients is instrumental in order to expand the correct use of surveillance in clinical practice and eventually improve HCC prognosis.

Core tip: This article deals with the role of surveillance for early diagnosis of hepatocellular carcinoma in patients at risk. It addresses several topics on this issue, including how to best perform surveillance (tools and interval), its results in terms of cancer stage, patient survival, cost-effectiveness, pitfalls and actual under-(mis-)use.

SURVEILLANCE AS A MEANS OF IMPROVING SURVIVAL OF PATIENTS AT RISK OF DEVELOPING HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is one of the leading malignancies worldwide, representing the fifth most common human cancer and the third cause of death from

cancer[1,2]. Patients diagnosed with HCC often have a dismal prognosis as it is diagnosed at late stages, when therapeutic approaches are limited, if applicable at all. Conversely, an early diagnosis of HCC allows the application of curative or effective treatments in most cases, improving the survival of these patients[3]. Therefore there is a need for early diagnosis of this tumor. Screening and surveillance for HCC applied to patients with chronic liver disease who are at risk of developing this cancer can indeed identify malignancies at early stages and improve patient survival, although there is still debate regarding the optimal screening and surveillance tools and the actual yield of surveillance[3-5]. This review addresses the current evidence supporting surveillance programs in patients at risk of developing HCC, the best way to perform surveillance, and the still unsolved nuances of this topic.

DIFFERENCE BETWEEN SCREENING AND SURVEILLANCE

Screening is the application of a test to detect a disease in a population which has no signs or symptoms of that disease, while surveillance is the periodic repetition of the screening test in the same population. Both screening and surveillance have the aim of detecting a disease before it becomes symptomatic, at an early time point of its natural history and when treatment is more effective, with the ultimate goal of reducing disease-specific mortality. Positive findings of screening or surveillance tests must entrain a pre-defined recall policy aimed at identifying true positive cases with additional diagnostic procedures. Screening and surveillance must fulfill the seven Prorok postulates[6] and, as outlined below, this is the case for the surveillance of patients at risk of developing HCC.

The disease must be common and with substantial morbidity and mortality

HCC is a common malignancy worldwide and its incidence is expected to rise in most Western world areas due to the aging of patients with chronic hepatitis C virus (HCV) infection, which is the main etiological factor of this tumor in developed countries[7,8]. Moreover, HCC is currently the main cause of death of patients with initially compensated liver cirrhosis[9,10]. Noteworthy, the incidence and mortality rates of HCC are very similar all over the world, thus emphasizing the high lethality rate of this tumor in the short term, especially when it is diagnosed at late stages precluding any effective treatment[2], although a favorable mortality trend has recently been observed in Europe[11-13].

The target population must be readily identifiable

More than 90% of HCCs develop in a cirrhotic liver, and the main causes of chronic liver disease in these patients are hepatitis B virus (HBV) or HCV infections, alcohol abuse and non-alcoholic fatty liver disease[14]. These diseases can be detected on the basis of patient history and/

or serological tests, thus making the target population for HCC surveillance readily identifiable.

Surveillance tests must have low morbidity, high sensitivity and high specificity

The American and European guidelines for HCC management recommend surveillance to be carried out by ultrasound examination of the liver (US) repeated every 6 mo[15,16]. This surveillance schedule has no morbidity and, when US is properly carried out, a fairly high sensitivity and specificity[17]. In particular, a recent meta-regression analysis has shown that US can identify subclinical HCCs with a sensitivity of 94%-95%, but this drops to 63% for early HCC, while specificity ordinarily exceeded 90%[18]. However, series coming from referral centers reported remarkably higher sensitivity figures (82%), even for early HCC[19,20]. Therefore, the availability of sonographers with expertise in this field is a mandatory prerequisite for a useful US-based surveillance for HCC[21]. The use of serum alpha-fetoprotein (AFP) as a surveillance test has an acceptable specificity but a poor sensitivity for early HCC since only a small fraction (10%-20%) of early cancers is associated with elevated AFP serum levels[15,16,19,22-24]. The combination of US and serum AFP assessment slightly increases (6%) the sensitivity of surveillance but almost doubles the cost for each small HCC detected due to a high number of false positives[18,25].

The surveillance test must be acceptable to the target population

A semiannual repetition of US is a non-invasive, easily-performed, and relatively low-cost surveillance schedule which is not a major obstacle for patient adherence. Rather, physician education and knowledge of the potential benefits of surveillance, and adequate operator training are areas where there is still room for improving the effectiveness of surveillance programs[26-29].

There must be standardized recall procedures

A positive result of the surveillance test must entrain a prompt activation of a pre-defined standardized algorithm (recall procedures) able to provide a definite diagnosis. Recall procedures for suspected lesions identified by US during screening or surveillance have to be consistently defined and involve radiological, contrast-enhanced imaging procedures or pathological evaluation of the lesion(s) relying on precise diagnostic criteria[15,16]. The diagnostic yield of these recall procedures has been independently confirmed, and allows an adequate evaluation of tumor extension that, in turn, has a pivotal role in driving the therapeutic strategy[21,30,31]. An inappropriate or delayed application of recall procedures is an important cause of surveillance failure[28].

There must be an acceptable and effective therapy

The goal of surveillance for HCC is to identify tiny lesions, amenable to curative treatments with the aim of improving patient survival. Surgical resection, percutane-

Table 1 Suggested thresholds of hepatocellular carcinoma incidence for the implementation of surveillance[15]		
Group of patients	Threshold incidence to implement surveillance (% per year)	Incidence of HCC
Surveillance recommended		
Asian male hepatitis B carriers over age 40	0.2	0.4%-0.6%/yr
Asian female hepatitis B carriers over age 50	0.2	0.3%-0.6%/yr
Hepatitis B carriers with family history of hepatocellular carcinoma	0.2	Incidence higher than without family history
African/North American Blacks with hepatitis B	0.2	Hepatocellular carcinoma occurs at a younger age
Hepatitis B virus carriers, cirrhosis	0.2-1.5	3%-8%/yr
Hepatitis C virus infection, cirrhosis	1.5	3%-5%/yr
Primary Biliary Cirrhosis, stage 4	1.5	3%-5%/yr
Genetic hemochromatosis, cirrhosis	1.5	Unknown, but probably > 1.5%/yr
Alpha 1-antitrypsin deficiency, cirrhosis	1.5	Unknown, but probably > 1.5%/yr
Other cirrhosis	1.5	Unknown
Surveillance benefit uncertain		
Hepatitis B carriers younger than 40 (males) or 50 (females)	0.2	< 0.2%/yr
Hepatitis C virus infection, stage 3 fibrosis	1.5	< 1.5%/yr
Non-cirrhotic non-alcoholic fatty liver disease	1.5	< 1.5%/yr

HCC: Hepatocellular carcinoma.

ous ablation, and liver transplantation (LT) are considered curative options for patients with small HCCs. The results of a randomized study carried out in China in HBV infection active carriers and of several cohort studies carried out in Western and Japanese patients with cirrhosis support the use of surveillance as a way of identifying early tumors amenable to curative treatment, and therefore of improving patient survival[19,24,32-34]. Notably, refinements in diagnostic techniques and patient management led to a progressive improvement in the survival of patients diagnosed with HCC during surveillance[35].

Surveillance should reduce disease-specific mortality
The ideal methodology for confirming that surveillance reduces the disease-specific mortality would be to perform a randomized, controlled trial comparing surveillance *vs* care-on-demand in at-risk patients. Two such studies had been performed in Chinese chronic HBV carriers with contrasting results[32,36]. In particular, despite a 40% reduction in the disease-specific mortality, the first trial was affected by a low degree (< 60%) of patient adherence to the semiannual surveillance program and by the LT unavailability, indicating that the reported figure was probably the "minimal" benefit achievable with surveillance in HBV patients[32]. The negative study was instead methodologically flawed by the fact that patients diagnosed with early HCC did not receive an effective treatment[36]. It is unrealistic to expect results on this topic from new randomized controlled trials, at least in the Western world, due to several reasons: (1) subjects in the control arm would frequently undergo abdominal US due to extrahepatic or liver disease-related reasons; (2) almost all the patients, if adequately informed on the risk-benefits of surveillance, would refuse to participate in the study[37,38]; and (3) this position would likely be shared by most clinicians. Thus, the belief that surveillance for HCC reduces the disease-specific mortality and the pertinent recommendations released by Western and Eastern international

guidelines mainly relies on the available proof-of-concept evidence, showing that US surveillance can detect small, asymptomatic tumors that are amenable to curative treatment while symptomatic HCCs are generally detected at an advanced stage, which greatly limits or even precludes any treatment. Pertinently, Western and Eastern cohort studies comparing the outcome of patients with HCC diagnosed during or outside surveillance programs consistently demonstrate that the assumed surveillance benefit holds true[4,24,32-34,38,39].

WHO SHOULD BE SURVEILLED?
In the Western world, surveillance is recommended for subjects at high risk of developing HCC such as patients with cirrhosis and certain categories of patients with chronic hepatitis, while Japanese guidelines extend this recommendation to all patients with chronic hepatitis[15,16,40]. An essential pre-requisite to perform surveillance is the absence of contraindications to treatment-either curative or palliative-once HCC is diagnosed. Thus, surveillance is useless in patients with Child-Pugh class C cirrhosis not listed for LT[41], as an early detection of HCC does not improve their survival due to the inapplicability of therapeutic options for malignancy other than LT and a strong competitive effect with cancer by liver failure as the death cause[42].

As mentioned before, surveillance should be cost-effective and one crucial determinant of cost-effectiveness (CE) is the disease incidence in the target population (see also the specific chapter below). Therefore, the selection of patients who should enter into surveillance programs for HCC is driven by their oncologic risk, which can be inferred from the incidence of HCC (Table 1). The incidence threshold that should trigger surveillance in patients with cirrhosis is 1.5% per year, while for patients with chronic hepatitis this drops to 0.2% per year[15]. It is important to note that these thresholds are not derived

from experimental data but they were proposed considering the results of CE analyses based on the Markov model showing an increase in survival of > 3 mo at a cost of less than 50000 USD per year of life gained[43,44].

Cirrhosis

According to the above mentioned thresholds, patients with cirrhosis are appropriate candidates for a cost-effective surveillance, as the annual incidence of HCC in cirrhotic patients with HCV or HBV infection is 1.5%-4.5% and 2.2%-4.3%, respectively, and it is approximately 2.6% in both alcoholic and non-alcoholic steatohepatitis cirrhosis[14,45-47]. Even cirrhotic patients with genetic hemochromatosis or primary biliary cirrhosis have an HCC risk high enough to implement surveillance, whereas the annual incidence of HCC reported in cirrhotic patients with autoimmune hepatitis is 1.1%, thus questioning the CE of surveillance in this category of patients[48-50].

Patients with HCV-related cirrhosis who cleared the infection with antiviral treatment represent a subset of patients with a decreased, but not abolished, risk of HCC[51,52]. Namely, the incidence rate of HCC per 100 person-years in Japanese patients with cirrhosis who achieved a sustained virological response (SVR) to antiviral treatment was 0.5% compared to 5% in patients without SVR and 8% in untreated cirrhotic patients, while a retrospective Italian study showed figures of 0.7% after SVR and 2% in non-responder patients[51,52]. It should be pointed out that HCC incidences observed after SVR do not cross the suggested CE threshold for surveillance in cirrhosis. Nevertheless, non-viremic HCV cirrhotic patients represent a peculiar population where mortality due to the complications of cirrhosis or liver failure is negligible and the chance of applying aggressive treatments for HCC is high[51,52]. The same applies to HBV patients effectively treated with antiviral nucleos(t)ide drugs in whom the risk of HCC remains as high as 1.3 per 100 person-years despite undetectable viremia[53,54].

To conclude, non-viremic HCV and HBV patients should continue (or start) to undergo surveillance if they were at high risk of developing HCC before starting antiviral treatment.

Non-cirrhotic chronic liver disease

Among pre-cirrhotic patients, those with chronic HBV infection have the highest risk of developing HCC, especially those with long-standing disease, who more likely acquired the infection perinatally, and those with persistent, high-load viral replication[55-57]. These features are frequent in Asian patients, and a study from China showed that patients with chronic hepatitis B without cirrhosis have an annual HCC incidence of 0.8%, thus exceeding the accepted threshold (0.2%) for a cost-effective surveillance[15,58]. African active HBV carriers or with a positive family history are also considered good candidates for surveillance, due to high HCC incidence. As the incidence of HCC in HBV-positive Western patients ranges from 0.1% to 0.4% per year[59,60], the latest

European guidelines for HCC management recommend the implementation of surveillance programs in the subgroups of HBV-positive patients with active hepatitis or a family history of HCC[16].

Although Japanese guidelines recommend HCC surveillance in chronic hepatitis C patients whereas American and European guidelines propose this procedure in those with advanced fibrosis, the evidence supporting these suggestions is less robust[15,16,40]. Indeed, on the one hand, a study carried out in Japan showed that the annual incidence of HCC in untreated patients with chronic hepatitis C increased with increasing fibrosis stage, being 0.5% in patients without or with mild fibrosis, and 5% in those with severe fibrosis[52]. On the other hand, a large, prospective study carried out in the United States to assess the incidence of HCC in patients with bridging fibrosis (Ishak stage 3 and 4) reported an incidence of 0.8% per year[61,62]. Importantly, in this cohort the absence of cirrhosis was assessed at enrollment and HCC was diagnosed after a median of 46.5 mo of follow-up, when cirrhosis had developed in 65% of these patients (15/23 patients) and thrombocytopenia was present in all but one patient[62]. These findings emphasize the difficulties in identifying a clear hallmark indicating the transition from a low to a high oncologic risk status. In an attempt to overcome this problem, either bed-side clinical scores or transient hepatic elastography have been proposed to stratify patients according to HCC risk[62-65].

Lastly, although HCC may also occur in non-cirrhotic patients with a non-viral chronic liver disease, exhaustive data on its incidence in these categories are not currently available, but it is unlikely for surveillance to be cost-effective in these settings.

HOW SHOULD SURVEILLANCE BE PERFORMED?

Imaging tools and expertise

In general, a surveillance test has to have a high sensitivity (to miss very few cancers) and an adequate specificity (to avoid unnecessary confirmatory testing). There is universal agreement that US is the imaging tool to be used for surveillance of HCC. A meta-regression analysis of several cohort studies set the sensitivity of US, as a surveillance test for HCC, at 94% for asymptomatic tumors and 63% for early HCC, with a specificity of > 90%[18]. The relatively low sensitivity of US for tiny lesions may be explained by the fact that this technique is highly dependent on both the operator expertise and the quality of US equipment. In fact, the presence of regenerative nodules and fibrous septa conferring a coarse echo-pattern to the cirrhotic liver makes it difficult to identify minute nodules. Therefore, US examination should be performed by skilled operators and with adequate instruments. In this case, the sensitivity for early-stage or small HCC ranges from 82% to 91%, and the mean size of HCCs detected during surveillance is < 2 cm, with only 1.4% of tumors > 3 cm[19,20,66,67].

Serum AFP

AFP is the serum tumoral marker most widely used in the surveillance for HCC[22]. Its levels are influenced by tumor size and aggressiveness, as well as by the etiology and activity of the liver disease[23,68-74]. These limitations affect the usefulness of AFP as a surveillance test for HCC, and its principal drawback is a poor sensitivity at cut-off levels ensuring an adequate specificity[22,75]. Namely, serum AFP levels are increased in a minority of early HCCs and, when elevated, tend to identify highly malignant cancers with a rapid growth rate[23,67-69,71-73]. Furthermore, AFP lacks specificity for HCC since abnormal levels can be caused by hepatitis activity flares in both HBV- and HCV-infected patients[70,74].

The combined use of US and AFP increases the sensitivity for early HCC by 6% compared to US alone, but also enhances the rate of false positive results, with detrimental consequences on direct and indirect costs for each early HCC detected[76-80]. In fact, while false positive results occur with US or AFP alone in 2.9% and 5.0% of cases, respectively, the figure rises to 7.5% on combining the two tests, and this drop of specificity translates into a cost of approximately 2000 USD per HCC identified with US alone as compared to 3000 USD with the combination of AFP plus US[25].

Inadequate sensitivity for early lesions and lack of specificity discourage the use of AFP as a screening and surveillance tool for HCC[23,78], so that the use of US alone in this setting has been recommended by Western guidelines for HCC management[15,16]. This suggestion, however, is not shared by the recently released Eastern guidelines[40,81], that continue to propose the combined use of US and sero-markers, such as AFP and des-gamma-carboxy prothrombin, aimed at maximizing the sensitivity of surveillance regardless of its negative impact on CE.

Special subgroups (patients on LT waiting list, patients with coarse liver echo-pattern, obese patients)

Patients on the LT waiting list represent a special subgroup where surveillance for HCC acquires additional clinical significance, as the identification of an HCC in these patients: (1) can hasten the urgency for LT by prioritizing the patient on the list; (2) alternatively, it may represent a reason for waiting list drop-out if the tumor burden exceeds the accepted criteria for LT[82-84]; (3) due to these reasons, it also impacts on the probability of the listed non-HCC patients to be transplanted[82,85]. Non-HCC patients listed for LT usually have an advanced cirrhosis which associates with a coarse liver echo-pattern, organ shrinkage and ascites, and these features may impair the US ability to detect (small) focal lesions[86]. Therefore, although there is no compelling evidence to support this suggestion, an HCC surveillance carried out with multiphasic computed tomography (CT) or magnetic resonance (MR) every 6 mo can be proposed for these patients; considering that the expected surveillance duration seldom exceeds 1 year, the detection of HCC is crucial to define the priority for LT (and hence to fairly allocate a limited therapeutic resource among oncologic and non-oncologic candidates), and the use of these techniques has been associated with a better CE ratio[21].

Patients with non-alcoholic liver cirrhosis represent a growing population at risk of HCC[87-89], and in most of them the presence of fatty liver and obesity may impair imaging resolution of liver US exploration. Although no formal studies have been carried out to address this issue, in some studies CT was purposely used instead of US for HCC surveillance in a minority of patients (3.3%) due to the presence of suboptimal US resolution because of a coarse liver echo-pattern or extreme obesity[67]. Instead, in a study carried out in the United States, the presence of an increased body mass index was not associated with a decreased sensitivity of US for HCC detection, although the robustness of this finding is flawed by the limited statistical power of the study and the overall poor quality of US results[90]. Thus, due to the growing prevalence of non-alcoholic liver disease, this is a field where prospective studies comparing US with other surveillance tools are urgently needed[91].

Optimal interval of surveillance (3 mo vs 6 mo vs 12 mo)

The surveillance interval should be dictated by the expected doubling volume time of the surveyed tumor, and not by the degree of the inherent risk of HCC. Median doubling volume time of untreated HCC is around 170 d, although there is a great inter-individual variability and the growth rate may be not constant over time[67,92]. This would indicate that the reference length of the surveillance interval is 6 mo. Increasing the length to 12 mo is indeed associated with a greater likelihood of missing early HCCs, reducing the applicability of effective treatments and thus worsening survival as compared to the semiannual surveillance schedule[34,93]. In fact, after correction for the lead-time bias, the survival of Child-Pugh class A or B patients with HCC identified during a semi-annual surveillance was significantly improved as compared to patients undergoing 12 mo surveillance[34]. Similar findings were obtained in Asian patients, in whom the survival benefit adjusted for lead-time bias was significantly greater when surveillance was carried out with an interval ≤ 6 mo as compared to > 6 mo[93]. Conversely, a randomized study prevalently including patients with alcoholic cirrhosis demonstrated that shortening the surveillance schedule to 3 mo was detrimental as it did not significantly increase the likelihood to detect small (≤ 3 cm) HCCs (79% *vs* 70%), amenability to curative treatment (62% *vs* 58%) and 5-year survival (85% *vs* 86%), whereas it led to a greater cumulative incidence of detected focal lesions that proved non-malignant during the follow-up, thus leading to an increased cost of recall procedures[94]. In this regard, it should be emphasized that the proposal of the Japanese and Asian guidelines to shorten the surveillance interval to 3 mo in patients at very high risk of developing HCC does not rely on experimental results or CE study models[40,81]. Thus, on the basis of the currently available evidence, a 6-mo interval should be recommended for HCC

surveillance[15,16].

DIFFERENCES BETWEEN EFFICACY AND EFFECTIVENESS

Efficacy is a measure of the degree to which one procedure obtains the expected result under standardized conditions, generally chosen to maximize the chance to observe the expected result. Effectiveness, instead, measures the extent of the benefit when the procedure is applied in clinical practice. Effectiveness not only depends upon the efficacy of the procedure but also on "external" non-standardized factors, such as physicians' (specific knowledge, convincement and recommendation) and patients' (acceptance and adherence) behavior, health system organization (timeliness of the recall policy, availability and accessibility of appropriate diagnostic tools and treatments, adequate follow-up), as well as economic, cultural and social influences. In the case of surveillance for HCC, it can be optimistically hypothesized that its effectiveness is affected by the following drawbacks: missed/unconvincing doctor recommendation (80%), limitations to surveillance access (90%), patient refusal (90%) or inadequate adherence (90%), untimely recall (by 90%), untimely availability of appropriate diagnostic and therapeutic options (90%) and improper follow-up (90%). Thus, assuming that the mentioned limitations are independent probabilities and the reduction in overall mortality of cirrhotic patients with HCC diagnosed during surveillance is 40% - according to the Italian Liver Cancer data (ITA.LI.CA)[34] - it can be calculated ($0.40 \times 0.80 \times 0.90 \times 0.90 \times 0.90 \times 0.90 \times 0.90 \times 0.90$) that the actual effectiveness of surveillance in cirrhosis drops to 17%.

Therefore, surveillance for early diagnosis of HCC is a typical example of "clinical nuance", whose basics tenets are that medical services and providers differ in the clinical benefit provided; hence, the benefit of the service depends on the person using it, as well as where and by whom the service is provided. As previously pointed out by our group, besides limited economical resources, a major flaw of surveillance for HCC is the "behavior hazard" of both clinicians (prescription and organization) and patients (adherence)[79]. These shortcomings explain the large gap between efficacy and effectiveness of surveillance of patients at risk of HCC, and indicate the road for reducing this gap and greatly improving the CE of the procedure without the need for diagnostic and therapeutic advancements.

COST-EFFECTIVENESS OF SURVEILLANCE

The economic aspect of HCC surveillance has also to be considered. Its CE is mainly determined by two features: the gain obtainable with surveillance in terms of quality-adjusted life-expectancy (effectiveness) and its total costs. In turn, these features are determined by two components each. Effectiveness strictly depends on HCC incidence and the actual possibility to submit patients diagnosed with HCC to potentially curative treatments; total costs result from the sum of the costs of surveillance test(s), tools utilized for tumor diagnosis and staging, and HCC treatment(s).

As mentioned above, from a CE standpoint US surveillance of cirrhotic patients should be started when the annual HCC incidence is expected to be at least 1.5%; however, it cannot be excluded that different surveillance strategies, and different surveillance intervals, can be more cost-effective in different clinical scenarios. For instance, available data suggest that the annual program of US surveillance (± AFP assessment) is cost-effective in patients with a tumor risk up to 3%-3.5% per year, while the semiannual program becomes more cost-effective in patients with a risk above these figures[44,76,95,96]. Indeed, the semiannual US strategy has been consistently reported to be the most effective program for an early tumor diagnosis but it inevitably increases direct and indirect costs with respect to programs with longer intervals. Considering this, a reasonable alternative from a CE perspective is the "AFP-triage strategy" that avoids US use in patients with normal AFP values. This strategy has been reported to be more cost-effective than semiannual US but with a lower efficiency in detecting HCC[97].

The second main determinant of surveillance effectiveness is the possibility to timely submit HCC patients to potentially curative treatments. While it is not possible to predict the tumor burden at presentation in the individual patient, it is intuitive that an advanced degree of liver dysfunction strongly limits-or even prevents-the therapeutic approach to the forthcoming HCC. The literature lacks specific analyses comparing the CE of surveillance *vs* no-surveillance in decompensated cirrhotic patients, also because surveillance is not currently recommended in patients with advanced cirrhosis not listed for LT. The only available evidence indicates that semiannual US surveillance can be more cost-effective than annual surveillance only if treatment can ensure a huge survival gain after HCC diagnosis, as in the case of LT[97], indirectly supporting the recommendation to keep, among Child-Pugh class C patients, only candidates for LT under surveillance.

The direct costs of surveillance test(s) are relatively low, as both US examination and AFP dosage are not high-cost procedures. It has been reported that costs for surveillance and tumor diagnosis are around 18000 USD per each potentially curable HCC detected, accounting for only 10%-20% of total costs of cancer management since the main determinant of costs is treatment[19,98,99]. Nevertheless, the CE of surveillance programs based on CT, MR or contrast-enhanced US (CEUS) has been tested with Markov model analyses and most of the studies found that their use raised costs, without a parallel significant increase in HCC detection, resulting in a higher incremental cost-effectiveness ratio (ICER) compared to US surveillance[76,82,100,101]. Thus, there is not sufficient evidence for adopting CT or MR as surveillance tests,

whereas the use of CEUS, although intriguing, requires further dedicated studies.

Another point that needs to be addressed is the use of AFP as a surveillance test. Sensitivity of AFP is reported to be around 60%, and its specificity is limited by the non-HCC related elevation of the marker due to hepatic necro-inflammation and regeneration occurring in active hepatitis or cirrhosis[15,16,70,71,74]. Consequently, the frequent false positive results of a periodic AFP measurement, entraining confirmatory tests, increase the total costs of surveillance based on serum AFP measurement[96].

As mentioned before, the main determinant of surveillance costs derives from the tumor treatment. For example, the inclusion of LT in the treatment algorithm, reimbursement of which can be up to 250000 USD (University of Alabama)[100], results in an up to 10-fold increase of the average cost-effectiveness when compared to scenarios where LT is not an option. Hepatic resection is another high-cost intervention, that can compete with percutaneous ablation in terms of both survival and CE. Available literature suggests a CE advantage for ablation in the case of single tumors ≤ 2 cm and 2-3 nodules each ≤ 3 cm, while surgery becomes more cost-effective for single tumors > 3 cm[102]. Thus, the type of treatment adopted, the proportion of patients undergoing each therapy and, more importantly, costs assumptions are the main sources of uncertainty for simulation models aimed at calculating the CE of surveillance for HCC. Therefore, it is advisable to propose prospective micro-costing analyses to refine this topic. Micro-costing studies collect data and values on the resources utilized for each patient so that, although time- and resource-consuming (expensive record keeping over time and use of database management), they allow a precise definition of costs. Only one prospective micro-costing study has been published, and this was more than 10 years ago[19]. Due to the changed scenario of HCC management since then, further similar studies are warranted.

To conclude, semiannual surveillance based on US achieves a higher detection rate of early HCC but at increased costs with respect to the annual program. From a CE perspective, alternative strategies, such as the semiannual AFP + annual US or the annual US (± AFP) schedules, could be proposed and tested in patients with a relatively low HCC incidence, such as young cirrhotic women or patients who have become non-viremic after (HCV- and HBV-infected) or during (HBV-infected) antiviral treatment.

WHICH IS THE BEST RECALL POLICY?

Recall policy is instrumental to the success of surveillance, since an abnormal surveillance test must promptly entrain a pre-defined strategy aimed at ruling in/out the presence of HCC and staging it. The diagnostic algorithm that composes recall procedures should be carried out within a reasonable time interval to allow timely and adequate treatment. Recall procedures greatly concur in diagnosing HCC at a very early (solitary, ≤ 2 cm) or early stage

(meeting the Milan criteria) that, in turn, allows application of curative treatment and eventually improves patient survival. It is recommended that patients are evaluated at a referral center with availability of all diagnostic techniques and therapeutic opportunities.

Importantly, any new lesion identified at screening or during surveillance as well as pre-existing lesions enlarging or changing their echo-pattern should be regarded as malignant unless otherwise demonstrated; however, as most nodules < 1 cm are non-malignant, the institution of recall procedures for these lesions would increase surveillance costs without clinical gain[94,103]. Therefore, these lesions should be strictly followed-up with US every 3 mo until an increase in size occurs (allowing a suitable definition of their nature with diagnostic techniques) or for one-two years[15,16,104]. This shortening of the interval between US scans ("enhanced" follow-up) is dictated by the knowledge that the volume doubling time of some HCCs may be as short as 30 d, and the main goal of surveillance is to detect HCCs ≤ 2 cm[89]. It has to be emphasized that the echo-pattern is not predictive of malignancy since, although HCC more often presents as a hypo-echoic lesion, it may be hyper-echoic or have a "target" appearance[105,106].

The recall strategy for lesions ≥ 1 cm relies on the use of dynamic, contrast-enhanced, multiphase, imaging techniques with vascular contrast media (CT, MR, CEUS) and overlaps with the diagnostic process. In cirrhotic patients, if the nodule shows the typical vascular patter *i.e.*, homogeneous contrast enhancement in the arterial phase (wash-in) followed by hypo-enhancement in the portal or venous phase (wash-out) - it can be regarded as HCC with no need for histological confirmation[20,21,30,31,107,108]. If the lesion does not display this typical pattern at the first imaging procedure, an alternative imaging technique can be performed, and if an atypical vascular pattern is found again, the lesion should undergo biopsy. It is recommended that histological samples are evaluated by an expert in liver pathology and, in the case of non-diagnostic pathological results, a follow-up with US every 3 mo should be implemented and the recall procedures repeated as soon as a nodule enlargement is observed (Figure 1).

When selecting the most rewarding imaging technique to be firstly performed in a patient with suspected HCC, it should be considered that MR has the highest sensitivity to detect the typical vascular pattern in tiny HCC (< 2 cm) and, using hepatocyte-specific contrast agents, it can provide important additional information in the so called "hepato-biliary phase" (hypo-intensity of the nodule) to suspect malignancy even in the absence of the wash-in phenomenon, a feature quite frequent in tiny lesions[21,109-112]. Discovering the malignant nature of nodules < 2 cm is indeed of paramount importance as, above this size, the prevalence of unfavorable prognostic factors, such as microscopic vascular invasion and satellites, greatly increases[59,112] (Figure 2).

The inclusion of CEUS among the imaging tech-

1	The disease must be common, with substantial morbidity and mortality
	Hcc is the fifth most common human cancer and the third cause of death from cancer
	The annual incidence of HCC in patients with compensated cirrhosis is 2-4%
	HCC is the main cause of death in patients with compensated cirrhosis

2	The targen population must be readily identifiable
	HCC develops in patients with cirrhosis in more than 90% of cases
	Hcc can develop even in pre-cirrhotic patients with HBV or HCV infection, or metabolic liver diseases
	The population at risk of HCC is usually identified on histological or clinical basis and patients are often referred to hepatology centers

3	Surveillance tests must have low morbidity, high sensitivity and high specificity
	Hepatic ultrasound, the recommended surveillance tool, has no morbidity
	The sensitivity of ultrasound for detection of preclinical and early HCC is 94-95% and 63%, respectively, while specificity is > 90%
	The diagnostic performance of ultrasound is greatly influenced by the operator expertise: in erpert hands ultrasound is an adequate surveillance test

4	The surveillance test must be acceptable to the target population
	A semiannual repetition of ultrasound is a non-invasive, easily-performed, and relatively low-cost surveillance schedule
	Adherence to the surveillance schedule is a function of patient education and physician awareness of the HCC risk
	Adequate operator training and access to the procedure are instrumental to the outcome of the surveillance process

5	There must be standardized recall procedures
	A positve result of the surveillance test has to timely entrain a pre-definite algorithm aimed at diagnosis comfirmation (recall procedures)
	Recall procedures involve radiological, contrast-enhanced imaging and/or pathological evaluation of the lesion (s) relying on precise diagnositic criteria
	The suitability of standardized radiological procedures has been comfirmed in independent series

6	There must be an acceptable and effective therapy
	Liver transplantation, surgical resection and percutaneous ablation are curative options for patients with small HCC, and can increase their survival
	Surveillance increases the amenability to curative treatments compared to patients diagnosed with a symptomatic tumour

7	Surveillance should reduce disease-specific mortality
	In the absence of adequately powered randomized clinical trials, simulations based on Markov model and cohort studies represent the best evidence supporting surveillance benefit
	Cohort studies showed that surveillance for HCC improves survival through an increased detection of tumors amenable to curative treatments. One randomized controlled trial performed in HBsAg carriers supports this information

Figure 1 Prorok's postulates: Paradigm of surveillance for early diagnosis of hepatocellular carcinoma.

niques of the recall policy is currently debated, due to the risk of misdiagnosis between HCC and intrahepatic cholangiocarcinoma (ICC)[15,16,113-115]. However, the recently released Italian recommendations for HCC management[104] have included CEUS in the recall algorithm due to: (1) its positive predictive value for HCC > 95% when a typical vascular pattern is observed; (2) the fairly low incidence of ICC in cirrhosis (1%-3% of newly diagnosed tumors); and (3) the fact that only half of small ICCs display a pattern typical for HCC at CEUS[115-118]. From a CE standpoint, however, it should be pointed out that, since a "panoramic" imaging technique is mandatory to correctly stage the tumor, CT or MR should be preferred, using CEUS as a second-line procedure in the case of inconclusive findings at radiological imaging techniques[105,114].

ACTUAL UPTAKE OF SURVEILLANCE AND LIMITATIONS TO ITS APPLICATION

Despite the available evidence that surveillance increases the survival of patients diagnosed with HCC, expanding the possibility to perform effective therapies, there is still controversy on its actual usefulness in clinical practice. Some recent studies, coming from the United States, have helped frame the receipt of HCC surveillance in everyday practice in this country and reported the obstacles to its utilization, providing hints on how to improve its uptake and outcome[116-121]. Indeed, initial reports showed that no more than 28% of patients diagnosed with HCC underwent at least 1 screening test in the 3 years preceding the diagnosis and, among them, 36% received AFP testing alone as a screening test[117]. However, this study did not report a measure of receipt of surveillance in the whole population of patients at risk. A subsequent study, performed on a larger and more representative sample, confirmed a low uptake of surveillance in patients diagnosed with HCC, showing that 17% and 38% of patients received consistent and inconsistent surveillance, respectively, before HCC detection, and demonstrated that being followed up by a gastroenterologist/hepatologist or an academic physician was associated with a higher likelihood of receiving surveillance as compared to patients followed by primary care physicians[118]. Thus, being followed by a specialist in liver disease is a key factor for the likelihood of receiving HCC screening and surveillance, a finding indirectly supported by the result of a self-reported use of
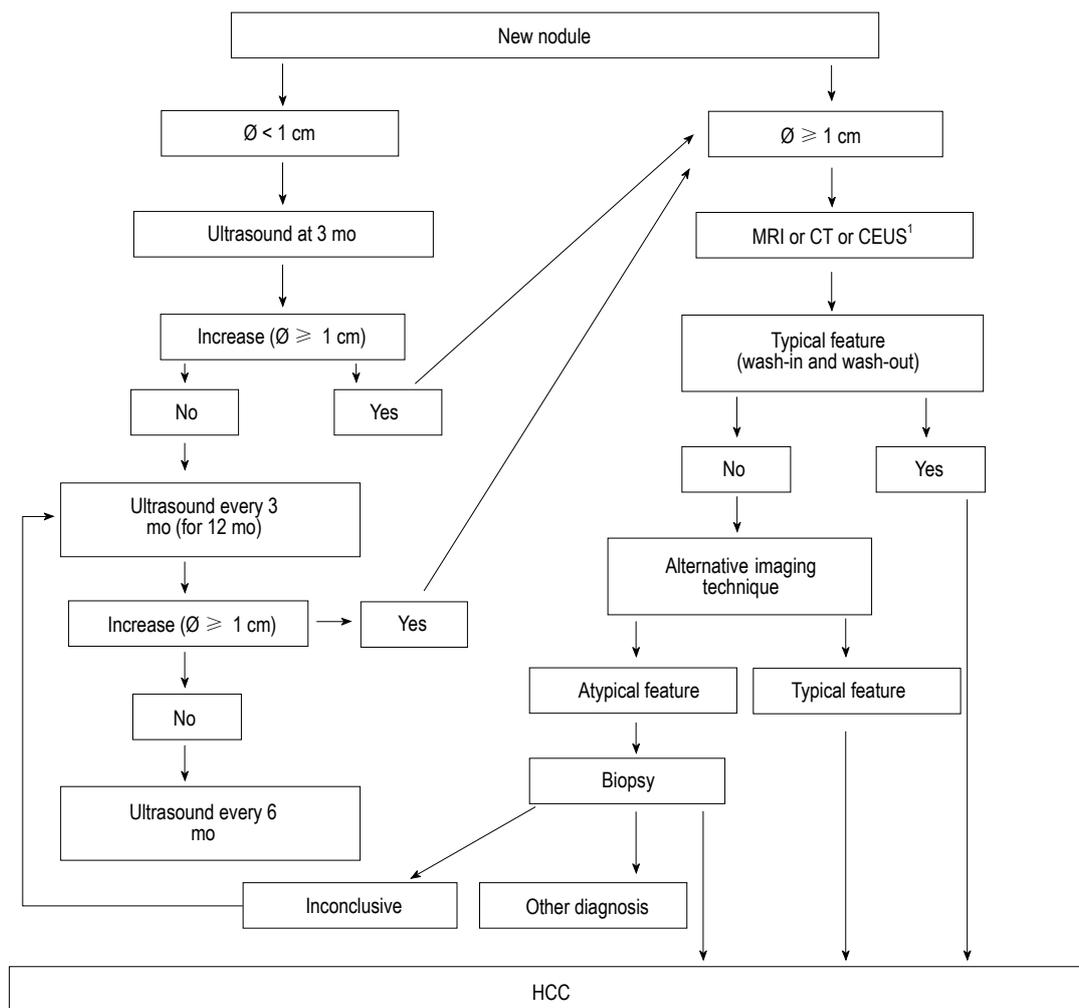
Figure 2 Recall policy and diagnostic algorithm proposed by the Italian Association for the Study of the Liver for cirrhotic patients with a nodule detected during ultrasound surveillance. [1]Note that, since magnetic resonance (MR) or computed tomography (CT) are anyhow needed for staging in the case of hepatocellular carcinoma diagnosis made by contrast-enhanced ultrasonography (CEUS), a pragmatic approach is to perform MR or CT as the first-line imaging technique for diagnosis, and to resort to CEUS when radiological imaging techniques provide inconclusive features (reprinted with permission)[102].

surveillance ranging from 71% to 84% among members of the American Association for the Study of the Liver and the Veteran Health Administration[121,122]. Moreover, an adequate surveillance was strongly associated with the local availability of all possible treatments for HCC[121], thus emphasizing the concept that patients at risk should be followed up and managed at referral centers with availability of multi-disciplinary resources to optimize the effectiveness of surveillance. These findings underscore that the patient's probability to be maintained under surveillance is strictly connected with specialist care and the possibility to receive treatment for HCC, and that effectiveness of surveillance is modest in decreasing HCC mortality when surveillance uptake is markedly low[119,121].

Lastly, longitudinal evaluation of the ITA.LI.CA database over 20 years showed an increase in the proportion of patients diagnosed with HCC during surveillance until 2002, followed by stationary figures over the subsequent 6 years, accounting for approximately 53% of these cases, but with a significant continuous shift to preference of the 6-mo interval[89]. These data, as well

as those coming from the United States, clearly reveal an insufficient and suboptimal use of surveillance in the real world of health care and should stimulate educational policies aimed at expanding the knowledge and the correct use of this tool for secondary prevention of HCC. Indeed, audits with identification of barriers to the application of surveillance and implementation of measures able to improve physician and patient education, together with system re-design, have led to a great increase in the application of adequate surveillance protocols for an early diagnosis of HCC[122].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Hepatocellular carcinoma in chronic hepatitis B patients under antiviral therapy

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Abstract

Patients with chronic hepatitis B are at increased risk of hepatocellular carcinoma (HCC), while the inhibition of viral replication can represent a reasonable target for HCC prevention. Interferon-α therapy results in decreased HCC risk, which is more evident in patients with high baseline HCC risk. The majority of chronic hepatitis B patients are treated with a nucleos(t)ide analogue (NA) for several reasons including the non-sustained response after interferon-α. The effect of the first licensed and low genetic barrier NA, lamivudine, on HCC incidence, has been repeatedly evaluated. Lamivudine, compared to no treatment, reduces the HCC incidence, which may increase again in cases with lamivudine resistance. Emerging data with the currently first-line NAs, entecavir and tenofovir, suggest that they also reduce the HCC incidence. The treatment benefit in reduction of the HCC incidence is always greater in patients with high baseline HCC risk, particularly cirrhotics, and without virological remission under entecavir/tenofovir. However, the HCC risk is not eliminated even in the vast majority of patients who remain in virological remission under entecavir/tenofovir. Therefore, patients at increased baseline HCC risk should continue to undergo HCC surveillance even if they have achieved complete long-term inhibition of viral replication and improvements in liver histology.

Core tip: Antiviral therapy reduces but does not eliminate the risk of hepatocellular carcinoma (HCC) in chronic hepatitis B patients with or without cirrhosis. The reduction of the HCC incidence under a high genetic barrier nucleos(t)ide analogue is higher in the vast majority of patients who will achieve virological remission compared to those who may maintain detectable viral replication. In current clinical practice, however, patients at increased baseline HCC risk should continue to undergo HCC surveillance according to the existing recommendations even if they have achieved complete long-term inhibition of viral replication and improvements in liver histology.

Vlachogiannakos J, Papatheodoridis G. Hepatocellular carcinoma in chronic hepatitis B patients under antiviral therapy. *World J Gastroenterol* 2013; 19(47): 8822-8830 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i47/8822.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i47.8822

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm and the third most frequent cause of cancer death[1]. It represents more than 90% of primary liver cancers and is a major global health problem. In most

cases, HCC develops within an established background of chronic liver disease. Following this, chronic hepatitis B virus (HBV) infection is a significant predisposing factor for the development of HCC and accounts for more than 50% of all cases[2]. The relative risk of HCC development is 100-fold higher for patients chronically infected with HBV versus those who are not infected. The risk is even higher for cases with high viral replication and/or HBV related cirrhosis[3].

In patients with cirrhosis, surveillance for HCC increases the possibility of an earlier diagnosis and improved survival[1]. However, screening programs are rather unsatisfactory and the prognosis remains poor because therapeutic interventions are rather ineffective in advanced stages[4]. Therefore, the development of preventive strategies is mandatory. HCC related to HBV can be prevented by vaccination. Nationwide vaccination of infants in Taiwan reduced the incidence of HCC in children aged 6-9 years from 0.52 per 100.000 for those born between 1974 and 1984 to 0.13 for those born between 1984 and 1986[5]. Nevertheless, the incidence of HCC is expected to increase during the next years because approximately 400 million people who are already chronically infected with HBV cannot benefit from immunization[6]. In patients with chronic HBV infection and high serum HBV DNA levels, viral replication can be inhibited by antiviral agents that prevent the progression of liver disease and perhaps the development of HCC in the long-term.

The current therapeutic options for patients with chronic hepatitis B include treatment with standard or PEGylated interferon-α (IFN-α), a drug with antiviral, immunomodulatory and perhaps antitumoral activities, and five oral nucleos(t)ide analogues (NAs) (lamivudine, adefovir, entecavir, telbivudine, tenofovir)[7]. In this review, we summarize the data on the impact of antiviral treatment in the prevention of HCC in patients with chronic HBV infection.

RATIONALE OF ANTIVIRAL TREATMENT FOR HCC PREVENTION

It is believed that persistent viral replication together with the resulting liver injury are key risk factors for HBV-related HCC[8,9]. More specifically, a direct linear relationship was reported between viral load and HCC risk[10]. Chronic HBV infection promotes viral induced immune response with release of cytokines and genotoxic reactive oxygen species leading to liver cell necrosis as well as to activation of liver fibrosis cascade. The ensuing acceleration of hepatocyte cell cycles and the increased risk of genetic alterations might culminate in malignant transformation of hepatocytes[11].

Moreover, the HBV sequences can integrate into cellular DNA and may modulate the expression of neighboring cellular genes in a cis-acting way[12]. The integration of HBV DNA may cause overexpression of those cellular genes which in turn contributes to the develop-

ment of carcinogenesis[13,14]. Furthermore, the viral protein HBx may play a crucial role in hepatocarcinogenesis because its trans-activation is involved in the function of a large number of signaling pathways and cellular genes that are involved in oncogenesis, proliferation, inflammation and immune responses[15]. Since all of the above mechanisms require the presence and replication of the virus, suppression of viral replication seems to be a reasonable target for the prevention of HCC.

There are additional important viral and host factors that may affect the risk of HCC development. Adequate evidence suggest that HBV genotype C is associated with more active and rapidly progressive liver disease including more frequent HCC development, compared to genotype B[16]. HBV genome mutations such as pre-S deletions, enhancer II mutations (T1653) and core promoter mutations (V1753, T1762 and A1764) have also been found to be associated with a higher HCC risk[17,18]. Moreover, older age, male gender, alcohol abuse and possibly metabolic syndrome also increase the risk of HCC[19,20].

Lastly, recent data from Eastern Asia showed that high levels of HBV surface antigen (HBsAg) (> 1000 IU/mL) in HBV e antigen (HBeAg) negative patients with low levels of HBV DNA (< 2000 IU/mL) is an independent risk factor for HCC development[21]. As HBsAg is mainly produced by the integrated form of HBV DNA, low viremic patients who have high HBsAg level might harbor more hepatocytes with HBV integration thus increasing genomic instability which play an important role in carcinogenesis.

IFN-α AND HCC

The usefulness of IFN-α in the prevention of HBV-related HCC has been investigated only with traditional IFN-α to date, as PEGylated IFN-α was licensed relatively recently and long-term follow-up studies have not been published yet. The IFN-α data on HCC prevention in patients with chronic hepatitis B have been conflicting so far and thus several meta-analyses have tried to elucidate this issue (Table 1). The first meta-analysis of 7 studies (2 Oriental-5 European) including 1505 patients with cirrhosis suggested a decreased incidence of HCC in IFN-α-treated patients (risk reduction-6.4%, $P < 0.001$)[22]. However, the pooled estimate in favor of IFN-α was a consequence of the two Oriental trials because the subgroup analysis of the five European studies found no benefit from IFN-α on the prevention of HCC (risk reduction-4.8%, NS). Sung *et al*[23] performed a meta-analysis of 12 randomized, case-control and cohort studies (1292 IFN-α treated and 1450 untreated patients) and showed that the HCC risk was reduced by 34% in IFN-α treated patients (RR = 0.66, 95%CI: 0.48-0.89). Subgroup analysis revealed a significant benefit in patients with early cirrhosis (RR = 0.53, 95%CI: 0.36-0.78) but not in patients without cirrhosis (RR = 0.72, 95%CI: 0.16-3.15). In addition, no difference was found in the HCC incidence in relation to virological response to therapy (RR = 0.76,

Table 1 Summary of meta-analyses evaluating the effect of antiviral treatment on the incidence of hepatocellular carcinoma in patients with chronic hepatitis B

1st author, year	No. of studies, total (used[1])	Total No. of patients, treated/ untreated	Treatment regimen	HCC cases, total *n*	HCC incidence	RD or RR	95%CI	*P* value
Cammà *et al*[22], 2001	7	853/652	IFN-α	122	Overall	RD = -6.4	-2.8--10	< 0.001
	(5)				European studies	RD = -4.8	-11.1-1.5	NS
	(2)				Oriental studies	RD = -8.0	-1.4--14.6	< 0.001
Sung *et al*[23], 2008	12	1292/1458	IFN-α	190	Overall	RR = 0.66	0.48-0.89	0.006
	(6)				Cirrhotics	RR = 0.53	0.36-0.78	0.001
	(3)				Non-cirrhotics	RR = 0.72	0.16-3.15	NS
	(4)				Virological responders	RR = 0.76	0.08-7.23	NS
	(4)				Non-virological responders	RR = 0.64	0.33-1.26	NS
Yang *et al*[24], 2009	11	1006/1076	IFN-α	178	Overall	RR = 0.59	0.43-0.81	0.001
Miyake *et al*[25], 2009	8	553/750	IFN-α	100	Overall	RD = -5.0	-9.4--0.5	0.028
	(3)				European studies	RD = -0.5	-4.9-4.0	NS
	(5)				Asian studies	RD = -8.5	-13.6--3.6	0.001
	(5)				Incidental rate of HCC ≥ 10%	RD = -9.4	-14.2--4.6	< 0.001
	(3)				Incidental rate of HCC < 10%	RD = -0.2	-4.3-4.7	NS
	(4)				HBeAg positive ≥ 70%	RD = -6.0	-11.8--0.2	0.043
	(3)				HBeAg positive < 70%	RD = -5.4	-15.4-4.6	NS
Sung *et al*[23], 2008	5	1267/1022	LAM	152	Overall	RR = 0.22	0.10-0.50	< 0.001
	(3)				Cirrhotics	RR = 0.17	0.04-0.79	0.020
	(2)				Non-cirrhotics	RR = 0.21	0.10-0.47	< 0.001
	(3)				Drug resistance	RR = 0.52	0.28-0.97	0.040
	(3)				Without drug resistance	RR = 0.37	0.17-0.77	0.008
	(3)				HBeAg positive	RR = 0.21	0.10-0.44	< 0.001
	(3)				HBeAg negative	RR = 0.25	0.06-1.06	NS
Papatheodoridis *et al*[38], 2010	21 (3)	3881/534	LAM	202	Treated *vs* untreated	2.8% (22/779) *vs* 6.4% (34/534)		0.003
	(3)				Treated in remission *vs* untreated	2.5% (9/353) *vs* 6.4% (34/534)		0.015
	(3)				Treated without remission *vs* untreated	2.8% (12/426) *vs* 6.4% (34/534)		0.016
	(10)				Treated in remission *vs* treated without remission	2.3% (23/982) *vs* 7.5% (64/852)		< 0.001
	(14)				Treated in remission under initial therapy *vs* treated in remission under rescue therapy	2.3% (23/982) *vs* 5.9% (19/320)		0.003
Singal *et al*[49], 2013	49 (6)	10025/3571	LAM or Other NAs[3]	808	LAM[2] *vs* untreated	RR = 0.48	0.38-0.61	< 0.001
	(49)				No difference between NAs[3]	Pooled HCC incidence rate: 1.3 (1.1-1.6) per 100 person-years		

[1]Number of studies included in each analysis; [2]In 6 studies including both LAM treated (*n* = 3306) and untreated patients (*n* = 3571); [3]In the 49 studies, there were 5946 patients treated with LAM, 1929 patients treated with adefovir, 879 patients treated with entecavir, 616 patients treated with telbivudine and 657 patients treated with tenofovir. IFN-α : Interferon-α; LAM: Lamivudine; NS: Non-significant.

95%CI: 0.08-7.23). In a more recent meta-analysis involving 11 studies (1006 IFN-α treated and 1076 controls), IFN-α reduced the risk of HCC in chronic hepatitis B patients by 41% compared to untreated controls[24]. Finally, Miyake *et al*[25] included 8 studies in a meta-analysis and found a preventive effect of treatment in favor of IFN-α (risk difference, -5.0%, *P* = 0.028) that was more pronounced in Asian patients, in patients with a baseline HCC risk (HCC risk in untreated cohorts) > 10% and in HBeAg positive patients (Table 1).

According to the aforementioned meta-analyses, IFN-α therapy appears to decrease the incidence of HCC, particularly in patients at high baseline risk for HCC development. It should be noted that the results of the individual studies should be interpreted with caution, as they were usually underpowered to capture relatively infrequent hard end-points such as HCC and they often tended to enroll subjects with less severe disease with low HCC risk. The effectiveness of IFN-α treatment was more evident in HBeAg positive patients suggesting that IFN-α may reduce the HCC risk more easily in patients with high viral replication and perhaps without HBV DNA integration into the host genome by accelerating the HBeAg seroconversion phase. There are no data on the impact of IFN-α-induced HBV DNA elimination in the reduction of HCC risk. In any case,

most of the patients with sustained response to IFN-α still have detectable HBV DNA by sensitive polymerase chain reaction (PCR) assays. However, residual viraemia in the absence of biochemical evidence of necroinflammatory liver activity seems to be of no clinical relevance, as the achievement of sustained biochemical remission in HBeAg negative patients has been associated with a significant decrease of the HCC incidence[26]. It should be noted that less than 30%-35% of patients who receive IFN-α achieve sustained responses[7,27,28]. Moreover, patients with advanced cirrhosis may experience severe liver decompensation during treatment with IFN-α[7,28]. Therefore, patients with contraindications to IFN-α including advanced liver disease as well as cases who do not achieve sustained off-treatment response after a course with IFN-α should receive therapy with a NA[7,28].

NAS AND HCC

Most patients are currently treated with oral NAs. These agents represent the first-line treatment option for the majority of chronic hepatitis B patients because of the relatively low efficacy and possible contraindications for or poor tolerance of IFN-α. In addition, they are used even in the majority of patients who may start with standard or recently PEGylated IFN-α and fail to achieve a sustained response[7,29,30]. Long-term therapy with NAs has improved the overall outcome of chronic hepatitis B and resulted in a substantial reduction in the need for liver transplantation[31]. The third generation NAs, entecavir and tenofovir, are currently recommended by the main treatment guidelines as the first-line NAs options[7,29,30] due to their high potency and high genetic barrier. Long-term monotherapy with entecavir or tenofovir achieves maintained on-therapy complete viral suppression in the vast majority of patients (> 95%), progressively increasing rates of HBeAg seroconversion in HBeAg positive cases and improvement of liver histology including reversion of histological cirrhosis in most cases[32-36]. Nevertheless, the effect of NAs on the prevention of HBV-related HCC is still unclear.

LOW-MODERATE GENETIC BARRIER NAS

Most of the published data on the effects of NAs on the HCC risk are derived from studies using lamivudine. In the only randomized, controlled clinical trial including 651 chronic hepatitis B patients (58% HBeAg positive) with biopsy-proven cirrhosis or advanced fibrosis, lamivudine was found to significantly reduce the risk of HCC compared to placebo (3.9% *vs* 7.4%, $P = 0.047$)[37]. When HCC cases diagnosed during the first year of treatment were excluded, the risk reduction was marginally non-significant ($P = 0.052$). It should be noted that the study was terminated early (after a mean duration of 32.4 mo) because of significant beneficial effects in the treatment group (7.8% developed cirrhosis complications *vs* 17.7% in the placebo group, $P = 0.001$). Therefore, it could be

argued that the early termination of the study probably made the effect of HCC prevention less obvious.

Sung *et al*[23] performed a meta-analysis of 5 studies involving 1267 treated patients (mostly with lamivudine) and 1022 controls (Table 1). They showed that the use of NAs reduced the HCC incidence by 78% (2.5% for NAs *vs* 11.7% for controls; RR = 0.22, $P < 0.001$). The HCC risk was found to be significantly reduced in patients with cirrhosis (NAs: 3.9% *vs* untreated controls: 22.4%; RR = 0.17, $P = 0.02$), in patients without cirrhosis (NAs: 1.8% *vs* untreated controls: 8%; RR = 0.21, $P < 0.001$) and even to patients who developed viral resistance (NAs: 3.3% *vs* untreated controls: 6.4%; RR = 0.52, $P = 0.04$). In addition, significantly lower HCC rates reported in treated than untreated HBeAg positive patients (1.7% *vs* 7.9%, $P < 0.001$), while there was only a numerical trend for reduced HCC rates in treated compared to untreated HBeAg negative patients (3% *vs* 10.5%, $P = 0.06$).

Papatheodoridis *et al*[38] performed another systematic review including randomized or observational cohort studies of adult patients with chronic hepatitis B and/or cirrhosis who received treatment with lamivudine and/or perhaps adefovir for a mean/median duration of ≥ 24 mo (Table 1). Twenty-one relevant studies (16 with NAs naïve patients-5 with lamivudine resistant patients) were identified including 3881 CHB patients (33% cirrhotics, 49% HBeAg positive). In the analysis of the 3 studies including both treated and untreated patients[37,39,40], HCC was detected significantly more frequently in untreated controls (34/534 or 6.4%) than in all treated patients (22/779 or 2.8%, $P = 0.003$) or in treated patients remaining in virological remission (9/353 or 2.5%, $P = 0.015$) or in treated patients with virological breakthroughs or no response (13/426 or 3%, $P = 0.016$). In the 16 studies including NAs naïve patients, the incidence of HCC was found to be higher in patients with than without cirrhosis (10.8% *vs* 0.5%, $P < 0.001$) and in patients with virological non-response or breakthroughs than in patients remaining in virological remission (7.5% *vs* 2.3%, $P < 0.001$). A higher incidence of HCC was also reported in studies with than those without regular HCC surveillance (6.6% *vs* 2.3%, $P < 0.001$), in studies including patients with a mean/median age ≥ 50 than < 50 years (6% *vs* 2.8%, $P < 0.001$) and in studies with predominantly (> 85%) HBeAg negative than predominantly HBeAg positive patients (5.5% *vs* 0.5%, $P < 0.001$).

In the 5 studies including patients with lamivudine resistance[38], HCC developed exclusively in cirrhotics (17.6% *vs* 0%, $P < 0.001$) and more frequently in patients with persistent viremia than in those who achieved virological remission (20.2% *vs* 5.9% $P < 0.001$). However, the induction of virological remission after rescue therapy was not found to be associated with a decreased HCC risk after the exclusion of 13 patients who had already developed HCC at the onset of the adefovir rescue therapy (5.9% *vs* 8.8%, $P = 0.466$). The cumulative HCC rate was significantly higher in patients with lamivudine resistance than in naïve patients regardless of liver disease

severity (7.1% *vs* 3.8%, *P* = 0.001) or among cirrhotics (17.6% *vs* 10.8%, *P* = 0.015).

In a more recent large Greek cohort study published after the latter meta-analysis, 818 HBeAg negative chronic hepatitis B patients with or without cirrhosis starting with lamivudine monotherapy were included[41]. During a median follow-up of 4.7 years, the HCC incidence was again higher in older patients and those with cirrhosis at baseline, but virological on-therapy remission was not found to decrease the incidence of HCC in all patients (*P* = 0.322) or in patients with cirrhosis (*P* = 0.327), while there was a trend for lower incidence in non-cirrhotic patients with than without maintained on-therapy remission (*P* = 0.076). In contrast, in another recent Japanese cohort study, maintenance of virological remission under lamivudine was reported to achieve significant reduction in the HCC incidence[42]. These seemingly conflicting results may be due to differences in patient characteristics (Caucasian or Asian patients, predominance of HBeAg negative or HBeAg positive patients, older or younger ages) as well as due to differences in the management of lamivudine resistance (prompt or no rescue therapy).

Despite the limitations of most cohort studies including heterogeneous patient populations, variations in treatment regimens and patient monitoring, differences in the definitions of response, wide range in the sensitivity of HBV DNA assays and different durations of follow-up, it is now widely accepted that even the administration of lamivudine, a low genetic barrier NA, significantly reduces the risk of HCC particularly in patients with cirrhosis and in those who achieve maintained virological remission. However, the risk of HCC remains high in patients with cirrhosis even if they achieve virological remission, particularly at older ages[2,4,38]. In addition, development of lamivudine resistance appears to be associated with an increased risk of HCC, which may not be reduced by an effective rescue therapy. The latter data in combination with the very high and progressively increasing rates of lamivudine resistance further discourage the use of lamivudine as first-line option for the treatment of chronic hepatitis B[7,29,30].

HIGH-GENETIC BARRIER NAS

There are only a few recent retrospective or prospective observational cohort studies that provide HCC data for patients treated with the high-genetic barrier NAs. Most of the available studies include patients treated with entecavir and only one patients treated with tenofovir that has been available in chronic hepatitis B for a shorter period.

In a retrospective study from Japan, Hosaka *et al*[43] compared the incidence of HCC in entecavir treated patients with a historical cohort of untreated HBV patients. They used a propensity score matching to eliminate the baseline differences resulting in a sample size of 316 patients per cohort (27% cirrhotics). The cumulative HCC incidence at 5 years was significantly lower in the entecavir treated patients than in untreated controls (3.7% *vs*

13.7%, *P* < 0.001). Cox regression analysis showed that entecavir reduced the HCC risk by 63% (HR = 0.37; 95%CI: 0.15-0.91). However, the benefit of entecavir in the reduction of cumulative HCC risk was significant only in cirrhotics (7% *vs* 39%, *P* < 0.001) but not in non-cirrhotics (2.5 *vs* 3.6%, *P* = 0.440).

The favorable effect of treatment with the high-genetic barrier NAs on the risk of HCC was also confirmed in other studies. Wong *et al*[44] performed a retrospective-prospective cohort study including 1446 NAs naïve or NAs experienced (28%) patients treated with entecavir and 424 historical untreated controls. Overall, there was no significant difference in the HCC rates between the entecavir treated patients and untreated controls. However, among patients with cirrhosis, entecavir significantly reduced the incidence of HCC compared to untreated cirrhotics (13.8% *vs* 26.4%, *P* = 0.049), while no difference was found in non-cirrhotics (3.3% *vs* 3.0%, *P* = non-significant).

In another study, Kim *et al*[45] used a prediction model to compare the incidence of HCC in 641 patients treated for 6 years with tenofovir in the tenofovir long-term registration trial with the predicted HCC rate estimated by the REACH-B risk calculator. The authors found that tenofovir reduced the HCC incidence compared to the predicted HCC risk. Specifically, there was a progressive divergence between the predicted and observed number of HCC cases after 3.3 years of follow-up with a standardized incidence ratio of 0.55 (95%CI: 0.32-0.94) at the latest follow-up (median: 5.52 years).

All the data summarized above show that treatment with a high-genetic barriers NA reduces the risk of HCC compared to no treatment with a more profound effect in cirrhotics. The lower benefit on the HCC risk in non-cirrhotic patients seems to be reasonably related to the low baseline HCC risk in this sub-group of patients. Therefore, great numbers of patients and long follow-up periods are required to provide the studies including non-cirrhotic patients with the appropriate power in order to detect a potential benefit on the HCC incidence from these agents.

The effect of entecavir on the risk of HCC has also been compared to the effect of lamivudine in some studies. In the study from Japan by Hosaka *et al*[43], the HCC incidence in the entecavir treated patients was compared to that in a historical cohort of 182 patients treated with lamivudine monotherapy without any rescue therapy in case of resistance. The reduction in the HCC incidence was greater in the entecavir treated than in non-rescued lamivudine treated cirrhotic patients (7% *vs* 22%, *P* = 0.043) but such an effect was not seen in non-cirrhotics (2.5% *vs* 4.9%, *P* > 0.05). On the contrary, an advantage of entecavir over lamivudine in the reduction of HCC risk was not confirmed in other studies. In a prospective study from Japan as well, Kobashi *et al*[46] assessed the incidence of HCC in 129 naïve patients (22% cirrhotics) treated with entecavir and 127 patients (27% cirrhotics) treated with lamivudine. After a mean follow-up of 4.25

years, HCC developed in 35 patients (11 on entecavir and 24 on lamivudine) with the 5-year cumulative HCC incidence being similar (12.4%) in the two groups (P = 0.680). Lamivudine resistance was developed in 60 (47%) of the 127 lamivudine treated patients and was associated with a significantly increased risk of HCC compared to patients without lamivudine resistance (P = 0.035). In a large nationwide prospective cohort study from Greece, Papatheodoridis *et al*[47] estimated the incidence of HCC in 321 HBeAg negative chronic hepatitis B patients (25% cirrhotics) treated with entecavir (86% naïve, 14% experienced) and compared it with the HCC incidence in a historical cohort of 818 patients treated with lamivudine and perhaps adefovir upon lamivudine resistance (26% cirrhotics). After a mean follow-up of 30 mo, 1.2% (4/321) of entecavir treated patients developed HCC with a trend for lower 5-year cumulative HCC incidence in the entecavir compared to the lamivudine group (4.8% *vs* 5.6%, P = 0.096). In the multivariate analysis, however, the HCC risk was independently associated with older age, male gender and cirrhosis but not with type of initial therapy. Finally, in a relatively small study from Turkey, Köklü *et al*[48] retrospectively analyzed the data from 227 patients (86% naïve, 14% experienced) with HBV cirrhosis (46% decompensated) who were treated with tenofovir (n = 72, 36% decompensated), entecavir (n = 77, 47% decompensated) or lamivudine (n = 74, 54% decompensated). The incidence of HCC was not statistically different between patients treated with newer antivirals (entecavir/tenofovir: 4% after 2 years of follow-up) and those treated with lamivudine (9% after 3 years of follow-up).

Given that the newer high-genetic barrier NAs achieve more potent and durable suppression of HBV replication and that lamivudine resistance has been associated with an increased risk of HCC, one would expect an advantage over lamivudine in the prevention of HCC development. However, the data from the currently available studies are limited and the findings appear to be inconsistent. Only one study reported a significant benefit in the reduction of the HCC incidence from entecavir over lamivudine without any rescue therapy upon resistance[43]. In contrast, three other studies and a recent meta-analysis reported no difference in the HCC rates between entecavir and lamivudine treated patients (Table 1)[46-49]. All these findings should be seen with caution, as they come from studies with low statistical power or different strategies for the management of lamivudine resistance (no rescue therapy, perhaps delayed rescue therapy, prompt onset of rescue therapy) that may be critical for the HCC risk. Moreover, these comparisons have limited practical value, as a high-genetic barrier NA should be used in any chronic HBV patient anyway because of their high potency and negligible risk of long-term resistance[7,28].

Other studies usually including NAs naïve and NAs experienced patients assessed the impact of entecavir on HCC development according to the induction of virological remission. Yang *et al*[50] investigated the risk of

HCC in 487 chronic hepatitis B patients (34% NAs experienced, 40% cirrhotics) treated with entecavir for ≥ 12 mo. HCC developed in 36 patients (7.4%). The risk of HCC was lower in patients with than without virological remission in both cirrhotics (HR = 0.21, 95%CI: 0.07-0.60) and non-cirrhotics (HR = 0.08, 95%CI: 0.01-0.50). In a multicenter European cohort (VIRGIL) study[51] including 372 entecavir-treated patients (26% cirrhotics, 63% NAs experienced), virological remission reduced the probability of a clinical event (HCC, hepatic decompensation or death) by 71% (HR = 0.29, 95%CI: 0.08-1.00, P = 0.05). The benefit of virological remission was significant only in patients with cirrhosis (HR = 0.22, 95%CI: 0.05-0.99, P = 0.04). Lastly, Kim *et al*[52] assessed the risk for development of HCC in 324 entecavir treated patients with HBV cirrhosis (32% decompensated). The 5-year cumulative incidence of HCC was 28.5% and patients with virological remission had significantly lower probability for development of HCC (RR = 0.056, P < 0.001).

There is a considerable amount of evidence that suppression of viral replication improves the outcome of chronic hepatitis B patients[7,29,30]. Since the risk of HCC is related to the viral load, reduction of viral load with therapy should presumably reduce the incidence of HCC[10]. This hypothesis is further supported by the results of the above single-arm studies in which long-term virological remission under entecavir was associated with a significant decrease in the incidence of HCC[50-52]. Again, the benefit on the reduction of the HCC incidence was more obvious in patients with cirrhosis who are at a high HCC risk if they remain untreated.

CONCLUSION

It is currently clear that antiviral therapy reduces but does not eliminate the risk of HCC in chronic hepatitis B patients with or without cirrhosis. Based on the standard IFN-α data, the currently used PEGylated IFN-α is also expected to reduce the incidence of HCC. Patients without a sustained off-treatment response after (PEGylated) IFN-α therapy should be treated with a NA, which represents the treatment option for the majority of chronic hepatitis B patients for several reasons[7,28]. Many data have shown that even treatment with lamivudine reduces the incidence of HCC, which may increase again in cases with untreated lamivudine resistance. Emerging data with the currently first-line NAs, entecavir and tenofovir, suggest that the risk of HCC is also reduced under long-term therapies with these agents. The treatment benefit in the reduction of the HCC incidence is always greater in patients with high baseline HCC risk, particularly those with cirrhosis. In addition, the reduction of the HCC incidence under a high genetic barrier NA is higher in the vast majority of patients who will achieve virological remission compared to those who may maintain detectable viral replication. Whether therapy with a high-genetic barrier NA offers an additional benefit on the reduction

of the HCC incidence compared to other NAs with low-moderate genetic barriers remains unclear, but it has no particular clinical interest, as monotherapy with entecavir and tenofovir represent the first-line NA choice for chronic hepatitis B patients anyway due to superiority of these agents in potency and resistance profile[7,28,32-36].

Since the risk of HCC is not eliminated even in patients who remain in virological remission under a high-genetic barrier NA, it has been suggested that HBV DNA might have already been integrated into the host genome before the onset of treatment resulting in genomic alterations and/or chromosomal instability[53]. Thus, the oncogenic process may have started before therapy and the liver may contain clones of cells carrying genetic abnormalities that predispose to cancer[54]. Given that the duration of most studies with the high-genetic barrier NAs does not exceed 4-6 years, it remains to be seen whether the HCC incidence will remain stable over time after 5-6 years of NA therapy. In current clinical practice, however, patients at increased baseline HCC risk should continue to undergo HCC surveillance according to the existing recommendations even if they have achieved complete long-term inhibition of viral replication and improvements in liver histology.

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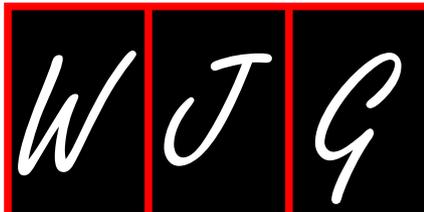
WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Hierarchical and selective roles of galectins in hepatocarcinogenesis, liver fibrosis and inflammation of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) represents a global health problem. Infections with hepatitis B or C virus, non-alcoholic steatohepatitis disease, alcohol abuse, or dietary exposure to aflatoxin are the major risk factors to the development of this tumor. Regardless of the carcinogenic insult, HCC usually develops in a context of cirrhosis due to chronic inflammation and advanced fibrosis. Galectins are a family of evolutionarily-conserved proteins defined by at least one carbohydrate recognition domain with affinity for β-galactosides and conserved sequence motifs. Here, we summarize the current literature implicating galectins in the pathogenesis of HCC. Expression of "proto-type" galectin-1, "chimera-type" galectin-3 and "tandem repeat-type" galectin-4 is up-regulated in HCC cells compared to their normal counterparts. On the other hand, the "tandem-repeat-type" lectins galectin-8 and galectin-9 are down-regulated in tumor hepatocytes. The abnormal expression of these galectins correlates with tumor growth, HCC cell migration and invasion, tumor aggressiveness, metastasis, postoperative recurrence and poor prognosis. Moreover, these galectins have important roles in other pathological conditions of the liver, where chronic inflammation and/or fibrosis take place. Galectin-based therapies have been proposed to attenuate liver pathologies. Further functional studies are required to delineate the precise molecular mechanisms through which galectins contribute to HCC.

Core tip: Galectins, a family of glycan-binding proteins, are involved in the pathogenesis of hepatocellular carcinoma (HCC). Up-regulation of galectin-1, galectin-3 and galectin-4 is observed in HCC cells, whereas galectin-8 and galectin-9 appear to be down-regulated in tumor hepatocytes. This altered expression correlates with tumor growth, HCC cell migration and invasion, tumor aggressiveness, metastasis, postoperative recurrence and poor prognosis. These galectins are also implicated in inflammation- and fibrosis-related liver pathologies.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents a global health problem. It is the fifth most common solid tumor and the third cause of cancer-related mortality per year[1]. HCC is most prevalent in Eastern Asia and sub-Saharan Africa; whereas the incidence in Europe and North America is considerably lower[2-4]. The etiology of HCC includes major risk factors such as infection with Hepatitis B or C virus (HBV, HCV), alcohol abuse or dietary exposure to aflatoxin[5-7]. Regardless of the carcinogenic insult, HCC usually develops in patients with cirrhosis due to chronic inflammation and advanced fibrosis[8]. Non-alcoholic steatohepatitis (NASH), a metabolic disorder resulting from insulin resistance syndrome that underlies fibrosis and cirrhosis, is emerging as another important risk factor for HCC[9,10].

During the past decade the management of HCC has significantly improved[11]. New advances in the field have led to a better knowledge and an earlier detection of this disease. Additionally, current therapies such as, resection, transplantation, ablation and chemoembolization, have provided benefit to patients diagnosed at early HCC stages improving and extending their survival[12-14]. However, most patients are diagnosed at advanced stages and therefore, they are not amenable to surgical treatment. Even after resection or transplantation, the prognosis remains unsatisfactory due to recurrence, metastasis and the development of new primary tumors[15-17].

Recent progress toward a better understanding of the molecular biology of HCC has allowed the development of molecular targeted therapies and has shed light on new systemic therapies for HCC. Several intracellular signaling pathways involved in abnormal proliferation, survival, differentiation, invasion and metastasis have been found to be dysregulated in HCC. Clinical trials are currently testing the potential use of inhibitors of the Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK), phosphatase and tensin homolog deleted on chromosome 10/phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin, transforming growth factor β (TGF-β), Wnt/β-catenin and epidermal growth factor receptor (EGFR) pathways, among others[18-20]. Sorafenib, a receptor tyrosine kinase inhibitor targeting vascular endothelial growth factor, platelet-derived growth factor and Raf signaling pathways prolongs survival in patients with advanced unresectable HCC[21,22]. Simultaneously, new immunotherapy strategies are being developed for the treatment of HCC, which could be administered in combination with conventional therapies in order to obtain a more favorable clinical

outcome[23]. Undoubtedly, the approval of oral administration of sorafenib highlights the the importance of elucidating the molecular mechanisms underlying HCC progression for the development of novel therapies.

Recently, there has been increasing evidence highlighting the involvement of galectins, a family of glycan-binding proteins, in the pathogenesis of HCC. In this review, we present emerging data showing that expression of some members of this family is altered in HCC cell lines and tissues compared to normal liver. These observations led to the proposition that galectins are potential prognostic biomarkers and therapeutic targets in HCC. We will discuss the possible roles of these proteins in HCC tumor transformation, progression, aggressiveness and metastasis. Moreover, we will highlight the involvement of galectins in other pathological settings of the liver, where chronic inflammation and/or fibrosis take place.

GALECTINS

Galectins are a family of evolutionary conserved glycan-binding proteins or lectins that recognize multiple *N*-acetyllactosamine (Galβ1,4GlcNAc) units on cell surface glycoconjugates. These animal proteins are defined by at least one carbohydrate recognition domain (CRD) with affinity for β-galactosides and conserved sequence motifs[24]. To date, fifteen galectins have been described in mammals and according to their structural characteristics they are classified into three groups: "proto-type" galectins (galectin-1, galectin-2, galectin-5, galectin-7, galectin-10, galectin-11, galectin-13, galectin-14 and galectin-15) contain one CRD and can dimerize; "tandem repeat-type" galectins (galectin-4, galectin-6, galectin-8, galectin-9 and galectin-12) contain two distinct CRD in tandem, connected by a linker peptide; and "chimera-type" galectin-3 which consists of unusual proline- and glycine-rich short stretches fused onto the CRD[25,26].

Some galectins (*e.g.*, galectin-1, galectin-3 and galectin-9) are widely expressed among different tissues including, immune cells, endothelial and epithelial cells, and sensory neurons (reviewed by[27-29]); whereas other family members have a more restricted tissue localization and compartmentalization (*e.g.*, galectin-7 is preferentially found in the skin, galectin-12 is abundantly expressed in adipose tissue, galectin-5 is restricted to rat reticulocytes, and galectin-10 is strongly represented in human but not mouse eosinophils)[27].

These lectins do not possess a signal peptide for export through the classical secretory pathway (Golgi-endoplasmic reticulum); however they are secreted to the extracellular milieu *via* a non-conventional poorly understood secretory pathway[30-32]. For instance, non-classical secretion of galectin-1 has been observed in skeletal muscle during *in vivo* development and in cultured myoblasts during differentiation[33]. Besides, secretion of galectin-3 from macrophages, renal and polarized intestinal epithelial cells has been detected[34,35]. There is also

evidence for secretion of galectin-9 in activated Jurkat T cells[36] and CD4 T cells expressing galectin-9 on the cell surface upon T cell receptor stimulation[37].

Through its binding to *N*-acetyllactosamine sequences, galectins form multivalent complexes with cell surface glycoconjugates and thus, transmit signals inside the cell[38-40]. Remarkably, it has also been demonstrated that galectin-1 can be internalized by Jurkat T cells in a carbohydrate-dependent mechanism, following dual pathways involving clathrin-coated vesicles and raft-dependent endocytosis[41]. Within the intracellular milleu, galectins bind to their ligands preferentially through protein-protein interactions, and regulate intracellular processes, including mRNA splicing, cell cycle progression, apoptosis, and cell proliferation[42].

Galectins have emerged as pivotal regulators of cellular physiology. Over the past decade, multiple biological functions have been reported for this protein family including roles in cell adhesion, migration, cytokine synthesis, and survival[43,44]. In fact, different members of the family have shown critical roles as mediators of acute and chronic inflammation[45,46]. Galectins are often aberrantly expressed in many different tumor types including astrocytoma, melanoma and prostate, thyroid, colon, head and neck, bladder, kidney, stomach, lung, bladder, uterine, breast and ovary carcinomas[27,47,48]. Moreover, mounting evidence indicates that these proteins play fundamental roles in cancer biology including tumor transformation, tumor growth, angiogenesis, migration, metastasis and tumor-immune escape[49-52]. Given these pleiotropic activities in the tumor microenvironment, galectins are being increasingly recognized as molecular targets for innovative cancer therapy[26,52-56].

In this review, we summarize the current data implicating galectins in HCC. Particularly, we focus our discussion on selected members of the family, including galectin-1, galectin-3, galectin-4, galectin-8 and galectin-9, which roles in HCC biology have been demonstrated.

GALECTIN-1

The first protein discovered within the galectin family was galectin-1. This galectin possesses one CRD and can form homodimers *via* non-covalent binding, which confers the ability to cross-link specific glycoconjugates[26,28]. Galectin-1 displays features of typical cytoplasmic proteins; it has been described in nucleus and cytoplasm and can translocate to the intracellular face of cellular membranes. Although galectin-1 lacks a recognizable secretion signal sequence, it is secreted through a non-conventional secretory pathway[31,32]; thus being detected on the extracellular side of cellular membranes as well as in the extracellular matrices (ECM) of various normal and neoplastic tissues[57].

While the role of galectin-1 within the intracellular milieu is often independent of its lectin activity, its extracellular functions are mostly dependent on the binding to *N*-acetyllactosamine units on cell surface glycoconjugates[28]. Intracellularly, galectin-1 is engaged in funda-

mental processes such as pre-mRNA splicing; and also it interacts with oncogenic H-RAS and contributes to its membrane anchorage, evidencing a key role for this galectin in driving tumor transformation (reviewed by[49,58]). In the extracellular space, galectin-1 binds to glycoconjugates on the cell surface, including different members of the integrin family and glycoproteins of the ECM such as laminin and fibronectin[59,60]. It is likely that the local abundance of galectin-1 in the tumor microenvironment may play a critical role during attachment or detachment of cancer cells throughout cancer progression[43]. Furthermore, galectin-1 promotes cell migration, a function that correlates with the ability of this protein to influence tumor progression, invasion and angiogenesis. However, the biological roles of galectin-1 appear to be tissue-specific as it also decreases cell migration of most immune cells providing a rational basis for its anti-inflammatory properties[43,45,55,61].

Expression of galectin-1 has been well documented in many different tumor types including astrocytoma, melanoma and prostate, thyroid, colon, bladder and ovary carcinomas[57,62]. Moreover, preferential accumulation of galectin-1 in the peritumoral stroma has been described for thyroid, head and neck, colon, ovary and prostate carcinoma[57]. Functions of galectin-1 during tumor progression have been largely documented in the literature. High levels of galectin-1 correlate with aggressiveness of tumors[63-67], and the acquisition of a metastatic phenotype[68-71]. This lectin plays a fundamental role in tumor angiogenesis by modulating endothelial cell biology[72,73] and its expression is induced by hypoxia[74,75]. Importantly, galectin-1 has been proposed to be a major immunosuppressive factor which contributes to tumor immunoevasive programs[76,77]. In fact, galectin-1 expression by tumor cells or by their surrounding stroma can regulate the function, fate and viability of infiltrating tumor-specific T cells[78].

Galectin-1 in HCC and in inflammation-associated liver injury

Galectin-1 gene *(LGALS1)* regulation was extensively studied using the well characterized system hepatoma x fibroblast hybrids. Activation of gene expression was achieved by treatment of galectin-1-non-expressing cells with the DNA demethylating agent azacytidine. The methylation status of the galectin-1 gene promoter was identified as a central mechanism that controls gene expression in normal tissues and also in transformed cells and tumors[27].

While in normal liver galectin-1 is expressed at low constitutive levels, in HCC its expression is dramatically up-regulated[79-82]. Gene expression profiling of normal and HCC human tissues using cDNA microarrays allowed the identification of *LGALS1* as one of the hallmark genes that are over-expressed in HCC, a phenomenon which was further confirmed by RT-PCR[79]. Kondoh *et al*[80] elucidated the molecular mechanism governing *LGALS1* gene expression in liver malignancy. This group investigated the methylation states of the
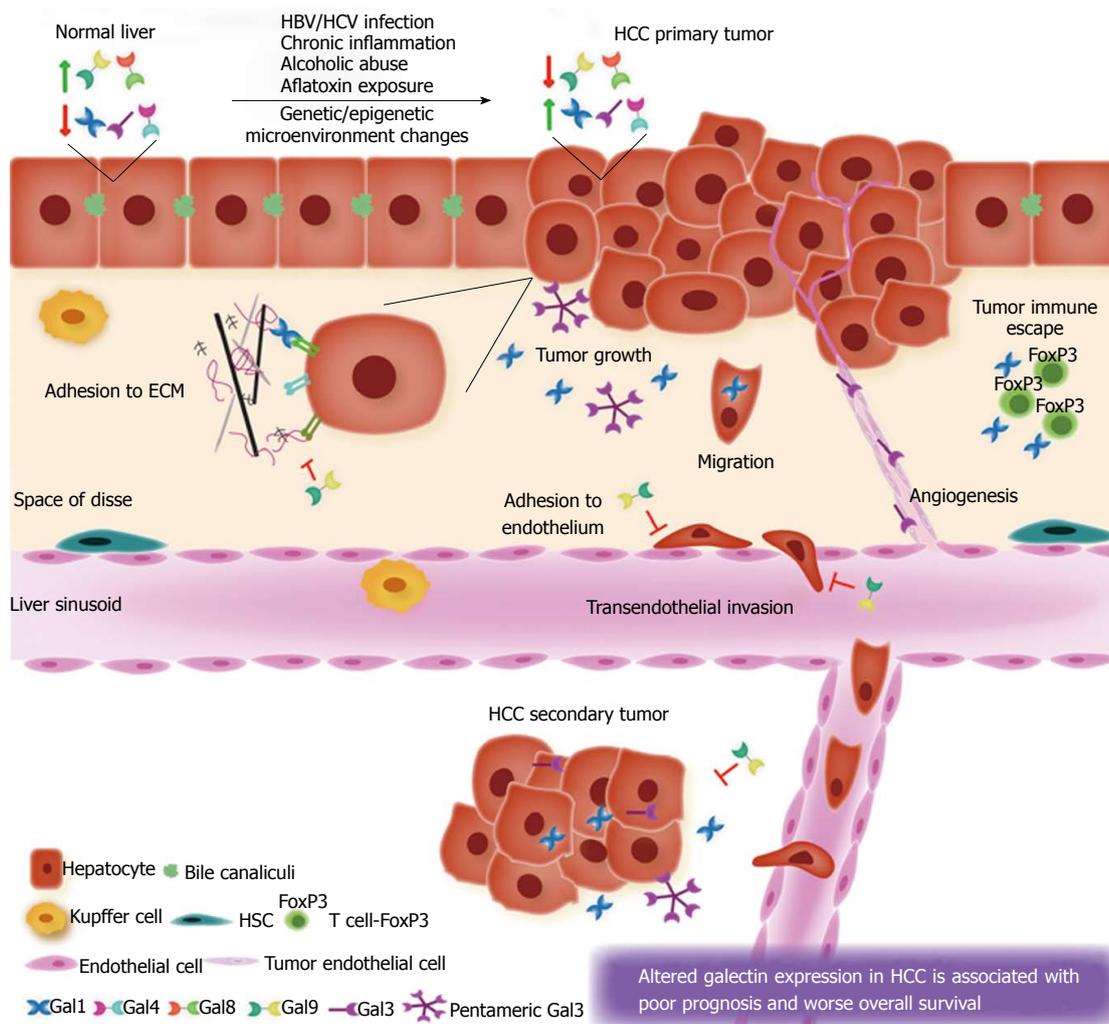
Figure 1 Galectins in hepatocellular carcinoma. In normal liver, galectin (Gal)-8 and galectin-9 are expressed in hepatocytes whereas galectin-1, galectin-3 and galectin-4 are not detectable. This expression pattern is altered in hepatocellular carcinoma (HCC) as galectin-1, galectin-3 and galectin-4 are up-regulated, whereas galectin-8 and galectin-9 are down-regulated in transformed hepatocytes. This aberrant expression favors tumor growth and hepatocyte adhesion to extracellular matrix (ECM), migration, adhesion to the endothelium, transendothelial invasion and metastasis. Galectin-3, normally absent in sinusoid endothelial cells, is up-regulated in tumor capillary endothelial cells, probably promoting angiogenesis. Increased expression of galectin-1 and lack of galectin-9 expression also contribute to tumor-immune escape. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

galectin-1 gene promoter in human HCC and adjacent non-tumor liver tissue, and in different HCC cell lines. Analysis of the methylation profile revealed that certain CpG dinucleotides surrounding the transcription start site of *LGALS1* promoter were frequently methylated in non-tumor liver, whereas these sequences were hypo-methylated in HCC tissues. Interestingly, using a mobility shift assay with nuclear extracts from three HCC cell lines (HLF, HuH7, and HepG2) as well as human embryonic primary liver (PL) cells, the authors showed specific interaction of a methylation-sensitive factor to the upstream and downstream regulatory elements which appear to be essential for the activation of the *LGALS1* gene in HCC cells[80]. Northern blot analysis demonstrated that galectin-1 mRNA was up-regulated in primary HCC in comparison to adjacent non-tumor liver tissues and human normal liver tissues. In fact, galectin-1 mRNA level was higher in the HuH-7 and HLF HCC cell lines as compared to HepG2 and PL cells[80].

Although over-expression of galectin-1 was observed in HCC *in vivo* as well as *in vitro*, the precise function of this endogenous lectin in liver pathophysiology remained uncertain for many years. However, emerging findings shed light to the role to the leading role of galectin-1 in HCC development and progression. Spano *et al*[81] reported that galectin-1 expression was significantly increased in HCC samples from patients with metastatic disease compared to those harboring a non-metastatic primary tumor. However, no significant associations were found with other parameters, although a trend toward an association between increased galectin-1 expression in HCC and vascular invasion was observed. Moreover, galectin-1 expression profile was also examined in human HuH-7 and JHH-6 HCC cells and human normal liver, cirrhotic tissue and HCC specimens using tissue microarrays. In all cases, increased expression of the *LGALS1* gene was confirmed in HCC. Furthermore, immunohistochemical analysis revealed a preferential accumulation of galectin-1

Table 1 Involvement of galectins in the pathogenesis of hepatocellular carcinoma

Galectin member	Expression	Function and/or effect	Model	Ref.
Galectin-1	Up-regulated (mRNA and protein) in HCC, secreted by tumor hepatocytes and accumulated in stroma surrounding HCC	Correlates with tumor aggressiveness, metastases and enhanced risk of post-operative recurrence	Human HCC tissues	[79-82]
		Favors HCC cell adhesion to ECM, cell migration and invasion	Human HCC cell lines	[81,85]
		Increases tumor growth and metastasis in draining-tumor lymph nodes	Nude mice injected with galectin-1 over-expressing HepG2 cells	[85]
		Possible role in the suppression of antitumor immune responses	Human HCC tissues	[82]
Galectin-3	Up-regulated (mRNA and protein) in HCC. Transactivation of murine *LGALS3* promoter can occur by HBV-X protein.		Human HCC tissues and cell lines	[79,124,125]
	High nuclear expression	Correlates with histological differentiation and vascular invasion	Human HCC tissues	[126]
	Up-regulated in HCC-associated capillary endothelial cells	Probably promotes angiogenesis	Tumor-associated endothelial cells isolated from rats	[128]
Galectin-4	Higher expression in HCC than normal tissues		Human HCC tissues and cell lines	[154]
Galectin-8	Diminished expression in hepatoblastoma and hepatocarcinoma		Human HCC tissues	[159]
Galectin-9	Downregulated in HCC	Galectin-9 suppression promotes cell proliferation and adhesion to ECM, tumor cell-endothelial cell adhesion and trans-endothelial invasion of HepG2 cells.	Human cell lines	[181]
		Downregulation of galectin-9 represents a risk factor for patient survival, correlates with tumor histopathological grade, vascular invasion and metastasis	Human HCC tissues	[181]

HCC: Hepatocellular carcinoma; ECM: Extracellular matrix; HBV: Hepatitis B virus.

in the delicate stroma tissue surrounding tumor hepatocytes of HCC tumors. The authors hypothesized that neoplastic hepatocytes secrete galectin-1 which is then accumulated in the stroma surrounding HCC (Figure 1 and Table 1).

The correlation between increased expression of galectin-1 in HCC and the presence of metastasis was validated by *in vitro* functional studies. Expression of *LGALS1* gene and secretion of galectin-1 protein were substantially up-regulated in JHH-6 (undifferentiated cells) and HuH-7 (differentiated cells). Notably, galectin-1 over-expression increased the migratory and invasive capacities of HuH-7 cells, and both processes were mediated by the stimulation of the Sky receptor tyrosine kinase (RTK) phosphorylation. Thus, similar to breast cancer[68], neuroblastoma[83], oral squamous cell carcinoma and lung adenocarcinoma[84], galectin-1 expression correlates with HCC tumor aggressiveness (Figure 1 and Table 1).

Under this scenario, we have focused our attention on the role of galectin-1 and its contribution to HCC development. In this regard, we examined the involvement of this galectin in HepG2 HCC cell adhesion and tumor growth[85]. We found that galectin-1 acts as a glycan-dependent matricellular modulator of HepG2 cell adhesion. We observed that galectin-1 favored cell adhesion to laminin, a polylactosamine-enriched glycoprotein and a major component of the ECM and basement membranes. Moreover, we demonstrated that the pro-adhesive effects of galectin-1 are specifically mediated by

α_1, α_2, α_3, α_v, and β_1 integrins and involve PI3K and/or ERK1/2 signaling pathways. Besides, galectin-1 over-expressing HepG2 cells showed an increased secretion of this lectin to the extracellular compartment and remarkably, we also found that exogenously added recombinant galectin-1 was internalized by HepG2 cells[85]. Hence, in accordance with Spano *et al*[81], galectin-1 secreted from HCC cells might exert its biological functions either by engaging cell surface receptors and transmitting signals inside the cell or through receptor-mediated internalization and endocytosis. However, because intracellular functions have also been described for this protein[42] a cell surface-independent mechanism responsible for galectin-1 functions cannot be excluded. We also found that galectin-1 up-regulation in the tumor microenvironment favored HCC growth *in vivo* and promoted a considerable increase in tumor metastasis. This effect was evident in draining-tumor lymph nodes of mice injected with galectin-1 over-expressing HepG2 cells[85]. Collectively, these results suggested the involvement of galectin-1 in neoplastic and inflammatory processes of the liver (Figure 1 and Table 1).

Compelling evidence indicates that high expression of galectin-1 predicts poor patient outcome in a variety of tumors. However, the prognostic value of this endogenous lectin in HCC patients remained elusive for many years. Recently, Wu *et al*[82] reported that elevated galectin-1 expression in HCC is significantly associated with tumor aggressiveness (vascular invasion, incomplete encapsulation, poor differentiation, and large tumor size) and enhanced

Table 2 Galectins in inflammation-associated liver injury

Galectin member	Experimental model	Role	Effects	Ref.
Galectin-1	Hepatitis induced by injection of Con A	Protective	Prevents both liver injury and T-helper cell liver infiltration, induces apoptosis of Con A-activated T cells, suppresses plasma levels of TNF and IFN-γ	[89]
	Inflammation-induced chronic cholestatic hepatitis at an early age, and HCC at later age (Mdr2-KO mice)	Protective	Galectin-1 is up-regulated in Mdr2-KO/B6 strain at early age	[91]
	Galectin-1-KO mice in the context of Con A-induced autoimmune hepatitis	Protective	Con A up-regulates galectin-1 in galectin-1-KO/B6 and Mdr2-KO/FVB strains. Endogenous galectin-1 selectively protects liver in the B6, but not in the FVB genetic background. It probably determines strain-specific differences in the course of chronic hepatitis and HCC development in the Mdr2-KO model	[91]
Galectin-3	NASH model Galectin-3-KO mice	Protective	Develops NAFLD/NASH spontaneously with aging	[140,141]
	CDAA diet-induced NAFLD/NASH in galectin-3-KO mice	Protective	Galectin-3 deficiency causes more severe hepatic injury and alterations in the expression of genes associated with carcinogenesis and lipid metabolism	[142]
	Atherogenic diet-induced NASH in galectin-3-KO mice	Promotes disease severity	Attenuates NASH: inhibits HSC-driven fibrosis, reduces inflammatory-cell infiltration and hepatocyte apoptosis, acts as a major scavenger receptor involved in ALE/AGE uptake by the liver	[143]
	Human liver tissues	Protective	Negative expression of galectin-3 in normal hepatocytes, strong staining for galectin-3 in hepatocytes from patients with steatosis hepatitis, hepatitis, cholestasis and cirrhosis	[145]
	Acute liver failure induced by APAP-hepatotoxicity in galectin-3-KO mice	Perpetuates liver injury	In wild type mice, galectin-3 is up-regulated in liver infiltrating macrophages. In galectin-3 deficient mice the pro-inflammatory M1-type macrophages subpopulation, the classical macrophage activation markers iNOS, TNF and IL-12 and pro-inflammatory chemokines are reduced	[147,148]
	Hepatitis induced by injection of Con A in galectin-3-KO mice	Pro-inflammatory	Galectin-3 deficiency reduces the number of T lymphocytes, B lymphocytes, dendritic cells, NK and NKT cells and enhances apoptosis of mononuclear cells	[149]
	Con A-induced liver injury in wild type mice pretreated with a selective inhibitor of galectin-3 (TD139)	Pro-inflammatory	TD139 attenuates liver injury, reduces the number of CD4^{+} and CD8^{+} T cells, favors the influx of IL-10-producing CD4^{+} T cells in the liver, decreases serum levels of IFN-γ, IL-17 and IL-4	[149]
Galectin-9	Blockade of the TIM-3/galectin-9 pathway using an anti-TIM-3 or anti-galectin-9 mAb in a context of liver IRI	Protective	Blockade of the TIM-3/galectin-9 pathway increases hepatocellular damage, local neutrophil infiltration, T cell and macrophage accumulation and liver cell apoptosis. Increases IFN-γ production by Con A-stimulated spleen T cells and augmented TNF and IL-6 production by Con A-stimulated macrophages/T cells	[190]
	Single injection of galectin-9 in the murine model of liver injury induced by Con A	Protective	Eliminates activated CD4^{+} effector T cells, prevents the synthesis and/or release of proinflammatory cytokine	[191]
	Mouse model of diet-induced NAFLD treated with galectin-9	Limits the inflammatory response	Induces apoptosis of NKT cells, also interacts with TIM-3-expressing Kupffer cells to induce secretion of IL-15, thus promoting NKT cell proliferation	[195]

ALE/AGE: Advanced lipoxidation and glycation end products; APAP: Acetaminophen; CDAA: Choline-deficient L-amino-acid; Con A: Concanavalin A; HSC: Hepatic stellate cells; IFN-γ: Interferon γ; IL: Interleukin; iNOS: Inducible isoform nitric oxide synthase; IRI: Ischemia and reperfusion injury; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; NK: Natural killer; NKT: NK T cells; TIM-3: T-cell immunoglobulin mucin domain 3; TNF: Tumor necrosis factor; HCC: Hepatocellular carcinoma; KO: Knockout.

risk of post-operative recurrence. Additionally, galectin-1 expression in HCC was also associated with early tumor recurrence (≤ 24 mo) and dissemination of primary tumor cells. Furthermore, a positive correlation was observed between galectin-1 expression and tumor-infiltrating FoxP3^{+} regulatory T cells (Tregs) in HCC samples from a large, random HCC cohort. In line with this evidence, it has been demonstrated that galectin-1 is a key regulator of murine CD4^{+}CD25^{+} regulatory Tregs[86] which play an essential role in suppression of anticancer immunity[87]. Taken this information into account it is possible to speculate that interaction between galectin-1 and Treg cells might play a role in the suppression of antitumor immune responses against HCC (Figure 1 and Table 1).

The immunomodulatory activities of galectin-1 in the liver were also investigated in a model of hepatitis induced by injection of concanavalin A (Con A) into mice, which leads to a dose-dependent injury in the liver[88]. T-cell activation is a crucial event in this model as shown by resistance to this inflammatory disease of mice lacking T and B lymphocytes. Furthermore, pretreatment with anti-interferon γ (IFN-γ) or anti-tumor necrosis factor (TNF) monoclonal antibodies conferred protection against Con A-induced liver injury, indicating that Th1-

Table 3 Galectins in fibrosis-related liver pathologies

Galectin Member	Expression	Function and/or effect	Model	Ref.
Galectin-1	Over-expressed in activated HSCs	Induces proliferation of HSCs *via* ERK 1/2 through CRD domain	HSCs activated *in vitro* (cultured on plastic for several days) and *in vivo* (isolated form rats treated with CCl_4 or with bile duct ligation)	[93,94]
	Positive in ICC cells, intracellular expression and secretion	Correlates with histologic dedifferentiation, vascular invasion, and lymph node metastasis	ICC tissue samples, CCKS1 cholangiocarcinoma cell line	[96]
Galectin-3	Over-expressed in activated HSCs	Induces proliferation *via* ERK 1/2 involving PKA and PKC pathways Dependent on CRD domain	HSCs activated *in vitro* (cultured on plastic for several days) and *in vivo* (isolated form rats treated with bile duct ligation)	[94]
		Intracellular Gal3 is required for activation of HSCs *via* TGF-β	HSCs activated *in vivo* (isolated form rats treated with CCl_4)	[132]
		Extracellular Gal3 required for activation of HSCs. Integrin and CRD dependent effect NFκβ induces expression and secretion of Gal3 in activated HSCs	HSCs activated *in vivo* (isolated from rats with bile duct ligation)	[134]
	Up-regulated in injured/cirrhotic hepatocytes	Poor liver function	Human fibrotic liver samples and extracts from rats treated with CCl_4	[124,133,136]
		Related to the preneoplastic and early neoplastic stages of ICC	ICC tissue samples	[96,137]
	Positive in ICC cells	Intracellular expression is associated with anti-apoptotic activity and resistance to chemotherapeutic agents	ICC cell lines	[138]

HSC: Hepatic stellate cells; CRD: Carbohydrate recognition domain; ERK: Extracellular signal-regulated kinase; ICC: Intrahepatic cholangiocarcinoma; PKA: Protein kinase A; PKC: Protein kinase C.

dependent cytokines are involved in this inflammatory disease. Interestingly, it has been demonstrated that galectin-1 exerts a protective role on Con A-induced autoimmune hepatitis in mice (Table 2)[89].

Recently, the protective role of galectin-1 in the liver inflammatory response was investigated using the Mdr2-knockout (Mdr2-KO) mice as a model of inflammation-induced chronic cholestatic hepatitis at an early age, and HCC at a later age, which together mimic the evolution of human disease[90]. Potikha *et al*[91] demonstrated that HCC development was retarded in Mdr2-KO/B6 strain compared to Mdr2-KO/FVB mice. Interestingly, up-regulation of galectin-1 transcript in the liver of Mdr2-KO/B6 mice was observed[91]. To highlight the relevance of the endogenous protein galectin-1-KO/B6 mice were used in the context of Con A-induced autoimmune hepatitis. The results demonstrated that endogenous galectin-1 selectively protects against Con A-induced liver injury in B6 mice (Table 2)[91].

Collectively, these data indicated that galectin-1 has an important role in HCC tumor growth, aggressiveness and metastasis (Figure 1 and Table 1). Moreover, they suggest that galectin-1 may act as a protective anti-inflammatory agent at early stages of the chronic liver pathology during inflammation-induced hepatocarcinogenesis, but as a pro-tumorigenic agent at late stages of the disease.

Galectin-1 in fibrosis-related liver pathologies

Hepatic fibrosis is the physiological result of the wound-healing response of the liver to repeated injury. This process is associated with an inflammatory response and a limited deposition of ECM. If the hepatic injury persists (*e.g.*, chronic viral hepatitis), and eventually the liver regeneration fails, hepatocytes are substituted with abundant ECM, including fibrillar collagen[92]. Kristense *et al*[93] conducted a proteome analysis on cellular and secreted proteins of normal (quiescent) and activated rat hepatic stellate cells (HSCs), the main ECM-producing liver cells. These researchers found that galectin-1 was up-regulated in both *in vivo* and *in vitro* activated HSCs, and in fibrotic liver tissues[93]. When the biological role of galectin-1 was investigated in HSCs, it was found that this lectin stimulated the proliferation rate and migratory activity of cultured HSCs through carbohydrate-dependent mechanisms (Table 3)[94]. These data clearly indicated that galectin-1 has an important role in the development of liver fibrosis.

By immunohistochemistry, galectin-1 expression was also assessed in the intrahepatic biliary tree. The intrahepatic biliary epithelial cells or cholangiocytes are involved in modifying the bile of canalicular origin. Cholangiocarcinoma occurs frequently associated with inflammation and fibrosis of bile ducts, and is caused by multiple factors including autoimmune, bacterial, congenital, drug, or viral agents[95]. In normal livers, Shimonishi *et al*[96] observed that intrahepatic bile ducts and hepatocytes did not express galectin-1. Remarkably, 73 % of the intrahepatic cholangiocarcinoma (ICC) samples analyzed were positive for galectin-1[96]. Expression of this lectin significantly correlated with histologic dedifferentiation of ICC,

vascular invasion, and lymph node metastasis of ICC[96]. These results suggest that galectin-1 over-expression in ICC cells is associated with neoplastic progression and tumor cell proliferation (Table 3).

These results highlight an important role of galectin-1 in chronically injured liver and its involvement in inflammation and fibrosis of bile ducts, thus providing the basis for the development of effective therapies based on the modulation of galectin-1-glycan interactions.

GALECTIN-3

Galectin-3 is the unique "chimera-type" galectin containing three structurally distinct domains, an atypical N-terminal domain that includes a serine phosphorylation site, important for the regulation of intracellular signaling, a collagen-like sequence sensitive to proteolysis by MMP-2 and MMP-9 matrix metalloproteinases and a C-terminus containing one carbohydrate-recognition domain (CRD) containing an Asp-Trp-Gly-Arg motif. This sequence motif is also present in members of the B-cell lymphoma 2 (Bcl-2) family of apoptosis regulators, and is responsible for the antiapoptotic activity of galectin-3[97]. In solution, galectin-3 largely occurs as a monomer[98]. Although in the absence of its binding partners it can form homodimers by self-association through its CRDs[99], in the presence of carbohydrate ligands, galectin-3 can polymerize up to pentamers through its N-terminal domain[99,100].

Galectin-3 is mainly localized at the cytoplasmic compartment, but it is also present within the nucleus, in the cell surface or in the extracellular space[29,101]. Translocation of this lectin from the cytoplasm to the nucleus is mediated by its N-terminal domain[102], whilst translocation from nucleus to the cytoplasm involves a nuclear export sequence located within its CRD[103] and occurs through nucleoporin NP98[104]. Notably, the N-terminal domain is also required for the secretion of the lectin to the extracellular milieu[105].

Galectin-3 has multiple and complex functions. In the cytoplasm, galectin-3 can bind to Bcl-2 and inhibit cellular apoptosis[97]. Also, it can interact with the activated K-Ras (K-Ras-GTP)[106,107] and affect Ras-mediated Akt signaling[108,109]. On the other hand, nuclear galectin-3 acts as a pre-mRNA splicing factor and is involved in spliceosome assembly[110] by forming protein complexes with Gemin4[111]. In the nucleus, Galectin-3 can also regulate gene transcription by enhancing transcription factor association with Spi1 and CRE elements in gene promoter sequences[29]. In addition, β-catenin, a molecule involved in Wnt signaling pathway, was also identified as a novel binding partner of galectin-3 in the nucleus[112].

On the other hand, extracellular galectin-3 mediates cell adhesion and activation and also acts as a chemoattractant for certain cell types[29]. It often forms multimers and thus, it cross-links cell surface ligands forming lattice-like structures which trigger cell signaling[29]. Galectin-3 has been shown to bind glycosylated components of the extracellular matrix, and cell-surface adhesion molecules like integrins[43]. Pro-apoptotic activity of extracellular ga-

lectin-3 was observed in several cell types, such as human T leukemia cell lines, human peripheral blood mononuclear cells, and activated mouse T cells[113].

Galectin-3 is widely expressed in human tissues, including immune cells, epithelial cells and sensory neurons (reviewed by[29]). This lectin regulates immune cell activities and contributes to immunosuppression as it induces monocyte and T-cell apoptosis, suppresses IL-15 production and inhibits B-cell differentiation[114,115]. In general, galectin-3 is a powerful pro-inflammatory signal as demonstrated by both *in vitro* and *in vivo* assays[29,116]. Extracellular galectin-3 has been demonstrated to activate and modulate the viability of immune and inflammatory cells, although the effects of Galectin-3 in T-cell survival are dependent on whether the protein is produced endogenously (anti-apoptotic) or is secreted to the extracellular medium (pro-apoptotic)[114,116].

Expression of galectin-3 and its intracellular distribution are frequently altered in cancer and pre-cancerous conditions[26], and it is evident that this lectin plays multiple roles in cancer pathogenesis, proliferation and spreading of metastasis[29,62,117]. Pre-clinical and clinical data indicate that expression of galectin-3 is associated with the carcinogenesis and malignant potential in melanoma, head and neck, thyroid, gastric, colon, uterine, and renal cancers[118]. In fact, galectin-3 contributes to tumorigenesis and tumor progression through several different mechanisms, including promotion of oncogenesis, angiogenesis, adhesion, invasion and metastasis[101,115].

The mechanisms of regulation of galectin-3 expression are still poorly understood. The promoter region of the human galectin-3 gene (*LGALS3*) contains several regulatory elements for activation by the SP1, AP-1, CREB, and NF-κB transcription factors[119]. In this regard, c-Jun, CREB, and NF-κB have been implicated in activation of the *LGALS3* gene[29,49]. Galectin-3 expression is also regulated by methylation of CpG islands in the promoter region. It has been demonstrated that demethylation of *LGALS3* promoter induces expression of galectin-3 in thyroid carcinoma[120,121]. Recently, Margadant *et al*[122] demonstrated that, in cells from epithelial origin, integrin β1 specifically triggers transcriptional activation of galectin-3 through a mechanism that involves demethylation of the *LGALS3* promoter. Further, it has been shown that the cell-surface glycoprotein MUC1 controls galectin-3 expression in an epigenetic manner in cancer cells, through a miRNA-dependent mechanism[123].

Galectin-3 in HCC

Hsu and colleagues demonstrated using immunohistochemistry and immunoblot analysis, that normal hepatocytes do not express galectin-3; however this galectin is prominently up-regulated in HCC tissues and in HCC cell lines[124]. Increased expression of galectin-3 in HCC was independent of whether the patients were previously exposed to hepatitis B virus (HBV). However, galectin-3 expression in HCC was positively influenced by HBV infection through a mechanism that included transactivation of the murine *LGALS3* gene promoter[124].

Accordingly, using cDNA microarray and gene expression profiling, Chung *et al*[79] reported the up-regulation of Galectin-3 in HCC human tissues with respect to their normal counterparts. Moreover, by analyzing gene expression patterns, Luo *et al*[125] also reported the over-expression of galectin-3 gene in HCC tissues respect to normal liver and adjacent non-tumoral tissues.

Interestingly, expression of galectin-3 correlated with histological differentiation and vascular invasion in HCC patients[126]. In particular, higher expression rate of nuclear galectin-3 denoted worse prognosis in this pathology and serum galectin-3 levels were found to be increased in HCC patients compared to those suffering chronic liver disease[126]. These results highlighted a central role for galectin-3 in HCC development and progression (Figure 1 and Table 1).

HCC is a hypervascular tumor in which angiogenesis plays a critical role. Tumor-associated capillary endothelial cells (TECs) in HCC are known to originate from liver sinusoid endothelial cells (SECs), which then undergo a capillarization process to become morphologically and functionally different TECs[127]. Using two-dimensional gel electrophoresis coupled to mass spectrometry, Jia *et al*[128] observed that galectin-3 is up-regulated in TECs, respect to SECs. This result validated by immunoblot and immunohistochemistry, demonstrated that galectin-3 is generally absent in liver SECs, but is significantly up-regulated in HCC TECs (Table 1)[128]. Further investigation is required to reveal whether galectin-3 produced in HCC TECs could influence HCC angiogenesis.

EGFR family is an important mediator of cancer cell transformation, proliferation, maintenance, and survival[129]. Paradoxically, high concentrations of epidermal growth factor (EGF) initiates different signaling cascades and mainly induces apoptosis of tumor cells expressing high levels of EGF receptor[130]. Recently, the role of galectin-3 in EGF-induced apoptosis on HepG2 cells was investigated[131]. Indeed, high concentrations of EGF inhibited proliferation and induced apoptosis of these cells, concomitantly with a reduced expression of galectin-3 at both mRNA and protein levels[131]. Also, high levels of EGF down-regulated the expression of cytoplasmic galectin-3. Remarkably, the reduced expression of galectin-3 in EGF-treated cells was associated with reduced phosphorylation of Akt and ERK. Moreover, over-expression of galectin-3 in HepG2 cells blocked EGF-induced growth inhibition and apoptosis[131]. Thus, cellular proliferation and/or apoptosis induced by EGF signaling pathway in HCC cells might rely on the expression levels of galectin-3.

Collectively, these results demonstrate that galectin-3 over-expression correlates with HCC progression (Figure 1 and Table 1) and suggest that this lectin could serve as a novel biomarker and therapeutic target in HCC.

Galectin-3 in fibrosis-related liver pathologies

Expression of galectin-3 is increased in liver fibrosis regardless of the initiating agent or disease process[94,132].

In vitro experiments and different experimental models of liver injury and fibrosis demonstrated that galectin-3 stimulated the proliferation rate of cultured activated HSCs and is also involved in myofibroblast activation, identifying galectin-3 as a potential therapeutic target in the treatment of liver fibrosis (Table 3)[94,132-134].

Liver fibrosis leads to progressive liver insufficiency, portal hypertension and ultimately to cirrhosis and/or HCC[135]. In patients with liver cirrhosis galectin-3 is not extracted by the liver[136], and also, its expression is induced in hepatocytes of cirrhotic liver[124,136]. Furthermore, galectin-3 was negatively associated with liver function in patients with alcoholic liver cirrhosis, an effect which might be partly explained by the impaired hepatic removal and/or by higher hepatic synthesis of galectin-3 (Table 3)[136].

As mentioned before, cholangiocarcinoma frequently occurs in a context of inflammation and fibrosis of bile ducts. Shimonishi *et al*[96] examined galectin-3 expression pattern in intrahepatic cholangiocarcinoma (ICC), and found that 93% of the ICC samples analyzed were positive for this lectin. The expression was more intense in well-differentiated ICC, and was significantly decreased in dedifferentiated areas or poorly differentiated ICCs, indicating that galectin-3 expression is rather related to the preneoplastic and early neoplastic stages of ICC, and tends to disappear at later stages of ICC (Table 3)[96,137]. Also, it has been demonstrated that galectin-3 played a role in apoptosis and response to chemotherapy in cholangiocarcinoma cell lines (Table 3)[138]. These results highlight the possibility of targeting galectin-3 as an alternative therapeutic approach in cholangiocarcinoma.

Galectin-3 in inflammation-associated liver injury

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as a condition in which excess fat accumulates in hepatocytes. NASH, a severe form of NAFLD in which inflammation and fibrosis of the liver take place, may eventually progress to end-stage liver disease and ultimately, to HCC[139]. Controversial results have been published on the effect of galectin-3 deficiency in models of hepatic steatosis/inflammation, with studies indicating either protection or increased disease severity in galectin-3 knock-out (KO) mice (Table 2)[140-143]. On one hand, it has been demonstrated that in choline-deficient L-amino-acid (CDAA) diet-induced NAFLD/NASH hepatic injury was more severe in galectin-3 KO mice, as compared to wild type mice[142].

On the other hand, Iacobini *et al*[143] reported a complete prevention or marked attenuation of NASH induced by an atherogenic diet in galectin-3 KO mice. In these animals, the earlier steps of NASH, *e.g.*, steatosis, hepatocyte injury, and inflammation, were dramatically influenced[143]. Further research is needed to elucidate the protective or promoting roles of galectin-3 in liver steatosis and inflammation.

Excess fatty acid oxidation and generation of reactive carbonyls with formation of advanced lipoxidation and

glycation end products (ALEs and AGEs, respectively) are involved in NASH. Several AGE-binding proteins have been identified including galectin-3, which has been widely recognized as an AGE receptor (AGE-R3)[144]. Butscheid *et al*[145] explored the expression of galectin-3 and RAGE, a member of the immunoglobulin superfamily which also serves as a receptor for AGEs, in specific cell types and histological structures of human liver biopsy specimens from patients with varying degrees of hepatic impairment (steatosis hepatitis, hepatitis, cholestasis and cirrhosis). They observed that when liver function is impaired and AGE levels rise, overexpression of galectin-3 appears to contribute to tissue protection (Table 2)[145].

Acetaminophen (APAP)-induced hepatotoxicity is a major cause of acute liver failure[146]. Evidence suggests that activated macrophages contribute to the pathogenic response to APAP and, two major phenotypically distinct subpopulations have been identified: classically activated (M1-type) macrophages which show pro-inflammatory function and alternatively activated (M2) macrophages which often display anti-inflammatory wound repair activities[147]. It appears that the outcome of tissue injury depends on which macrophage subpopulation predominates. In wild type mice, galectin-3 is markedly up-regulated in macrophages infiltrating the liver 48-72 h after APAP administration[147]. Interestingly, loss of galectin-3 resulted in reduced hepatotoxicity and decreased expression of proinflammatory mediators[148]. Taken together, the data suggest that galectin-3 plays a key role in promoting late pro-inflammatory responses, classical macrophage activation and perpetuating injury in the liver following APAP intoxication (Table 2).

Supporting these findings, Volarevic *et al*[149] showed that galectin-3 deficiency leads to a marked attenuation of Con A-induced hepatitis. This effect was associated with a decreased number of effector cells in the liver. Moreover, pretreatment of wild type mice with a selective inhibitor of galectin-3 (TD139) attenuated Con A-induced liver injury and reduced the number of CD4+ and CD8+ T cells (Table 2)[149]. Hence, galectin-3 plays an important pro-inflammatory role in Con-A-induced hepatitis and may function as a potential target for therapeutic intervention in acute liver diseases.

GALECTIN-4

Galectin-4 is a "tandem-repeat" galectin, which possesses two CRDs and is primarily expressed in epithelial cells along the gastrointestinal tract[150]. Recently, this lectin has been reported as a major component of lipid rafts in brush border membranes of small intestinal epithelial cells[151]. In a human colon adenocarcinoma cell line, galectin-4 has been proposed to play an important role in the apical delivery of proteins[152].

Galectin-4 expression is altered in human malignancies[46,62,150]. Although controversial data has been published, it is apparent that galectin-4 is significantly down-regulated in colon adenocarcinoma. In fact, it has

been recently demonstrated that galectin-4 functions as a tumor suppressor in this type of malignancy[153]. In contrast, galectin-4 expression is higher in HCC[154] and gastric cancer cell[155], as compared to their corresponding normal tissues, suggesting a context-dependent role of galectin-4 in tumor development and progression. Kondoh *et al*[154] identified several cDNAs that were differentially expressed in surgically resected human HCC as compared to non-tumor liver and normal liver tissues[154]. Non-tumor liver tissues were obtained from patients that suffered cirrhosis associated with HCV infection and, from patients suffering liver cirrhosis but in the absence of HCV or HBV infection. Normal liver tissues that were used as controls were obtained from patients who died of pancreatic carcinoma and subarachnoid bleeding. Interestingly, one of the genes differentially expressed was the galectin-4 gene (*LGALS4*). Northern blot analysis revealed that galectin-4 mRNA was more abundant in HCCs than in adjacent non-tumor liver tissues or normal liver tissues from non-HCC patients[154]. When HCC cell lines were analyzed (HuH-7 and HepG2 cells), the levels of galectin-4 mRNA were undetectable or low in rapidly growing cells. However, the levels of this lectin increased considerably in HuH-7 cells growing at a higher cell density, although the expression of galectin-4 did not increase in HepG2 cells. Furthermore, the expression of galectin-4 mRNA was also induced in HuH-7 cells cultured with low concentration serum (0.1%)[154]. Thus, although the precise roles of galectin-4 in HCC remained to be elucidated, these results show a possible association between galectin-4 expression and liver malignancy. Functional studies will provide insight to further understand the role of galectin-4 in HCC biology.

GALECTIN-8

Galectin-8 is another member of the "tandem-repeat"-type family of galectins, which possesses two CRDs and thus, behaves as a bivalent molecule. The galectin-8 gene (*LGALS8*) encodes numerous mRNAs (most likely seven) generated through alternative splicing, mostly in intron VIII[156]. Because the N-terminal domain of galectin-8 intrinsically dimerizes[157], cleavage of the linker region between galectin-8N and galectin-8C may allow the possibility to dissect potential signaling pathways initiated by each separate domain[46].

This lectin has been initially cloned from a rat liver cDNA library[158]. Using Northern analysis it was established that galectin-8 mRNA is highly expressed in lungs and, to a lesser extent in the liver, kidneys, spleen, hindlimb and cardiac muscles in the rat[158]. The role of galectin-8 has been mostly investigated in relation to tumor malignancy[62,156] in a variety of different tumors from different origin[62,159]. Immunohistochemical studies revealed that galectin-8 expression is increased in cancerous versus normal tissues in the lung, bladder, kidney, prostate and stomach. However, in the liver and also in large intestine, pancreas, larynx and skin, immunohistochemical analysis revealed decreased expression of this lectin in cancerous

versus normal tissues, suggesting tissue-specific regulation of galectin-8 expression in cancer[159]. In normal and cirrhotic livers, the staining intensities of galectin-8-positive cells appeared to be moderate to strong. On the contrary, in hepatoblastomas and hepatocarcinomas the staining intensity of positive cells was weak to moderate. Collectively, these experiments revealed tissue-specific regulation of galectin-8 expression upon malignant transformation of various tissue types of epithelial origin. Further investigation is necessary to further delineate the functional roles of galectin-8 in liver carcinogenesis and to determine if galectin-8 downregulation is associated with poor prognosis of HCC.

GALECTIN-9

Galectin-9 is a "tandem-repeat" galectin originally isolated from mouse embryonic kidney cells[160]. Galectin-9 consists of two different CRDs joined by a flexible peptide linker, with 39% amino acid sequence homology. The C-terminal CRD and the N-terminal CRD share high affinity for both branched N-glycans and repeated oligo-lactosamines. Further, the N-CRD exhibits striking affinity for the Forssman pentasaccharide and polymerized *N*-acetyllactosamine[161,162]. Alternative splicing leads to the formation of three splice variants that vary only in the length of the peptide linker. The 35.9 kDa medium-sized isoform (galectin-9M) corresponds to authentic galectin-9 whereas the long and small-sized isoforms (galectin-9L and S) have a 32-amino acid insertion and a 12-amino acid deletion, respectively in the linker peptide[36]. The length of this region influences the rotational flexibility of the two CRDs in the space, impacting on galectin-9 valency[163].

Human galectin-9 was first identified as a tumor antigen in Hodgkin's lymphoma, a condition characterized by abundant blood and tissue eosinophilia[164] and it is widely distributed within the immune system. This galectin is known to play a variety of cellular roles, including modulation of cell differentiation, adhesion, aggregation, and cell death[165]. Through modulation of cell signaling, this lectin can regulate multiple physiological and pathological processes such as immunity, inflammation, and cancer.

Galectin-9 has been identified as a ligand for the T-cell immunoglobulin mucin domain 3 (TIM-3), a membrane glycoprotein expressed on the surface of Th1, Th17 and citotoxic T cells, as well as in natural killer (NK) cells, monocytes, dendritic cells, macrophages and mast cells (reviewed by[166]). The galectin-9/TIM-3 pathway plays a dual role in immunity. On one hand, it favors a pro-inflammatory response, induces maturation of monocyte-derived dendritic cells, and through this process, enhances Th1-type immune responses[167]. On the other hand, galectin-9 contributes to apoptosis of thymocytes and peripheral T cells, implicating a dual role of the Galectin-9/TIM-3 axis in both T-cell maturation and negative regulation of T-cell-mediated immune reactions[168,169].

Blocking or activation of the Galectin-9/TIM-3 signaling pathway has been found to affect the evolution of many diseases, including autoimmune diseases, allergic disorders, graft rejection and anti-viral immunity (reviewed by[170]). Due to its potent roles in T cell suppression, galectin-9 has been considered as a therapeutic candidate for autoimmune and inflammatory diseases[167,171].

Although most studies indicate that TIM-3 is involved in galectin-9 mediated signaling in T cells, multiple mechanisms and alternative receptors have been also proposed for this lectin[163,172,173]. More recently, a publication by Leitner *et al*[174] suggested that TIM-3 does not act as a receptor for galectin-9. These controversial results emphasize the involvement of distinct glycosylated receptors in galectin-9 effects.

In spite of considerable evidence indicating the role of galectin-9 in tumor biology and inflammation, the mechanisms governing expression of this protein are poorly understood. So far, IFN-γ has been shown to induce galectin-9 expression in fibroblasts[175], endothelial cells[176] and on Kupffer cell[177]. Additional modulators of galectin-9 include interleukin-1β (IL-1β) and interleukin-5 (IL-5) in astrocytes[178] and eosinophils[179] respectively. Interestingly, decreased galectin-9 expression typically correlates with tumor progression and metastasis formation in various types of cancer[166].

Galectin-9 in HCC and in HCV/HBV infection-associated HCC

Galectin-9 has been identified as a possible prognostic marker in breast cancer, melanoma, and oral squamous cell carcinoma[180]. Most recently, Zhang *et al*[181] examined the relationship between galectin-9 expression and HCC, using an *in vitro* approach and immunohistochemistry on HCC tissues. The authors found that silencing galectin-9 expression in HepG2 HCC cells through siRNA-mediated strategies resulted in a weakened cell aggregation and increased proliferation and adhesion to ECM[181]. Also, galectin-9 suppression increased tumor cell-endothelial cell adhesion and trans-endothelial invasion of HepG2 cells. Additionally, downregulation of galectin-9 in human HCC tissue specimens represented a significant risk factor for patient survival and significantly correlated with the histopathologic grade of the tumor, lymph node metastasis, vascular invasion and intrahepatic metastasis[181]. These results emphasized an anti-metastasic role for galectin-9 in HCC (Figure 1 and Table 1).

T-cell responses are regulated by multiple mechanisms to maintain homeostasis and to prevent exuberant tissue inflammation and autoimmune disease. Whilst these regulatory mechanisms are critical to terminate excessive inflammatory responses, they can excessively constrain antiviral immunity in settings of persistent viral infection[182]. Galectin-9 is present at significantly higher levels in sera from patients infected with HCV or HBV compared to normal healthy controls[177,182,183]. Galectin-9 is expressed mainly in Kupffer cells[177,182], but is also present in inflammatory leucocytes and hepatocytes[183]. Re-

cently, it has been reported that progression to persistent infection of HCV was accompanied by increased plasma levels of galectin-9[184].

In patients chronically infected with HCV or HBV, multiple regulatory mechanisms act in concert to induce failure of the immune response and facilitate viral persistence. Interestingly, it has been demonstrated that galectin-9 plays a key role in limiting T-cell responses in the liver and facilitating the establishment of viral persistence. Galectin-9 induces the secretion of pro-inflammatory cytokines from monocytes and macrophages[177] that can further amplify immunopathology associated with HCV/HBV infection. As a counter-effect, galectin-9 induces TIM-3-mediated apoptosis of effector T cells[177,182] and favors the expansion of Tregs[177,184,185] thereby attenuating adaptive immune responses.

Li *et al*[186] studied the relevance of galectin-9 in patients with HBV-associated HCC. By flow cytometry analysis, the authors found that tumor cells and T cells expressed low amounts of galectin-9 while dendritic cells expressed moderate levels of this protein and Kupffer cells showed the highest expression in HBV-associated HCC tissues in comparison to non-tumor adjacent tissues[186]. The authors also observed that in HBV-positive patients the percentage of galectin-9$^+$ Kupffer cells was higher in tumor tissues than in normal adjacent tissues. However, in HBV-negative patients the expression of galectin-9 in Kupffer cells was negligible in both HCC and adjacent tissues. Interestingly, IFN-γ derived from tumor-infiltrating T cells contributed to the increased galectin-9 expression in the HCC microenvironment[186]. In addition, high numbers of TIM-3$^+$ T cells were detected in HBV-associated HCC, which expressed senescence markers and exhibited decreased proliferative ability and impaired effector function when compared with TIM-3$^-$ T cells. Therefore, the TIM-3/galectin-9 signaling axis mediates T-cell dysfunction and predicts poor prognosis in patients with HBV-associated HCC[186].

Although these data indicates a major role for galectin-9 in regulating liver immune responses, the observation that this galectin predominantly dampens immune function seems hard to reconcile with the poor outcome in patients with low galectin-9 expression. Possibly, galectin-9 expression is lost during the course of tumorigenesis, enabling tumor cells to metastasize more easily while alternatives modes of escape are being developed[180] (*e.g.*, the up-regulation of galectin-1) (Figure 1 and Table 1). A better understanding of the mechanisms underlying galectin-9 functions is required to elucidate its possible role as a promising target in HCC.

Galectin-9 in inflammation-related liver pathologies

The ischemia and reperfusion injury (IRI), an inflammatory event controlled by an exogenous antigen-independent insult that stimulates innate immunity, remains a critical problem in clinical organ transplantation. Liver IRI occurs frequently after major hepatic resection or liver transplantation. It has been demonstrated that CD4$^+$ T cells are the key mediators of IRI-triggered liver inflammation[187]. Kupffer cells release pro-inflammatory mediators such as TNF and IL-6[188], and CD4$^+$ T cells amplify Kupffer cell activity[189]. In this context, blockade of the TIM-3/galectin-9 pathway exacerbated local inflammation and liver damage (Table 2)[190]. These results suggest the importance of TIM-3/galectin-9 signaling in the maintenance of liver homeostasis and controlling dysregulated liver immune response, for example during IRI.

Similar results were observed in the murine model of liver injury, Con A-induced hepatitis, where T cell activation plays a crucial role. Blockade of TIM-3 using an anti-TIM-3 Ab resulted in more severe liver damage. On the contrary, biochemical and histopathological data indicated that a single injection of galectin-9 was sufficient to protect mice against Con A-induced hepatitis (Table 2)[191].

Another progressive inflammatory liver disorder is autoimmune hepatitis (AIH), where a defective control of CD4$^+$ T cells takes place. Liberal *et al*[192] showed that patients with AIH had reduced levels of TIM-3 and galectin-9 on effector CD4$^+$ T cells and Treg cells, respectively, as compared to healthy individuals[192]. Reduced signaling of the TIM-3/galectin-9 axis contributed to impaired control during AIH by rendering effector cells less prone to Treg cell control and Tregs less capable of suppressing effector responses.

A distinct subset of cells, referred as NKT cells has been characterized by the expression of a semi-invariant T cell receptor (TCR) and surface antigens typical of natural killer (NK) cells. These cells exhibit features of both cell types and act as a bridging system between innate and adaptive immunity[193]. NKT cells are particularly enriched within the liver and regulate immune responses through rapid secretion of large amounts of both Th1 and Th2 cytokines following stimulation[194]. The TIM-3/galectin-9 signaling pathway also plays a critical role in the homeostasis of hepatic NKT cells. It has been demonstrated that galectin-9 limits the inflammatory response in a mouse model of diet-induced nonalcoholic fatty liver disease (NAFLD) (Table 2)[195].

In summary, these observations validated the relevance of the TIM-3/galectin-9 signaling axis in maintaining a balanced local immune microenvironment in the liver. Dysregulation of this axis can lead to a chronic inflammatory liver disorder which can eventually develop into an HCC.

CONCLUSION

Because of their roles in tumor progression, galectins have evolved as promising targets for cancer therapy. A variety of studies revealed the involvement of this evolutionarily conserved protein family in murine and human cancers[26,52-56]. Modified citrus pectin, peptides, anti-galectin neutralizing antibodies and chemical inhibitors that antagonize galectins CRDs have been demonstrated the ability to reduce tumor volume, metastasis, angiogenesis,

potentiate immune responses and increase host survival in various tumor-type models[73,75,196-198].

Current literature shows that the "proto-type" galectin-1, the "chimera" galectin-3 and "tandem-repeat" galectin-4 are increased in HCC cells compared to their normal counterparts. On the other hand, expression of "tandem-repeat" galectin-8 and galectin-9 is decreased in tumor hepatocytes. The aberrant expression (up- or down-regulation) of these galectins correlates with tumor growth, HCC adhesion, migration and invasion, tumor aggressiveness, metastasis, postoperative recurrence and poor prognosis (Figure 1 and Table 1). It is noteworthy that galectins also play key roles in other liver pathologies associated with chronic inflammation and fibrosis (Tables 2 and 3). Although research in this field is just beginning, the role for these galectins in HCC biology is substantiated by a wide range of accumulating evidence from animal models and human samples. Further functional studies are crucial to delineate the precise mechanisms by which galectins promote liver carcinogenesis, HCC progression, aggressiveness, inflammation and metastasis. Hopefully, in a near future, galectin-based therapies can be developed for the treatment of HCC, liver-associated fibrosis and liver chronic inflammatory disorders.

ACKNOWLEDGMENTS

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Cellular reprogramming and hepatocellular carcinoma development

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers, and is also the leading cause of death worldwide. Studies have shown that cellular reprogramming contributes to chemotherapy and/or radiotherapy resistance and the recurrence of cancers. In this article, we summarize and discuss the latest findings in the area of cellular reprogramming in HCC. The aberrant expression of transcription factors OCT4, KLF4, SOX2, c-MYC, NANOG, and LIN28 have been also observed, and the expression of these transcription factors is associated with unfavorable clinical outcomes in HCC. Studies indicate that cellular reprogramming may play a critical role in the occurrence and recurrence of HCC. Recent reports have shown that DNA methylation, miR-NAs, tumor microenvironment, and signaling pathways can induce the expression of stemness transcription factors, which leads to cellular reprogramming in HCC. Furthermore, studies indicate that therapies based on cellular reprogramming could revolutionize HCC treatment. Finally, a novel therapeutic concept is discussed: reprogramming control therapy. A potential reprogramming control therapy method could be developed based on the reprogramming demonstrated in HCC studies and applied at two opposing levels: differentiation and reprogramming. Our increasing understanding and control of cellular programming should facilitate the exploitation of this novel therapeutic concept and its application in clinical HCC treatment, which may represent a promising strategy in the future that is not restricted to liver cancer.

Key words: Reprogramming; Hepatocellular carcinomas; Cancer stem cells; Transcription factor; Therapeutics

Core tip: Cellular reprogramming contributes to chemoresistance and radioresistance and cancer recurrence in hepatocellular carcinoma (HCC). Recent findings on cellular reprogramming in HCC are summarized and discussed, including stemness transcription factors, DNA methylation, miRNAs, tumor microenvironments, and signaling pathways. The novel therapeutic concept of reprogramming control therapy is also described, which may be a promising strategy for HCC therapy in the future.

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INTRODUCTION

Liver cancer is one of the most common tumors worldwide. An estimated 749000 new liver cancer cases and 695000 cancer deaths occurred worldwide in 2008[1]. Half of these cases and deaths were estimated to have occurred in sub-Saharan Africa and Southeast Asia. Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, which accounts for 70%-85% of the total liver cancer burden worldwide[2].

Reports have shown that tumor recurrence[3] and patient survival[4,5] are correlated with HCC differentiation. Based on the Edmondson-Steiner's classification, HCC can be graded from I to IV: well-differentiated (grade I), moderately differentiated (grade II), poorly differentiated (grade III), and undifferentiated (grade IV) HCC[6]. The prognosis of poorly differentiated carcinoma is worse than that of well-differentiated carcinoma[4], and the five-year survival of patients with poorly differentiated HCC is significantly worse than that of patients with moderately or well-differentiated HCC[7]. Ample evidence demonstrates that the poor prognosis and low five-year survival with poorly differentiated carcinoma are correlated with the expression of specific genes[4,8,9] and signal pathway activation[10,11], which can increase the resistance to chemotherapeutic drugs and the frequency of HCC recurrence.

Evidence shows that aggressive poorly differentiated human cancers express high levels of embryonic stem cell-like genes, suggesting that reprogramming to a more dedifferentiated state occurs during tumor progression[12]. Moreover, if different reprogramming factors are activated, cancer cells can form well-differentiated and poorly differentiated sarcomas[13]. Poorly differentiated cancers have a higher content of prospectively isolated cancer stem cells than well-differentiated cancers[14]. These data support the view that cancer is a reprogramming-like disease and that cancer stem cells (CSC) may arise through a reprogramming-like mechanism before initiating tumor formation and progression in HCC. Therefore, understanding the role of cellular reprogramming may facilitate the development of new therapeutic strategies for HCC.

CELLULAR REPROGRAMMING AND CANCER STEM CELLS

Cancer stem cells

Classical tumor formation theory, *i.e.*, clonal evolution theory, suggests that each cell in a tumor is biological homogeneous[15], whereas the alternative theory considers that the cells within a tumor are not identical, which is also known as tumor heterogeneity[16]. In the alternative

Table 1 Expression of transcription factors in various cancer types	
Type of cancer	**Transcription factors**
Breast cancer	*NANOG*[22], *SOX2*[23], *OCT4*[24] and *KLF4*[22]
Colorectal cancer	*NANOG*[25], *SOX2*[26] and *OCT4*[26]
Gastric cancer	*NANOG*[27], *SOX2*[27] and *OCT3/4*[27]
Hepatic cancer	*NANOG*[28], *SOX2*[29], *OCT4*[29] and *KLF4*[30]
Lung cancer	*NANOG*[31], *SOX2*[32] and *OCT4*[33]
Esophageal cancer	*NANOG*[34], *SOX2*[35], *OCT3/4*[35] and *LIN28*[36]
Ovarian cancer	*OCT4*[37] and *LIN* 28[38]

theory, all of cell types can arise from a signal cell, known as a CSC, which has the potential for self-renewal and differentiation[17]. Ample evidence supports a major role for the CSC model in tumor heterogeneity. Lapidot *et al*[18] first demonstrated a critical role for CSC in human acute myeloid leukemia, where leukemic stem cells (LSC) initiated human acute myeloid leukemia after transplantation into SCID mice. The existence of LSC prompted further research into other types of cancer. CSC have recently been identified in several solid tumors, including breast, brain, colorectal, pancreas, liver, melanoma, and prostate cancers[19]. CSC possess the properties of normal stem cells, *i.e.*, self-renewal and differentiation. Self-renewal enables CSC to produce another CSC with essentially the same developmental and replication potential, which can increase the capacity for self-protection against drugs, toxins, and radiation. Differentiation involves the production of different types of cancer cells that trigger tumor initiation, maintain tumor growth, and finally form a bulk tumor.

Cancer development

Studies have shown that reprogramming factors have specific expression signatures in human tumors (Table 1) and that the expression levels of these factors are correlated with the differentiation grades of tumor. Ben-Porath *et al*[12] found that poorly differentiated tumors preferentially overexpressed embryonic stem cell (ESC) genes. Moreover, the activation targets of reprogramming factors, such as *NANOG*, *OCT4*, *SOX*2 and *c-MYC*, are more frequently overexpressed in poorly differentiated tumors than well-differentiated tumors[12]. Chiou *et al*[20] reported that the expression levels of *NANOG*, *OCT4* and *CD133* were correlated with a poor survival prognosis in patients with oral squamous cell carcinoma. Reprogramming factors also play essential roles in maintaining the properties of CSC in tumors. Silencing the expression of Oct-4 in CD133+ lung cancer can significantly inhibit the capacity for self-renewal, enhance CD133+ cell differentiation into CD133- cells, and reverse the effects of chemotherapy or radiotherapy[21]. These data suggest that reprogramming factors play critical roles in the origin and development of CSC.

Origin of CSC

Studies have shown that the occurrence of CSC is related

to cellular reprogramming, but the origin of CSC remains a conundrum. However, important new evidence has demonstrated that there are two possible routes for CSC emergence.

First, CSC may arise from normal stem cells (SC) that lose the ability to regulate proliferation. Kim *et al*[39] showed that SC are more readily reprogrammed into induced pluripotent stem cells (iPS) compared with somatic cells. *OCT4* and either *KLF4* or *c-MYC* are sufficient to generate iPS from neural SC[39], which suggests that SC can be reprogrammed, and the process may be much easier than reprogramming mature cells. Riggi *et al*[40] successfully reprogrammed mesenchymal SC (MSC) into Ewing sarcoma cancer SC by inducing the expression of the ESC genes *OCT4*, *SOX2* and *NANOG* using the *EWS-FLI1* fusion gene. Chiba *et al*[41] reported that normal SC can be transformed into CSC after overexpressing the *BMI-1* gene, which had the potential for tumor formation.

The alternative theory hypothesizes that CSC may be reprogrammed from somatic cells, which acquire the capacities for self-renewal and tumor initiation after genetic lesions. After forcing the expression of exogenous OSKM (OCT4, SOX2, KLF4, MYC) in the human somatic fibroblast line TIG1, Nagata *et al*[42] isolated induced cancer SC (iCSC) from cell populations with the capacity for self-renewal. The lack of a functional RB1 can also trigger reprogramming, which generates cells with the properties of CSC from mouse fibroblasts[43]. Therefore, studies suggest that CSC can be reprogrammed from somatic cells. Moreover, the dedifferentiation of tumor cells may also lead to stemness property of cells. Recent studies suggest that tumor cells could also be a source of CSC. The expression of the reprogramming factors, *OCT4* and *NANOG*, was detected in poorly differentiated lung adenocarcinoma, whereas ectopic expression of *OCT4* and *NANOG* increased the proportion of the CD133-expressing subpopulation, sphere formation, and enhanced drug resistance in lung adenocarcinoma[44]. Similar results were also observed in melanoma and colon cancer[45,46]. For example, exogenous expression of the *OCT4* gene or the transmembrane delivery of OCT4 protein promoted the dedifferentiation of melanoma cells into CSC-like cells by the induced expression of endogenous *OCT4*, *NANOG* and *KLF4*[45]. Su *et al*[46] showed that HT29/CD44⁻ cells can be reprogrammed into CSC with significantly increased expression levels of *c-MYC*, *STAT3*, *SOX2* and *OCT4* by the CD44-SRC-integrin axis.

CELLULAR REPROGRAMMING OF HCC

Related factors

Transcription factors: Recently, it was demonstrated that forced expression of combinations of four transcription factors, *i.e.*, *OCT4*, *KLF4*, *SOX2*, and *c-MYC* or *OCT4*, *SOX2*, *NANOG* and *LIN28*, can reprogram somatic cells into iPS that closely resemble ESC[47-50]. In-

creasing evidence has demonstrated that aberrant expression of reprogramming factors may confer primitive and aggressive traits, which are associated with unfavorable clinical outcomes in HCC. *OCT4*, *NANOG* and *SOX2* have been detected in HCC cell lines and in tumor specimens from patients with HCC, and Oct4 could play a significant role in activating the Wnt/β-catenin and transforming growth factor-β (TGF-β) signaling pathways[51]. Huang *et al*[29] demonstrated that SOX2- and OCT4A-positive expression were significantly associated with an aggressive phenotype in HCC. *SOX2* or *OCT4A* are independent prognostic factors for HCC, but the coexpression of *SOX2/OCT4A* has the poorest prognosis in HCC[29]. Increased expression of Nanog is also correlated with a poorer clinical outcome in HCC, whereas the overexpression of NANOG in NANOG⁻ cells increases the capacity for self-renewal by the insulin-like growth factor receptor (IGF1R) signaling pathway in HCC[28]. Of interest, expression of the pluripotent transcription factor KLF4 is decreased or lost in primary HCC[30]. The loss of KLF4 expression is also significantly associated with poor survival in HCC[30]. Evidence suggests that KLF4 is a putative tumor suppressor gene. The enforced restoration of KLF4 expression markedly inhibits cell migration, invasion, and growth *in vitro*, and significantly attenuates tumor growth and metastasis in HCC animal models[30,52]. Reprogramming factors are expressed preferentially in hepatocellular carcinoma SC (HCSC). Expression levels of *CD44*, *OCT4* and *BMI1* were specifically upregulated in CD45⁻CD90⁺ cells isolated from the tumor tissues and blood samples of patients with HCC compared with those in CD45⁻CD90⁺ cells isolated from normal livers[53]. Ma *et al*[54] found that CD133⁺ HCC cells expressed consistently higher mRNA levels of β-catenin, *OCT-3/4*, *BMI*, *SMO*, and *NOTCH-1* than CD133⁻ HCC cells.

DNA methylation: Epigenetic studies have demonstrated that specific DNA methylation patterns, including global hypomethylation and promoter hypermethylation, may be early events in HCC[55]. A genome-wide DNA methylation microarray analysis showed that side population (SP) cells had a different DNA methylation status compared with non-SP cells in HCC[56]. Recent discoveries have shown that DNA methylation is an essential epigenetic mechanism during iPS reprogramming[57]. Demethylating agents and demethylase proteins may activate pluripotent gene promoters, thereby facilitating cellular reprogramming and ultimately enhancing the efficiency of iPS generation. Wang *et al*[58] found that chemoresistant cells exhibited increased expression levels of *OCT4* in HCC, whereas the expression of *OCT4* was regulated by DNA methylation. More recent reports have shown that the expression of OCT4 is associated with the protein level of lipid storage droplet (LSD) in pluripotent cancer cells and human testicular seminoma tissues[59]. CD133 expression is also regulated by DNA methylation in HCC[60]. The elevated expression of CD133 is associated with the demethylation of Line-1 in HCC[60]. More-

Figure 1 The process of cellular reprogramming and potential therapies in hepatocellular carcinoma. The endogenous and exogenous interferences such as DNA methylation, microenvironment factors, microRNAs (miRNAs) and signaling activation (see text for details) could induce the reprogramming of hepatic cells and stem/progenitor cells, result in tumor initiation, an excess of self-renewal and chemo/radio-resistance, and form HCC. Reversely, the differentiation induction including demethylation, miRNAs, RNAi and signaling inhibition, will be the potential therapies for HCC. Additionally full reprogramming induction might offer us a novel way to treat HCC. The reprogramming approach would help to induce the partially reprogrammed cells to transform in full reprogrammed cells, like induced pluripotent stem cells, which can be induced to various types of differential somatic cells. HCC: Hepatocellular carcinoma.

over, TGF-β-1 can inhibit the expression of DNA methyltransferases (DNMT)1 and DNMT3β, thereby leading to significant demethylation in the CD133 promoter-1 in CD133⁻ Huh7 cells[61]. Studies of MSC have shown that methylation of the tumor suppressor genes, *HIC1* and *RASSF1A*, is sufficient to successfully reprogram the MSC into cancer stem/initiating cells[62]. These studies suggest that the demethylation of reprogramming factors and/or methylation of tumor suppressor genes contribute to reprogramming in HCC and to the origination of HCSC.

MicroRNAs: MicroRNAs (miRNAs) are well-characterized regulators of development and differentiation[63]. Studies have demonstrated that specific miRNAs have high expression levels in ESC and that they play a critical role in the control of pluripotency-related genes[64,65]. The clusters of miRNA-302s/367s[66] or miRNA-302s/369s/200c[67] can directly reprogram mouse and human somatic cells to pluripotency and increase the expression levels of OCT4 and SOX2. Studies have shown that miRNA-302 is a direct target of OCT4 and SOX2 in human ESC[68], whereas miRNA-302 and OCT4/SOX2 may work as a positive feedback system in cellular reprogramming. Moreover, the reprogramming miRNA-302 is highly expressed in a rare subpopulation of glioma cell lines. miR-302 expression causes tumorsphere formation and significant upregulation of pluripotent genes[69]. Results indicate that miRNAs participate in the neoplastic transformation of HCSC in HCC. In total, 68 miRNAs have been found to be overexpressed, whereas 10 miRNAs were underexpressed in a SP of HCC cells compared with fetal liver cells[70]. miRNA can also regulate the expression of cancer SC markers in HCC. OCT4 was regulated by miRNA-145 in T3A-A3, which are CSC-like cells[71], whereas miRNA-148 attenuated the expression of CD90 and CD44 in HCC[72]. miRNA-181 family members were highly expressed in (epithelial cell adhesion molecule⁺ (EpCAM⁺AFP⁺) HCC cells, and the inhibition of miRNA-181 led to a reduction in the quantity of EpCAM⁺ HCC cells and their tumor-initiating ability[73]. These reports suggest that miRNAs are potential factors in the reprogramming of HCC (Figure 1).

Microenvironment: Microenvironment plays a role in HCC, although its role during cellular reprogramming remains unclear. Hypoxia is a well-known characteristic of the tumor microenvironment, including HCC. In the emerging field of induced pluripotency, Yoshida *et al*[74] have shown that hypoxia can significantly improve the generation of iPS colonies following reprogramming. Seven hypoxia-related prognostic genes, *i.e.*, *CCNG2*, *EGLN3*, *ERO1L*, *WDR45L*, *FGF21*, *MAT1A* and *RCL1*, which were dysregulated in HCC, were associated with chronic hypoxia, and were correlated with a poor prognosis in HCC[75]. *CCNG2*[76] and *EGLN3*[77] were upregulated in CSC, whereas MAT1A deficiency increases the expression of CD133⁺ HCSC[78]. Mathieu *et al*[79] showed that hypoxia by hypoxia-inducible factor (HIF) could induce a hESC-like transcriptional program, including induction of the reprogramming factors, *OCT4*, *NANOG*, *SOX2*, *KLF4*, *cMYC* and miRNA-302, in 11 cancer cell types, including HCC. Haraguchi *et al*[80] reported that CD13 is a marker for semiquiescent CSC in human liver cancer cell lines, where the expression of CD13 is accompanied by the expression of carbonic anhydrase 9 (CA9), a hypoxia marker in HCC.

The tumor environment is always characterized by inflammation. Interleukin (IL)-6, an inflammatory cytokine, led to HCC from an IL-6-driven transformed SC with inactivated TGF-β signaling[81]. Moreover, a subset of highly chemoresistant and invasive HSC were screened that had aberrant expression levels of cytokine IL-6 and TWIST. The secretion of IL-6 and TWIST can significantly increase the expression levels of let-7 and miR-181, which contribute to chemoresistance and cell invasion in HCC[82].

Both of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are the major etiological agents of chronic liver disease and HCC. *In vitro* and *in vivo* studies have shown that OCT4, NANOG, KLF-4, β-catenin and (EpCAM) are activated by HBx, and the upregulated expression of multiple stem genes demonstrates that HBx contributes to hepatocarcinogenesis, at least partly, by promoting changes in gene expression, which are characteristics of CSC[83]. Moreover, HCV can also induce the cancer stem cell-like signatures in cell culture and mouse

model.

Signaling pathways

Reprogramming is likely to induce drastic molecular changes that involve the upregulation of pluripotent genes and the repression of differentiation genes. Thus, signaling pathways have profound effects on the reprogramming of somatic cells into iPS[84]. A class comparison analysis showed that 793 genes were differentially expressed in hepatic stem cell-like HCC (HpSC-HCC) and mature hepatocyte-like HCC (MH-HCC)[85]. A pathway analysis indicated that differentially expressed genes were significantly associated with SC signaling pathways, including Wnt/β-catenin, TGF-β and ERK/MAPK signaling[85]. These results suggest that signaling pathways have significant effects on cell reprogramming in HCC.

Wnt/β-catenin: It is well-known that Wnt/β-catenin signaling can control ESC self-renewal and the maintenance of stemness[86], and it also regulates the expression of *ESC* genes[87]. Furthermore, it may contribute to the reprogramming of somatic cells in pluripotent cells[88]. Yamashita *et al*[89] identified a novel prognostic HCC subtype based on EpCAM expression, which resembled hepatic progenitor cells with activated stem cell markers and Wnt/β-catenin signaling. The expression of EpCAM was associated with the activation of Wnt/β-catenin signaling[89]. Similar results were reported by Yang *et al*[90] who found that OV6^{+} cancer cells could endogenously activate Wnt/β-catenin signaling in HCC. Expression of OV6 increases after the activation of Wnt/β-catenin signaling, whereas inhibition of Wnt/β-catenin signaling leads to a decrease in the proportion of OV6^{+} cells in HCC[90]. Moreover, the activation of Wnt/β-catenin signaling could be inhibited by silencing the expression of *OCT4*, with a reduction in *WNT-10b* and *β-catenin* and an increase in TCF3[51]. These results indicate that Wnt/β-catenin signaling may be an essential part of cellular reprogramming and the maintenance of stem-like characteristics in HCC.

TGF-β: TGF-β signaling pathway has been reported in many cellular processes in adult organisms and the developing embryo, including cell growth, differentiation, apoptosis, and homeostasis. Ichida *et al*[91] demonstrated that TGF-β signaling is involved with cellular reprogramming. The inhibition of TGF-β signaling can promote the completion of reprogramming by the induction of Nanog[91]. Recent studies have shown that the TGF-β signaling pathway can regulate cellular reprogramming in HCC. HCSC exhibit the unexpected loss of Transforming growth factor beta receptor Ⅱ, which could lead to inactivation of the TGF-β signaling pathway[81]. Toll-like receptor 4/NANOG-dependent tumor-initiating stem-like cells (TICs) were also detected with an inactivated TGF-β signaling pathway. Restoration of the TGF-β signaling pathway can inhibit the expression of pluripotent genes, including *NANOG, CD133, OCT4* and *SOX2*, as

well as tumorigenesis and abrogate the chemoresistance of TICs[92].

Mitogen-activated protein kinase/ERK kinase: The mitogen-activated protein kinase/ERK kinase (MAPK/ERK) signaling pathway has been detected in mouse ESC[93]. During reprogramming, the inhibition of MAPK/ERK could promote the transformation of pre-iPS into ground state pluripotent SC, which are cells associated with inhibition of the glycogen synthase kinase-3 (GSK3) signaling pathway[94]. It has been reported that CD133^{+} HCC exhibit a substantial increase in MAPK/ERK pathway activation[95,96] and that activation of the MAPK/ERK pathway can enhance proliferation, tumor angiogenesis, and initiate tumors in CD133^{+} HCC. Moreover, MAPK inhibition using the MAPK kinase 1 (MEK1) inhibitor PD98059 leads to a significant increase in TGF-β-induced apoptosis in CD133^{+} HCC[97].

In addition to these signaling pathways, the BMI-1 and Insulin-like growth factor-1 signal pathways also play key roles during cellular reprogramming in HCC. BMI-1 expression was highly correlated with the CSC phenotype in CD133^{+} HCC cells, and a modification in BMI-1 expression resulted in a similar change in the maintenance of a CD133 subpopulation in HCC[98]. Insulin-like growth factor (IGF2) and IGF1R can be upregulated in NANOG[+] CSC, and a specific inhibitor of IGF1R signaling may significantly inhibit self-renewal and NANOG expression in HCSC, thereby indicating that IGF1R signaling participates in NANOG-mediated cellular reprogramming in HCC[28].

POTENTIAL THERAPIES BASED ON CELLULAR REPROGMMING

The detection and treatment of HCC have greatly improved with the advances in medicine; however, HCC remains largely incurable due to tumor recurrence. Conventional anticancer approaches, surgical resection, chemotherapy, and radiotherapy are primarily directed at bulk tumor populations. However, these strategies are frequently ineffective because of resistance to drugs and/or radiation[99]. Increasing evidence indicates that cellular reprogramming is involved with self-renewal, drug and/or radiation resistance, and tumorigenicity in HCC, and the concept of using precancerous cells and their progeny, CSC, in cancer therapy could provide unique insights into early cancer diagnosis, treatment, and preventive therapy[100]. Cellular reprogramming could also be a potentially useful therapeutic target in HCC.

Inhibition of reprogramming

Methylation: Given the essential role of DNA methylation during cellular reprogramming in HCC, DNA methylation may be a therapeutic target in HCC. Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that catalyzes the addition of methyl groups to H3K27, and the blocking of H3K27 methylation leads to a sig-

nificant reduction in TF-induced reprogramming[101]. 3-deazaneplanocin A, an S-adenosylhomocysteine hydrolase inhibitor, is an efficient inhibitor of the function of EZH2, which reduces the levels of H3K27 me3 in HCC cells, thereby reducing the number of EpCAM+ cells and the self-renewal capacity of these cells[102]. Lysine-specific histone demethylase 1 (LSD1) is a histone demethylase, and specific small bioactive inhibitors of LSD1 can enhance H3K4 methylation, derepress epigenetically suppressed genes, and inhibit the proliferation of pluripotent cancer cells, including teratocarcinoma, embryonic carcinoma, seminoma, and ESC[59]. All these studies suggest that methylation of histone 3 may be a potential target in HCC therapy.

miRNA: It is known that miRNAs are involved with the reprogramming of HCC and that they directly regulate the expression of reprogramming factors; however, miRNA can also act as a barrier during reprogramming. Evidence suggests that miRNA-34 is a reprogramming suppression miRNA, which can repress the expression of pluripotent genes, including *NANOG*, *SOX2* and *MYCN*[103]. The expression of pluripotent genes in HCC can also be downregulated by miRNAs. miRNA-145 can directly target OCT4 to arrest the cell cycle and inhibit the tumor growth of T3A-A3[71]. Moreover, miRNAs can regulate self-renewal, differentiation, and chemoresistance in HCSC. The inhibition of let-7 increases the chemosensitivity to sorafenib and doxorubicin by directly targeting SOCS-1 and Caspase-3, whereas silencing of miR-181 expression leads to a reduction in the motility and invasion by directly targeting RASSF1A, TIMP3, and nemo-like kinase in CD133+ HCC[82]. Zhang *et al*[104] demonstrated that overexpression of miR-150 downregulates c-Myb protein levels and leads to a significant reduction in CD133+ cells, which is accompanied with significant inhibition of cell growth and tumorsphere formation. Ma *et al*[105] reported that antagonizing miR-130b reduces the resistance to chemotherapeutic agents, leads in the loss of *in vivo* tumorigenicity, and inhibits self-renewal in CD133+ TICs through TP53INP1 silencing.

Silencing of transcription factors

Using chemotherapeutic drugs to select chemoresistant cancer cells in HCC, Wang *et al*[58] showed that chemoresistant cells exhibit CSC features with dramatically increased Oct4 levels and a highly activated OCT4-TCL1-AKT-ABCG2 pathway. OCT4 knockdown and/or AKT pathway inhibition can reduce the resistance to chemotherapy both *in vitro* and *in vivo*[58]. Oikawa *et al*[106] focused on Sal-like protein 4 (SALL4) and found that elevated expression of SALL4 in tumors is associated with poor survival in HCC. The silencing of SALL4 expression significantly inhibits *in vitro* and *in vivo* tumor growth with increased differentiation[106]. Yamashita *et al*[85] suggested that RNAi-mediated knockdown of EpCAM can reduce self-renewal, tumorigenicity, migration, and drug resistance in HCC cells. Haraguchi *et al*[80] demonstrated that CD13 could ROS-induced DNA damage after genotoxic

chemotherapy or radiation stress and protect cells from apoptosis. The combination of a CD13 inhibitor and the genotoxic chemotherapeutic agent fluorouracil (5-FU) drastically reduces the tumor volume in mouse xenograft models[80].

Regulating signaling pathways

Reports have shown that the abnormal activation and/or inhibition of signaling pathways in CSC, as well as the regulation of signal pathways, may be effective approaches to HCC therapy. Yamashita *et al*[89] found that TCF/β-catenin binding inhibitors were much more sensitive to EpCAM+ HCC than EpCAM− HCC, and they significantly inhibited the growth of EpCAM+ HCC. CD133+ HCC cells that survived chemotherapy had increased preferential expression levels of proteins involved with the AKT/PKB and BCL-2 pathways. AKT/PKB pathway-related cell survival proteins significantly reduce after treatment with an AKT1 inhibitor. Coincubation of an AKT1 inhibitor with DOX or 5-FU almost completely inhibits the preferential survival effect induced by CD133+ cells in HCC[107]. HCSC also exhibit an inactivated TGF-β signaling pathway[81]. A CD133+ population demonstrated significant resistance to TGF-β induced apoptosis compared with CD133− cells in HCC, whereas the MEK1 inhibitor PD98059 leads to a significant increase in TGF-β-induced apoptosis in CD133+ cells[97].

Differentiation induction

Given that the formation of tumors involves various cancer cells that differentiate from CSC, it is expected that CSC will become less malignant if forced to differentiate into mature cells. Tang *et al*[81] demonstrated that IL-6 can drive the differentiation of HCC from hepatic stem/progenitor cells with inactivated TGF-β signaling. Chow *et al*[108] found that MYC-driven tumors contains a subset of cells (SP cells), which are characterized by Hoechst 33342 efflux. SP tumor cells exhibit markers of hepatic stem cells and chemoresistance, whereas chemoresistance is lost when SP tumor cells differentiate into non-SP tumor cells[108]. This suggests that the differentiation of hepatic CSC may be a possible therapeutic approach. Recently, Yamashita *et al*[109] identified an oncostatin M (OSM) receptor in EpCAM+ HCSC. OSM treatment induced hepatocytic differentiation in EpCAM+ HCSC with a reduction of SC-related gene expression and an increase in albumin expression. Furthermore, a combined treatment with OSM and 5-FU eliminated HCSC and non-CSC subpopulations in an efficient manner[109]. A recent study showed that bone morphogenetic protein 4, a critical molecule in hepatogenesis and hepatic stem cell differentiation, can also promote differentiation and inhibit self-renewal in CD133+ HCSC with a high exogenous dose[110].

Full reprogramming induction

iPS can be generated from normal tissues by the expression of defined transcription factors, as well as from malignant cells[111]. After transformation with four ectopic

reprogramming factors, *i.e.*, OCT4, KLF4, SOX2 and c-MYC, the chronic myeloid leukemia (CML) cell line KBM7 could be reprogrammed into iPS[112]. Moreover, Kumano *et al*[113] induced iPS in samples isolated from patients with CML sensitive to imatinib. This report was the first example of the reprogramming of human primary cancer cells into iPS. In principle, CSC can also be reprogrammed into iPS using four or less reprogramming factors. Kim *et al*[39] showed that iPS could be reprogrammed from adult neural SC using only two reprogramming factors. This indicates that the number of reprogramming factors could be reduced using somatic cells that express appropriate levels of complementary factors endogenously. Studies have shown that HCSC exhibit the endogenous expression of *SOX2*, *C-MYC*, *NANOG* and *OCT4*, and that these endogenous reprogramming factors could facilitate the reprogramming of CSC into iPS, which may reduce the recurrence of HCC.

PERSPECTIVE

In this study, we reviewed the expression of transcription factors detected in HCC and summarized the complex mechanisms that contribute to cellular reprogramming in HCC, which then lead to the acquisition and maintenance of self-renewal and stemness features by a population of cancer cells, thereby resulting in the generation of HCSC. There are numerous potential applications of cellular reprogramming in regenerative medicine and cancer therapy. However, we showed that the knowledge obtained through studies of the molecular and cellular mechanisms that underlie reprogramming in HCC will also have deep implications for our understanding and the treatment of HCC, as well as other types of cancer. Furthermore, we also should refine the theory for application since the non-stem cell mediated, mature hepatocyte-derived HCC emerged in mice[114-116].

Recognizing the role of cellular reprogramming in HCC suggests a novel therapeutic concept: reprogramming control therapy. Based on reprogramming in HCC studies, a possible reprogramming control therapy could be developed that targets two opposing: differentiation (or dereprogramming) and reprogramming (or dedifferentiation). The differentiation approach would focus on the differentiation of reprogrammed cells in HCC. Reprogrammed cells exhibit stem cell-like characteristics, including the expression of stemness genes and the activation of specific signaling pathways. Modifications of gene expression and/or signaling pathways could induce the reprogrammed cells to differentiate into mature somatic cells with impaired self-renewal and reversed chemoresistance and/or radioresistance. The reprogramming approach would help to induce the partially reprogrammed cells in HCC to transform in full reprogrammed cells, such as iPS, which can be redifferentiated into various types of mature cells. *In vitro* experiments and mice model studies have shown that these theoretical therapeutic approaches may have applications in future HCC therapy. Increased knowledge and control of cellular programming could lead to the development of this novel therapeutic concept and its application in clinical HCC therapy, which may be a promising strategy in the future.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Anti-viral therapy to reduce recurrence and improve survival in hepatitis B virus-related hepatocellular carcinoma

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improved the survival rate of patients with hepatocellular carcinoma (HCC). However, hepatitis B virus (HBV)-related HCC has a much higher recurrence rate. In this article, we describe strategies for reducing recurrent HCC using anti-viral therapy for HBV infection.

Abstract

Hepatocellular carcinoma (HCC) is the most common malignancy and the third leading cause of cancer death worldwide. Chronic infection with hepatitis B virus (HBV) and hepatitis C virus accounts for approximately 75%-80% of HCC cases worldwide. In particular, chronic HBV infection is a predominant risk factor for HCC in Asia and Africa. Hepatic resection and radiofrequency ablation are increasingly used for the curative treatment of HCC, and good local control can be achieved. However, the high rate of recurrence is a major obstacle to improving prognosis. A high viral load of HBV DNA is the most important correctable risk factor for recurrence. Furthermore, interferon and/or nucleotide analogues may decrease HBV DNA. Therefore, these drugs may decrease recurrence. In this article, treatment strategies for HBV-related HCC are described in order to reduce recurrence and improve survival.

Core tip: Recent advances in treatment modalities have

INTRODUCTION

Hepatic resection or liver transplantation provide a complete curative treatment for hepatocellular carcinoma (HCC)[1,2]. In addition, regional ablation therapy including radiofrequency ablation (RFA) is now increasingly used for the curative treatment of HCC, and good local control can be achieved[3-5]. However, these techniques are unsatisfactory due to a high post-treatment recurrence rate[6]. It was reported that up to 70% of patients relapse within 5 years after curative treatment[7].

This high rate of recurrence is a major obstacle to improving prognosis. Therefore, antiviral and anti-inflammatory therapies both before and after curative treatment may be crucial in preventing HCC recurrence and improving survival. Current approved medications for chronic hepatitis B treatment are interferon-α (IFNα) and nucleotide analogues (NAs), including lamivudine (LVD), entecavir (ETV), tenofovir disoproxil fumarate, adefovir-dipivoxil (ADV), and telbivudine[8]. However, despite curative treatment of HCC, the 5-year recurrence rate remains high, at 70%-80%[9]. The mechanisms of HCC recurrence differ greatly from those of other carcinomas in terms of the high rate of intrahepatic metastases and multicentric carcinogenesis against a background

of viral liver disease. Whether antiviral therapy after treatment of HCC can prevent recurrences is thus an important issue. Interferon (IFN) therapy in hepatitis C virus (HCV)-related HCC has been reported to reduce recurrence rates and contribute to survival, and its significance in preventing secondary carcinogenesis[10-14] including improvement of hepatic functional reserve[15] has been established.

The treatment of hepatitis B virus (HBV)-related HCC has centered on nucleic acid analogues to reduce viral load and inactivate hepatitis, however, treatment with IFN, similar to that in type C hepatitis, has recently attracted attention. Nucleic acid analogues and IFN may act together, but therapeutic strategies for preventing secondary carcinogenesis after treatment of HBV-related HCC remain unclear. This paper reviews the clinical evidence regarding treatment from the perspective of preventing secondary carcinogenesis, including reducing recurrence rates and improving prognosis after curative treatment of HBV-related HCC.

ROLE AND MECHANISM OF HBV DNA LOAD IN RECURRENT HCC

The mechanism of hepatocarcinogenesis by HBV includes direct malignant transformation and other indirect effects. With regard to direct malignant transformation, HBV gene integration into the host hepatocyte genome causes changes in host gene expression and properties, facilitating hepatocarcinogenesis[16,17].

Hence, as an indirect effect, persistent infection by HBV leads to hepatocyte destruction and regeneration, increasing genetic instability[18]. Epidemiological studies have examined differences in carcinogenesis due to HBV DNA load[19], however, the mechanisms by which HBV DNA load causes differences in malignant transformation remain unclear.

HBV DNA load has been shown to play a role in carcinogenesis in patients with type B chronic liver disease, and more recently, HBV DNA load has also been reported to be involved in recurrence after curative treatment of HCC.

In a retrospective study of 72 patients with hepatic resection for HBV-related HCC, Hung et al[20] reported that patients with a high serum HBV DNA load at the time of tumor resection showed a significantly higher recurrence rate, compared to patients with a low viral load. Multivariate analysis showed that a high HBV DNA load, alpha-fetoprotein level, tumor size, and age were factors contributing to recurrence. Xia et al[21] reported that high serum hyaluronic acid and HBV viral load are the main prognostic factors of local recurrence after complete radiofrequency ablation of hepatitis B-related small HCC.

Because HBV DNA load changes with the administration of antiviral drugs, patients with a high viral load at the time of HCC treatment who receive antiviral drugs subsequently show differences in HBV DNA load compared to those who do not receive such treatment. Kim et al[22] analyzed the patients excluded from antiviral drug therapy. After the patients treated with antiviral drugs were excluded, recurrence-free survival rates in a total of 157 patients with HBV-related HCC who underwent hepatic resection were compared between 89 patients with a persistently low HBV DNA load and 68 patients with a persistently high viral load. Recurrence-free survival rates were better in the persistently low HBV DNA load group compared to the high level group.

MECHANISM OF ANTIVIRAL DRUGS IN PREVENTING RECURRENT HCC

NAs preparations

The direct antitumor activity of nucleotide analogues has not been reported. Lamivudine has no inhibitory effects on integrated HBV DNA, thus there is no suppressive effect on de novo carcinogenesis due to HBV gene integration into the host genome[23,24].

Considering that HBV DNA load is related to HCC recurrence, the prevention of recurrence by antiviral drugs, rather than direct antitumor effects, is due to a reduction in HBV DNA load which improves hepatocyte destruction and regeneration and reduces genetic instability, thus decreasing HCC recurrence rates. Hosaka et al[25] reported that HBV core-related antigen levels were independent risk factors for HCC recurrence. In addition, Chuma et al[26] reported that recurrence was significantly lower in patients who received lamivudine before the development of HCC.

In a retrospective study by Kubo et al[27] of 24 patients with HBV-related HCC who underwent liver resection, a difference in recurrence-free survival rates was seen between 14 patients who received lamivudine and 10 patients who did not. Multivariate analysis also showed that lack of antiviral therapy and multiple tumors were factors related to recurrence-free survival rates.

In another retrospective study of 49 patients who underwent curative treatment for HBV-related HCC (liver resection, 31 patients; RFA, 18 patients), Kuzuya et al[28] examined cumulative recurrence rates of HCC in 16 patients who received lamivudine and 33 patients who did not. There was no significant difference between the two groups. Although there was no significant difference in HCC recurrence rates, hepatic functional reserve was improved and survival was better in the lamivudine group. In the lamivudine group, hepatic functional reserve was significantly better at the time of HCC recurrence, a higher percentage of patients were able to undergo curative treatment, and prognosis tended to be better (Table 1).

Other studies[29,30] have reported significantly larger remnant liver volume and better prognosis after liver resection in lamivudine-treated groups, and that lamivudine improves liver function and reduces deaths due to liver failure. Lamivudine after treatment of HCC may not prevent cancer recurrence, but may contribute to an improved prognosis by maintaining hepatic functional

Table 1	Studies in which Nucleoside analogues were administered after treatment for hepatitis B virus-related hepatocellular carcinoma					
Authors	Treated *vs* Untreated	Treatment	Observation time	HCC Tx	Recurrence	Survival
Kubo *et al*[27]	14 *vs* 10	LVD	1117 d (median)	Ope	NA	Tumor-free survival (*P* = 0.0086)
Kuzuya *et al*[28]	16 *vs* 33	LVD	38.0 mo *vs* 32.6 mo (median)	Ope/RFA	NS (*P* = 0.622)	NS (*P* = 0.623)
Li *et al*[29]	43 *vs* 36	LVD with/without ADV	12 mo	Ope	NS (*P* = 0.077)	Overall survival (*P* = 0.0094)
Piao *et al*[30]	30 *vs* 40	LVD	24 mo	Ope/RFA	NS	NS (*P* = 0.12)
Wu *et al*[31]	518 *vs* 4051	LVD/ETV/Telbivudine	2.64 yr	Ope	*P* < 0.001	*P* < 0.001

HCC: Hepatocellular carcinoma; LVD: Lamivudine; ETV: Entecavir; ADV: Adefovir-dipivoxil; NS: Not significant; NA: Not analyzed; Tx: Treatment.

Table 2	Studies on the effects of interferon on hepatitis B virus-related hepatocellular carcinoma after treatment					
Authors	Treated *vs* Untreated	Treatment	Observation time	HCC Tx	Recurrence	Survival
Someya *et al*[38]	11 *vs* 69	IFNα	16 yr	Ope/RFA	*P* = 0.013 (High AST group)	NA
Lai *et al*[39]	35 *vs* 36	IFNα	30 mo	Inoperable	*P* = 0.001 (Tumor regression)	*P* = 0.047
Lo *et al*[40]	40 *vs* 40	IFNα	60 mo	Ope (Stage Ⅲ/ⅣA)	*P* = 0.031	NS (*P* = 0.311)
Sun *et al*[41]	118 *vs* 118	IFNα	36.5 mo (median)	Ope	*P* = 0.048	*P* = 0.0003
Chen *et al*[42]	106 *vs* 109	IFNα	63.8 mo (median)	Ope	NS (*P* = 0.766)	NS (*P* = 0.826)

HCC: Hepatocellular carcinoma; IFN: Interferon; NS: Not significant; NA: Not analyzed; Tx: Treatment.

reserve[28]. Wu *et al*[31] recently reported that NAs were important in preventing recurrences after liver resection (Table 1).

At present, opinion is divided regarding whether administration of NAs after HCC treatment prevents HCC recurrence[32]. However, NAs may improve prognosis by improving hepatic functional reserve. NAs treatment was able to improve survival post-HCC treatment compared with no NAs therapy[33]. Recently, ETV therapy was found to be more effective with a rapid reduction in viral load compared with LVD. ETV is safe and well-tolerated during long-term treatment[34]. Furthermore, ETV has a higher genetic barrier to resistance[35]. ETV treatment might have potent protective effects against recurrence of HCC.

EFFECTS OF IFN IN PREVENTING RECURRENCE AFTER CURATIVE TREATMENT FOR HCC

Basic research has shown that IFN has antiviral effects, antitumor effects against HCC[36,37], and inhibits the proliferation of cancer cells. In a retrospective study by Someya *et al*[38] evaluating IFN therapy in patients after curative treatment for HCC who also had HBV-related cirrhosis, uni- and multivariate analysis showed that IFN prevented recurrences, especially in the group with high aspartate transaminase. In addition, in a randomized controlled trial (RCT) of high-dose IFN in patients with HCC who could not undergo surgery, the IFN-treated group showed a significantly higher rate of ≥ 50% tumor size reduction compared to the control group[39]

(Table 2).

Furthermore, RCTs have been conducted to investigate the effects of IFN in preventing recurrences in patients after treatment for HCC. Lo *et al*[40] conducted a RCT in 40 patients with HBV-related HCC after curative hepatic resection. They compared a group treated with IFN-α2b 10 MU/m^2, three times weekly, for 12 wk and a non-treated control group. The 1- and 5-year survival rates in the IFN group were 97% and 79%, respectively, compared to 85% and 61% in the control group (*P* = 0.137). Multivariate analysis showed that IFN therapy may lower the risk of death. In a subgroup analysis, the 5-year survival rate in stage Ⅰ/Ⅱ patients did not differ between the IFN and control groups, but with IFN therapy in stage Ⅲ/ⅣA patients, early recurrence of HCC was prevented, and the 5-year survival rate improved from 24% to 68% (*P* = 0.038). Sun *et al*[41] also compared an IFN group and control group after HCC surgery in a randomized study. IFN therapy was reported to be useful, with significant increases both in median overall survival and median disease-free survival times. However, the results of a recent phase Ⅲ randomized study of IFN-α2b after curative resection for HBV- and HCV-related HCC conducted in Taiwan showed no prevention of HBV or HCV recurrence[42] (Table 2).

Therefore, the effects of IFN therapy after curative treatment of HCC remain unclear. Pegylated (PEG)-IFN has superseded conventional IFN due to a higher response rate and once weekly administration instead of daily or three times a week. Recently, it was reported that high levels of hepatitis B surface antigen (HBsAg) increase HCC development among hepatitis B envelope an-

tigen (HBeAg)-negative patients with a low viral load[43]. A recent study clearly showed that the rates of HBsAg clearance after PEG-IFN treatment are substantial and durable in HBeAg-negative patients. Rates of HBsAg clearance were shown to increase further during long-term follow-up, with 12% of patients achieving HBsAg clearance at 5 years post-treatment[44]. Better results are anticipated in the future using PEG-IFN.

CONCLUSION

Patients with high HBV DNA levels at HCC onset show significantly higher HCC recurrence rates compared to patients with low HBV DNA levels. In patients with high HBV DNA levels, the administration of antiviral drugs relatively early during treatment is recommended to prevent HCC recurrences. However, to more accurately evaluate the effects of antiviral therapy in preventing HCC recurrence, large-scale studies in more patients should be conducted.

Persistent viral suppression by antiviral therapy can inhibit carcinogenesis. Treatment with PEG-IFN results in a higher virological therapeutic response compared with conventional IFN. In addition, ETV, which has become a drug of first choice instead of LVD, has a very low resistance mutation rate, thus long-term viral suppression is possible. The long-term therapeutic effects of PEG-IFN and ETV are currently uncertain, but equal or better efficacy than conventional IFN or lamivudine for the prevention of carcinogenesis is expected. Future research should be aimed at clarifying the effects of antiviral therapy in HBV-related HCC.

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TOPIC HIGHLIGHT
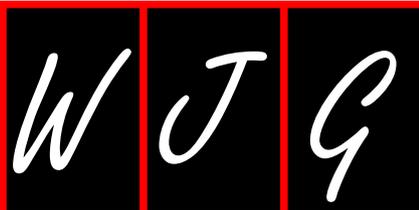
WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Risk prediction of hepatitis B virus-related hepatocellular carcinoma in the era of antiviral therapy

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Abstract

Hepatocellular carcinoma (HCC) is a grave primary liver cancer that has a limited therapeutic option because it is generally diagnosed later in an advanced stage due to its aggressive biologic behavior. The early detection of HCC has a great impact on the treatment efficacy and survival of patients at high risk for cancer. Potential host, environmental, and virus-related risk factors have been introduced. Hepatitis B virus (HBV) is a major cause of end-stage liver diseases such as liver cirrhosis or HCC in endemic areas, and its serologic or virologic status is considered an important risk factor. HCC risk prediction derived from the identification of major risk factors is necessary for providing adequate screening/surveillance strategies to high-risk individuals. Several risk prediction models for HBV-related HCC have been presented recently with simple, efficient, and readily available to use parameters applicable to average- or unknown-risk populations as well as high-risk individuals. Predictive scoring systems of risk estimation to assess HCC development can provide the way to an evidence-based clinical approach for cost- and effort-effective outcomes, capable of inducing a personalized surveillance program according to risk stratification. In this review, the concepts and perspectives of the risk prediction of HCC are discussed through the analysis of several risk prediction models of HBV-related HCC.

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Key words: Hepatocellular carcinoma; Hepatitis B virus; Chronic hepatitis B; Risk prediction; Risk factors

Core tip: This review shows the concepts and perspectives of the risk prediction of hepatitis B virus-related hepatocellular carcinoma. Accurate risk scoring systems to predict hepatocellular carcinoma (HCC) development, derived from independent risk factors integrated in aspects of host, environment, and virus, are necessary for performing the strategic processes such as screening/surveillance, diagnosis, and treatment in high-risk individuals of HCC. Globally standardized consensus for HCC risk prediction models should be established on the basis of simplicity, assessability, and reproducibility of the model characteristics available in real clinical setting.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most aggressive malignant neoplasms and a leading cause of cancer-related morbidity and mortality[1]. During the last

few decades, the incidence rate of HCC has increased in most developed countries, but its mortality rate has decreased[2]. Tumor diagnosis at an advanced stage and accompanying chronic liver diseases, including liver cirrhosis, are major limitations of curative management in many cases. The accurate selection of high-risk groups and adequate screening/surveillance programs for HCC detection at an early stage may provide clinical strategies capable of overcoming "tumor diagnosis at an advanced stage"[3]. The early detection of HCC in populations and individuals at high risk is critical in providing curative treatments and in consequently acquiring a survival benefit, which has been validated through a randomized controlled trial of screening for HCC[4].

The hepatitis B virus (HBV) genome consists of partially double-stranded DNA of approximately 3200 base pairs with four overlapping open reading frames encoding the envelope (S), core (C), polymerase (P), and X proteins (Figure 1).

Chronic HBV infection is usually characterized by the presence of hepatitis B surface antigen (HBsAg) in the serum for at least 6 mo after exposure to the virus. Patients with chronic HBV infection have a more than 100-fold increased risk of HCC occurrence compared with uninfected individuals[5]. Therefore, HBV-infected patients have been considered a high-risk group of HCC and regarded as candidates for a precise application of screening/surveillance strategies scheduled by using risk weight-based stratification. In addition to the possession of HBsAg itself, the following HBV-associated biomarkers affecting liver disease progression to cirrhosis and HCC have been suggested: serum titer of HBsAg, hepatitis B e antigen (HBeAg), serum level of HBV DNA, HBV genotype, and HBV mutations[6]. In recent years, the evolution of antiviral therapeutics for chronic HBV infection is a result of the clinical efforts to reduce the development of HCC.

In this article, we discuss the host, environmental, and virus-related risk factors associated with the development of HBV-related HCC and present the risk prediction systems for the development of HCC based on stratification of scoring estimation derived from independent risk factors.

RISK FACTORS

Host factors
The following potential host factors for HCC occurrence in HBV-infected individuals have been suggested based on demographic, clinical, and epidemiologic investigations: male gender, increasing age, genetic susceptibility and family history of HCC, obesity, diabetes, coexistent alcohol consumption or smoking, high serum alanine aminotransferase (ALT) activity, low serum albumin, low platelet counts, high serum alpha-fetoprotein level, and accompanying liver cirrhosis[6]. In relation to these risk factors, several study groups have strongly recommended HCC surveillance strategies in men > 40 years old and
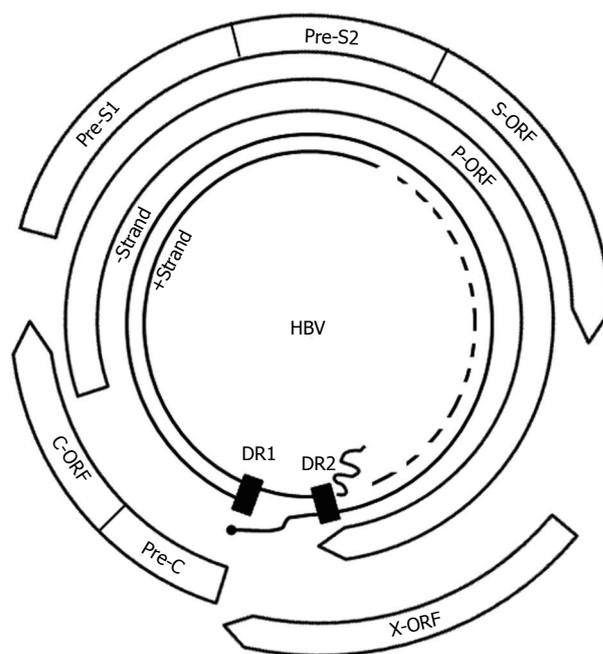
Figure 1 Representative scheme of the hepatitis B virus genome. Hepatitis B virus (HBV) genome consists of partially double-stranded DNA with four overlapping open reading frames. ORF: Open reading frame; DR: Direct repeat.

women > 50 years old with chronic hepatitis B who have a family history of HCC and accompanying cirrhosis, considering different ethnicities[3,7-10]. Liver cirrhosis, irrespective of etiology, is the most important and independent risk factor for the development of HCC, accounting for 73% to 85% of patients with HCC in HBV-endemic areas[2]. Among the biochemical risk factors, ALT is the most readily available in clinical fields, and its serum level above the upper limit of normal (ULN) is considered an independent risk factor for HCC even in average-risk populations without HBsAg, to say nothing of high-risk subjects seropositive for HBsAg. Subjects in the upper range (0.5-1 × ULN or approximately 25-40 IU/L) of the normal limit of serum ALT levels were reported to be at an increased risk of HCC compared with subjects in the lower range (< 0.5 × ULN or < 25 IU/L)[11,12]. The risk factors mentioned previously are simple to measure, easy to administer, and convenient to apply clinical parameters useful for constructing risk prediction models of HCC.

Environmental factors
Environmental risk factors are difficult to define clearly in clinical settings. Ethnic susceptibility, alcohol consumption, cigarette smoking, co-infection with other viruses, and chemical carcinogens including aflatoxin were representative of environmental factors. Ethnicity is considered a relative risk factor for HCC. Africans, African Americans, and Asians are included in populations for HCC surveillance in HBV-endemic regions[3,7-9]. In these regions, virus infection mainly occurs through perinatal transmission vertically or horizontally[13], resulting in sus-

ceptibility to disease chronicity and relative intractability to antiviral therapy because of long-standing periods of infection. Simultaneous co-infections by other viruses such as hepatitis C virus, hepatitis D virus, and human immunodeficiency virus may be additional risk factors, but they have not been established definitely. Aflatoxin is well known to be a carcinogen capable of developing HCC. Aflatoxin B1 is a representative genotoxic hepatocarcinogen that induces the transversion of guanine (G) to thymine (T) in codon 249 of exon 7 of the *p53* tumor suppressor gene in human hepatocytes (the so-called stop-codon mutation), resulting in the substitution of arginine (A) to serine (S). Mutations of *ras* oncogenes are also found in aflatoxin B1-induced HCC, but are less frequent than the *p53* mutation[14]. These environmental factors are not usually involved in constructing risk prediction models of HCC because of the lack of quantitative assessment as independent risk factors.

Virologic factors

Virologic risk factors have been considerably investigated in cases with hepatitis virus-associated HCC. The clinical implications of the serum HBV DNA level for liver disease progression are recognized in HBV-infected patients. A stepwise increase of the serum HBV DNA level is associated with a corresponding linear increase in the cumulative incidence of HCC as well as the progression of HBV-related liver disease to liver cirrhosis or hepatic decompensation regardless of the serum ALT activity, HBeAg status, and presence of cirrhosis[15,16]. Therefore, the serum HBV DNA level is a major independent virologic risk factor. Furthermore, inactive carriers with chronic HBV infection, who are seronegative for HBeAg have serum levels of HBV DNA less than 4 log copies/mL and serum ALT activity within the normal limit and do not have chronic hepatitis, cirrhosis, or HCC either histologically or clinically, are at risk for HCC and liver-related death compared with individuals not infected with HBV[17]. Hepatitis B viral load has been reported to be a risk factor for post-treatment recurrence of HCC[18]. In these backgrounds, antiviral therapy with nucleoside/nucleotide analogs in patients with HBV-associated liver disease is a main pivot to control HCC development and recurrence. In fact, lamivudine therapy has reduced the incidence of HCC in patients with compensated cirrhosis when viral suppression was sustained[19]. Recently, the quantitative assessment of serum HBsAg has been suggested as a new tool for determining HCC development and for predicting the response to antiviral therapy[20-22]. However, circulating HBsAg in blood, a component of the HBV envelope proteins, is originated from non-infectious viral particles as well as intact Dane particles with viral infectivity; the clinical impact of the serum HBsAg level on HCC development and the antiviral response in patients with chronic HBV infection should be ascertained prospectively. On the other hand, the serum level of HBsAg, like the HBV DNA level, may fluctuate in the natural course of chronic HBV infection[23,24], and

changing patterns of the serum HBsAg level through long-term regular monitoring might determine whether the changes could affect the disease progression in HBV-infected patients. Besides these virologic factors, HBeAg/anti-HBe status, HBV genotype, basal core promoter mutations/precore mutations or mutations relevant to deletions within pre-S region, and co-infection with other viruses can be considered risk factors for HCC[25-28]. Among these virologic risk factors, the serum HBV DNA level and HBeAg status are the most valuable and available parameters capable of constructing risk prediction models of HCC. However, the serum HBsAg level, HBV genotype, and HBV mutations are not readily available in clinical settings. The measurement of these factors tends to be required for specific situations such as academic approaches to antiviral therapy, epidemiologic investigations, or scientific interest. Therefore, the application of virologic factors for building risk prediction models of HCC should be granted as evidence-based as a matter of prudence even if most virologic factors provide important information for HCC risk stratification.

RISK PREDICTION SYSTEMS

Risk prediction systems capable of estimating the strength of HCC development are clinically important for identifying patients at high risk who should participate in a scheduled surveillance program. To construct readily available prediction systems of HCC, the risk factors for HCC mentioned previously should be independently established through statistical verification. Statistical techniques adopted in the process should be reliable and reasonable to identify an objective recognition. Next, selected risk factors should be integrated and organized under the consideration of demographic and epidemiologic differences of developing HCC, inducing a systematic stratification of scoring estimation derived from independent risk factors for HCC. Finally, constructed risk prediction systems should be validated internally or externally, which makes individualized surveillance strategies possible. Cancer risk weighed-oriented scoring estimation could provide the advantage in aspects of cost- or effort-effectiveness through a tailored approach to cancer surveillance.

Several predictive scoring systems for the development of HBV-related HCC have been introduced recently (Table 1). Yuen *et al*[11] for the first time deduced and validated the risk score (*i.e.*, the GAG-HCC score) with sensitivity > 84% and specificity > 76% to predict the 5- and 10-year risks for the development of HCC based on age, gender, HBV DNA levels, core promoter mutations, and cirrhosis; they concluded that the risk score could be used to identify high-risk patients with chronic hepatitis B (CHB) for screening and treating HCC. They emphasized the significance of this study with a valuable approach excluding the patients who received any type of established management for CHB capable of affecting the occurrence of HCC. Wong *et al*[29] included two prospective

Table 1 Risk prediction models of hepatocellular carcinoma in patients with chronic hepatitis B virus infection

Ref.	No. of participants/ validation	Parameters of HCC prediction	Risk weights for parameters	Range of weights	Year of risk prediction
Lee *et al*[32], 2013	2227/1113	Age	[0-6]	0-19	5, 10 and 15
		Sex	[0-2]		
		ALT	[0-2]		
		Family Hx of HCC	[0-2]		
		HBeAg/HBV DNA/ HBsAg/Genotype	[0-7]		
Wen *et al*[12], 2012	[1]298051	Age	M1, 2, 3, 4: [0-6]	M1: -1-12	5 and 10
		Sex	M1, 2: [0-2], M3, 4: [0-1]		
		Smoking	M1, 3, 4: [0-1]	M2: 0-23	
		Alcohol	M1, 3, 4: [0-1]		
		Physical activity	M1, 3, 4: [-1-0]	M3: -1-23	
		DM	M1: [0-2], M3, 4: [0-1]		
		ALT	M2: [0-2], M3, 4: [0-1]	M4: -1-30	
		AST	M2: [0-13], M3: [0-12], M4: [0-7]		
		HBV	M4: [0-4]		
		AFP	M4: [0-8]		
Yang *et al*[30], 2011	3584/1505	Age	[0-6]	0-17	3, 5 and 10
		Sex	[0-2]		
		ALT	[0-2]		
		HBeAg	[0-2]		
		HBV DNA	[0-5]		
Yang *et al*[31], 2010	2435/1218	Age	M1, 2, 3: [0-6]	M1: 0-17	5 and 10
		Sex	M1, 2, 3: [0-2]		
		Alcohol	M1: [0-1], M2, 3: [0-2]	M2: 0-20	
		ALT	M1: [0-3], M2: [0-2], M3: [0-1]		
		Family Hx of HCC	M1, 2, 3: [0-2]	M3: 0-20	
		HBeAg	M1: [0-3]		
		HBeAg/HBV DNA	M2: [0-6]		
		HBeAg/HBV DNA/ Genotype	M3: [0-7]		
Wong *et al*[29], 2010	1005/424	Age	[0-3]	0-43.5	5 and 10
		Alb	[0-20]		
		Bil	[0-1.5]		
		HBV DNA	[0-4]		
		Cirrhosis	[0-15]		
Yuen *et al*[11], 2009	[2]820	Age	With core promoter mutations	-	5 and 10
		Sex	Age (in years) + 16 sex (male = 1; female = 0) +		
		HBV DNA	3 HBV DNA levels (Log copies/mL) + 19 core		
		Core promoter mutations	promoter mutations (mutant = 1; wild-type = 0)		
		Cirrhosis	+ 30 cirrhosis (presence = 1; absence = 0)		
			Without core promoter mutations		
			Age (in years) + 14 sex (male = 1; female = 0) + 3		
			HBV DNA levels (Log copies/mL) + 33 cirrhosis		
			(presence = 1; absence = 0)		

[1]The number of participants in a subcohort without hepatitis C virus test results was 298051, with being randomly and equally split into a training set and a validation set. [2]The risk score was assessed by the leave-one-out cross-validation. HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; DM: Diabetes mellitus; AFP: Alfa fetoprotein; Bil: Bilirubin; Alb: Albumin; M: Model.

cohorts in their study: a training cohort (1005 patients) and a validation cohort (424 patients). A predictive scoring system ranging from 0 to 43.5 was constructed by using five independent risk factors: age, albumin, bilirubin, HBV DNA, and cirrhosis. They concluded that the classification of HCC risk to low-, medium-, and high-risk groups based on this scoring system was accurate in predicting HCC development. They insisted that the score was derived from clinical parameters routinely measurable in large prospective cohorts for a long-term period, and the validation was established with high accuracy in another sizable cohort. Yang *et al*[30] enrolled 3584 pa-

tients from the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL-HBV) study cohort as a development cohort of risk estimation. Male gender, older age, elevated serum ALT activity, HBeAg positive, and higher serum HBV DNA titer were identified as independent risk factors for HCC; a 17-point risk score from these risk factors was developed. They interpreted that this simple-to-use risk score based on noninvasive clinical variables could accurately predict the risk of HCC in patients with chronic hepatitis B. They also depicted easy-to-use nomograms to accurately predict the risk of HCC in CHB patients,

facilitating risk communication between clinicians and patients[31]. This Risk Estimation for Hepatocellular Carcinoma in Chronic Hepatitis B study suggested that clinicians could make evidence-based decisions about clinical management. Wen *et al*[12] developed five simple risk prediction models with one-by-one escalating manner based on clinically available data from a prospective cohort of 428 584 general subjects. Age, sex, health history, HBV and hepatitis C virus (HCV) status, and serum ALT, aspartate aminotransferase, and alpha-fetoprotein levels were determined to be statistically significant independent predictors of HCC risk. They concluded that prediction models using transaminase data were best able to predict HCC risk even among subjects with unknown or HBV- or HCV-negative infection status. The significance of this study is that setting up a simple, easy-to-administer risk prediction model applicable even in low-risk, average-risk, or unknown-risk subjects as well as a high-risk population was attained. Lee *et al*[32] most recently developed risk prediction models of HCC by integrating host and HBV profiles after identifying independent risk factors such as older age, male, HBeAg, HBV genotype C, and increasing levels of ALT, HBV DNA, and HBsAg associated with an increased risk of HCC. They concluded that the categorization into low, medium, and high HCC risk could enable physicians to estimate the 5-, 10-, and 15-year risk of HCC with excellent accuracy and discriminatory ability. Two points are noteworthy in this study. One was the introduction of the quantitative serum HBsAg level in the analytic process to derive HCC risk models. The serum HBsAg level was determined to be an independent risk factor of HCC development as well as a response assessment to antiviral therapy. The other was the construction of a prediction model of cirrhosis risk, which was not developed before in patients with chronic HBV infection. Because cirrhosis is a precancerous lesion, the development of a cirrhosis risk prediction model has a valuable impact on the selection of candidates for a scheduled surveillance program according to the risk stratification of HCC.

In summary, accurate prediction models of HCC development constructed from readily available clinical and laboratory variables are necessary for performing strategic processes such as screening/surveillance, diagnosis, and treatment in high-risk patients of HCC. A globally standardized consensus for cancer risk prediction models should be established based on simplicity, assessability, and reproducibility of the model characteristics available in real clinical settings. Hereafter, generalized authorization of risk prediction models needs to be confirmed by using internal and external validation with a prospective manner in different populations of regions with epidemiologic versatility of HCC.

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TOPIC HIGHLIGHT

WJG 20ᵗʰ Anniversary Special Issues (1): Hepatocellular carcinoma

Target genes discovery through copy number alteration analysis in human hepatocellular carcinoma

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Abstract

High-throughput short-read sequencing of exomes and whole cancer genomes in multiple human hepatocellular carcinoma (HCC) cohorts confirmed previously identified frequently mutated somatic genes, such as *TP53*, *CTNNB1* and *AXIN1*, and identified several novel genes with moderate mutation frequencies, including *ARID1A*, *ARID2*, *MLL*, *MLL2*, *MLL3*, *MLL4*, *IRF2*, *ATM*, *CDKN2A*, *FGF19*, *PIK3CA*, *RPS6KA3*, *JAK1*, *KEAP1*, *NFE2L2*, *C16orf62*, *LEPR*, *RAC2*, and *IL6ST*. Functional classification of these mutated genes suggested that alterations in pathways participating in chromatin remodeling, Wnt/β-catenin signaling, JAK/STAT signaling, and oxidative stress play critical roles in HCC tumorigenesis. Nevertheless, because there are few druggable genes used in HCC therapy, the identification of new therapeutic targets through integrated genomic approaches remains an important task. Because a large amount of HCC genomic data genotyped by high density single nucleotide polymorphism arrays is deposited in the public domain, copy number alteration (CNA) analyses of these arrays is a cost-effective way to reveal target genes through profiling of recurrent and overlapping amplicons, homozygous deletions and potentially unbalanced chromosomal translocations accumulated during HCC progression. Moreover, integration of CNAs with other high-throughput genomic data, such as aberrantly coding transcriptomes and non-coding gene expression in human HCC tissues and rodent HCC models, provides lines of evidence that can be used to facilitate the identification of novel HCC target genes with the potential of improving the survival of HCC patients.

Core tip: In addition to detecting somatic mutations in cancer genomes with high-throughput short-read sequencing technologies, analysis of copy number alteration in hepatocellular carcinoma (HCC) cancer genomes genotyped by high density single nucleotide polymorphism arrays is a cost-effective approach to reveal genome-wide somatic alterations accumulated during tumorigenesis. Integration with other genomic data from HCC tissues derived from high-throughput short-read sequencing, proteomics, epigenomics and transcriptomics could provide lines of evidence to identify common and novel HCC genes for potential clinical applications.

INTRODUCTION

Human hepatocellular carcinoma (HCC) is the fifth leading cause of cancer mortality, causing an estimated half a million deaths annually[1,2]. Risk factors for developing HCC include hepatitis infection, obesity, alcoholism and consumption of aflatoxin-contaminated food. Due to the rising incidence of hepatitis C infection, HCC is one of the fastest-growing cancers in the United States and Western countries, and the incidence is expected to continue to increase[3]. Surgical resection is the most successful treatment for early stage HCC. However, fewer than 30% of HCC patients are qualified for curative resection owing to liver dysfunction and cirrhosis. Moreover, frequent tumor recurrence is observed even after curative resection.

Recent successes in cancer targeted therapy arising from the identification of somatic alterations and their specific inhibitors are associated with reduced side effects and prolonged patient survival. Many of these FDA-approved inhibitors are small molecules or monoclonal antibodies against cancer-specific tyrosine kinase mutations, including Imatinib mesylate (Gleevec) for fusion oncogene Bcr/Abl-positive chronic myelogenous leukemia[4], Gefitinib (Iressa) or Erlotinib (Tarceva) for epidermal growth factor receptor mutated non-small cell lung cancer[5] and Trastuzumab (Herceptin) for HER2/neu receptor amplified and overexpressed breast cancer patients[6]. Although no specific drug target has been identified for HCC, FDA approved the multi-kinase inhibitor sorafenib for treatment of advanced HCC, due to a favorable overall patient survival[7]. However, HCC patients receiving sorafenib showed marginal benefits, with a prolonged survival of 3-4 mo on average[8,9]. With limited improvement of HCC patient survival, identification of recurrent and altered somatic genes through integrated genomic approaches is vital to better understand HCC molecular tumorigenesis, to develop early diagnostic markers and methods, and to find additional druggable targets for the improvement of HCC management.

MUTATED HCC GENES WITHIN RECURRENT ALTERED CHROMOSOME LOCI

In HCC, many tumor suppressor genes and oncogenes were identified based on recurrent genetic lesions, including loss of *TP53* (17p13)[10], *RB* and *BRCA2* (13q)[11], and amplification of *c-myc* (8q24)[12] and *ERBB2*

(17q12-q21)[13]. Epigenetic mechanisms also contribute to HCC progression, such as CpG island hyper-methylation of *p16* (*INK4a*) and *COX2*[14-16], as well as altered expression of microRNAs[17,18]. Conventional point mutation is another common mechanism to alter cancer gene functions. In HCC, frequent point mutations of *TP53* and *β-catenin* are involved in key pathways of hepatocarcinogenesis[19,20]. Other studies have reported mutations in *M6P/IGF2R*[21], *BRCA2*[22], *Smad2/4*[23], *HCCS1*[24], *PTEN*[25] and *Axin1*[26].

Recently developed high-throughput short-read sequencing technologies were used to identify somatic mutations in HCC cancer genomes at genome-wide scales. These studies confirmed that *TP53* and *CTNNB1* (encoding for β-catenin) are the most frequent recurrent mutations in human HCC. In addition, moderate mutation frequencies were identified in multiple HCC cohorts for several novel genes, including epigenetic and chromatin remodeling genes (*ARID1A*, *ARID2*, *MLL* and *MLL3*) and members of a number of oncogenic pathways (*RPS6KA3*, *JAK1* and *KEAP1*)[27-32]. These results suggested that aberrant pathways involved in cell cycle regulation, oxidative stress, chromatin remodeling and oncogenic signaling, such as Wnt/β-catenin, JAK/STAT and Akt/mTOR, play critical roles in the process of HCC tumorigenesis. Nevertheless, HCC remains a highly lethal cancer due to the lack of biomarkers for early diagnosis, molecular classification and efficient therapeutic interventions. Efforts to develop specific inhibitors for these aberrant pathways and reveal better therapeutic targets in HCC are urgently needed.

HIGH DENSITY SINGLE NUCLEOTIDE POLYMORPHISM ARRAYS FOR ANALYSIS OF RECURRENT COPY NUMBER ALTERATIONS

Copy number alterations (CNAs), distinguished from germ line transmitted copy number variations, account for some of the genetic diversity of populations, in addition to the accumulated genomic DNA changes during tumor progression. CNAs are important subclasses of somatic mutations, with aberrant chromosomal regions of amplification or deletion commonly associated with overexpressed oncogenes or loss of tumor suppressor genes, respectively[33]. With the comprehensive annotation of human genome in the last decade, the mutated cancer genes could be aberrant protein-coding and non-coding genes such as small microRNAs or long non-coding RNAs within the CNA regions[34].

Copy number alterations in cancer cells can be detected by conventional karyotyping and chromosomal in situ hybridization technologies. To profile CNAs in cancer genomes compared to the genomes of adjacent normal cells, comparative genome hybridization (CGH) technology was used to identify copy number changes in karyotypes from breast cancer cell lines and primary blad-
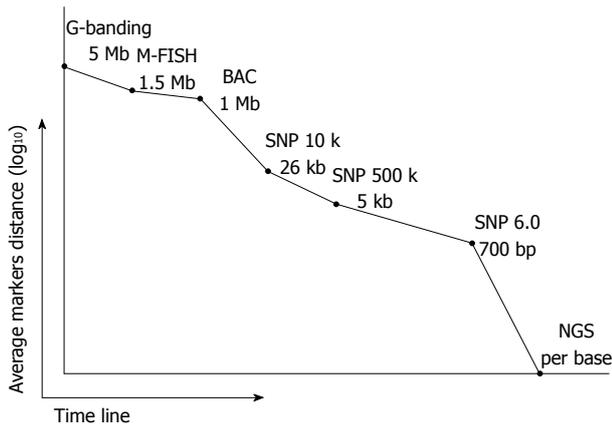
Figure 1 Timeline and average marker distance of technologies for the detection of copy number alterations.

Table 1 Copy-number altered regions in genomes of hepatocellular carcinoma cell lines

Cytoband	Start (Mb)	End (Mb)	Known cancer genes	Novel candidates genes
Amplicons				
1q21.2-22	150.07	151.89	*SHC1, CKS1B, ADAM15*	*CREB3L4, RAB1, mir190b, S100A14*
3p26.1-25.3	6.90	9.43		*LMCD1*
3q26.2-26.31	170.07	170.24		
	170.28	170.99	*EVI1, MDS1, TERC*	
	171.21	173.50		*FNDC3B*
5p15.33-12	0.40	45.14	*TRIO, AMACR, DAB2*	*LPCAT1, SEMA5A, CDH12*
7p22.2-14.3	4.15	32.10	*RAC1, ETV1, CHN2*	
7p12.1-11.2	52.79	55.17	*EGFR*	
	56.00	56.53		
8p11.21	40.44	40.62		
8q24.21	129.21	129.29		
11q13.2-13.3	65.85	66.44	*RIN1, BRMS1*	*SLC29A2*
	67.58	67.71		
	67.91	69.35	*LRP5, CCND1, ORAOV1*	*FGF4, FGF3*
12p12.1	24.36	25.54	*BCAS1, K-ras*	
20q13.31	53.94	53.96		
Homozygous deletions				
2q22.1	141.72	141.80	*LRP1B*	
7q21.11	77.96	78.04		*MAGI2*
9p23	9.42	9.46		*PTPRD*
	11.90	12.00		
9p21.3	21.85	21.90	*MTAP, CDKN2A*	
	24.27	24.84		
13q12.11	18.98	20.44	*TPTE2, Tg737*	

der tumors[35]. With the availability of genomic resources (*e.g.*, BAC clones) and array technologies (*e.g.*, high-density oligonucleotide probes), array-based CGH (aCGH) technologies not only examine genome-wide CNAs in high resolution but also allow researchers to pinpoint and profile the non-random CNAs for identification of novel aberrant cancer genes (Figure 1)[36-38]. The recently developed high-throughput short-read sequencing technology might become an alternative and effective approach to simultaneously detect CNAs and other classes of somatic mutations at the single nucleotide level[39,40]. Nevertheless, the availability of thousands of cancer genomes genotyped by high-density SNP arrays from various tumor samples and cancer cell lines at both NCBI GEO (Gene Expression Omnibus) and EBI ArrayExpress databases is a critical resource for *in silico* analysis of CNAs[41,42]. Moreover, integrated genomic analysis with both high-throughput short-read sequencing technology and high-density SNP genotyping arrays to comprehensively profile and validate recurrent CNAs of cancer genomes are promising approaches for the identification of novel cancer genes[40].

DIFFERENT TYPES OF CANCER MUTATIONS EMBRACED IN CNAS LOCI

To identify novel diagnostic and therapeutic target genes, CNA analysis of cancer genomes genotyped using commercial high-density SNP arrays from your own experiments or downloaded from public domains is a powerful and cost-effective approach. First, to discover putative tumor suppressor genes, we overlapped homozygous deleted regions from multiple samples to narrow down the common deleted regions by using high-density SNP genotyping arrays. As shown in Figure 2, the homozygous deleted region at chromosome 13q12.11 in SK-hep1 cells could be refined from 1.88 to 1.46 Mb to facilitate the identification of candidate tumor suppressor genes[38,43]. Second, for the identification of candidate oncogenes in HCC, the most common approach is to integrate data

from genomic experiments in order to reveal genes residing in overlapping amplicons with up-regulated gene expression. For instance, *FNDC3B, SLC29A2, Ago2, IER3* and many others were identified as putative oncogenes due to their genomic DNA amplification and mRNA overexpression in HCC tissues[38,44-47]. When ectopically expressed, these putative oncogenes in HCC cells commonly show malignant phenotypes using various functional assays and facilitated tumor progression *in vitro* and *in vivo*.

Third, CNA analysis allows the identification of HCC genes with attributes of genomic DNA amplification, mRNA overexpression and recurrent point mutations, such as the putative metastatic HCC oncogene with LMCD1 mutations at E135K (in 3/48 cases) and K237R (in PLC/PRF/5 cells)[44]. When these mutations were expressed in HCC cells, HCC cell migration capability was enhanced in association with cortical actin accumulation and lamellipodial extension. Moreover, the overexpression of the LMCD1 E135K mutation in HCC cells significantly promoted systemic lung metastasis in a murine tail vein injection model. Table 1 summarizes some novel HCC genes in association with overlapping amplicons and homozygous deletions in HCC cell lines. Finally, CNA analysis detects differences in copy number (*i.e.*, dosages), such as amplifications and deletions. Therefore,
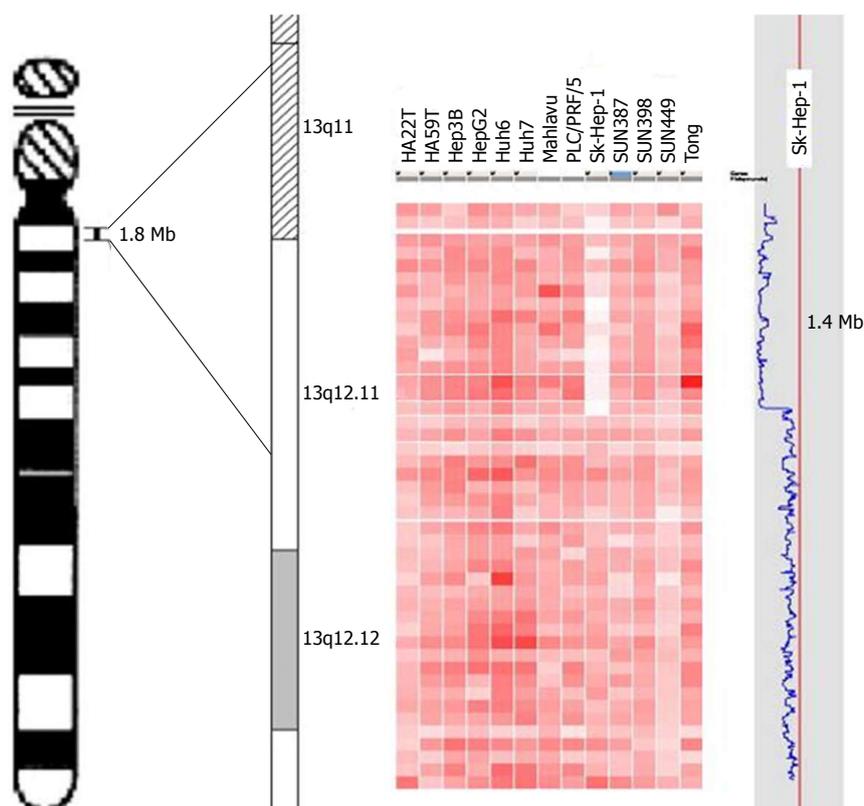
Figure 2 Refinement of homozygous deletion by copy number alteration analysis at chromosome 13q12.11 in hepatocellular carcinoma cells.

Table 2 List of some integrated cancer genomic databases

Database	Project	Website	Ref.
cBioPortal for cancer genome	Project provides visualization, analysis and download of large-scale cancer genomic data sets	http://www.cbioportal.org/public-portal/	Cerami *et al*[57]
COSMIC	Catalogue of somatic mutations in cancer	http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/	Forbes *et al*[58]
ICGC	International Cancer Genome Consortium provides tools for visualizing, querying and downloading the data.	http://dcc.icgc.org/	Joly *et al*[59]
TCGA data portal	A platform for researchers to search, download, and analyze data sets generated by TCGA	https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp	
Tumorscape	High-resolution copy number data collected from multiple cancer types	http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf	
UCSC cancer genome browser	A set of web-based tools to display, investigate and analyze cancer genomic data and associated clinical information	https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/	Goldman *et al*[60]

COSMIC: Catalogue of somatic mutations in cancer.

it will not reveal balanced translocation but will detect sudden dosage changes for unbalanced translocation. Using CNA analysis and high-density SNP arrays, *PAX5* fusion genes were identified with a variety of partner genes, including *ETV6*, *FOXP1*, *AUTS2*, and *C20orf112,* in pediatric acute lymphoblastic leukemia (ALL)[48].

INTEGRATED HCC CANCER GENOMIC DATABASES WITH CNAS

Integrated data derived from multiple genomic approaches could potentially avoid pitfalls of data inconsistency usual with the single genomic approach and provide lines of evidence to validate target genes embraced in the aberrant genomic loci from the level of DNA and RNA to protein. For these advantages, several user-friendly HCC databases were constructed, including OncoDB.HCC, HCCnet, dbHCCvar, CellMinerHCC, HCC-M, and EHCO[49-54]. However, only OncoDB.HCC integrated genomic alteration data to prioritize HCC cancer genes for further expression and functional validations in HCC cell lines and tissues. Nevertheless, recent international efforts at applying high-throughput short-read sequencing technologies and CNA analysis of cancer genomes in multiple cancer types, including HCC, comprehensively cataloged different types of somatic mutations and revealed genetic heterogeneity even from the same subtype

of cancer. Table 2 lists common open-access integrated cancer genome databases for downloading and visualizing cancer genomic data[55,56].

CONCLUSION

As discussed in this review article, an integrated genomic approach is an effective and essential method of identifying novel HCC genes. With the availability of a tremendous amount of high-throughput short-read sequencing data and SNP array data from cancer genomes deposited in the public domain, integrated genomic approaches, including CNA analysis, are the most cost-effective approach for revealing HCC driver genes for improving HCC therapy.

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TOPIC HIGHLIGHT
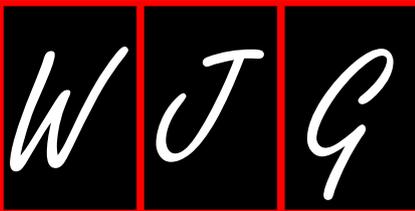
WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Mitochondrial DNA alterations and mitochondrial dysfunction in the progression of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is ranked third in mortality among cancer-related diseases. Mitochondria are intracellular organelles that are responsible for energy metabolism and cellular homeostasis, and mitochondrial dysfunction has been regarded as a hallmark of cancer. Over the past decades, several types of mitochondrial DNA (mtDNA) alterations have been identified in human cancers, including HCC. However, the role of these mtDNA alterations in cancer progression is unclear. In this review, we summarize the recent findings on the somatic mtDNA alterations identified in HCC and their relationships with the clinicopathological features of HCC. Recent advances in understanding the potential roles of somatic mtDNA alterations in the progression of HCC are also discussed. We suggest that somatic mtDNA mutations and a decrease in the mtDNA copy number are common events in HCC and that a mitochondrial dysfunction-activated signaling cascade may play an important role in the progression of HCC. Elucidation of the retrograde signaling pathways in HCC and the quest for strategies to block some of these pathways will be instrumental for the development of novel treatments for this and other malignancies.

Core tip: In this review, we summarize the recent findings on the somatic mtDNA alterations identified in hepatocellular carcinoma (HCC) and their relationships with the clinicopathological features of HCC. We suggest that somatic mtDNA mutations and a decrease in the mtDNA copy number are common events in HCC and that a mitochondrial dysfunction-activated signaling cascade may play an important role in the progression of HCC. Elucidation of the retrograde signaling pathways in HCC and the quest for strategies to block some of these pathways will be instrumental for the development of novel treatments for this and other malignancies.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the one of most common cancers worldwide and is ranked third with respect to mortality[1,2]. There are approximately 434000 new cases of HCC per year[3]. Several risk factors have been suggested to be involved in the development of HCC, including aflatoxin exposure, alcohol consumption, chronic inflammation associated with viral hepatitis and familial tendency[4-8]. Moreover, inflammation and oxidative stress have been suggested to contribute to the carcinogenesis of HCC[9-11].

In the 1930s, the German biochemist Warburg[12] proposed that tumor cells prefer to utilize glycolysis rather than respiration as a primary energy source, even in the presence of abundant oxygen. This phenomenon was termed "aerobic glycolysis" or the "Warburg effect". He further proposed that defects in energy metabolism, especially in the mitochondria, are involved in the initiation or progression of cancer[13].

Mitochondria are cytoplasmic organelles that play multiple roles in energy metabolism and cellular homeostasis, including the generation of ATP *via* respiration and oxidative phosphorylation (OXPHOS), the production of reactive oxygen species (ROS), metabolic homeostasis, and the initiation and execution of apoptosis[14,15]. These roles are executed by proteins that are encoded by genes in the nucleus and mitochondria. Mitochondrial DNA (mtDNA) is a 16.6-kb, double-stranded circular DNA that contains genes for 22 transfer RNAs, 2 ribosomal RNAs and 13 polypeptides that comprise the respiratory enzyme complexes[16]. In addition to the coding region, mtDNA contains a non-coding region called the "D-loop", which is approximately 1.1 kb, encompasses nucleotide position (np) 16024-np 576, and controls the replication and transcription of the mtDNA[17].

Due to its lack of protective histone proteins, a limited DNA repair system and its spatial proximity to a high level of ROS, mtDNA sustains a 10-fold higher level of damage than that of nuclear DNA (nDNA)[18-20]. Somatic mutation and damage to mtDNA can lead to impairment of the OXPHOS system and enhanced ROS generation, which in turn accelerates the occurrence of DNA mutations. This scenario has been proposed to contribute to the initiation and progression of tumors[21,22].

Over the past decade, somatic mtDNA mutations have been identified in several types of cancer, including HCC[23-27]. Some of the acquired mtDNA mutations have been suggested to cause mitochondrial dysfunction, increase the production of ROS, and promote tumor growth[28,29].

In this article, we review the recent findings on somatic mtDNA alterations in HCC. In addition, we discuss the potential roles of mtDNA alterations and mitochondrial dysfunction in the progression and metastasis of HCC.

SOMATIC MITOCHONDRIAL DNA ALTERATIONS IN HCC

Over the past decade, several types of somatic mtDNA
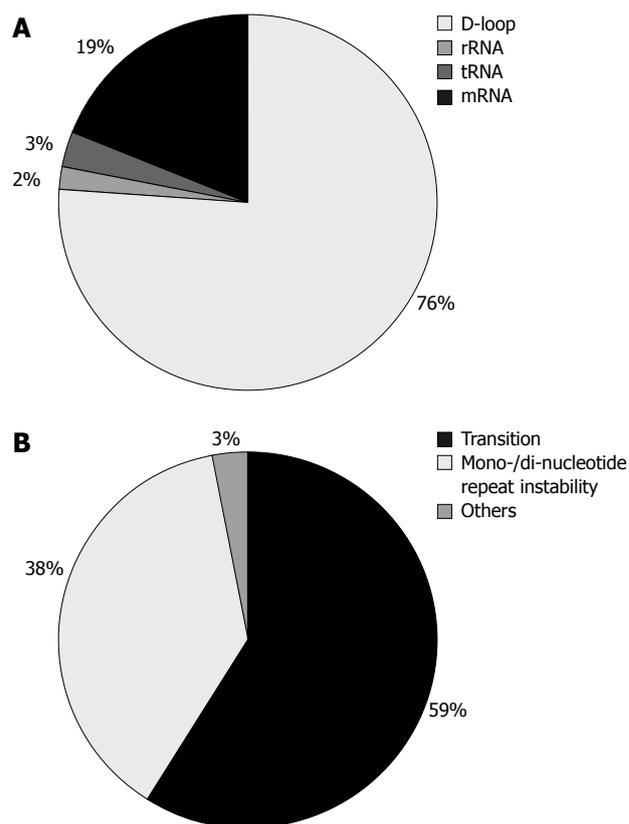
Figure 1 The location distribution of the identified somatic point mutations (A) and the types of somatic point mutations (B) in the mitochondrial DNA in hepatocellular carcinoma. Data adapted from Yin *et al*[29] and Wong *et al*[30].

alterations have been identified in human HCC. These mtDNA alterations include point mutations, deletions, insertions and copy number changes.

Point mutations

Screening for somatic point mutations in the whole mitochondrial genomes of HCC samples[29,30] revealed that approximately 52% of HCC patients carry at least one homoplasmic or heteroplasmic point mutation in their tumor tissue mtDNA. Of the identified point mutations, 76% are located in the D-loop region, 2% are located in rRNA genes, 3% are located in tRNA genes and 19% are located in mRNA genes (Figure 1A). The incidence and location distribution of the point mutations are consistent with those observed in other cancer types[25].

The D-loop region is a hot spot for somatic mtDNA mutations in HCC and other cancers. It was reported that the D-loop region of mtDNA, especially the mononucleotide repeat in the np 303-309 poly-C sequence, is the most susceptible site to oxidative damage compared with the other regions of the mtDNA, implying that oxidative damage contributes to point mutations in the D-loop and/or the instability of the mononucleotide or dinucleotide repeats in the mtDNA. However, the unique G-to-T transversion caused by oxidative DNA damage is not detected in HCC[29,30]. Among the mtDNA mutations that have been identified in HCC, approximately 59% are transition mutations (G/A-to-A/G or C/T-to-T/C) and

38% are mono- or di-nucleotide instabilities (Figure 1B), suggesting that oxidative damage *per se* is not the major factor responsible for point mutations in HCC. The presence of hepatitis B infection, liver cirrhosis, alcohol abuse or their combination may affect the qualitative changes in the mtDNA in HCC[30].

Because the D-loop region controls the replication and transcription of the mtDNA, mutations in the D-loop region may influence the mtDNA copy number and the expression of the mitochondrial genome[31]. It has been shown that the occurrence of point mutations in the D-loop, especially near the replication origin of the heavy-strand (O_H) of the mtDNA, affects the mtDNA copy number in HCC[32]. In addition, Nishikawa *et al*[33] reported that the number of mtDNA mutations in the D-loop region is positively correlated with a poor HCC differentiation grade. These findings suggest that the somatic mutations in the mtDNA D-loop region may affect mitochondrial function by decreasing the mtDNA copy number and/or transcription in HCC, thereby leading to HCC progression.

Among the mutations in the coding region, the nonsense mutation G3842A creates a premature stop codon and the missense mutations T6787C, G7976A, G9267A, and A11708G result in amino acid substitutions in the highly evolutionarily conserved regions of the affected mitochondrial genes. Moreover, the base-pair deletion and insertion 11032delA and 12418insA may lead to a frame-shift mutation, and the tRNA mutations T1659C in tRNA^{Val} and G5650A in tRNA^{Ala} may alter the tRNA structure and were shown to associate with mitochondrial disorders[29]. Therefore, these mtDNA point mutations may result in mitochondrial dysfunction in HCC.

Deletions

Among the large-scale deletions identified in the mtDNA in different cancer types[32,34-37], the 4977-bp deletion is the most common mtDNA deletion in tumors[23,38-43]. Consistent with findings in other types of cancer, the incidence of the 4977-bp deletion and its accumulation level are lower in the malignant tissues than the non-tumor tissues of HCC patients[23,39]. Moreover, gender and a long-term history of alcohol consumption in HCC patients may affect the accumulation of the 4977-bp-deleted mtDNA[23]. Although the role of mtDNA deletion in HCC is unclear, it has been suggested that the observed decrease in mtDNA with a deletion is the result of the tumor cells adapting to a new microenvironment during hepatocarcinogenesis[25,44].

In addition, a 50-bp deletion was previously reported in one HCC patient[32]. This deletion is flanked by a 9-bp direct repeat in the D-loop region of the mtDNA. The mtDNA deletion appeared to be homoplasmic in the HCC tissue but was not detected in the corresponding non-tumor liver tissue. The tumor-specific accumulation of this deletion does not seem to be similar to that of the 4977-bp deletion in cancers. Because this deletion

partly truncates the regulatory region of the mtDNA, the mtDNA copy number in the HCC tissue was found to be significantly reduced compared with that in the non-tumor liver tissue[32]. This mtDNA deletion may lead to mitochondrial dysfunction *via* mtDNA depletion and/or impairment of the transcription of mitochondrial genes.

Insertions

Two small insertions (approximately 260 bp and approximately 520 bp) have been identified as a tandem duplication and a tandem triplication and are flanked by two poly-cytosine (poly-C) sequences at np 303-309 and np 568-573 in the D-loop region of the mtDNA in various human cancers, including HCC[35]. This tandem duplication or triplication was detected in approximately 4% of HCCs and is highly correlated with the presence of length variation in the poly-C at np 568[44]. However, these insertions have also been found in somatic tissues in elderly subjects and, thus, are not specific for cancer tissues.

Copy number changes

A decrease in the mtDNA copy number is a common event in HCC[23,32,45,46]. Over 60% of HCCs have a lower mtDNA copy number than their corresponding non-tumor liver tissues. As mentioned above, it was observed that the reduction in the mtDNA copy number is associated with point mutations located near the replication origin in the D-loop region of the mtDNA[32]. Moreover, it was suggested that the decrease in the mtDNA copy number in HCC may be related to or result from the altered expression of genes involved in mitochondrial biogenesis, such as peroxisome proliferator-activated recept activator-1 (PGC-1) and mitochondrial single-stranded DNA binding protein (mtSSB)[23]. These results suggest that the mtDNA mutations in the D-loop region and the impairment of mitochondrial biogenesis contribute to the decrease in the mtDNA copy number in HCC[34].

The reduction in the mtDNA copy number seems to be more frequently observed in female patients with HCC compared with male patients with HCC[23]. This difference between male and female HCC patients could be a result of clinical manifestation, progression and/or mortality rate[23]. Yamada *et al*[46] showed that the low mtDNA copy number in HCC is significantly correlated with large tumor size and liver cirrhosis. In addition, HCC patients with a lower mtDNA copy number in their tumors tend to show poorer 5-year survival compared with patients with a higher mtDNA copy number[46]. It was also suggested that hepatitis B infection, liver cirrhosis, and alcohol abuse affect quantitative changes in the mtDNA in HCC[29]. Recently, it was reported that there is an association between the mtDNA content in the peripheral blood leukocytes and hepatitis B virus-related hepatocellular carcinoma[47], which suggests that the mtDNA copy number in the peripheral blood leukocytes could be used as a predictor of HCC occurrence.

POTENTIAL ROLES OF MITOCHONDRIAL DNA MUTATIONS AND MITOCHONDRIAL DYSFUNCTION IN HCC PROGRESSION

Several types of somatic mtDNA alterations have been identified in HCC, but the roles of these mtDNA alterations in HCC progression are unclear. Evidence from several lines of research has substantiated the pathological role of mtDNA mutation and mitochondrial dysfunction in HCC.

The majority of the somatic point mutations in the mitochondrial coding region and the decrease in the mtDNA copy number may cause mitochondrial dysfunction in HCC. These findings provide a molecular basis for the Warburg effect. In addition, it has been shown that the low mtDNA copy number in HCC is significantly correlated with large tumor size, liver cirrhosis, and poor 5-year survival[46]. Therefore, it is possible that mtDNA mutations and a decrease in the mtDNA copy number and, thereby, mitochondrial dysfunction modify the progression of HCC.

In the human SK-Hep1 hepatoma cell line, mtDNA depletion was demonstrated to induce resistance to oxidative stress and chemotherapeutic agents through an adaptive increase in the expression of manganese superoxide dismutase (MnSOD) and other antioxidant enzymes[48]. Moreover, chloramphenicol was found to inhibit mitochondrial protein synthesis in human hepatoma HepG2 cells and to render these cancer cells resistant to mitomycin-induced apoptosis[49]. Using similar approaches, respiratory inhibitors and an uncoupler of mitochondrial respiration as well as inhibitors of mtDNA replication or protein synthesis in the mitochondria were found to induce mitochondrial dysfunction and cisplatin resistance in human hepatoma HepG2 cells and to promote cell migration in other hepatoma cells *via* a paracrine signaling pathway[50]. It was further demonstrated that the mitochondrial dysfunction-induced upregulation of amphiregulin contributes to the cisplatin resistance and cell migration of hepatoma cells[50]. In addition, these treatments also induced changes in the expression of genes that affect the metastatic ability of cancers, including the integrin pathway, the PDGF signaling pathway and the cadherin signaling pathway[51]. On the other hand, the overexpression of PGC-1 in HepG2 cells was found to elevate mitochondrial protein expression and to reduce cell mobility *via* increased E-cadherin expression[52,53]. These findings support the hypothesis that mtDNA mutations and mitochondrial dysfunction contribute to the malignant progression of HCC.

Mitochondrial dysfunction increases ROS production and Ca^{2+} mobilization and reduces ATP generation, which may be involved in the malignant changes induced by mtDNA mutations and mitochondrial dysfunction in HCC. It has been demonstrated that antioxidants and calcium chelators can block mitochondrial dysfunction-induced amphiregulin expression and prevent cisplatin resistance and cell migration[50]. In addition, it was re-

cently demonstrated that mitochondrial dysfunction-reduced intracellular ATP content represses the protein expression of hypoxia-inducible factor-1 (HIF-1) through the activation of the AMP-activated protein kinase (AMPK)-mTOR pathways in HepG2 cells[54]. HIF-1 is a nuclear transcription factor that plays a crucial role in cancer progression, including angiogenesis, invasion and metastasis[55]. These findings suggest that mitochondrial dysfunction regulates nuclear gene expression and phenotypic changes to face the different microenvironments of HCC. Therefore, the activation of retrograde signaling from the mitochondria to the nucleus may play an important role in the malignant progression of HCC.

Consistent findings were observed in other types of cancer. It has been reported that in some cancer cell lines, a pathogenic mtDNA mutation (*e.g.*, the T8993G transversion) promotes tumor growth in nude mice by preventing apoptosis[56-58]. Moreover, it was shown that the heteroplasmic 12418insA mutation, which has been identified in HCC[29] and other cancers[24,26,59], impairs mitochondrial respiratory function and promotes tumorigenesis by enhancing ROS production[60]. In addition, ROS-generating mtDNA mutations have been demonstrated to regulate tumor cell metastasis[61]. It was also shown that mtDNA depletion or mitochondrial dysfunction can enhance invasive phenotype changes[62-64] or chemo-resistance in some specific types of cancer[65]. The underlying mechanisms have been suggested to involve the communication between the mitochondria and the nucleus called "retrograde signaling"[66,67]. Several biomolecules have been identified to be involved in this signal transduction, including calcineurin, NFAT, ATF2, Akt, and NFκB/Rel, which then affect the expression of an array of nuclear genes[68,69]. The detailed mechanisms by which mtDNA mutations and mitochondrial dysfunction affect HCC progression await further investigation.

Although mtDNA alterations have been identified in HCC, it remains controversial whether mtDNA alterations are correlated with the initiation and progression of HCC. To dissect the role of mtDNA alterations in HCC, a larger sample size of HCC is required in future research. Moreover, some lines of evidence suggest that mitochondrial dysfunction and the dysfunctions caused by mtDNA alterations have the potential to contribute to tumor progression. However, whether a specific mtDNA mutation plays a driving force or is an indirect consequence of HCC progression requires further evaluation. Therefore, it is important to develop a strategy to dissect the role of specific mtDNA mutations in cancer progression and/or to exclude non-causal epiphenomena.

Because the coordination between the nDNA and mtDNA is important for the maintenance of mitochondrial structure and function[14,17], mutations in the nDNA-encoded genes that are responsible for mtDNA integrity and/or mitochondrial function may play an important role in tumorigenesis and cancer progression. For example, it was recently reported that defects in P53[70], mitochondrial DNA polymerase[71], and mitochondrial

deacetylase SIRT3[72] may affect mtDNA integrity and promote tumorigenesis. In addition, not only mtDNA mutations but also mitochondrial dysfunction caused by nDNA mutations, oncogenes, and tumor microenvironments (hypoxia and inflammation) are suggested to underlie energy metabolism reprogramming (or the Warburg effect)[73,74]. In summary, the interaction between mtDNA and nDNA may play an important role in the initiation and progression of HCC.

CONCLUSION

Several types of mtDNA alterations, including point mutations, deletions, insertions and copy number changes, have been identified in HCC. Somatic point mutations and depletions are the two most common of these mtDNA alterations in HCC. The low mtDNA copy number in HCC has been shown to be significantly correlated with large tumor size, liver cirrhosis, and poor 5-year survival[46]. However, the presence of somatic mtDNA point mutations in HCC does not seem to correlate with the patient's age or sex, the tumor size or grade, hepatitis virus infection, or the patient's survival[29,30]. This finding may result from the possibility that mtDNA point mutations do not always play a similar role in HCC progression. In addition, the heteroplasmic or homoplasmic level of the same mtDNA mutation may result in different consequences in tumorigenesis[60]. Therefore, the role of the specific mtDNA mutation and its level during HCC progression warrant further study.

The majority of the point mutations in the coding region of the mtDNA and the decrease in the mtDNA copy number likely cause mitochondrial dysfunction in HCC. These findings have provided solid evidence to substantiate the mechanism by which mitochondrial dysfunction is involved in metabolic reprogramming or the "Warburg effect" in cancer. Several lines of evidence have important implications in the pathological role of mtDNA mutation and mitochondrial dysfunction in HCC. Pharmacologic approaches to induce mitochondrial dysfunction can enhance chemo-resistance and promote metastasis, which may contribute to the malignant progression of HCC. Thus, the increased ROS production and Ca^{2+} mobilization and the reduced ATP generation induced by mitochondrial dysfunction may be involved in the malignant changes of HCC. However, the detailed mechanism by which mtDNA mutation and mitochondrial dysfunction affect HCC progression remains unclear. Elucidation of the retrograde signaling pathways in HCC and the search for strategies to block these pathways will be important for the development of novel treatments for this and other malignancies.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Prevention of hepatocellular carcinoma in chronic viral hepatitis B and C infection

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Abstract

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality worldwide, with the majority of cases associated with persistent infection from hepatitis B virus (HBV) or hepatitis C virus (HCV). Natural history studies have identified risk factors associated with HCC development among chronic HBV and HCV infection. High-risk infected individuals can now be identified by the usage of risk predictive scores. Vaccination plays a central role in the prevention of HBV-related HCC. Treatment of chronic HBV infection, especially by nucleoside analogue therapy, could also reduce the risk of HBV-related HCC. Concerning HCV infection, besides the advocation of universal precautions to reduce the rate of infection, pegylated interferon and ribavirin could also reduce the risk of HCV-related HCC among those achieving a sustained virologic response. Recently there has been mounting evidence on the role of chemopreventive agents in reducing HBV- and HCV-related HCC. The continued advances in the understanding of the molecular pathogenesis of HCC would hold promise in preventing this highly lethal cancer.

Core tip: Hepatocellular carcinoma (HCC), with the majority of cases associated with infection from hepatitis B virus (HBV) or hepatitis C virus (HCV), is the most common primary liver tumor. We introduced risk factors and risk predictive scores associated with HCC development among chronic HBV and HCV infection for its early diagnose and prevention. Vaccination plays a central role in the prevention of HBV-related HCC. Treatment of chronic HBV infection, especially by nucleoside analogue therapy, could reduce the risk of HBV-related HCC. Pegylated interferon and ribavirin could reduce the risk of HCV-related HCC. Chemopreventive agents in reducing HBV- and HCV-related HCC were also discussed.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver tumor, and represents the third leading cause of cancer death worldwide. It is the fifth most common cancer in men and seventh in women, accounting for 7% of all cancers[1]. Hepatocarcinogenesis

is a multistep process mainly associated with persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV)[2], which affects more than 350 and 170 million individuals respectively worldwide. HCC is highly prevalent in regions endemic for chronic HBV and HCV infection[3].

The incidence of HCC continues to increase worldwide, with a unique geographic, age, and sex distribution. The most important risk factor associated with HCC is liver cirrhosis, which is again predominantly caused by chronic HBV or HCV infection. Primary prevention in the form of HBV vaccination has led to a significant decrease in HBV-related HCC, and the antiviral therapy for chronic HBV and HCV infection also reduce the incidence of HBV- and HCV-related HCC[4].

China has one of the highest carrier rates of HBV in the world，reaching nearly 10% of the general population. The disease burden of HBV infection and HCC is also believed to be among the world's largest, and that of HCV infection is likely to be substantial as well[5].

RISK PREDICTION

An important component of HCC prevention is the identification of high-risk HBV- and HCV-infected individuals, who will benefit from various chemopreventive therapies discussed below. Several natural history studies have identified important risk factors for HCC among patients with chronic hepatitis B (CHB) and chronic hepatitis C (CHC), with risk predictive scores also designed for practical usage.

Risk factors and prediction scores: CHB
A study evaluating the relationship between serum HBV DNA level and risk of HCC demonstrated that the incidence of HCC among CHB patients increased with serum HBV DNA level. Elevated serum HBV DNA level ($\geqslant$ 2000 IU/mL) is a strong risk predictor of HCC independent of hepatitis B e antigen (HBeAg)-positivity, serum alanine aminotransferase levels, and liver cirrhosis[6]. Subsequent studies also showed patients with moderate levels of serum HBV DNA (60-2000 IU/mL), when compared to individuals not infected with HBV, still had a substantial increased risk of HCC and liver-related death[7].

Besides serum HBV DNA levels, other host- and viral-related factors could also predispose to HCC. A meta-analysis found HBeAg-positive non-cirrhotic patients, when compared to HBeAg-positive cirrhotic patients, had a significantly reduced HCC risk after antiviral therapy[8]. HBV genotype also plays a role; HBV genotype C is closely associated with HCC especially in cirrhotic patients aged > 50 years[9]. An observation study in Hong Kong also found genotype C HBV infection to be an independent risk factor for HCC development when compared with genotype B[10].

Several clinical scoring systems have been developed for the prediction of HCC in CHB, as depicted in Table 1. These scoring systems are based on the longitudinal follow-up of treatment-naïve CHB patients for 5 years or more. Two common parameters used are age and serum HBV DNA levels. Other parameters used include gender, serum alanine aminotransferase levels, serum albumin, HBeAg status, presence of cirrhosis and presence of core promoter mutations[11-15]. Risk prediction is now also possible for CHB patients undergoing nucleoside analogue (NA) therapy. A recent study investigated the risk of HCC among a large population of CHB patients treated with entecavir. Older age and presence of cirrhosis were independently associated with HCC in the entire cohort; advanced age and hypoalbuminemia were associated with HCC in patients without cirrhosis. The risk scores accurately predict which patients with CHB treated with entecavir would have a higher chance of developing HCC[13].

Risk factors: CHC
When compared to CHB, fewer clinical scoring systems have been developed for the prediction of HCV-related HCC. These are as depicted in Table 1. The majority of HCV-related HCC develop in patients with established cirrhosis. In a study investigating prognostic risk factors for HCV-related HCC, among 913 patients followed up for at least 3 years, age, male sex, portal hypertension, hepatic inflammation, and iron storage were significant risk factors for HCV-related HCC[16]. In a meta-analysis involving HCV-infected persons, sustained virologic response (SVR) was associated with reduced risk for HCC[17]. Even transient virologic control among patients with subsequent relapse after treatment, was associated with a lower risk of the development of HCC[18].

Prediction of HCV-related HCC may be enhanced by the development of related markers. Signal transducer and activator of transcription 1 and phosphatase and tensin homolog are associated with early growth response protein 1 signaling, which potentially promotes angiogenesis, fibrogenesis, and tumorigenesis in HCV-related HCC. This approach has potential for the early diagnosis and possible prevention of HCC. The corresponding serum markers found can help to predict high-risk groups for HCC[19].

Host factors
HCC is more common in HBV carriers with a family history of HCC. In a study of 5238 HBV carriers (553 with HCC and 4685 without HCC), the risk of HCC was significantly higher in those with a family history of HCC, with a multivariate-adjusted rate ratio for HCC of 2.41 compared with HBV carriers without a family history[20]. If the carriers had two or more affected family members, the risk was even higher with the ratio increased to 5.55. It is therefore recommended to begin surveillance in adults once a family history of HCC has been identified. A recently published study also included the presence of family history, besides traditional viral-related parameters as a component for risk prediction[15].

Table 1 Risk factors and prediction scores for hepatitis B virus- and hepatitis C virus-related hepatocellular carcinoma

Risk factors	HBV-related HCC	HCV-related HCC
Increased age	√[11-15]	√[16]
Male gender	√[11,12,15]	√[16]
Increased serum HBV DNA levels	√[11,12,14,15]	
Presence of cirrhosis	√[12-14]	
Increased serum ALT concentration	√[11,15]	
HBeAg positivity	√[11,15]	
Presence of core promoter mutations	√[12]	
Presence of virological remission after 24 mo	√[13]	
Presence of hypoalbuminemia	√[13]	
Decreased serum albumin	√[14]	
Increased serum bilirubin	√[14]	
HBV genotype C	√[15]	
Presence of HBsAg	√[15]	
Family history of HCC	√[15]	
Presence of portal hypertension		√[16]
Presence of hepatic inflammation		√[16]
Increased iron storage levels		√[16]
Presence of sustained virological response		√[17]
Presence of complete viral suppression		√[17]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase.

Table 2 Antiviral interventions for prevention of hepatitis B virus- and hepatitis C virus-related hepatocellular carcinoma

Antiviral interventions	HBV-related HCC	HCV-related HCC
IFN: IFN-α	+/-[28,29]	√[46]
Pegylated IFN		√[47]
NAs: Lamivudine	√[36]	
Entecavir	√[37,38]	
Ribavirin		√[47]
Vaccination	√[21,22]	
Screening of blood product	√[27]	√[27]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; IFN: Interferon; NA: Nucleotide analogs.

Table 3 Chemopreventive agents for hepatitis B virus- and hepatitis C virus-related hepatocellular carcinoma

Chemopreventive agents	HBV-related HCC	HCV-related HCC
Statins	√[42]	
Antidiabetic medications	√[42]	√[42]
Aspirin	√[41,53]	√[53]
Propranolol		√[51]
FASN		√[52]
Dietory agents: Coffee	√[54]	√[54]
Vitamin E	√[54]	√[54]
Vitamin D		√[50]
Fish oil (n-3 PUFA)	√[55-57]	√[55-57]
Phytochemicals: Resveratrol	√[43]	
EGb	√[44]	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PUFA: Polyunsaturated fatty acid; FASN: Fatty acid synthase; EGb: Extract of ginkgo biloba leaf.

PREVENTION OF HBV-RELATED HCC

HBV infection is the major cause of HCC. Vaccination against HBV is instrumental in the prevention of HCC, and is recommended for all newborns and individuals who are at increased risk for infection. Studies in Taiwan, where universal HBV vaccination was introduced in 1984, have documented a significant decrease in the incidence of HCC in both children and adolescents after the introduction of HBV vaccination as discussed below[21,22].

In patients already chronically infected with HBV, antiviral treatment could prevent disease progression to cirrhosis or HCC. Additionally, periodic surveillance using ultrasonography and serum α-fetoprotein every 3-6 mo for earlier detection of HCC is also important so that curative treatments (*e.g.*, hepatic resection) can be offered[23].

The antiviral interventions and chemopreventive methods to prevent HBV-related HCC are summarized in Tables 2 and 3 respectively.

Vaccination

Vaccination plays a central role in HBV prevention strategies worldwide, and a decline in the incidence and prevalence of HBV infection following the introduction of universal HBV vaccination programs has been observed in many countries[24]. Control and significant reduction in incidence of new HBV infections as well as HCC have been repeatedly reported in countries in East Asia and Africa[25].

A study of the incidence of HCC in children in Taiwan from 1981 to 1994 showed that the average annual incidence of HCC in children 6-14 years of age declined from 0.70 per 100000 children (between 1981 and 1986), to 0.57 per 100000 (between 1986 and 1990), and to 0.36 per 100000 (between 1990 and 1994). The corresponding rates of mortality from HCC had also decreased. The incidence of HCC in children 6-9 years of age declined from 0.52 per 100000 (for those born between 1974 and 1984) to 0.13 per 100000 (for those born between 1984 and 1986). Since the institution of Taiwan's program of universal HBV vaccination from 1984, the incidence of HCC in children has declined dramatically[22]. The risk of developing HCC for vaccinated cohorts was statistically significantly associated with incomplete HBV vaccination. The prevention of HCC by HBV vaccination extends from childhood to early adulthood. Failure to prevent HCC results mostly from unsuccessful control of HBV infection by highly infectious mothers[21].

Antiviral therapy: Interferon and NAs

DNA integration of hepatitis viruses alters the function of critical genes, promoting malignant transformation of virus-infected liver cells[26]. Treatment of CHB infection aims to control viral replication and prevent the development of complications. There are currently seven

drugs available for the treatment of CHB, five NAs and two interferon (IFN)-based therapies. Long-term treatment with NA is often required, and the decision to treat is based on the clinical assessment including the phase of CHB infection and the presence and extent of liver damage[24].

Concerning IFN therapy, a study involving 641 biopsy-proven CHB patients treated with IFN-α2b were followed up for a median period of 113 months. Although HCC occurred less frequently in biochemical responders than in non-responders, virologic response is not associated with decrease in HCC development. Poor biochemical response, as well as older age and a higher serum AFP level remain independent predisposing factors of HCC development in CHB patients treated with IFN-α[27]. In addition, a study about the long-term effects of IFN-α in Chinese patients showed that IFN-α was of no long-term benefit in inducing HBeAg seroconversion or in the prevention of HCC and other cirrhosis-related complications[28].

On the contrary, nearly all the studies showed that NA is able to reduce HCC[29]. Many randomized controlled trials showed that lamivudine, one of the earliest oral NAs for antiviral therapy in HBV infection, can reduce disease progression in HBV-related cirrhosis and HCC[29-34]. A recent study followed up 293 CHB patients without HCC who were treated with lamivudine for a mean duration of 67.6 mo. In cirrhotic patients, the attainment of maintained viral response (defined as HBV-DNA levels of < 4.0 log copies/mL) during lamivudine treatment was revealed to reduce the risk of HCC development. No significant reduction was observed in the non-cirrhotic group[35].

Entecavir is a potent NA with high genetic barrier to resistance, and prolonged treatment results in regression of fibrosis, hence is currently recommended as first-line antiviral therapy for CHB. In a study of CHB patients with liver cirrhosis, entecavir therapy reduces the risks of hepatic complications, HCC, liver-related and all-cause mortality of CHB patients with liver cirrhosis in 5 years, particularly among those who had sustained viral suppression[36]. In another multicentre cohort study, 372 entecavir-treated patients followed up for a mean duration of 114 mo were investigated. Clinical events were defined as development of HCC, hepatic decompensation or death. Virological response to entecavir (HBV DNA < 80 IU/mL) was associated with a lower probability of disease progression in patients with cirrhosis, suggesting that complete viral suppression is essential for NA treatment, especially in patients with cirrhosis[37].

A meta-analysis investigating the effects of IFN or NA on the risk of developing HCC in CHB patients shows that, the reduction in HCC is more significant among patients with early cirrhosis than among non-cirrhotic patients. Five studies (*n* = 2289) compared patients treated by NA with control. The risk of HCC after treatment is reduced by 78%. HBeAg-positive patients have a more significant reduction in HCC risk with treatment.

Patients without cirrhosis benefit more from NA than those with cirrhosis, although resistance to NA blunts the benefit of treatment[8].

In summary, while the evidence of the efficacy of IFN in preventing HBV-related HCC remains conflicting, there is a gradual accumulation of evidence supporting the positive effect of NA on reducing HBV-related HCC.

Chemoprevention

The observation that anti-platelet therapy inhibits or delays immune-mediated hepatocarcinogenesis suggests that platelets may be one of the key players in the pathogenesis of HBV-associated liver cancer and that immune-mediated necroinflammatory reactions may be an important cause of malignant transformation during chronic hepatitis[38]. A prospective study on 300504 patients with chronic liver disease showed that aspirin users had statistically significant reduced risks of incidence of HCC and mortality due to chronic liver disease compared to those who did not use aspirin[39]. Further studies are needed to confirm this finding and clarify its underlying mechanism.

A study concerning the association between the use of statins in HBV-infected patients and the risk of HCC shows that statin use may reduce the risk for HCC in HBV-infected patients in a dose-dependent manner[40]. This may be related to the effect of statins in reducing fatty change in the liver, and requires future validation studies to confirm the findings.

There are also several investigational drugs which could have potential for chemoprevention against HBV-related HCC. Resveratrol is a natural polyphenol that has beneficial effects across various disease models. In an animal study investigating the efficacy of resveratrol against HBV-related HCC in HBV X protein (HBx) transgenic mice, resveratrol had a pleiotropic effect on HBx transgenic mice in terms of the down-regulation of lipogenesis, the promotion of transient liver regeneration, and the stimulation of antioxidant activity. Furthermore, at later precancerous stages, resveratrol delayed HBx-mediated hepatocarcinogenesis and reduced HCC incidence from 80% to 15%. The potential mechanisms for resveratrol on HCC prevention might be associated with its effects of stimulating the activity of Ampk and SirT1, and downregulating the expression of the lipogenic genes, Srebp1-c and peroxisome proliferator-activated receptor gamma. The decrease in Srebp1-c further downregulates the expression of its target genes, Acc and Fas[41]. Several other studies demonstrated resveratrol downregulates cyclin D1 as well as p38 MAP kinase, suppresses Akt and Pak1 expression and activity, and increases ERK activity, suggesting that growth inhibitory activity of resveratrol is associated with the downregulation of cell proliferation and survival pathways, and sensitization to apoptosis[42]. Resveratrol also acts as an inhibitor for sirtuins. Overexpression of SIRT1 in cancer tissue has been demonstrated to promote mitotic entry of liver cells, cell growth and proliferation, and inhibit apoptosis related to the PTEN/PI3K/AKT signaling pathway[43,44].

A study in China suggested that extract of Ginkgo Biloba leaf (EGb) could reduce the incidence of the HCC with HBV transgenic mice. The reason may be that EGb could reduce liver HBx, p53, Bcl-2 protein expression in HBV transgenic mice[45]. These investigational products would need confirmation in human clinical trials in the future.

PREVENTION OF HCC RELATED TO HCV

With the commencement of successful vaccination programs against HBV, CHC is now emerging as an important cause of chronic liver diseases. The drive of carcinogenesis during HCV infection is thought to result from the interactions of viral proteins with host cell proteins. Thus, the induction of liver mutation phenotypes through the expression of HCV proteins provides a key mechanism for the development of HCV-associated HCC. With the emerging importance of CHC, mechanisms of HCV-associated hepatocellular carcinogenesis should be clarified to provide insight into advanced therapeutic and preventive approaches to decrease the incidence and mortality of HCC[46].

Strategies aimed at eliminating the virus may provide opportunities for effective prevention of the development of HCC. The first step is to encourage universal precautions to reduce infections transmitted *via* different modalities *e.g.*, iatrogenic routes, sharing of intravenous needles etc and further implementation of universal screening of donated blood products. Concerning therapy for HCV, pegylated IFN plus ribavirin therapy is effective at reducing the risk of HCC in patients with CHC who achieve SVR.

The effects of antiviral therapy and chemopreventive measures in preventing HCC are mentioned in Tables 2 and 3 respectively.

Antiviral therapy: IFN and ribavirin

Current strategies to reduce HCC incidence in CHC patients include prevention of cirrhosis development by avoiding metabolic, pharmacological, or social factors associated with accelerated progression of liver disease, or through virus eradication by IFN-based treatments. Moreover, a successful antiviral treatment has positive impact on the rate of HCC development in patients who are already cirrhotic[1].

Combination of pegylated IFN and ribavirin therapy is recommended for antiviral therapy worldwide, and is effective in reducing the rate of recurrence of HCV-associated HCC after curative resection or transplantation[47]. The pooling of data from the literature suggests a preventive effect of antiviral therapy on HCC development in patients with HCV-related cirrhosis, but the preventive effect is limited to those achieving SVR[48]. However, some HCV mutations, such as the amino acid substitution M91L, are associated with treatment failure and a poor prognosis[47].

There is a recent study of the effect of pegylated IFN and ribavirin treatment of CHC on the incidence of HCC. After a median observation period of 3.6 years, a significantly lower rate of HCC incidence was noted in patients achieving SVR when compared to non-virological responders. A similarly lower rate of HCC incidence was noted among cirrhotic patients achieving SVR (18.9%) when compared to cirrhotic non-virological responders (39.4%)[18].

A meta-analysis study has been performed recently with the data sources from MEDLINE, EMBASE, CINAHL, the Cochrane Library, Web of Science, and the Database of Abstracts of Reviews and Effectiveness from inception through 2012, to systematically review observational studies to determine the association between response to HCV therapy and development of HCC among persons at any stage of fibrosis and those with advanced liver disease. Among HCV-infected persons, there is moderate-quality evidence demonstrating SVR to be associated with reduced risk for HCC; SVR after treatment among HCV-infected persons at any stage of fibrosis is associated with reduced HCC[17].

Chemoprevention

Vitamin D insufficiency has been associated with the occurrence of various types of cancer. A recent study aimed to determine the relationship between genetic determinants of vitamin D serum levels and the risk of developing HCV-related HCC. The data suggest a relatively weak but functionally relevant role for vitamin D in the prevention of HCV-related hepatocarcinogenesis[49].

Propranolol has antioxidant, anti-inflammatory, anti-angiogenic properties and antitumoral effects and therefore is potentially active in the prevention of HCC. A retrospective long-term observational study suggests that propranolol treatment might decrease HCC occurrence in patients with HCV cirrhosis[50]. These findings also need to be verified by prospective clinical trials.

Understanding the interplay between the viral and cellular components of the HCV replication complex could provide new insight for prevention of the progression of HCV-associated HCC. Fatty acid synthase (FASN) is found to interact with NS5B. FASN may thereby serve as a target for the treatment of HCV infection and the prevention of HCV-associated HCC progression[51]. Thus, understanding the molecular mechanisms, which are implicated in the development of HCC during the course of HCV infection, may help to design a general therapeutic protocol for the treatment and for its prevention.

PREVENTION OF HCC RELATED TO HBV AND HCV COINFECTION

HBV and HCV coinfection is not uncommon with an estimated 7-20 million infected individuals worldwide[52]. A community-based prospective cohort study evaluating HCC development in HBV and HCV co-infected subjects found the hazard ratios (HRs) of HBV monoinfection, HCV monoinfection, and HBV/HCV coinfection were 17.1, 10.4 and 115.0, respectively. Different geno-

types and multiplicative synergistic effect of HBV and HCV coinfection on HCC risk was observed. Infection with HCV genotype 1 (HR = 29.7) and mixed infection with genotype 1 and 2 (HR = 68.7) significantly elevated HCC risk, much higher than HBV infection. The effect of different HCV genotypes and the multiplicative synergistic effect of HBV/HCV coinfection on HCC risk underline the need for comprehensive identification of hepatitis infection status in order to prevent and control HCC[53].

Pegylated interferon-alpha plus ribavirin should be recommended in patients with dominant HCV replication. However, HBV rebound may occur after elimination of HCV with anti-HBV treatment required. These therapeutic measures may contribute to the prevention of HCC this special group of patients[52].

OTHER POTENTIAL CHEMOPREVENTIVE METHODS

The use of aspirin, but not nonsteroidal anti-inflammatory drugs, is associated with a decreased risk of HCC and death from chronic liver disease in the National Institutes of Health-AARP Diet and Health Study of patients between the ages of 50 and 71 years[39]. However this study does not provide information on the HBV and HCV status of its participants, and would need confirmation by future studies specifically for the HBV- and HCV-infected population.

More recent data have suggested dietary factors, including increased intake of coffee[54], unsaturated fatty acids and fish to be protective against HCC. Subjects with known HBV or HCV status, and subjects who were anti-HCV and/or hepatitis B surface antigen positive were analysed. Consumption of n-3 polyunsaturated fatty acid (PUFA)-rich fish or n-3 PUFAs, particularly eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid, appears to protect against the development of HCC, even among subjects with HBV and/or HCV infection[55], probably through dampening the inflammation in the liver and decreasing formation of tumor necrosis factor (TNF)-α, and through simultaneously inhibition of COX-2 and beta-catenin[56,57]. The findings also point to a potential anticancer role for the n-3 PUFA-derived lipid mediators 18-HEPE and 17-HDHA, which can down-regulate the important proinflammatory and pro-liferative factor TNF-α.

CONCLUSION

Clinical experts evaluated ten previously identified dimensions of HCC control: clinical education; risk assessment; HBV strategy; HCV strategy; life-style risk factors; national statistics; funding for screening; funding for treatment; political awareness; and public awareness. Of these strategies, the most significant needs in regional efforts to control HCC are political awareness, public awareness, and life-style risk factors[58].

HCC is a challenging malignancy of global importance. As HCC is strongly associated with chronic viral hepatitis, prevention against the infection is crucial for prevention against HCC. Vaccination against HBV in the newborns and early childhood is highly effective to lower infection rates substantially. For HCV, universal precautions when dealing with human blood, education on high-risk behaviours and screening programs for blood donors can reduce infection rates. Although prevention and treatment of CHB and CHC have been improved within the last decades even in high-risk countries, further effective and sustainable reduction of these infections is still needed[26].

Antiviral therapies for CHB and CHC, while important, can only reduce but not completely eliminate HCC. Improvement in identification of infected persons, accessibility of care and affordability of treatment are needed for antiviral therapy to have a major impact on the global incidence of HCC[59]. Further advances in our understanding of the molecular pathogenesis of HCC hold promise in improving the diagnosis and treatment of this highly lethal cancer[4].

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TOPIC HIGHLIGHT

Effects of antiviral therapy on preventing liver tumorigenesis and hepatocellular carcinoma recurrence

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Abstract

Chronic hepatitis B virus (HBV) infection is the key driving force of liver disease progression, resulting in the development of hepatic dysfunction, cirrhosis and hepatocellular carcinoma (HCC). The primary aim of therapy is to suppress or eliminate HBV replication to reduce the activity of hepatitis, thus reducing the risk of, or slowing the progression of, liver disease. Nucleos(t)ide analogues (Nucs) may result in rapid suppression of HBV replication with normalization of serum transaminases and restore liver function, thus increasing survival in patients with hepatic decompensation. Long-term Nuc therapy may result in histological improvement or reversal of advanced fibrosis and reduction in disease progression, including the development of HCC. The long-term benefits of a finite course of interferon (IFN)-α therapy also include a sustained and cumulative response, as well as hepatitis B surface antigen seroclearance and reduction in the development of cirrhosis and/or HCC. Pegylated IFN and newer Nucs may achieve better long-term outcomes because of improved efficacy and a low risk of drug resistance.

However, treatment outcomes are still far from satisfactory. Understanding the effects of anti-HBV treatment against HCC incidence and recurrence after hepatectomy or liver transplantation is required for further improvement of outcome.

Core tip: Chronic hepatitis B virus (HBV) infection is the key driving force of hepatocellular carcinoma (HCC). In this review, we discussed the mechanism of HBV induction of HCC and described the current trends in anti-HBV therapy. The associations of anti-HBV therapy with prevention of HCC incidence and recurrence after curative operations were also summarized. Moreover, based on our center's experiences, a standardized antiviral strategy was suggested which greatly benefited those patients who underwent hepatectomy and liver transplantation with regard to better clinical results.

INTRODUCTION

Hepatitis B virus (HBV) is a member of the Hepadnaviridae family, which includes small enveloped DNA viruses. HBV infection affects > 2 billion people worldwide and is a significant cause of liver cirrhosis and hepatocellular

carcinoma (HCC), which increases morbidity and mortality in these patients[1]. HBV targets and replicates in hepatocytes, and the risk of developing HCC among HBV carriers is 10 to 100-fold greater compared with that in uninfected people[2]. Treatment with antiviral drugs such as nucleos(t)ide analogues (Nucs) or interferon (IFN)-α may result in rapid suppression of HBV replication and thus reduce the progression of fibrosis and the development of HCC[3]. Although localized or systemic radiation and chemotherapy have been used to eliminate the tumor mass, surgical resection or liver transplantation are still the most effective treatments, but relapse is common[4]. It is widely accepted that comprehensive treatment is required for prevention of HBV-associated HCC development and recurrence. Thus, this review highlights the mechanism of HBV induction of HCC and discusses the current trends in anti-HBV therapy for prevention of HCC and its recurrence.

HBV INFECTION INDUCES CHRONIC INFLAMMATION AND CANCER TRANSFORMATION

HBV persistently replicates in immortalized hepatocytes *in vitro* without overt cellular damage or death, implying that the viruses are not directly cytopathic, and the pathogenesis of hepatitis is immune mediated[5,6]. Liver injury in response to inflammatory hepatitis elicits an inflammatory response in non-parenchymal cells (NPCs), such as myeloid Kupffer cells and hepatic stellate cells. Toll-like receptor-nuclear factor (NF)-κB signaling activation may trigger an innate immune response and inhibit virus replication in HBV-transgenic mice[7]. NPCs secrete NF-κB-regulated hepato-mitogens [*e.g.*, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and hepatocyte growth factor], which promote compensatory proliferation of quiescent hepatocytes carrying HBV-induced mutations. This process allows for the transmission of genetic alterations to daughter cells, thereby favoring liver neoplastic progression. Alternatively, autocrine secretion of transforming growth factor (TGF)-β by hepatocytes induces cell survival and proliferation in the absence of liver damage and independent of NPC-mediated secretion of hepato-mitogens. Increased proliferation is then followed by dysplasia, adenoma, and HCC formation[8,9]. In conclusion, NF-κB activation-associated carcinogenesis most likely depends on downstream hepato-mitogen release and death-driven compensatory proliferation[9,10].

HBV X (HBx) protein is encoded by the smallest HBV open reading frame and is 154 amino acids in size, with a molecular weight of approximately 17.5 kDa. HBx can localize to the mitochondria where it acts as an adaptor or kinase activator to influence signal transduction pathways such as: protein kinase C, Janus kinase/signal transducer and activator of transcription (JAK/STAT), phosphoinositide 3-kinase, stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), Ras-Raf-

mitogen-activated protein kinase (Ras-Raf-MAPK), and extracellular signal-regulated kinase (ERK). This may provide a unified mechanism by which HBx exerts many of its pleiotropic activities, including transcription, cell cycle control, and apoptosis[11-14]. It is also reported that HBx can activate NF-κB directly, which could be partially *via* upregulated inhibitor of NF-κB kinase activity and the mammalian target of rapamycin (mTOR) pathway[15]. As mentioned above, various inflammatory cytokines, including TNF-α, IL-1α, IL-1β, IL-6 and IL-8, which play an important role in the inflammation-carcinogenesis axis of the liver, are NF-κB activation-mediated, and IL-6 is thought to be one of the most important[16]. Recently, our group found that IL-22 could also promote HCC *via* STAT3 activation, suggesting that inflammatory cytokines have also attracted considerable attention as mediators of the association between inflammation and hepatocarcinogenesis[17].

Furthermore, HBx could interfere with the antitumor immune response *via* other inflammatory cells. Infected intrahepatic natural killer cells are also known to induce cytolytic activity without IFN-γ production, suggesting that hepatocellular killing occurs without virus clearance[18]. Dendritic cells may be infected with HBV, which will cause defective chronic HBV infection, resulting in poor adaptive immunity[19]. CD4+ CD25+ FOX3P+ regulatory T cells could be induced by HBx-stimulated production of TGF-β1, and their crosstalk with Th17 cells may contribute to an immune tolerance-clearance balance in the liver[20].

MECHANISMS OF HBV ONCOGENESIS

Increasing evidence suggests that HBV contributes to HCC by directly modulating pathways that may promote the malignant transformation of hepatocytes. Firstly, HBV insertions are associated with host large genetic alterations: deletions, duplications and chromosomal translocations. These events could either induce chromosome changes or act "in cis" on the expression or function of nearby cellular genes that contribute to chromosome instability[21,22]. For instance, integration of HBx gene fragments (316-462/262-462 bp) could directly transform human immortalized normal liver L02 cells in studies using a cell model. Further, these integrations could be detected in five of 44 clinical HBV-positive HCC tissues[23]. Integration at specific sites in host genes may contribute to a growth advantage in a clonal cell population but subsequent additional mutations will eventually accumulate. Evidence was first provided in two independent HCCs, with retinoic acid receptors and cyclin A being targeted by HBV integration in tumors[24]. Recently, more genes involved in cell survival, proliferation and immortalization were also reported as the HBV integration target, such as human telomerase reverse transcriptase (hTERT, a regulator of telomerase), platelet-derived growth factor receptor, calcium signaling-related genes, and ribosomal protein genes[25].

Although it is suggested that upregulated expression of HBx and HBV S proteins is associated with hepatocarcinogenesis in transgenic mouse models, the exact mechanism remains unclear[26]. It is worth noting that hepatocytes in cirrhotic livers display decreased proliferation rates with a dominant replicative senescence phenotype characterized by critically shortened telomeres and permanent cell cycle arrest[1]. However, during hepatocyte proliferation, low or absent telomerase activity in cirrhotic liver is associated with upregulated HBx or pre-S2 protein[27]. In a study of 55 HCC and 17 chronic hepatitis patients, hTERT was positive in 81% of HCCs, and the mean telomere length in HCC was significantly shorter compared with that in chronic hepatitis[28].

HBx is also suggested to have the ability to induce direct chromosomal instability by interfering with the mitotic checkpoints[29,30]. HBx induces epigenetic changes, including DNA methylation aberration, histone modification and miRNA expression. Jiang *et al*[31] reported that increased miR-22 is associated with HCC development in male patients. Xu *et al*[32] also suggested that suppression of miR-148a upon HBx activation can enhance tumorigenesis. Moreover, HBx binds and inactivates p53, and interacts with DNA damage-binding protein 1 (DDB1, the DNA repair protein), which may affect repair functions and allow the accumulation of genetic changes, and also confer resistance against nucleolar stress and anticancer drugs[33].

CURRENT OPINION IN ANTIVIRAL THERAPY

Despite dramatic improvements in the treatment of patients against HBV over the past decade, treatment of chronic HBV infection is currently based on two different strategies: (1) IFN-α or thymosin-a1 (T-a1) aimed at inducing a sustained antiviral response; and (2) oral anti-HBV Nucs to achieve long-term complete suppression of HBV replication[34].

The first strategy is typically used in patients with less advanced liver disease, with high alanine aminotransferase (ALT) and not too high HBV DNA replication. It is particularly successful in younger patients and in those infected with HBV genotype A or B. Since the first introduction of IFN-α in 1976, the long-term benefit of IFN therapy has included a sustained and cumulative immune response. T-a1 is an immunomodulator that triggers maturational events in lymphocytes and T-cell function. It can promote reconstitution of immune defects and promote disease remission and cessation of HBV replication in patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B, without significant side effects[35,36]. Eighteen patients with HBeAg-positive and serum HBV DNA-positive chronic hepatitis B received 6 mo of treatment with 1.6 mg subcutaneous T-a1 twice weekly[37]. They achieved better HBV loss and seroconversion than 30 patients receiving 6 mo of 3-5 MU subcutaneous IFN-α (injection daily for 15 d, then three times weekly).

The results of this trial indicate that T-a1 is of potential interest in patients with anti-HBe- and HBV DNA-positive chronic hepatitis B.

The introduction of 12-kDa linear polyethylene glycol (PEG) for IFN-α2b and 40-kDa branched PEG for IFN-α2a has allowed weekly rather than daily or three times weekly injection[34]. This has had a significant impact on the tolerability and ease of use. In addition, for patients with HBeAg-positive chronic hepatitis B, Pegylated IFN (PEG IFN)-α2a offers superior efficacy over lamivudine, on the basis of HBeAg seroconversion, HBV DNA suppression, and hepatitis B surface antigen (HBsAg) seroconversion[37]. Overall, PEG IFN-α is an ideal treatment strategy in selected patients with HBeAg-negative chronic hepatitis B, because of its well-recognized and predictable safety profile and unique mechanism of antiviral activity leading to long-lasting immune control.

For high HBV DNA levels, Nucs are typically adopted for patients with more advanced liver disease, and for those who have failed or cannot tolerate IFN therapy. However, the main limitation is the development of resistance: for example, after 5 years of therapy with lamivudine (L-nucleoside), 76% of patients developed resistance. Telbivudine, another L-nucleoside, is more potent than lamivudine but resistance still developed in 25% of HBeAg-positive and 11% of HBeAg-negative patients after 2 years. Adefovir, an acyclic phosphonate, is relatively weak, but is effective against lamivudine- and telbivudine-resistant mutations, and it should be used in combination rather than substituted. Resistance to adefovir develops relatively slowly, rising to 29% for HBeAg-negative patients after 5 years, but more rapidly when used alone for lamivudine-resistant HBV. Currently, the two first-line Nucs are entecavir and tenofovir. Entecavir, a cyclopentane (D-nucleoside), is very potent, with 94% of patients having undetectable HBV DNA after 5 years. Resistance develops in only 1.2% of treatment-naïve patients. Tenofovir, another acyclic nucleotide, is more potent with less renal toxicity compared to adefovir. It is effective against lamivudine-resistant mutations when used alone. No resistance to tenofovir has been described after its use for 3 years or longer, often for patients with human immunodeficiency virus/HBV co-infection[38].

In conclusion, for patients with HBeAg-positive chronic hepatitis B, PEG IFN-α offers superior efficacy on the basis of HBeAg seroconversion, HBV DNA suppression, and HBsAg seroconversion. As a result of these features, new therapeutic regimens based on combinations of PEG IFN-α and third-generation Nucs such as entecavir and tenofovir are being developed to increase the rate of HBsAg seroclearance, which remains the ideal endpoint in all HBeAg-negative chronic hepatitis B patients.

ANTIVIRAL THERAPY SUPPRESSES THE CHRONIC INFLAMMATION-CANCER TRANSITION

A prospective cohort study with 11 years follow-up

showed that HBV DNA concentration > 10^4 copies/mL is an especially strong predictor of risk of developing HCC in individuals aged ≥ 30 years, independent of the level of serum ALT[39]. It is accepted that anti-HBV therapy can improve the outcome of chronic HBV infection in terms of HCC incidence.

In a randomized controlled trial of 101 male patients in the Taiwan region, cumulative incidence of HCC development was significantly decreased in the IFN-α-treated group (1 of 67 patients) than in the control group (4 of 34 patients), at 1.1-11.5 years after the end of therapy[40]. In addition, a retrospective study suggested that natural lymphoblastoid IFN-α (IFN-α nl) and IFN therapy may provide better long-term beneficial effects than placebo in terms of HBV clearance, reduction of HCC, and prolonged survival. HCC was detected in 1.5% of the IFN-α nl group, 3.7% of the IFN-α2a group and 14.7% of the control group.

As for the long-term benefits of Nucs, in a randomized control trial, HCC occurred in 3.9% of patients treated with lamivudine and 7.4% of those in the placebo group in a total of 651 patients (HR = 0.49, P = 0.047)[41]. A retrospective multicenter study of 377 Japanese patients receiving lamivudine treatment for up to 96 (23.1 ± 19.0) mo showed a marked reduction in the incidence of HCC compared with a historical control group matched for age, sex, liver fibrosis score, albumin level and platelet count (0.4% per year *vs* 2.5% per year, P < 0.001)[42]. In another study of 656 HBeAg-negative patients (54% had chronic hepatitis, 30% had cirrhosis), lamivudine (median 22 mo, range 1-66) was highly effective in reducing viral load in HBeAg-negative patients, and HBV suppression reduced the development of HCC and disease worsening in patients with cirrhosis[43]. A Korean study also showed a reduced incidence of HCC in patients with compensated cirrhosis who received lamivudine therapy (4.9%) compared to untreated patients or patients treated with lamivudine who had viral breakthrough (11.8%) or a suboptimal response (19.4%)[44]. In a recent systemic review, Papatheodoridis *et al*[45] reviewed 21 studies that included 3881 treated and 534 untreated patients and found that HCC developed less frequently in Nuc-treated patients (2.8% *vs* 6.4%, P < 0.003).

In a systematic review of 11 studies of the effect of IFN and Nuc therapy on the outcome of HBV infection over the past 10 years, Sung *et al*[46] indicated that IFN-α or Nuc treatment significantly reduced the risk of HCC. Although IFN benefited patients with cirrhosis, Nucs benefited those with non-cirrhosis and HBeAg-positive infection. From the experiences mentioned above, sustained HBV suppression induced by IFN-α and Nuc therapy may be necessary to reduce the development of HCC in HBV-infected patients.

EFFECT OF ANTIVIRAL THERAPY ON HCC RECURRENCE

Does anti-HBV therapy decrease the risk of HCC recurrence after the most effective methods to reduce tumor burden: partial hepatectomy or liver transplantation? As suggested previously, the 5-year overall survival for all early HCC patients was 58% (transplantation: 63%; resection: 53%)[47]. Huang *et al*[48] reported that patients with HBV reactivation after liver resection have a higher liver failure rate, lower 3-year disease-free survival rate, and lower overall survival rate than those without reactivation (11.8% *vs* 6.4%, P = 0.002, 34.1% *vs* 46.0%, P = 0.009, and 51.6% *vs* 67.2%, P < 0.001, respectively).

Exploratory subset analysis showed that adjuvant IFN-α had no survival benefit for pTNM stage I/II tumor (5-year survival 90% in both groups; P = 0.917) but prevented early recurrence and improved the 5-year survival of patients with stage III/IVA tumor from 24% to 68% (P = 0.038)[49]. Lee *et al*[50] also reported that metastasis-associated protein 1-positive HCC recurred postoperatively in 26 of 93 patients (28%), although the PEG IFN group had significantly lower overall cumulative recurrence rates than the control group (7% and 14% *vs* 24% and 34% at 1 and 2 years, respectively; P < 0.05). In addition, the 1- and 2-year cumulative survival rates were higher in the PEG IFN group compared with the control group (100% *vs* 93% and 100% *vs* 87%, respectively; P < 0.05). In a report of 237 HCC patients after hepatectomy treated with IFN-α or placebo within comparable clinicopathological parameters, the median overall survival was 63.8 mo in the IFN-α group and 38.8 mo in the placebo group (P = 0.0003), and the median disease-free survival period was 31.2 *vs* 17.7 mo (P = 0.142)[51]. Chen *et al*[52] showed that adjuvant IFN-α2b treatment was associated with a significantly higher incidence of leukopenia and thrombocytopenia and did not reduce postoperative recurrence of viral hepatitis-related HCC.

Regarding the effect of Nucs on HCC recurrence, Anselmo *et al*[53] suggested that hepatitis B immunoglobulin (HBIg) and lamivudine treatment markedly reduced HBV recurrence rate and significantly improved 1- and 3-year recurrence-free survival rates after liver transplantation. Chan *et al*[54] also reported that the 1-, 3- and 5-year disease-free survival rates in patients treated with lamivudine or entecavir were 66.5%, 51.4% and 51.4% compared with 48.9%, 33.8% and 33.8%, respectively, in the control group. Kubo *et al*[55] reported that the tumor-free survival rate after hepatectomy was significantly higher in the lamivudine than the control group. Recently, multivariate analysis showed that HCC recurrence after transplantation was markedly associated with HBV reinfection. However, HBIg was associated with worse survival as well as HBV reinfection and HCC recurrence (P = 0.002, P < 0.001 and P < 0.001, respectively)[56].

In our center (Liver Transplantation Center, The First Affiliated Hospital of Nanjing Medical University), we suggest that HBsAg-positive patients who have > 10^4/mL or 10^3-10^4/mL HBV DNA copies with impaired liver function, must take lamivudine after curative surgery. Moreover, for those who have HBV YMDD mutation during initial treatment, entecavir and/or adefovir dipivoxil should be used as the replacement. If drug resistance
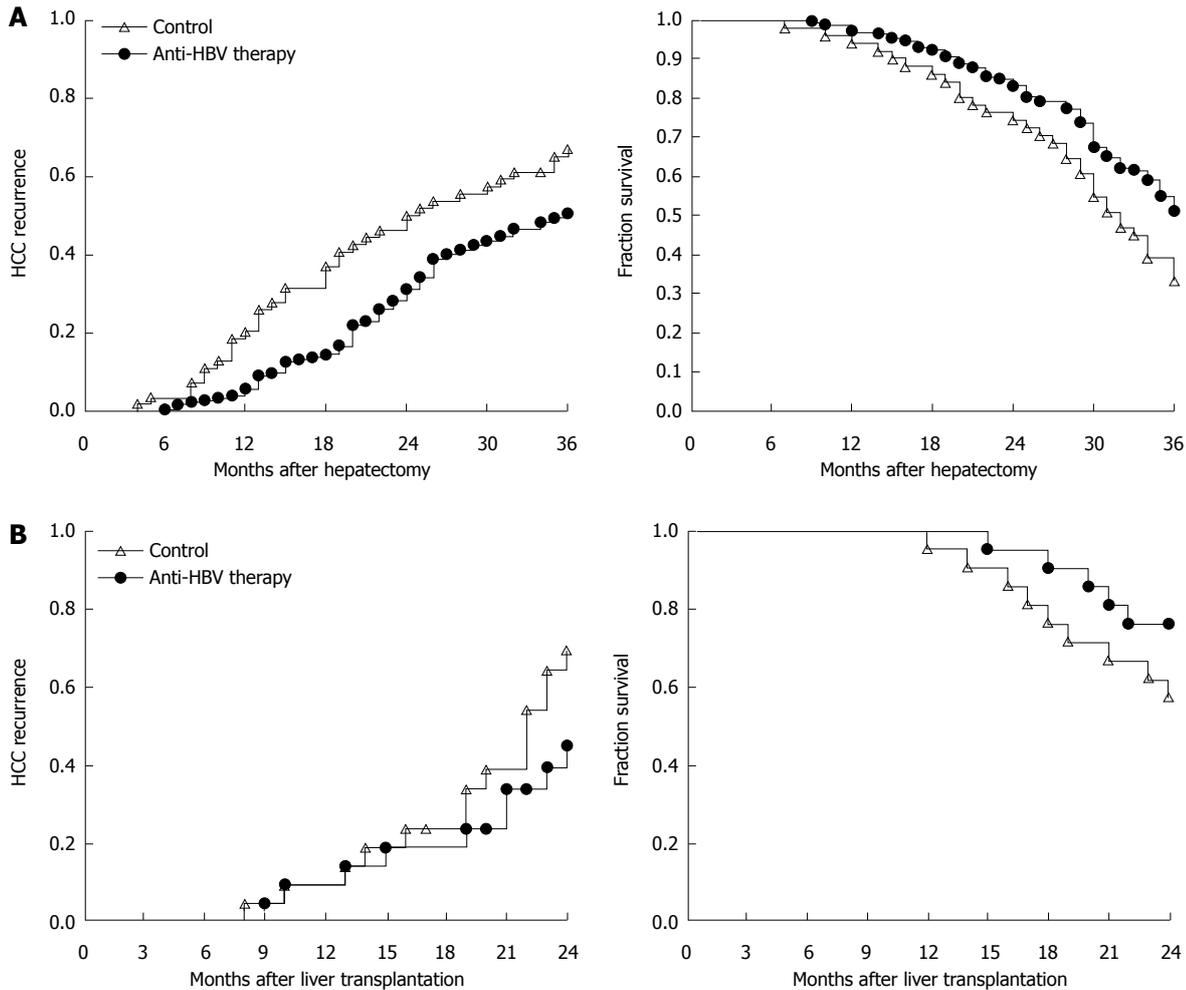
Figure 1 Comparison of hepatocellular carcinoma recurrence and outcome in patients who received anti-hepatitis B virus therapy or placebo after hepatectomy or liver transplantation. A: From September 2009 to May 2010, 224 HCC patients who received partial hepatectomy due to HBV-related HCC were enrolled. HCC recurrence and 3-year overall survival rate in patients with anti-HBV treatment (n = 173) and patients without standardized anti-HBV treatment (n = 51) were monitored for at least 3 years. Left: log-rank test, P = 0.013; right: log-rank test, P = 0.006; B: From January 2010 to August 2011, 42 HCC patients within Milan criteria who received liver transplantation were enrolled. HCC recurrence and 2-year overall survival rate in patients with anti-HBV treatment (n = 28) and patients without standardized anti-HBV treatment (n = 14) are shown. Left: log-rank test, P = 0.031; right: log-rank test, P = 0.045. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

occurs, tenofovir disoproxil fumarate could be used instead. In our randomized controlled clinical study, we verified that standardized anti-HBV therapy could significantly improve the outcome and decrease the recurrence of patients who underwent partial hepatectomy and liver transplantation (Figure 1).

CONCLUSION

This literature review describes two different aspects of the tumorigenesis of chronic HBV infection: the direct mechanism by which HBV DNA and its main product HBx induce host DNA instability; and HBV infection-associated liver inflammation and imbalanced immuno-regulation. We also briefly introduce the current strategy against HBV infection and show that timely usage of Nucs and immunomodulatory agents can eventually prevent further disease progression, including HCC, in patients with chronic HBV infection. Long-term studies

will probably confirm that new antiviral drugs such as entecavir, tenofovir and telbivudine can offer even more opportunities for reducing disease progression than lamivudine therapy does.

For patients who undergo hepatectomy or liver transplantation as curative treatment for HCC, tumor recurrence must be monitored by ultrasound and α-fetoprotein assay. More importantly, from our experience, HBV replication should also be monitored because sustained HBV activation or relapse is significantly related to HCC development and recurrence. Standardized anti-HBV treatment can ultimately delay HCC recurrence and benefit survival.

Since PEG-IFN, as the newly introduced IFN, offers a better opportunity to suppress HBV replication in patients who do not have cirrhosis or fibrosis, it should provide promising prospects in reducing HCC development and recurrence. While in many third world countries, lamivudine is still the first-line drug, mass usage of

newly developed IFN could be used more frequently in the future and show better prospects.

In conclusion, developing safe and affordable agents, as well as management strategies to improve sustained or maintained HBV suppression, should be the ultimate goals in the management of chronic HBV infection.

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TOPIC HIGHLIGHT
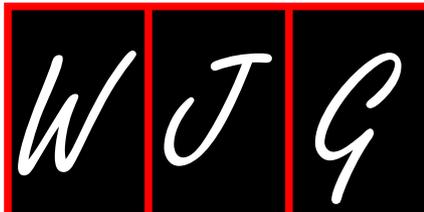
Exploitation of host clock gene machinery by hepatitis viruses B and C

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Abstract

Many aspects of cellular physiology display circadian (approximately 24-h) rhythms. Dysfunction of the circadian clock molecular circuitry is associated with human health derangements, including neurodegeneration, increased risk of cancer, cardiovascular diseases and the metabolic syndrome. Viruses triggering hepatitis depend tightly on the host cell synthesis machinery for their own replication, survival and spreading. Recent evidences support a link between the circadian clock circuitry and viruses' biological cycle within host cells. Currently, *in vitro* models for chronobiological studies of cells infected with viruses need to be implemented. The establishment of such *in vitro* models would be helpful to better understand the link between the clock gene machinery and viral replication/viral persistence in order to develop specifically targeted therapeutic regimens. Here we review the recent literature dealing with the interplay between hepatitis B and C viruses and clock genes.

Core tip: New antiviral strategies have been developed, including the interferon/ribavirin-free therapy, to control hepatitis viruses replication. Although, IFN-free regimens have generated excitement among scientists, for the reason that they are better tolerated, they are not still able to completely eradicate the viruses. Here we underline the circadian relationship between host cell and hosted hepatitis viruses, that has to be taken into account in order to optimize the timing of therapeutic regimens, not only to minimize the pharmacological agents' toxicity but also to improve the efficacy of treatment modalities through optimized timing of therapeutic regimens, targeting in a better way virus replication.

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INTRODUCTION

Viruses are among the most important human carcinogens[1]. Numerous mechanisms have been described to be dysregulated by viruses, always focusing on the impairment of the most well known tumor suppressors and/or oncogene proteins and their signaling pathways[2,3].

It has been already established that alteration of the circadian clock molecular circuitry is involved in carcinogenesis. Circadian defects have also been associated with liver diseases, including hepatocellular carcinoma (HCC)[4,5], a condition in which viruses play a role in disease pathogenesis and progression.

Specifically, basic cell functions and processes, such as cell division, proliferation, growth, differentiation, autophagy, apoptosis and metabolism, show time-related fluctuations, and when the period of oscillation is about 24 h the rhythmicity is defined as circadian[6-10]. At the cellular level, circadian rhythmicity is driven by a molecular clockwork comprised of a translational-transcriptional feedback loop realized by a set of genes, called core clock genes, coding for proteins that in turn suppress gene expression in a cycle that completes itself each day. Clock genes are transcriptionally activated by the transcription factors circadian locomotor output cycle kaput (CLOCK) and aryl hydrocarbon receptor nuclear translocator-like (ARNTL). The latter two protein heterodimerize and bind to the E-box enhancer elements in the promoters of the Period (*PER* 1, 2 and 3) and Cryptochrome (*CRY* 1 and 2) genes. The *PER* and *CRY* mRNAs are translated into PER and CRY proteins that form a repression complex, which in turn translocates back into the nucleus, interacts directly with CLOCK and ARNTL blocking their activity[4,11-13].

Among the processes regulated by the clock gene machinery are pathways of cell metabolism and vesicle trafficking, suggesting the potential role for the circadian clock circuitry in the regulation of viral expression/replication[14]. A relationship between circadian dysfunction and tumorigenesis has also been found at both the cellular and the organismal levels, indicating that the circadian clock may impact on the development of cancer[15-17], a disease also influenced by viruses. Recently, scientific evidences support a functional connection between viral expression/replication and circadian dysfunction in the pathogenesis of liver diseases[14,18-20]. However, whether the circadian clock directly regulates viral cell cycle in mammalian cells, or whether viruses may play a role in the cycling of mammalian cell clocks is not yet totally clear.

The implication of viral expression/replication and circadian dysfunction in the pathogenesis of liver diseases suggests that a functional connection between these two processes may exist as it has been already showed[14,18-20]. Nevertheless, the relationship between circadian cycles and viral expression/replication is an intriguing area for future study and it has implications for multiple human diseases. The study of new causes which are able to influence the clock genes expression are under investigation as disruption of biological clocks is implicated in a variety of disorders including fatty liver disease, obesity and diabetes[21,22]. Exciting data reported the influence of hepatitis B and C viruses on the hepatic clock genes[18,19], demonstrating for the first time that these viruses are able to impair the inner molecular clockwork, presumably to better exploit the host-cell replication machinery. Hepatotropic viruses impair also liver functions, and this effect may be a cause or a consequence of the disruption of the inner cellular biological clock. At the present, the relationship between hepatitis viruses expression/replication and the circadian clock is poorly understood. Here we review the scientific reports addressing the interaction between hepatitis B and C viruses and the molecular clockwork.

LIVER AND CLOCK GENES

The liver plays an important role in maintaining energy homeostasis within the organism. The major biochemical reactions occurring within the liver are involved in glucose breakdown/genesis, which is strictly linked to fatty acid metabolism (biosynthesis/beta oxidation). All these biochemical reactions and the metabolic networks must be finely coordinated in order to avoid unnecessary interference between the pathways[21]. To this end, reactions are separated locally and temporally. Hepatic metabolic functions show rhythmic fluctuations with 24-h periodicity[23], driven by molecular clockworks ticking through translational-transcriptional feedback loops and operated by a set of genes, called clock genes, encoding circadian proteins[4]. In the absence of environmental cues, specifically light:dark cycle, it has been demonstrated that rhythmic food intake influences the hepatic circadian oscillator[23,24]. Hence, the clock genes oscillations are not phase locked but are flexible to enable adjustment to the changing environments[23].

In the liver, gene expression profiling has shown that transcriptional processes display approximately 24-h rhythmicity and have a crucial role in metabolic processes. Energy and nutrient homeostasis at both cellular and organismal levels is guaranteed by nearly constant adjustments of metabolic gene expression, and the transcriptional networks that regulate glucose and lipid metabolism are sensitive to nutritional status, responding to diverse physiological signals[25]. The fractions of cyclic transcripts depending on systemic signals and local oscillators amount to approximately 14% and 86%, respectively. The systemically regulated liver genes include immediate early genes (IEG), which convey rhythmic signals to core clock genes of hepatocyte oscillators and thus are involved in the synchronization of liver clocks, and tissue specific output genes, directly participating in rhythmic liver physiology and metabolism. The IEG

class contains several heat shock protein genes, known to be regulated by heat shock transcription factor 1 (HSF1) and target genes of serum response factor 1 (SRF1), and these immediate early transcription factors (IETFs) act as sensors of blood-borne signals, driving the synchronization of circadian clocks[26]. Metabolite sensing is linked to transcriptional responses in hepatocytes by nuclear receptors through switching between co-activator and co-repressor recruitment[27]. Nuclear hormone receptors comprise a unique class of transcriptional regulators that are capable of sensing the concentrations of metabolites, including lipids, oxysterols, heme, and bile acids[28]. An important role in the control of glucose, lipid, and mitochondrial oxidative metabolism is played by the expression of co-regulators, in particular the PGC-1α, which is highly responsive to nutritional status and other physiological signals[29]. The cross-talk between circadian rhythms and metabolism is operated also by the peroxisome proliferator-activated receptors (PPAR), in particular α and γ[30]. Both factors are already known to be dysregulated by hepatitis B and C viruses. PPARα regulates transcription of genes involved in lipid and glucose metabolism upon binding of endogenous free fatty acids[31]. PPARγ binds eicosanoids deriving from either omega-3 (ω-3) or omega-6 (ω-6) fatty acids and their oxidized counterparts, is rhythmically expressed, its expression is regulated by PER2 and in turn directly regulates ARNTL transcription[32]. The clock gene machinery drives the expression of a large array of enzymes involved in lipid metabolism, controls lipogenesis and regulates triglyceride packaging into chylomicrons (globules that transport dietary lipids) at the level of the intestine, whereas in the liver, clock disruption triggers lipid accumulation[33-35]. In liver ARNTL and CLOCK control gene expression of enzymes involved in glucose and lipid homeostasis, as well as in bile acid and apolipoprotein biosynthesis[36]. Diurnal oscillation characterizes a number of proteins involved in lipid metabolism [such as hepatic cytochrome P450 cholesterol 7 α-hydroxylase, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, or apolipoprotein AIV] show in both humans and rodents. REV-ERB α links the clock with the master pathway of hepatic lipid metabolism, is involved in bile acid synthesis and sterol regulatory element-binding protein (SREBP) signaling and SREBPs control both fatty acid and sterol biosynthesis through modulation of rate-limiting enzymes in these pathways[35]. Diurnal variations hallmark also glucose metabolism, and the rate-limiting enzymes for gluconeogenesis, glycolysis, glycogenesis and glycogenolysis show circadian variations of activity, determining the circadian rhythmicity of hepatic glucose production and glycogen content. The biological clock drives the circadian regulation of hepatic gluconeogenesis by CRY 1 and CRY2 *via* inhibition of cAMP signaling in response to G protein coupled receptor (GPCR) activation[37], and controls hepatic glycogen synthesis through transcriptional activation of glycogen synthase (GYS2) by CLOCK[38], and the disruption or mutation of the clock genes CLOCK and ARNTL results in disorders of glucose homeostasis[39,40].

HEPATITIS B VIRUS AND CLOCK GENES

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family, which causes persistent liver infections[41]. With more than 2 billion people being infected worldwide and 400 million suffering from chronic hepatitis B, HBV infection is one of the most significant public health problems. Despite the advance of modern medicine in the development of new antiviral drugs, HBV infection remains a leading cause of liver cirrhosis and cancer[3].

HBV genome is a partial double-stranded DNA that replicates through the reverse transcription of pregenomic RNA[42]. The analysis of the entire sequence of HBV-DNA, constituted by a circular incomplete double-strand DNA molecule, of 3182 bp in length[43], reveals four Open Reading Frames (ORFs), overlapping each other, necessary for transcription and expression of HBV proteins. These ORF are named: ORF S, ORF C, ORF P and ORF X[44] and they encode for four proteins with specific structure and function[45]. HBV biology and life cycle were already described[46]. The X protein (encoded by ORF X), remains partially explored and its function needs to be established[47]. Cultured hepatocytes overexpressing the *X*-gene, reveal a crucial role of the X protein in trans-activating viral and cellular genes[48]. Moreover, some authors associated HBx protein with HCC due to its property of impairing cellular proliferation[49], although the X protein cannot induce infection by itself.

One study reported the ability of the HBx protein in modulating the clock genes in LO2 cells[19]. Cultured LO2 cells stably overexpressing the HBx protein displayed higher mRNA and protein levels of the CLOCK gene whilst ARNTL resulted to be decreased as compared to control cells. The authors suggest that the impairment of circadian rhythm of liver cells due to HBx expression may be one of the reasons leading to liver cancer development. It remains to elucidate how HBV impairs the clock gene machinery and to confirm the effect on liver cancer progression due to impairment of the cellular molecular clockwork by HBx.

HEPATITIS C VIRUS AND CLOCK GENES

Hepatitis C virus (HCV) is a hepatotropic virus belonging to the Flavivirus family. It is estimated that 170 million people worldwide are infected with HCV[50]. In the majorities of the cases, HCV infection leads to severe liver diseases and is considered one of the major risk factors for HCC development[51].

HCV genome consists in a positive-stranded RNA of approximately 9.6 kb, coding for a single polyprotein of about 3000 amino acids, processed co- and post-translationally by cellular and viral proteases cleaving it into three structural (core, E1 and E2), seven nonstructural (NS2,

NS3, NS4A, NS4B NS5A and NS5B) mature proteins and an ion channel (p7)[52]. Despite the small sequence divergences HCV is classified into six major genotypes (further divided into different subtypes)[50]. Overwhelming lines of evidence have indicated that the pathogenicity of HCV and its effect on disease progression and treatment is genotype dependent[50].

We used two different *in vitro* models to investigate the relationship between HCV and clock genes, the OR6 cells harboring HCV replication and the Huh-7 cells expressing the HCV core proteins of genotype 1b or 3a. In both cases it was found that HCV down-regulated the expression of two crucial clock proteins CRY2 and PER2.

CRY2 protein is involved in NF-κB activation and pro-inflammatory processes[53], (see next section for discussion), while the role of PER2 on HCV replication is particularly interesting, as this circadian protein regulates the rhythms of IFNγ signaling, critical for innate and adaptive immunity against infection[54,55]. Exogenous overexpression of PER2 protein in OR6 cells hampered HCV-RNA replication, and consistently, PER2 overexpression influenced the HCV-dependent altered expression of Interferon stimulated genes (ISG) products (OAS1, Mx1, IRF9). PER2 potentiated the expression of OAS1 which activates RNase L resulting in viral RNA degradation and inhibition of viral replication[56].

Of note, when experiments were performed, cells were synchronized using serum shock procedure, a method previously reported to induce circadian gene expression in mammalian cultured cells[57], before RNA extractions at regular time points over 28 h period. This approach allows assessing differences in the time-related fluctuation of expression.

CROSS-TALK BETWEEN THE BIOLOGICAL CLOCK, HEPATITIS VIRUSES AND IMMUNITY

Hepatic injury in HCV infection is not only directly induced by viral cytopathic effects, but is principally related to host immune responses. Viral persistence is influenced by dynamic restriction of the host's immune response, and the strength of immune response determines resultant acute viral clearance opposed to chronic persistence, leading to pathogenic mechanisms potentially responsible for HCC onset and progression during chronic hepatitis virus infection. Chronic immune-mediated liver cell injury triggers the development of HCC in the absence of viral transactivation, insertional mutagenesis, and genotoxic chemicals[58]. Circadian patterns of immune function have been maintained throughout evolution, are driven by the clock gene machinery, and the magnitude of immune response depends in part on the circadian timing of antigen challenge[59,60]. Alterations in the circadian regulation of the immune system may therefore lead to viral persistence or reactivation. The components of the immune system show time related variations with

a period of 24 h. In particular, the levels of leukocyte populations in the blood of humans and rodents are characterized by circadian variations. Natural killer (NK) cells are critical for immune surveillance against viral infections and their function is under tight circadian control. NK cells bear no antigen receptor and therefore belong to the innate immune system, however they share several features with highly differentiated T lymphocytes, such as a high tissue migratory potential and the production of granzyme B and perforin, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granular macrophage cell stimulating factor, allowing immediate cytotoxic effector defense in the periphery[61]. Circadian expression of negative and positive components of the molecular clock, as well as cytokines and cytolytic factors, are evident in NK cells, and perturbations of daily rhythms caused by external and internal stressors may compromise the first line of defense against infections[61,62]. In NK cells, expression of cytokines (IFN-γ and TNF-α) and cytolytic factors (granzyme B and perforin) are highly synchronized, peaking approximately during the middle of the active period in rats, and NK cell cytotoxic activity peaks at similar circadian phases. Similarly, NK cytotoxicity is maximal during periods of wakefulness in humans[60]. The clock genes drive circadian rhythmicity of NK cell function. Alterations of the molecular clockwork modify the harmonized expression of NK cell cytolytic factors. In particular, knock-down of Per2 or Arntl in rat-derived RNK16 NK cells changes in a diverse way the expression of genes encoding IFN-γ, TNF-α, granzyme B, and perforin[54]. Furthermore, knock-down of Per2 or Arntl changes protein levels of granzyme B and perforin, but not of IFN-γ and TNF-α[63,64]. In addition, distorted rhythms of granzyme B and perforin as well as altered rhythm and low levels of IFN-γ, together with changes in the rhythm of Arntl and Per2, were evidenced in Per2 mutant mice[62].

In the human blood, higher counts of total lymphocytes, T lymphocytes and B lymphocytes have been consistently observed in the night time, and when T lymphocyte subsets are considered, CD4+ (T helper) and CD8+ (cytotoxic) naive, central memory and effector memory T lymphocytes show peak numbers in the night, while CD4+ effector T cells show no rhythm and CD8+ effector T cells show a low amplitude rhythm with a peak in the day[65,66]. T and B lymphocytes are involved in the adaptive (*i.e.*, antigen-specific) immune response, whereas granulocytes, monocytes and NK cells mainly belong to the innate (*i.e.*, not antigen-specific) immune system. In rodents higher numbers of total leukocytes and of lymphocytes were reported in the day, while in humans higher levels in the counts of innate immune system cells were reported in the daytime or late day[67]. Hence, both nocturnal rodents and diurnal humans show higher lymphocyte counts during the rest period, and peaks of other cell types (granulocytes, neutrophils, monocytes) were found in the day in rats, while highest NK cell numbers were observed at the end of the night,
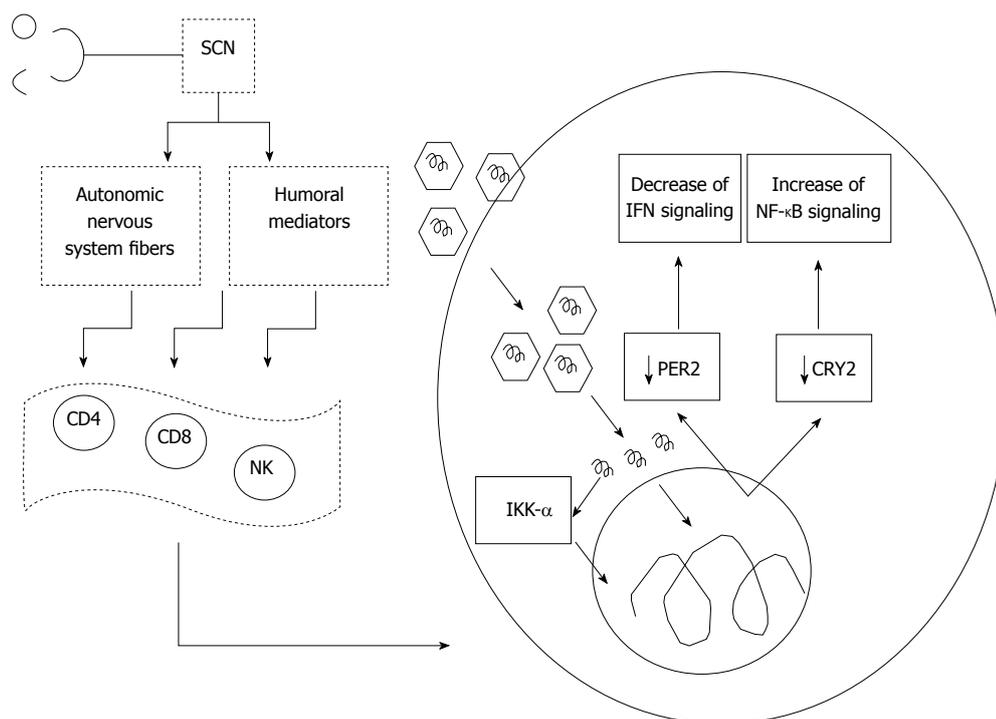
Figure 1 Scheme rendering the interplay between the circadian clock circuitry, the immune system and the alterations induced by hepatitis C virus on the clock gene machinery and downstream signaling pathways. SCN: Suprachiasmatic nuclei.

i.e., at the beginning of the activity period.

Cellular immune rhythms are synchronized by the mammalian central pacemaker located in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus via time dependent changes in the activity of the sympathetic nervous system (SNS), in the release of hormones (growth hormone, prolactin, melatonin, cortisol) and in behavior that is linked to the sleep-wake cycle[65,68,69]. The rest period is characterized by peak levels of pro-inflammatory hormones like growth hormone, prolactin (and melatonin in humans) and pro-inflammatory cytokines like interleukin (IL)-1 and TNF-α. Besides, T helper (h) 1 and Th2 responses are likewise highest during sleep[70]. During the active period the hypothalamus-pituitary-adrenal axis becomes activated and cortisol suppresses pro-inflammatory cytokine production, CD4$^+$ T cell numbers and allergic reactions[71]. Disruption of this temporal organization of the immune system can lead to immunodeficiency and/or exceeding immune reactions (*e.g.*, low grade systemic inflammation).

Oscillation across the day was observed also for the levels of cytokines and other effector molecules, in particular serum levels and *in vitro* production of IFN-γ, tumor necrosis factor TNF-α, IL-1, IL-2, IL-6 and IL-12 were all shown to present a rhythm in humans, with a peak generally observed at night or in the early morning[60]. Immune rhythms are influenced by hormone rhythms (*e.g.*, cortisol, melatonin, norepinephrine), and in humans the rhythms of naive, central memory, and effector memory T cell counts are regulated by cortisol, whereas numbers of CD8$^+$ effector T cells follow changes in endogenous epinephrine[65,72-74].

The presence of biological clocks in immune cells and lymphoid organs drives rhythms in the functions of cells within the immune system, but on the other hand immune responses and mediators influence behavioral and molecular circadian rhythms[54,62]. Whether circadian disruption of cellular-mediated immunity or neuroendocrine-immune interaction lead to viral reactivation is unclear.

The cross-talk between the clock and innate immune functions is mediated among other circadian factors by CRY2, which transcriptionally regulates STAT3 and hampers activation of NF-κB signaling by negatively regulating the cAMP-PKA pathway[53]. Interestingly, we reported a severe down-regulation of CRY2 in OR6 cells replicating HCV genotype 1b[18], which could induce increase of cytokine production related to NF-κB signaling pathway[53]. This mechanism could enhance the effects deriving from direct activation of NF-κB by the HCV core protein, which may bind to the death domain of tumor necrosis factor receptor 1 (TNFR1) and to the cytoplasmic tail of lymphotoxin-beta receptor, with resistance to TNF-α-induced apoptosis, suggesting a mechanism by which HCV may evade the host's immune surveillance leading to viral persistence and possibly to hepatocarcinogenesis[75]. On the other hand, HCV infection, and in particular core nonstructural protein (NS)4B and NS5B, reduce TNF-α-induced phosphorylation of IκB kinase (IKK, α, β and γ) and inhibitor of NF-κB (IκB), which are upstream regulators of NF-κB activation. HCV plays a role in immune-mediated liver injury in HCV infection also inhibiting nuclear translocation of NF-κB and expression of NF-κB-dependent anti-apoptotic proteins, such as B-cell lymphoma-extra large (Bcl-xL), X-linked

inhibitor of apoptosis protein (XIAP), and the long form of cellular-FLICE inhibitory protein (c-FLIP)[76]. Furthermore, a crucial host factor for HCV is represented by IKK-α (Figure 1). HCV interacts with DEAD box polypeptide 3, X-linked (DDX3X) through its 3' untranslated region, and activates IKK-α, which translocates to the nucleus and induces a CBP/p300-mediated transcriptional program involving sterol regulatory element-binding proteins (SREBPs). HCV infection in this way utilizes a NF-κB-independent and the kinase-mediated nuclear function of IKK-α: making use of this intrinsic innate pathway and taking control of lipogenic genes and lipid metabolism, enhances core-associated lipid droplet formation to facilitate viral assembly, which in turn may contribute to high chronicity rates and the pathological hallmark of steatosis in HCV infection[77].

CONCLUSION

Up to date only few studies reported the influence of viruses on the clock gene machinery. Further studies are required to investigate the relationship between viruses and the clock genes as they could lead to new therapeutic strategies for future treatment options. Performing cell synchronization may be useful to observe *in vitro* differences in time related patterns of expression[18]. Consequently, we recommend a better set-up of the experiments and cell synchronization before investigating the biological clock at the molecular level, considering that single cells in culture are asynchronous and this may conditionate the results.

As for the new therapeutic strategies that can be developed based on the circadian regulation of viral replication, circadian rhythm-based treatments (*i.e.*, chronotherapies), have been employed against several different pathological conditions[78,79]. Standard therapy for HCV patients involves administration of IFN-α and ribavirin (a nucleoside analogue)[50,56]. Recently, an interferon/ribavirin-free therapy based on newly identified and efficacious protease inhibitors (telaprevir, boceprevir) promisingly entered into the clinic to treat HCV patients[80]. In light of these findings, if the new strategies to inhibit viral replication take in consideration the circadian relationship between host cell and hosted viruses, this could not only minimize the pharmacological agents' toxicity but can also improve the efficacy of treatment modalities through optimized timing of therapeutic regimens, targeting in a better way virus replication. As already suggested, administration of nucleoside analogues to inhibit viral DNA replication can be matched to parallel the diurnal peaks[14] considering the circadian pattern of host cell proliferation and differentiation.

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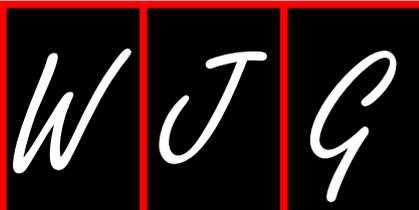
TOPIC HIGHLIGHT

Hepatitis C virus-related mixed cryoglobulinemia: Is genetics to blame?

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Abstract

Mixed cryoglobulinemia (MC) is the extrahepatic manifestation most strictly correlated with hepatitis C virus (HCV) infection; it is a benign autoimmune and lymphoproliferative disorder that evolves to lymphoma in 5%-10% of cases. MC is reputed to be a multistep and multifactorial process whose pathogenicity is still poorly understood. It is still unknown why only some chronically infected HCV patients develop MC and only some of these exhibit systemic symptoms (MC syndrome). Several studies have investigated the pathogenetic basis of MC and the most recent ones suggest that the virus is able to trigger such a disorder only in the presence of genetic factors that are still unknown. Here, we try to clarify the complex relationship between HCV-related MC and the host's genetic background. The data that we report are heterogeneous and sometimes even conflicting. Therefore, large, multicenter studies are clearly needed. The identification of a characteristic genetic signature of cryoglobulinemic patients would be an important step toward a personalized approach in their clinical care. The new wide-ranging genomics technologies will hopefully help to resolve these complex issues.

Key words: Hepatitis C virus; Mixed cryoglobulinemia; Genetics; Viral pathogenetic factors; Host pathogenetic factors

Core tip: Mixed cryoglobulinemia (MC) is the extrahepatic manifestation most strictly correlated with hepatitis C virus (HCV) infection; it is a benign autoimmune/lymphoproliferative disorder that evolves to lymphoma in 5%-10% of cases. MC pathogenesis is still poorly understood. Several studies have tried to clarify the pathogenetic basis of MC and have suggested that HCV can trigger such a disorder only in the presence of still-undetermined genetic factors. Here, we attempt to clarify the relationship between HCV-related MC and the host's genetic background. The data that we report are heterogeneous and sometimes conflicting, so large, multicenter studies are clearly needed.

INTRODUCTION

Mixed cryoglobulinemia (MC) is the extrahepatic manifestation most strictly correlated with hepatitis C virus

(HCV) infection[1], as well as being an autoimmune and B cell lymphoproliferative disorder that evolves to lymphoma in 5%-10% of patients. Defined as a systemic vasculitis, MC is caused by intravascular immune complexes named cryoglobulins (CGs). The term "mixed" refers to the simultaneous involvement of immunoglobulin G (IgG) and IgM in generating the CGs that can include partially monoclonal (type Ⅱ MC) or totally polyclonal (type Ⅲ MC) immunoglobulins. The IgM has rheumatoid factor activity and is produced by clonally expanded autoreactive B cells[2-5].

The pathogenesis of MC is still poorly understood, although it is certain that several subsequent events contribute to disease onset, when they occur in a favorable host genetic substrate[1,6-8]. The reasons why only some chronically infected HCV patients develop MC and why only some of these exhibit systemic symptoms, the so-called MC syndrome (MCS), are unknown. One of the most obvious explanations, the genetic factor, has only recently been seriously contemplated, when the impact of this disease on chronic HCV infection and its role in predisposing to lymphoid malignancies has been recognized. Since then, several studies have tried to clarify the complex pathogenesis of MC and the most recent have focused on genetics.

Together with genetic predisposition, epigenetic factors such as the expression of specific miRNAs can be a major contribution to the pathogenesis of HCV-related lymphoproliferative disorders[9]. In particular, miR-26b is downregulated in peripheral blood mononuclear cells from HCV-related MC but totally restored after complete virological and clinical response to anti-HCV therapy[10,11]. However, this review focuses on the numerous attempts to define the specific genetic background predisposing to development of MC.

We try to clarify this topic by reporting all the attempts to define the genetic basis of HCV-related MC, starting from studies that failed to attribute a direct role in triggering this condition to viral factors, and ending with studies proposing an association between some particular host genetic variants and the development of MC. Other studies have shown a relationship between chronic HCV infection and lymphoma or other autoimmune diseases, which are worth considering for their resemblance to MC.

MC AND HCV FACTORS

Viral genotype and MC

Since the mid-1990s, several studies have analyzed the relationship between HCV factors, such as genotype and viremia, and MC susceptibility. Although results are often conflicting, most studies conclude that the distribution of viral genotypes in MC patients without clinical manifestations does not significantly differ from those observed in HCV patients with no evidence of lymphoproliferation[12-14]. The patients in the cited papers had asymptomatic MC and, as speculated by Sinico *et al*[14], these studies leave open the possibility that HCV genotype or subtype

could influence progression to symptomatic MC. However, the analysis of 60 MC patients, including 22 with symptoms, reported by Frangeul *et al*[15], did not show a significant association between MCS and HCV genotype.

Specific HCV hypervariable region 1 and 2 mutations and MC

Some authors have thoroughly investigated the possible role of mutations in the N-terminal hypervariable regions 1 and 2 (HRV1 and HRV2) of the E2 envelope glycoprotein in predisposition to MC.

The initial results about the relationship between E2 mutational pattern and MC pathogenesis suggest an association of particular HVR1 variants (insertion at codon 385 and deletion at codon 384) with type Ⅱ MC[16]. The authors focused on 385 insertions responsible for improved ability of E2 to bind the HCV putative receptor CD81, with consequent higher stimulation of CD81 itself leading to augmented lymphoproliferation[16].

Another attempt, published some years later, did not confirm these data, but correlated different viral mutations with MC (positions 389 and 398 for HVR1 and positions 474, 493 and 497 for HVR2)[17]. Conversely, a study published by Rigolet *et al*[18], after an accurate approach of cloning and sequencing HVR1 regions isolated from HCV-positive MC patients, clearly concluded that any particular motif of E2 coding sequence could be associated with MC. These data were confirmed in a study conducted on a population of 80 MC patients by Bianchettin *et al*[7]. A similar experimental plan and accurate statistical and bioinformatic approaches suggested that MC arose by as-yet-unidentified host rather than virus-specific factors, meaning that attention should be focused on the host.

Convincing proof of the role played by host genetics in determining HCV-related MC onset appeared in an epidemiological study by Pozzato *et al*[19], which demonstrated that there were ethnic differences in the prevalence of asymptomatic HCV-associated monoclonal B-cell expansion. Based on an observational suspicion of a high prevalence of MC in Italy versus a low prevalence in Japan, the authors investigated 60 Italian and 44 Japanese HCV patients and concluded that there were no differences in the two groups apart from ethnicity. This clearly suggests that HCV is able to induce B-cell expansion only in the presence of unidentified genetic factors.

MC AND GENETIC FACTORS

MC and HLA polymorphisms

The first studies regarding the host genetic factors conditioning susceptibility to development of MC during chronic HCV infection analyzed human leukocyte antigen (*HLA*) gene cluster variants. *HLA* gene products are responsible for presenting viral antigens to T cells, therefore, it has been speculated that some HLA variants could be implicated in driving the immune response against the virus to produce autoreactive antibodies (the CGS). An early attempt to investigate the genetic predisposition to MC was published even before the discovery

of HCV and HLA class Ⅱ polymorphisms. Migliorini *et al*[20] did not find any association between MC and either class Ⅰ or class Ⅱ HLA molecules. Since then, several studies and some controversial data have been published. Ossi *et al*[21], studying 16 MC patients, showed a higher expression of HLA-B51 and B35 antigens, previously correlated with other autoimmune disorders, as well as the presence of HLA-A9 with its A24 split in 50% of the same population.

An almost contemporary study performed in a large cohort of multi-transfused patients, including 116 HCV-positive ones, showed no association between a specific HLA pattern and MC. The authors conclude that the HLA class Ⅱ DR2 subtype (DRB1*1601, DQB1*0502), which is characteristic of multi-transfused patients who maintain HCV negativity after years of blood transfusions, could be considered as a sort of protection against HCV infection[22].

A meticulous study, mostly for the accuracy of the statistical analysis, showed a higher frequency of HLA-B8 and HLA-DR3 in a group of 25 HCV-positive cryo-patients[23]. The odds ratio was also calculated and the highest corresponded to the presence of both B8 and DR3, suggesting the existence of an HLA-B8-DR3 haplotype associated with HCV-infected MC patients. These results were partially confirmed in a Chinese study in which HLA-DR3 was significantly associated with the presence of HCV-related cryoglobulinemia that was mostly asymptomatic[24].

The absence of an association between HLA and MC was demonstrated by another Italian group. Analysis of HLA-DRB1 alleles in 46 patients with HCV infection concluded that HLA class Ⅱ polymorphisms did not distinguish patients with MC from those without MC[25].

Cacoub *et al*[26] also evaluated *HLA-DRB1* and *HLA-DQB1* polymorphisms in a cohort of 76 symptomatic or asymptomatic MC patients. Multivariate logistic regression analysis of several features indicated the presence of HLA-DR11 as a positive predictor of MC, together with the already known female sex and age. The same HLA class Ⅱ alleles were evaluated in another study that focused on the association between particular HLA-DR-DQ combinations and HCV-positive non-Hodgkin's lymphoma (NHL) with and without a background of MC[27]. Various HLA Ⅱ associations have been found for HCV-positive NHL in the presence of MC (higher frequency of DR5-DQ3 HLA) and for HCV-positive and MC-negative NHL (higher frequency of DR1-DQ1), suggesting the presence of alternative pathogenetic processes for similar but different HCV lymphomas.

MC and cytokine mutations

Alterations in the cytokine/chemokine patterns, also involving proinflammatory and Th1 chemokines, have been demonstrated in MC and other extrahepatic disorders induced by HCV infection[28]. These previous studies have investigated genetic variants of this complex class of immune response regulators.

Several studies have shown that interleukin (IL)-10

may be involved in the pathogenesis of lymphoid disorders; moreover, three different mutations in the IL-10 promoter (-1082G→A, -819C→T and -592C→A) were associated with higher IL-10 production. In a study by Persico *et al*[29], conducted on 270 well-characterized patients with NHL and/or HCV-related chronic hepatitis, a high prevalence of IL10-1082GG genotype was significantly associated with NHL in HCV-infected patients.

Polymorphisms of inflammatory chemokines are also significantly correlated with the outcome of HCV infection, because chronic hepatitis itself is closely associated with inflammation.

Recent reports have shown high levels of a B-cell-specific cytokine, namely B-cell-activating factor (BAFF; or B lymphocyte stimulator), in the serum of HCV patients with lymphoproliferative disorders but could not define the mechanisms underlying this phenomenon[30-33]. BAFF is a tumor necrosis factor α family member and a key regulator of B-cell differentiation, survival, and immunoglobulin secretion, and the mutated genotype of its promoter (-871T) is associated with higher BAFF mRNA levels in monocytes[34,35]. Two consecutive studies conducted on a well-characterized MC population indicated a significantly higher prevalence of T allele homozygosity in patients with MCS, as well as the presence of the T allele (homozygous TT plus heterozygous TC) compared to HCV carriers without MC[8,36]. These results are consistent with the serum BAFF levels found in the different groups. Therefore, the transcriptional activation induced by the BAFF promoter variant could be considered one of the mechanisms contributing to the pathogenesis of HCV-related lymphoproliferative disorders.

MC and IgG Fc receptors

Two independent studies have evaluated the role of the genetic variability of IgG Fc receptors (FcGRs) in the susceptibility to MC during the course of HCV infection. The FcGRs, present on leukocytes, are responsible for the clearance of immune complexes, phagocytosis, antibody-dependent cellular cytotoxicity, and regulation of the release of inflammatory mediators and B-cell activation, mainly in phagocytes. Their polymorphic variants are associated with reduced affinity for immune complexes, autoimmune diseases, and cancer[37]. In the first study, Vassilopoulos *et al*[38] analyzed a cohort of HCV patients with different autoimmune/lymphoproliferative disorders, including MC, discriminating between symptomatic and asymptomatic individuals and investigating FcRⅢ A and the *NA1/NA1 FcGRⅢ B* genotypes. They did not find any increased frequency of particular alleles in the autoimmune manifestations group compared to historical controls. In the second study, a more numerous cohort of cryoglobulinemic patients was evaluated. Despite the wider and better characterized MC population, this recent screening of FcGR2A 131R/H, FcGR2B 232 I/T, FcGR3A 176 V/F and FcGR3B NA1/NA2 confirmed the previous results, with the distribution of *FcGR* genotypes not being significantly different compared to the controls[8]. We reported in 21 HCV-MC patients treated

Table 1 Association between hepatitis C virus-related lymphoproliferative disorders and host genetic factors

Factors	References
HLA polymorphisms	
HLA-A9	[21]
HLA-B8	[23]
HLA-DR3	[23,24]
HLA-DR11	[26]
HLA-DR5-DQ3	[27]
Cytokine mutations	
IL-10 promoter (-1082GG)	[29]
BAFF promoter (-871T)	[8]
Sporadic associations	
Fibronectin Msp I and HaeIIIb	[40]
CYP27B1	[41]

HLA: Human leukocyte antigen; IL: Interleukin; BAFF: B-Lymphocyte activating factor.

with rituximab (anti-CD20 monoclonal antibody) that the response was strictly related to the F allele homozygosity of FcGR3A, suggesting that this genotype could be involved in response to rituximab therapy.

Sporadic associations

The role of mutations within Fas and Fas-L genes has been described in mice with an increased prevalence of autoimmune manifestations, therefore, some authors have postulated that such mutations could be related to autoimmune diseases and lymphoproliferation. Results obtained from a small number of patients with Sjögren's syndrome or type Ⅱ MC do not support such a hypothesis, suggesting that the germline mutations of the Fas receptor and its ligand are probably not involved in the pathogenesis of HCV-related type Ⅱ MC[39].

A possible relationship between two fibronectin polymorphisms (called *Msp* I and *Hae*Ⅲb) and type Ⅱ MC has been investigated, in order to define the risk of lymphoma development. Fabris *et al*[40] analyzed 74 patients with MC, including 21 who developed B-cell NHL and 72 with HCV-negative and MC-unrelated NHL. None of the major MC-related clinical manifestations was significantly linked with a particular allele or genotype of the *Msp* I and *Hae*Ⅲb fibronectin gene polymorphisms. However, the two genetic sites seem to confer an independent increased risk of NHL in MC patients.

As a result of the critical role of vitamin D in the regulation of the immune system, the analysis of the serum vitamin D status in HCV-infected patients with extrahepatic manifestations seems particularly interesting. Terrier *et al*[41] found a strong association between low serum levels of vitamin D and the presence of MC and systemic vasculitis in patients with chronic HCV infection. Regarding the B-cell compartment, they observed significant correlations between serum 1,25-dihydroxyvitamin D and the B-cell activation status.

Lange *et al*[42] previously found that 1,25-dihydroxyvitamin D serum concentrations were higher in HCV patients with *CYP27B1* AA genotype compared to patients

with *CYP27B1* AC or CC genotype, thus, it is conceivable that MC patients harbor these latter genotypes. Unfortunately, no further studies have been published on this topic but an abstract of Terrier Benjamin *et al*[43] reports an exactly opposite association between phenotype and genotype in patients with HCV-related systemic vasculitis.

Recent important advances in the HCV field strongly suggest that the polymorphic variants of the *IL-28B* gene should be analyzed. Indeed, in 2009 several independent studies have shown that single nucleotide polymorphisms near the IL-28B coding region are closely associated with HCV clearance. IL-28B is involved in innate immunity and a recent study evaluated the influence of these genetic variations in the development of HCV-related MC[44]. The allele distribution reported in the study was similar in patients with or without MC, and does not support the hypothesis that these polymorphisms influence the development of MC.

The associations between HCV-related lymphoproliferative disorders and host genetic factors are summarized in Table 1.

CONCLUSION

It is clear from the reports described in this review that the role of genetics in HCV-related MC is a current and compelling research topic. Each patient is genetically unique, which can affect the evolution of chronic HCV infection towards benign lymphoproliferation predisposing to lymphoma. The identification of a characteristic genetic signature of cryoglobulinemic patients could be a step towards personalized approaches in the clinical care of HCV infection, which are useful for targeted follow-up of high-risk individuals. The above data are heterogeneous and sometimes even conflicting, thus, there is a clear need for multicenter studies including large numbers of patients, and the future application of the new genomic and proteomic wide-range technologies will surely assist in this direction.

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TOPIC HIGHLIGHT
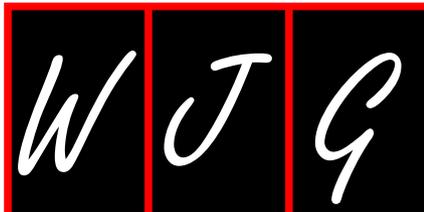
WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Immunological alterations in hepatitis C virus infection

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Abstract

A higher prevalence of immunological processes has recently been reported in patients with hepatitis C virus (HCV) infection, focusing the attention of physicians and researchers on the close association between HCV and immune disorders. HCV lymphotropism represents the most important step in the pathogenesis of virus-related immunological diseases and experimental, virologic, and clinical evidence has demonstrated a trigger role for HCV both in systemic autoimmune diseases, such as rheumatoid arthritis, Sjögren syndrome, hemolytic anemia and severe thrombocytopenia, and in organ-specific autoimmune diseases, such as autoimmune hepatitis, thyroid disorders and diabetes. This review will outline the principal aspects of such HCV-induced immunological alterations, focusing on the prevalence of these less characterized HCV extrahepatic manifestations.

Key words: Hepatitis C virus; Immune disorders; Cytopenia; Extrahepatic manifestation; Autoantibody

Core tip: Hepatitis C virus (HCV)-infected lymphoid tissue of the host represents a site for the persistence of HCV infection which exerts a chronic stimulus to the immune system, facilitating clonal B-lymphocyte expansion and consequent wide autoantibody production, including cryo- and non-cryo-precipitable immune complexes which may lead to organ- and non-organ-specific immunological alterations. This review outlines the principal aspects of such HCV-induced immunological alterations, focusing on the prevalence of these less characterized HCV extrahepatic manifestations.

INTRODUCTION

Autoimmunity and viral infections are closely related, and the hepatitis C virus (HCV), is recognized as one of the viruses most often associated with autoimmune features. For this reason HCV is not only associated with chronic hepatic inflammation but also an array of extrahepatic complications. In the majority of these associated extrahepatic manifestations, the pathogenic mechanism appears to be immunologically driven, with many having features of autoimmunity. HCV infection has been associated with both organ-specific [thyroiditis, diabetes, autoimmune hepatitis (AIH)] and systemic autoimmune diseases and this association has generated growing interest in recent years since it is often observed in patients with chronic HCV infection.

PATHOGENESIS OF HCV RELATED IMMUNE DISORDERS

HCV lymphotropism represents the most relevant step in the pathogenesis of virus-related immunological dis-

orders[1]. Indeed, infected lymphoid tissue of the host represents a site for the persistence of the HCV infection[2-6]. HCV exerts a chronic stimulus to the immune system, facilitating the clonal B-lymphocyte expansion and consequent wide autoantibody production, including cryo- and non-cryoprecipitable immune complexes[3,7-9] which may lead to organ- and non-organ-specific immunological alterations[3,7,8,10]. The first step is translocation, demonstrated in a high percentage of HCV-infected patients, with consequent Bcl-2 proto-oncogene activation, antiapoptotic activity and prolonged survival of lymphocytes[3,7,9,10]. Besides, the identification of HCV envelope protein E2 able to bind the CD81 molecule expressed on both hepatocytes and B-lymphocytes seems to be crucial for HCV-driven autoimmunity[3,7,9,10].

Dysregulation of cytokine networks skewing regulatory T-cells to a Th2 phenotype, which may be associated with enhanced humoral immune responses and autoantibody production has also been related to the expansion of autoantibody-producing B-cells and chronic lymphoproliferation in HCV infection[11]. HCV infections induce a massive chemokine and cytokine burst and therefore recruit leukocytes to the site of infection with the goal to stop viral spread. This excitation of the human defense system could stimulate a potentially self-reactive lymphocytes inducing autoimmunity in susceptible individuals[11].

Many studies have linked Th1 immune response with HCV infection[12], mixed cryoglobulinemia (MC)[13] and organ specific autoimmune disorders[14]. These findings suggest that a possible common immunological Th1 pattern could be the pathophysiological base of the association of autoimmunity related HCV infections.

Several studies have shown an increased expression of interferon-gamma (IFN-γ), and IFN-γ inducible chemokines (C-X-C motif chemokine 10 - CXCL10), in hepatocytes and in lymphocytes of HCV-infected patients[12,15,16], which are directly related to the degree of inflammation and an increase in circulating levels of IFN-γ and CXCL10[17,18].

Furthermore, it has been shown that NS5A and core proteins, alone or by a synergistic effect with Th1 cytokines [IFN-γ and tumor necrosis factor-α (TNF-α)], are capable of upregulating CXCL10 and monokine induced by gamma interferon (MIG) gene expression and secretion in cultured human hepatocyte derived cells. These data suggest that CXCL10 produced by HCV-infected hepatocytes could play a key role regulating T-cell trafficking into a Th1-type inflammatory site by recruiting Th1 lymphocytes, that secrete IFN-γ and TNF-α, with a synergistic effect on CXCL10 secretion by hepatocytes, thus perpetuating the immune cascade[19].

HCV AND SYSTEMIC AUTOIMMUNE DISEASES

Mixed cryoglobulinemia

MC is the most well documented extrahepatic manifestation of HCV infection[2,20]. MC, which is defined by documenting cryoprecipitates in serum (Ig precipitates from serum at temperatures under 37 ℃ and dissolves upon re-warming), is characterized by the presence of circulating immunocomplexes produced by a benign proliferation of B-cells. MC represents the link between HCV and various autoimmune and lymphoproliferative disorders. Although serum cryoglobulins (CGs) are frequently present in patients with chronic HCV[3-5,21,22], in many of them CGs are present at low levels and symptoms are often absent or very mild. Only about 5% of HCV-infected subjects have clinically overt MC syndrome.

HCV-related arthritis

Chronic oligo-polyarthritis during chronic HCV infection is often associated with MC but can also represent an independent entity. Indeed, it is not rare to observe a simple association between HCV infection and classical rheumatoid arthritis (RA) that can co-exist by chance or can be related to the ability of HCV to act as a trigger of the immune disease in individuals genetically predisposed to RA.

A polyarthritis, which is often non-erosive and rarely progressive, and involves small joints is the most common kind of arthritis associated with HCV chronic infection without the coexistence of cryoglobulinemia. Instead, 40%-80% of HCV-infected patients with MC[23] are reported to have a bilateral and symmetric arthralgia, which is non-deforming and includes mainly the knees and hands, and, more seldom, the elbows and ankles. Rheumatoid factor (RF) activity is found in 70%-80% of MC patients but is not correlated with the presence of articular disease, as patients chronically infected with HCV in the absence of HCV-MC or RF may have prominent articular symptoms. Usually there is no evidence of joint destruction, and antibodies to cyclic citrullinated peptide, which are highly specific to rheumatoid arthritis, are absent[24]. These evidences suggest that HCV infection should be considered in the differential diagnosis of patients with atypical arthritis.

Sjögren syndrome

Another autoimmune condition associated with HCV is a chronic lymphocytic sialoadenitis similar to sialoadenitis associated with idiopathic Sjögren syndrome (SS), which has been reported in approximately 50% of patients with HCV infection[25].

Some authors have distinguished the HCV-related sicca syndrome from Sjogren's syndrome based on several differences, including absence of anti-SSA and anti-SSB antibodies, pericapillary and non pericanalary lymphocytic infiltration, lack of glandular canal damage, high prevalence of mixed cryoglobulinemia (50%), hypocomplementemia (51%), and systemic vasculitic manifestations (58%)[25-28]. Moreover, the lymphocytic type of the infiltrate in the minor salivary gland shows a predominance of CD8 lymphocytes which is not observed in primary SS[29]. Although the possible etiopathogenetic

role of HCV in SS remains a controversial issue[27], the explanation for this extrahepatic manifestation could be a cross reactivity between the HCV envelope and host salivary tissue which lead to an immune reaction directed against salivary glands[26]. The correct classification of patients with sialoadenitis related to HCV chronic infection have important clinical, prognostic and therapeutic implications since it may evolve into a B cell malignant lymphoma, especially in the presence of MC[10,30].

HCV related cardiac disorders

Several observations suggest that HCV infection is an important cause of a variety of otherwise unexplained heart diseases. Indeed, it was reported that (+) or (-) chain HCV-RNAs can be detected in the biopsied myocardial tissue or in the autopsied heart suggesting that HCV might proliferate in the myocardium[31], resulting in induction of cardiomyopathy. Frustaci *et al*[32] have shown that HCV replicates in myocardial tissue of patients with myocarditis, and that HCV infection may contribute to the development of an autoimmune myocarditis, frequently associated with myocardial antibodies and responsive to immunosuppressive therapy. In 2000, Matsumori suggested that some specific HCV clones with high affinity for the heart can develop and cause cardiomyopathy[33] and in 2006, in a large study involving more than 1000 patients, the same group identified anti-HCV antibodies, HCV RNA, NT-proBNP, and cardiac troponin I and T in sera stored for up to 17 years, and found the anti-HCV antibodies were more prevalent in patients with myocarditis than in the general US population[34]. These results suggest that in regions where its prevalence is high, HCV infection may be an important cause of myocarditis and heart failure. Moreover, the same authors concluded that NT-proBNP is a more sensitive marker of myocardial injury than cardiac troponins in patients with heart failure from HCV myocarditis. More recently, other studies confirmed that NT-proBNP is a sensitive biomarker for identifying patients with heart failure caused by HCV-related myocarditis[35,36]. Antonelli *et al*[36] assessed serum NTproBNP in 50 HCV-positive patients and in 50 sex- and age-matched controls. HCV patients showed significantly higher mean NT-proBNP level than controls[35]. This result was confirmed by the same group in another study where TNF-α was also found to be higher in HCV+ patients with respect to controls, suggesting the presence of subclinical cardiac dysfunction[36].

AUTOIMMUNE CYTOPENIAS IN PATIENT WITH HCV INFECTION

Hemolytic anemia and severe thrombocytopenia were the most frequent cytopenias observed in patients with HCV infection. The different types of immune-mediated cytopenias may be severe and clinically significant.

Hemolytic anemia

Although autoimmune hemolytic anemia (AHA) has frequently been reported in association with HCV in the setting of interferon (IFN) treatment[37,38], it has also been observed as an isolated extrahepatic manifestation. The existence of AHA in patients with chronic hepatitis was first described in 1951, when Hyman *et al*[39] described AHA in 3 patients with chronic liver involvement. In 1973, Panush *et al*[40] described a patient with chronic active hepatitis who presented with AHA with a positive Coombs test, who responded to treatment with steroids. In 1982, Portell *et al*[41] reported 5 patients with chronic hepatopathy (3 with active chronic hepatitis and 2 with cirrhosis) and a positive Coombs AHA, with positive ANA in 4 and sicca syndrome in 1. In 2001, 2 cases of HCV infection associated with Coombs-positive AHA, in the absence of treatment with IFN, were reported by Srinivasan[42] and Chao *et al*[43], respectively. In 2003, Ramos-Casals *et al*[44] presented the largest series of cases of HCV-related AHA not treated with antiviral therapy. Seventeen HCV patients, mostly women with a mean age of 56 years, presented a high level of association with autoimmune diseases, with cryoglobulinemia as the most frequent immunologic marker. Most patients had a history of liver cirrhosis and even if they had a good response to corticosteroids, the prognosis was poor (56% mortality).

HCV-associated immune thrombocytopenic purpura

Although thrombocytopenia during the course of chronic liver disease is usually attributed to hypersplenism, an autoimmune mechanism has been suggested as playing a role in some patients with HCV infection. This hypothesis is based on the observation of a greater prevalence of thrombocytopenia and antiplatelet antibodies in HCV patients compared with HBV patients[45], and of the frequency of HCV infection seen among patients initially diagnosed with idiopathic thrombocytopenic purpura (ITP)[46-48]. The pathophysiology of infection-related ITP involves diverse immunologic pathways as well as non-immune mechanisms that accelerate platelet destruction and/or decrease platelet production.

High affinity binding of HCV to the platelet membrane with subsequent binding of anti-HCV antibody might lead to phagocytosis of platelets[49]. Dysregulation of the host immune system triggering the production of autoantibodies against platelet glycoproteins has also been postulated[45,50]. However there have been conflicting data on the presence of specific antibodies in platelets in patients with HCV-related ITP[45,50-52].

Thrombocytopenia in HCV patients may be present even in the absence of clinically evident liver disease or splenomegaly and may be mistakenly diagnosed as primary chronic immune thrombocytopenic purpura (CITP)[48,53]. Six cross-sectional studies have reported serologic evidence of HCV infection in 20% of patients with a clinical diagnosis of CITP[48,53-57], and in the largest series published to date, HCV antibodies were identified in 30% of

250 patients fulfilling the American Society of Hematology criteria for CITP[54]. There were significant differences in the demographic characteristics of patients with HCV-positive compared with patients with HCV-negative CITP. Patients positive for HCV were older and the incidence was distributed equally between the sexes compared with the female predominance in HCV-negative CITP.

ORGAN-SPECIFIC AUTOIMMUNE DISEASES

Thyroid disorders and HCV

Autoimmune thyroid involvement and hypothyroidism were significantly more frequent in patients with chronic hepatitis C (CHC) than in comparison groups such as patients with viral hepatitis B or D[58-60] or normal subjects[61,62]. The most frequent thyroid disorder in this setting is the presence of circulating anti-thyroid antibodies which is more commonly reported in female subjects[58]. The prevalence of abnormally high levels of anti-thyroid antibodies observed in these patients ranges from 2% to 48%[58,61,63,64], with heterogeneous geographic distribution[65]. These discrepancies are related to variable genetic predisposition and environmental co-factors, such as iodine intake or other infectious agents[66]. The evidence of a subclinical hypothyroidism was observed in 2%-9% of patients with chronic HCV infection, particularly in those patients with MC[59,60,62,63,67], and these patients seem to be susceptible to Hashimoto's autoimmune thyroiditis and Grave's disease when treated with interferon.

Antonelli *et al*[21] in 2004 analyzed 630 consecutive patients affected by CHC compared with a large control group of subjects from iodine-deficient and sufficient areas and with 86 patients with chronic hepatitis B. They demonstrated that patients with CHC were more likely to have hypothyroidism, anti-thyroglobulin and anti-thyroid peroxidase antibodies than any of the other groups. The same group evaluated thyroid function, the presence of thyroid autoantibodies, thyroid nodules and thyroid cancer, in 93 HCV + MC consecutive patients matched by sex and age to 93 patients with CHC without MC and 93 healthy (HCV-negative) controls. Subclinical hypothyroidism and thyroid autoimmunity were significantly more frequent in HCV + MC patients than in HCV-negative controls. Moreover, serum thyroid peroxidase antibodies were also significantly more frequent in HCV + MC patients than in CHC patients. Finally, the prevalence of thyroid nodules was not significantly different in the three groups[68]. In conclusion, pooling all data about HCV-positive patients (with CHC or HCVAb positivity) and using as control healthy subjects and HBV-infected patients, there was a significant increase in the prevalence of both thyroid autoimmune disorders (OR = 1.6; 95%CI: 1.4-1.9) and hypothyroidism (OR = 2.9; 95%CI: 2.0-4.1)[69].

Some authors have reported that patients with chronic HCV have a higher prevalence of papillary thyroid carcinoma[70,71]. In 2002, the prevalence of thyroid cancer in a series of 94 HCV-related mixed cryoglobulinemic patients was investigated[70]. A control group was obtained from a sample of the general population (2401 subjects) who had undergone thyroid ultrasonography. The prevalence of thyroid nodules was higher, although not significantly so, in control subjects than in MC patients but 2 patients with papillary thyroid cancer were found in the MC series, while no case was observed among controls.

A more recent study[71] prospectively investigated the prevalence and features of thyroid cancer in 308 patients with CHC in comparison with 2 large sex- and age-matched control groups from the general population with different iodine intake. Thyroid status was assessed by measurement of circulating thyroid hormones and auto-antibodies, thyroid ultrasonography, and, when indicated, fine-needle aspiration cytology. The authors have found that circulating thyrotropin, anti-thyroglobulin, and anti-thyroperoxidase antibodies levels, and the prevalence of hypothyroidism were significantly higher in HCV patients and 6 cases of papillary thyroid cancer were detected among HCV patients, whereas only 1 case was observed in controls, suggesting a high prevalence of thyroid papillary cancer in HCV patients. Because of this high prevalence of thyroid disorders, the guidelines on management of CHC recommend investigation of thyroid function, including free T4 and TSH in all patients, and since interferon-based therapy could exacerbate thyroid dysfunction, thyroid function tests should be fully evaluated prior to initiating HCV treatment.

Diabetes mellitus and HCV

Data from the literature have shown a higher incidence of type 2 diabetes mellitus with chronic HCV when compared with patients with other liver disorders[72-74]. In a large study[75] involving 229 consecutively recruited MC-HCV patients compared with 217 sex- and age-matched controls without HCV infection, the prevalence of type 2 diabetes was significantly higher in MC-HCV patients than in controls. Moreover, MC-HCV diabetic patients more often had non-organ-specific autoantibodies than non-diabetic MC-HCV patients.

Another study conducted in 2005 by the same group[22], established the prevalence and clinical phenotype of type 2 diabetes in a large series of non-cirrhotic HCV patients. The prevalence of type 2 diabetes was significantly higher in HCV patients compared with control subjects or non-cirrhotic HBV patients. Moreover, type 2 diabetic HCV patients had a significantly lower BMI than type 2 diabetic control subjects and significantly higher BMI than non-diabetic HCV patients. In contrast, no association with diabetes mellitus type 1 has been identified[22,72,73,76-78]. The association between chronic HCV and diabetes mellitus seems to be independent of the severity of the liver disease and is associated with insulin-resistance, but not with the presence of pancreatic anti-insulin antibodies[79]. In contrast, interferon treatment of HCV has been associated with the appearance of diabetes mellitus type 1 and development of anti-

pancreas autoimmunity[80-82].

AIH and HCV infection

Finally, an intriguing, still controversial aspect is the possible etiopathogenetic role of HCV in AIH[3,6,8,9,65]. Patients with AIH may present with mixed cryoglobulins, HCV infection, and extrahepatic manifestations such as thyroiditis, sicca syndrome, and arthritis[6], while patients with HCV infection show one or more non-organ-specific auto-antibodies. The antigenic target specificity of HCV-related autoantibodies shows only quantitative differences compared with those associated with "primary" AIH[8].

In clinical practice, the search for serum autoantibodies should be limited to cases for whom treatment with IFN is planned. An exception may be cases where clinical data (female gender, young age), high biochemical activity (transaminase-globulins) and histological aspects (interfaces hepatitis) of liver disease may suggest the presence of AIH with superimposed HCV infection.

The heterogeneous geographical distribution of HCV-associated AIH[65] suggests a possible involvement of various pathogenetic co-factors; among these, HCV might trigger a particular AIH clinico-serological subset, which is prevalent in specific geographical areas.

CONCLUSION

In the case of patients with chronic HCV infection, the possible existence of extrahepatic manifestations should be taken into account and an accurate analysis of clinical and anamnestic data is recommended. Some patients may display the entire complex spectrum of HCV-related disorders which could be mild for many years and progress, generally during a long follow-up, to more severe systemic manifestations. In the last few years, very consistent data have been accumulated through different *in vivo* and *in vitro* models, suggesting that a more accurate characterization of the modalities and consequences at the molecular level of HCV infection of lymphatic cells may be of great importance in the future for the clarification of the pathogenesis of several pathological manifestations of HCV.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Interleukin 28B polymorphisms as predictor of response in hepatitis C virus genotype 2 and 3 infected patients

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Abstract

Single nucleotide polymorphisms near the interleukin 28B (*IL-28B*) gene have been identified as strong predictors of both spontaneous or Peg-interferon (Peg-IFN) and ribavirin (RBV) induced clearance of hepatitis C virus (HCV). Several studies have shown that, in patients with genotype 1 (GT-1), rs12979860 C/C and rs8099917 T/T substitutions are associated with a more than two-fold increase in sustained virological response rate to Peg-IFN and RBV treatment. Although new treatment regimens based on combination of DAA with or without IFN are in the approval phase, until combination regimens with a backbone of Peg-IFN will be used, we can expect that IL28B holds its importance. The clinical relevance of IL28B genotyping in treatment of patients infected with HCV genotype 2 (GT-2) and 3 (GT-3) remains controversial. Therefore, after a careful examination of the available literature, we analyzed the impact of IL28B in GT-2 and -3. Simple size of the studies and GT-2 and GT-3 proportion were discussed. An algorithm for the practical use of IL28B in these patients was suggested at the aim of optimizing treatment.

Core tip: The clinical relevance of interleukin 28B genotyping in patients with hepatitis C virus genotype 2 and 3 infection is debated. In this critical analysis of studies performed so far, it was shown that this genetic tool may help in optimizing treatment of genotype 3 patients, whilst it plays a marginal role in genotype 2 infected patients management.

INTRODUCTION

In patients with hepatitis C virus (HCV) genotype 1 (GT-1), the standard treatment based on dual combination of Pegylated interferon (Peg-IFN) and ribavirin (RBV) has been replaced by the triple combination regimen including a protease inhibitor; on the contrary, in patients with HCV genotype 2 (GT-2) and genotype 3 (GT-3), it continues to represent the standard of care[1,2].

Interleukin28B (*IL-28B*) genotype is a strong predictor of response to IFN-based treatment in GT-1[3-6], but at a first glance, genetic analyses so far conducted in GT-2 and -3 patients provided ambiguous results[7-14]. The studies published so far may have bias but we should also bear in mind that identification of response predictors assumes a different relevance in different HCV genotypes. Indeed, high rates of sustained virologic response (SVR) achieved in GT-2 infected patients make response predictors of marginal interest and limit their use to the identification of patients who may take

Table 1 Prevalence and impact of interleukin 28B rs12979860 in studies combining genotype 2 and genotype 3

Study	No. of patients	Prevalence of *IL-28B* CC genotype	Treatment duration (wk)	RVR in *IL-28B* CC genotype	SVR in *IL-28B* CC genotype
Mangia *et al*[10]	268	41%	12-24	59%	79%
Sarrazin *et al*[11]	267[1]	43%	24-48	51%	47%
Lindh *et al*[13]	341	44%	12-24	67%	70%
Bitetto *et al*[19]	101	37%	24	na	78%

[1]Follow-up information not available in all patients. RVR: Rapid virological response; SVR: Sustained virological response; IL: Interleukin.

profit from a treatment of short duration whilst. On the contrary, in GT-3 patients, the unsatisfactory rate of SVR reported even with IFN-free regimens, induce a continuous search of predictors of response[15].

A detailed examination of the studies on IL28B in GT-2 and GT-3 patients suggests that there are valid explanations for the contrasting results on SVR association. In our opinion, the analysis of genetic predictors performed in mixed cohorts, incorporating GT-2 or -3 in different proportions may be the first responsible of these contrasting results[10,11]. Additional confounders are the different treatment regimens of the patients included in these studies, heterogeneous in terms of duration and intensity[11,14], and, more importantly, the different populations of patients evaluated in these retrospective genetic analyses that combines naive and previous treatment-experienced[16].

Finally, the use of either rs8099917 SNP located 7.6 kb upstream the *IL-28B* gene or rs12979860, located 3.2 kb upstream the open reading frame of IL28B gene may be an additional source of confusion. Indeed, these two SNPs showed similar distribution in Caucasian patients but different frequency and, consequently, lower strength of association in races other than Caucasian.

To overcome some of these issues, two meta-analyses on the role of IL28B in GT-2 and 3 have been recently published[17,18]. However, as happened with (the) single studies, these meta-analyses reached contrasting results. In the study by Chen *et al*[17] no association between SVR and *IL-28B* CC was found in the subgroup of GT-2 and 3, although it was shown that TT at rs8099917 SNP is associated with a favorable response in GT-2 Asian subjects. In the second meta-analysis, the Authors reached the conclusion that the favorable *IL-28B* CC genotype is a statistically significant predictor of SVR and rapid virological response (RVR) in Caucasian patients treated with Peg-IFN and ribavirin for 24 wk, with the exception of Asian patients with GT-2 achieving higher rates of RVR when carrying the favorable *IL-28B* genotype[18].

DETAILED ANALYSIS OF THE STUDIES

Data summarizing the results of the studies on rs1297860 in HCV mono-infected patients are reported in Table 1. Studies including GT-2 and -3 lumped together[10,11,13,19] and studies investigating cohorts of patients with GT-3 alone were separately analyzed[12,14,20,21]. Results by genotype were provided by a large study from our group investigating 710 patients[21]. Another study focused on viral kinetics of IL28B polymorphisms by GT-1 *vs* GT-2 and -3[20].

Studies combining GT-2 and-GT-3

The results of the largest studies on IL28B treatment response prediction in GT-2 and GT-3 are here analyzed. Combined results for GT-2 and GT-3 together are generally provided. Stored DNA samples from 268 Caucasian patients enrolled in a multicenter controlled trial from Italy were tested for rs12979860. Patients were randomized to Peg-IFN and RBV for standard (24 wk) or variable (12/24 wk) treatment duration on the basis of RVR. Two hundred and thirteen patients were GT-2 and 55 GT-3 infected[10]. *IL-28B* CC-type was present in 37% of patients. Rates of SVR were 82% in patients with CC-type, 75% in CT and 58% in TT. The CC-type resulted an independent predictor of SVR, but the predictive role was largely driven by the capability of predicting SVR among patients without RVR. Like in GT-1 (22), among the 165 (61%) patients with HCV RNA undetectable at week 4, *IL-28B* genotype was not predictive of SVR[10].

These findings reinforce the concept that a week 4 undetectable HCV RNA is the strongest predictor of SVR to Peg-IFN and RBV treatment; at the same time, they suggest that the clinical relevance of *IL-28B* genotype for GT-2 and -3 is far from being borderline, as it can help in selecting patients that may be interferon insensitive at baseline.

The results reported by Sarrazin *et al*[11] regarding 267 patients (GT-2 = 77, GT-3 = 190), among which only 205 received treatment, are apparently in contrast with our conclusions. The Authors showed an association between *IL-28B* CC-type and SVR in the subgroup of patients with RVR. No association was observed in patients without RVR[11]. However, despite a lower rate of RVR in observed in this study than in others (40%), only 11 patients without RVR were analyzed (CC = 3, CT = 4 TT = 3). Therefore, a type II error cannot be ruled out. Moreover, if we analyze CC *vs* CT plus TT patients who completed the treatment, we can observe a trend toward a statistically significant association with SVR in the subgroup of patients without RVR (*P* = 0.08).

If we consider the results of the previously reported studies on GT-2 and -3 in comparison with those attained in patients with G1 infection[22], we could hypothesize that the lower the rate of RVR, the stronger the association between SVR and CC-type (Figure 1).

Similar considerations apply to the study by Lindh *et al*[13]. In this study, 341 White patients with GT-2 and
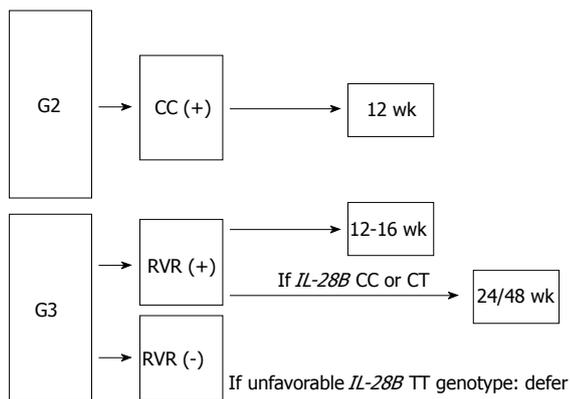
Figure 1 Algorithm for clinical application of interleukin 28B genotyping in hepatitis C virus GT-2 and GT-3 infected patients. RVR: Rapid virological response; IL: Interleukin.

Table 2 Prevalence and impact of interleukin 28B rs12979860 in studies analyzing genotype 3 separately

Study	No. of patients	Prevalence of *IL-28B* CC Genotype	Treatment duration (wk)	RVR in *IL-28B* CC genotype	SVR in *IL-28B* CC genotype
Scherzer *et al*[12]	71	38%	2	78%	76%
Moghaddam *et al*[14]	281	46%	14-24	84%	77%
Fattovich *et al*[20]	55	51%	24	86%	87%
Mangia *et al*[21]	470	41%	12-24	80%	84%

RVR: Rapid virological response; SVR: Sustained virological response; IL: Interleukin.

-3 were evaluated. The RVR rate was 61%; 134 patients did not achieve RVR. Patients were subdivided according to a treatment duration of 12 or 24 wk (n = 166 and n = 175, respectively). When patients were considered overall, a significant association between IL28B and RVR was shown. However, association between CC and SVR was significant for patients treated for 24 wk (P = 0.02), but not for those receiving a short course of treatment with Peg-IFN alpha-2a and fixed 800 mg doses of RBV. Among patients without RVR, CC genotype was represented in 35%, CT in 47% and TT in 15%. In the subgroup of patients without RVR treated for 24 wk, SVR rates were higher for patients with CC as compared to CT and TT (74% *vs* 59% and 29%, respectively).

The study by Bitetto *et al*[19] evaluated 101 patients with GT-2 and -3 as part of a larger cohort of patients with different HCV genotypes. In this study no association with either RVR or SVR was demonstrated among GT-2 and -3. In particular, 78% of IL28B CC infected with GT-2 and GT-3 combined together and 88% of IL28B CT/TT achieved viral clearance after treatment.

Studies evaluating GT-3 separately
Two studies focused on GT-3 only; Scherzer *et al*[12], evaluated a small cohort of 71 patients, while Moghaddam *et al*[14] studied 281 patients (Table 2)[20]. Data on 475 GT-3 were separately available also in the study by Fattovich *et al*[20] and in our prospective cohort of 710 patients with GT-2 and GT-3[21]. These studies are analysed below.

Scherzer *et al*[12] investigated both rs12979860 and rs8099917 SNPs in a cohort of patients originally randomized 1:1 to 800 or 400 mg of RBV in combination with Peg-IFN alpha-2a. The results of this study might be limited by the small sample size, moreover the low dosages of RBV (used) may impact the generalizability of the conclusions. In the final analysis, only patients who completed the treatment were considered, they were 37 and 31 in each arm, respectively. A CC genotype was identified in 38% of patients, but no association with SVR was observed. Indeed, 19/25 (76%) CC and 34/44 CT and TT combined together (77.3%) achieved SVR[12]. As shown in patients with genotype 1 infection[22], higher

levels of baseline HCV RNA were associated in this study with CC genotype as compared to CT or TT[12].

A larger study investigating IL28B in GT-3 has been published by Moghaddam *et al*[14]. The Authors evaluated both rs12979860 and rs8099917 SNPs in DNA extracted from plasma of 281 GT-3 patients representing 51% of patients enrolled into two previous clinical trials, a non-randomised and a randomised one[23,24]. Authors demonstrated rates of SVR comparable between CC, CT and TT (77% *vs* 81% and 96%), whereas a statistically significant association between RVR and favorable genotypes was shown. Indeed, 84% of CC-type, 62% of CT and 56% of TT achieved RVR (OR = 3.3, 95%CI: 1.9-5.8). Genotyping rs8099917 SNP, the results were not different (OR = 2.7, 95%CI: 1.6-4.7). In this study, the exclusion of a number of patients who did not fit the inclusion criteria may have represented a bias. Moreover, the association analysis between the different host genotypes and SVR should have been adjusted for confounders, as for example an uneven distribution of patients with favourable genotypes across the different treatment arms. Strikingly, in this study the frequency of CC genotype was 8%-9% higher than in other studies[10].

Negative results were reported also by Fattovich *et al*[20] in a retrospective cohort study on Italian patients. This study offered the possibility to a separate analysis of GT-2 and GT-3 but the overall number was not higher than 159 including 104 GT-2 and 55 GT-3. No association with RVR was reported in 24 of 28 patients with IL28CC and GT-3 in comparison to 20 of 27 CT/TT (P = 0.31). Similarly, the results were not different for 20 of 23 GT-3 CC and 28 of 32 subjects with CT/TT who achieved SVR (P = 0.79).

The Write study with IL28B available in 93.7% of 710 GT-2 and GT-3 patients is the largest series prospectively evaluating for IL28B. Results of this study including 475 GT-3 showed that while within GT-2 no association between IL28B CC and SVR (90.3% for CC *vs* 82.0% for non CC, P = 0.15) can be observed, within GT-3, the association between *IL-28B* CC and SVR is highly significant (84% *vs* 60%, P < 0.001).

These results demonstrate that when the sample size is adequate, the association between IL28B and RVR or

SVR can be appreciated. Therefore, it may be rational to evaluate *IL-28B* genotyping in patients with GT-3, unless further evidence suggest otherwise. Despite the occurrence of side effects or poor tolerability, patients who bear a favorable *IL-28B* genotype should not discontinue treatment. At the same time, an unfavorable *IL-28B* genotype in patients with GT-3 infection may suggest to defer treatment in waiting for more efficacious drugs.

DISCUSSION

After a careful analysis of the available data, a few aspects deserve consideration. Sample size is one of the most relevant issues in genetic studies as the power of the single study is influenced by the effect size and by the frequency of the minor allele[25]. The effect size of IL28B is large, yet the frequency of the minor allele for SNP rs1297860 ranges between 8% and 16% across the studies evaluating GT-2 and -3[10,12]. Although with such variability, it might be difficult to establish a minimum sample size valid across the studies, the risk for many of them to be underpowered is not negligible[26]. With the assumption of a 0.37 frequency of CC-type and an expected rate of SVR of 0.68 in CT patients, more than 520 patients are required to detect an odds ratio of at least 1.8. Therefore, study's conclusions should be based on studies with large sample size.

A further consideration owes to be made, the role of predictive factors is not absolute, but it depends on the efficacy of treatment. With about 80% of SVR attained in patients with GT-2 treated with P/R combination it is easy to understand that the sensitivity of the *IL-28B* genotype for the prediction of SVR in patients with GT-2 is limited and it is easy to understand that combining together GT-2 and GT-3 the sensitivity of the *IL-28B* genotype for the prediction of SVR is no higher than 40%-47%[10,11]. The lower rate of SVR in patients with GT-3 only put things in a different context suggesting that the combination of unfavorable IL28B and advanced fibrosis may represent a good reason to defer treatment based on Peg-IFN backbone due to the expectancy of a very poor response. Waiting for alternative treatment may be in these case a more reasonable choice.

Based on these considerations, we have imagined an algorithm for the management of patients with chronic GT-2 and GT-3 infection including IL28B and on treatment response (Figure 1). Our proposal is to perform *IL-28B* genotyping in patients with GT-3 at the aim of encouraging them to treatment, when undecided, to establish the duration of treatment and to decide not to treat those with very poor likelihood of SVR.

In conclusion, in easy to treat GT-2 patients IL28B may be considered as an additional not essential predictor of shortened treatment duration, while in GT-3, genotyping of *IL-28B* polymorphisms may be used to convince skeptical patients, to maintain on treatment those who are at risk of withdrawing because of side effects and to defer treatment in patients with low likelihood of response.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Post-translational modifications of hepatitis C viral proteins and their biological significance

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Abstract

Replication of hepatitis C virus (HCV) depends on the interaction of viral proteins with various host cellular proteins and signalling pathways. Similar to cellular proteins, post-translational modifications (PTMs) of HCV proteins are essential for proper protein function and regulation, thus, directly affecting viral life cycle and the generation of infectious virus particles. Cleavage of the HCV polyprotein by cellular and viral proteases into more than 10 proteins represents an early protein modification step after translation of the HCV positive-stranded RNA genome. The key modifications include the regulated intramembranous proteolytic cleavage of core protein, disulfide bond formation of core, glycosylation of HCV envelope proteins E1 and E2, methylation of nonstructural protein 3 (NS3), biotinylation of NS4A, ubiquitination of NS5B and phosphorylation of core and NS5B. Other modifications like ubiquitination of core and palmitoylation of core and NS4B proteins have been reported as well. For some modifications such as phosphorylation of NS3 and NS5A and acetylation of NS3, we have limited understanding of their effects on HCV replication and pathogenesis while the impact of other modifications is far from clear. In this review, we summarize the available information on PTMs of HCV proteins and discuss their relevance to HCV replication and pathogenesis.

Core tip: Post-translational modifications (PTMs) are an important step in protein maturation and associated with protein function, activity and/or protein life span. PTMs of viral proteins are often essential for regulation of processes involved in viral infections and can be crucial for infectious virion production. Moreover, identification of PTM sites in viral proteins is particularly useful for the development of antiviral drugs. This overview on PTMs of hepatitis C virus (HCV) proteins discusses how PTMs affect HCV replication and virus-induced pathogenesis.

INTRODUCTION

Hepatitis C virus (HCV), a member of the genus *Hepacivirus* within the family *Flaviviridae*, is able to establish chronic infection in humans, which eventually leads to liver cirrhosis, hepatocellular carcinoma (HCC) and liver

failure[1,2]. Approximately 3% of the world population are infected with HCV. However, no effective vaccine has been developed and the current antiviral treatments have some limitations[3,4]. In order to develop efficient antiviral therapies, a complete understanding of viral pathogenesis and virus-host interactions is fundamental. Like other positive-stranded RNA viruses, HCV hijacks the host cell's translation machinery for producing viral proteins[5]. Thereby, post-translational modifications (PTMs) of virus encoded proteins occur as a rather natural step during the cell's general protein synthesis process, but concurrently encompass impact on viral replication and infectivity. In this review, we will start with a discussion on the proteolytic cleavage of HCV polyprotein, and give an overview on PTMs of HCV proteins and discuss their influence on viral replication and pathogenesis.

PROTEOLYTIC CLEAVAGE OF HCV POLYPROTEIN

HCV genome consists of a 5'-untranslated region (UTR), a large open reading frame (ORF) encoding a polyprotein precursor of about 3000 amino acids and a 3'-UTR[6]. Proteolytic processing of the HCV polyprotein giving rise to single viral proteins represents an initial step in viral protein modification. There are nine defined proteolytic cleavage sites within the HCV polyprotein precursor, resulting in the generation of at least ten non-overlapping proteins, including structural proteins core, E1 and E2, and nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Additional viral protein products might be produced by alternative ORFs discovered within the HCV genome[7-10].

Proteolytic processing of the polyprotein precursor occurs co- and post-translationally involving cellular as well as viral proteases[11]. The structural proteins are cleaved off the polyprotein precursor by host cellular signal peptidase (SP) located in the endoplasmic reticulum (ER) of the host cell, while the nonstructural proteins are released from the polyprotein precursor by viral proteases NS2-3 and NS3-4A[9]. The core protein is found to be additionally cleaved inside the ER membrane by host cellular signal peptide peptidase (SPP), thus yielding the mature core variant[12]. This step leads to the release of core from the ER and its trafficking to lipid droplets (LDs) which are believed to serve as a platform for HCV particle assembly[13]. E2, p7 and NS2 are first generated as an E2-p7-NS2 precursor protein. Remarkably, the E2-p7-NS2 precursor is proteolytically processed at the p7-NS2 junction efficiently whereas the E2-p7 junction gets cleaved less frequently, hence resulting in the presence of E2 and p7 proteins as well as the non-cleaved E2-p7 variant in infected cells[14]. The NS2-3 autoprotease cleaves at the NS2-NS3 junction and the NS3-4A protease cleaves at the sites between NS3 and NS4A, NS4A and NS4B, NS4B and NS5A, and NS5A and NS5B. Proteolytic processing by the NS3-4A complex follows a certain order that the cleavage first happens *in cis* at the NS3-NS4A

junction, then rapidly *in trans* at NS5A-NS5B followed by proteolysis at NS4A-NS4B, and finally at NS4B-NS5A. NS3 to NS5B mainly function in HCV genome replication[8,15]. Proteolytic processing releases structural and nonstructural HCV viral proteins that take part in different stages of HCV life cycle.

POST-TRANSLATIONAL MODIFICATIONS OF HCV PROTEINS

Core protein
HCV core protein is the most conserved viral protein among different HCV genotypes. It constitutes the viral nucleocapsid that encapsidates the viral RNA genome, and is essential for virus particle assembly[16,17]. HCV core also possesses several regulatory functions, such as cellular transcription, virus-induced transformation, signal transduction, steatosis and HCC. Moreover, core is significantly involved in virus-mediated pathogenesis. It is able to modulate apoptosis and cell growth, but also up-regulates reactive oxygen species (ROS) production and has a possible immunoregulatory role[16,18].

The complete core protein is composed of three domains: an N-terminal hydrophilic domain that is essential for RNA binding and homo-oligomerization, a C-terminal hydrophobic domain that associates with LDs and is involved in proper folding, and a hydrophobic signal sequence tail that can target E1 to the ER membrane[16,19-21]. Unlike other HCV proteins, core protein liberation from HCV polyprotein precursor needs sequential proteolytic processing. Following cleavage from HCV polyprotein at the core-E1 junction by host cellular SP, the immature core protein is additionally cut by SPP within its hydrophobic C-terminus to release mature N-terminal amino acids 173-179 core protein and dissociate core from the ER membrane[16,22]. The exact C-terminus of mature core has not been identified yet in mammalian cells, even though it was reported to be Phe[177] or Leu[179] in insect cells[23]. This further processing by SPP relies on previously correct cleavage by SP and the sequential processing controls viral protein production rate[24]. Only the mature form of core can attach to LDs and interact with NS5A that transports HCV genome RNA to core[25,26]. Therefore, core maturation by SPP cleavage plays a critical role in virus assembly and regulation of HCV life cycle.

It has been demonstrated that disulfide bonds in nucleocapsid proteins of viruses with icosahedral structure play a role in virus assembly and capsid structure stabilization[27]. Since HCV virion is packaged into a similar spherical structure, its nucleocapsid might resemble the same organization[28]. Mutation analysis discovered that mature core formed a dimeric membrane protein which was linked by disulfide bond at Cys[128]. This disulfide bond formation stabilizes capsid structure and strengthens the interaction between core and membranes, and is critical for virus assembly and virion production. However, the disulfide bond in core has no effect on HCV RNA rep-

lication, the association with LDs or other functions of core[27]. Because of the low mutation rate of Cys[128], drugs targeting Cys[128] disulfide bond formation may be considered as a candidate to inhibit HCV virion formation.

Phosphorylation is a common type of PTMs, which is also observed in HCV core protein. The phosphorylation of core by protein kinases A (PKA) at Ser[53] and Ser[116] and by protein kinases C (PKC) at Ser[53] and Ser[99] was reported both *in vitro* and in Huh-7 and HepG2 cells. However, Ser[99] and Ser[116] are the major and predominant sites with low phosphorylation efficiency[29,30]. The phosphorylation at these two sites is critical for inhibition of HBV gene expression and replication in Huh-7 cells, but the detailed *trans*-suppression mechanism of HCV core remains unclear. The same study showed that only truncated core is phosphorylated by PKC suggesting structural conformation might be a prerequisite for phosphorylation[30]. The phosphorylation at Ser[116] by PKA is shown to be responsible for the repressive activity of core on cyclin-dependent kinase inhibitor (CKI) p21 promoter[31]. Reduced p21 may interfere with p53 driven repair mechanism in cell cycle, which may facilitate tumorigenesis. Another role of phosphorylation of core might be involved in modulating nuclear localization of core, although controversial results have been reported[29,30]. Nuclear core is involved in regulating host gene transcription[32].

Moreover, HCV core also undergoes several other types of PTMs. For example, the ubiquitination of core protein by E3 ubiquitin ligase E6AP preferentially at N-terminal lysine residues induces the degradation of core in the cytoplasm by the ubiquitin-proteasome pathway, which could control HCV virion production and have an antiviral effect. The interaction region between core and E6AP is located between amino acids 58 to 71 of the core protein, which are highly conserved in all HCV genotypes[33-35]. Palmitoylation of core at Cys[172] plays a vital role in targeting the core to smooth ER and ER-associated LDs, but does not affect SPP proteolytic cleavage-induced maturation and LD accumulation. Importantly, it also affects HCV assembly and production[36].

Envelope glycoproteins E1 and E2

HCV envelope glycoproteins E1 and E2 play an important role in virus entry and immune evasion[37]. In infected cells, E1 and E2 are either found as noncovalent heterodimers, which are mainly localized to the ER, or as disulfide-linked aggregates, which were originally thought to represent misfolded protein complexes[38-41]. Heterodimers and oligomers of E1 and E2 are also found in infectious virus particles, whose structure is stabilized by disulfide bonds[38,42,43].

Both E1 and E2 proteins consist of a large N-terminal ectodomain and a C-terminal hydrophobic transmembrane anchor. PTMs of HCV envelope proteins include the attachment of glycans and the formation of disulfide bridges[44,45]. Glycans attached to HCV envelope proteins were shown to modulate virus entry by modifying their receptor binding affinity or fusion activities. They are also involved in protein folding and play a key role in immune evasion by masking potential antigenic sites from binding of neutralizing antibodies[44,46,47]. Because glycosylation sites within HCV glycoproteins are rather highly conserved, glycosylation mutants are considered as immunogens to induce a potent antibody response against HCV[48].

There are four to five N-glycosylation sites in E1 and up to 11 N-glycosylation sites in E2[44,47,49,50]. N-linked glycosylation occurs at asparagine residues and the consensus sequence is Asn-X-Ser/Thr[44,51]. Mass spectrometric analysis of E2 revealed that this protein is mainly modified by high-mannose type oligosaccharides and more complex glycan types are observed for just two glycosylation sites within E2[52]. E1 is believed to be modified only by high-mannose type oligosaccharides since a restricted localization of E1/E2 heterodimers to the ER is confirmed by immunofluorescence[49]. However, more complex type glycosylations generally occur in the cis-Golgi compartment, where indeed a small population of E2 protein has been detected by immunofluorescence[53]. On the other hand, the attachment of complex glycans can happen during the release of viral particles via the exocytotic pathway, which involves the Golgi apparatus. Interestingly, due to the differences in the assembly process, more mature glycoproteins containing complex type glycans could have been observed with HCV pseudoparticles (HCVpp) compared to cell culture-derived HCV particles (HCVcc)[38]. HCVpp is found to assemble in post-Golgi compartments[54], while HCVcc assembly takes place in ER-derived compartments[55]. HCVpp glycoproteins might also be more accessible to Golgi glycosyltransferases than HCVcc glycoproteins, which are components of high-order virion complexes[38]. Differences in the glycosylation pattern of HCVpp and HCVcc might be relevant for studying HCV immune evasion strategies.

Furthermore, the carbohydrate composition of envelope glycoproteins vary to some extent depending on the cell line the virus infected[56]. Changes in the glycosylation pattern of HCV glycoproteins have a major impact on virus particle assembly, entry and immunogenicity[44,50], thus affecting virus pathogenesis and virulence. Mutations of glycosylation sites N1 and N4 in HCV glycoprotein E1 (E1N1, E1N4) as well as N8 and N10 in HCV glycoprotein E2 (E2N8, E2N10) strongly interfere with the incorporation of both envelope proteins into HCVpp, suggesting the importance of these sites for protein folding and E1/E2 heterodimerization[42,44]. Additionally, mutation of glycosylation site E2N2 or E2N4 leads to the decreased infectivity of HCVpp, confirming a role of both glycans in virus entry[44]. Moreover, glycans at positions E2N1, E2N6 and E2N11 are shown to decrease the binding affinity of E2 to the cluster of differentiation 81 (CD81) receptor and to reduce the sensitivity of pseudotyped HCV particles to antibody neutralization, hence contributing to humoral immune evasion[47]. These findings are supported and extended by studies with HCVcc glycosylation mutants[42]. Apparently, glycosylation sites

E2N1, E2N2, E2N4 and E2N6 seem to surround the CD81 receptor binding site within E2, therefore "protecting" this site from recognition by neutralizing antibodies. Helle *et al*[42] provided structural evidence for glycans attached to HCV envelope proteins to modulate the humoral immune response.

Besides N-glycosylation, little information is available on O-glycosylation of HCV E1 or E2. Supposedly, there is one potential O-glycosylation site within E1 and four potential O-glycosylation sites within E2[48]. So far, 3 O-linked glycosylation sites in E2 have been shown to be important for HCV entry, with two of them apparently decreasing E2 binding affinity to CD81 receptor[48].

Virion-associated HCV glycoproteins are assembled into large oligomeric protein complexes which are stabilized by disulfide bonds[38,42]. These complexes are able to bind conformation-sensitive neutralizing antibodies and recombinant CD81[38], and therefore can be considered functionally significant rather than the result of a misfolding event.

Proper folding of glycoprotein E1 is dependent on E2 coexpression and *vice versa*[57,58]. E1 and E2 consist of eight and 18 highly conserved cysteine residues, respectively[43]. Structural information is mainly available for HCV E2 protein, where nine intramolecular disulfide bonds have been identified[59]. Because of the difficulties in expressing E1 in the absence of E2, disulfide arrangement of cysteine residues in E1 has not been determined[43]. Beside their apparent impact on virus particle structure and infectivity, it is conceivable that disulfide-linked glycoprotein oligomers may play an active role in HCV budding by assisting protein-protein interactions[38]. Furthermore, it is possible that the presence of disulfide bridges in HCV envelope proteins could be responsible for the lack of sensitivity of HCVcc to low-pH treatment[60]. This suggests their direct influence on virus internalization by affecting the presentation of HCV fusion peptide[38]. Additionally, the impact of disulfide rearrangement and the oxidation state of cysteine residues in E1 and E2 glycoproteins on HCV entry and membrane fusion was confirmed by Fraser *et al*[43]. Here the presence of free thiol groups has been shown to be essential for HCV infectivity.

Altogether, PTMs of HCV glycoproteins by glycosylation and disulfide bond formation have a strong impact on several steps in viral life cycle, more specifically entry, fusion of viral membrane with the host cell's endosomal membrane and budding.

p7

HCV p7 represents a small integral membrane protein which is able to oligomerize and form proton channels within the HCV particle envelope. The precise role of p7 in HCV life cycle has not been determined, even though it has been shown to be essential for infection, but not for viral replication[61,62]. Due to incomplete or delayed proteolytic processing, the generation of a p7 species linked to the E2 glycoprotein has been observed. The role

of E2-p7 precursor during HCV infection is not known so far. However, it is speculated that E2-p7 might be involved in regulating the production of native p7 and formation of ion channel complexes[63]. The optimal cleavage at the E2-p7 junction is shown to be important for virus production probably due to the increased NS2-associated virus assembly complex formation in close proximity of LDs. It also enhances NS2 interaction with NS3 and E2, but does not affect HCV genome replication[64].

Structural analysis revealed that HCV p7 protein consists of two membrane-spanning α-helices connected by a short cytoplasmic loop[65]. PTMs of p7 have not been demonstrated.

NS2

HCV NS2 is a transmembrane protein. Together with the N-terminal domain of NS3, NS2 forms the NS2-3 autoprotease. The NS2-3 cysteine autoprotease is a zinc-dependent metalloprotease that cleaves the HCV polyprotein at the NS2-NS3 junction. After its self-cleavage from NS3, NS2 is quickly degraded[66,67]. Like p7, NS2 is known to be essential for virus assembly. Even though NS2 is part of the HCV replication complex, which is composed of NS2, NS3, NS4A, NS4B, NS5A and NS5B, NS2 is not essential for virus replication[62,68,69]. The interaction of NS2 with E1, E2, NS3 and NS5A results in co-localization of these viral proteins to dot-like structures near LDs, which are the sites for virus particle assembly[64,70]. Moreover, proper cleavage at the NS2-NS3 junction is important for an active HCV replication complex formation, but is not required for NS3 protease activity[71,72]. Other functions linked to NS2 include the inhibition of apoptosis and modulation of host cellular gene transcription[73-76].

The highly hydrophobic N-terminus of NS2 consists of three trans-membrane segments which form the protein membrane binding domain[77]. No attachment of fatty acids or prenyl groups by modifications typically involved in membrane targeting, like farnesylation, myristoylation, palmitoylation or prenylation[78], has been associated with membrane anchoring of NS2 so far. Though NS2 is located to the ER membrane, the protein is not glycosylated[79]. The protease activity of NS2 is located within its C-terminal domain, which is able to homodimerize and thus creates two composite active sites[80]. Regarding the role of NS2 in HCV particle formation, the overall structural integrity rather than the protease activity of NS2 itself appears to be crucial[81,82].

The C-terminal globular domain of NS2 facing towards the cytoplasm of the infected cell was shown to be modified by phosphorylation. Phosphorylation of NS2 is presumably mediated by host cellular casein kinase 2 (CK2)[79]. NS2 is a short-lived protein that is rapidly degraded by the proteasome. Proteasome-mediated degradation of NS2 is regulated in an ubiquitin-independent manner by phosphorylation within its C-terminal domain. Ser[168] as part of a CK2 consensus recognition site (Ser/Thr-X-X-Glu) is shown to be vital for NS2 degra-

dation. It is highly conserved between all HCV genotypes and single point mutation of Ser[168] confers resistance to NS2 degradation[79]. Therefore, phosphorylation of NS2 is strongly connected to its abundance within the host cell and can have a strong impact on HCV pathogenesis, more particularly on assembly and virion production.

NS3-4A complex

HCV NS3-4A is a noncovalent complex composed of the serine protease NS3 and its cofactor NS4A[83]. The NS3-4A mediated cleavage releases NS3, NS4A, NS4B, NS5A and NS5B from the HCV polyprotein in a specific order. The NS3-4A protease complex also has three identified cellular targets so far, including mitochondrial antiviral signaling protein (MAVS), T-cell protein tyrosine phosphatase (TC-PTP) and toll/IL-1 receptor homology domain-containing adaptor inducing IFN-β (TRIF), which may be involved in the development of persistent infection and HCC[10,15]. Therefore, the NS3-4A protease is a prime target for antiviral drug design. For example, the two recently approved direct-acting antivirals (DAAs), telaprevir and boceprevir, are oral NS3-4A protease inhibitors[4,84].

NS3 protein consists of an N-terminal serine protease domain with its catalytic triad composed of His[57], Asp[81] and Ser[139], and a C-terminal RNA helicase/NTPase domain. The NS3 helicase/NTPase couples NTP hydrolysis to unwind extensive RNA secondary structure and is important for RNA replication and virus assembly[85,86].

The two domains of NS3 can function independently from each other, and the reason for their physical linkage remains unclear[83,85]. The intracellular NS3 protease shows structure homology with extracellular serine proteases, but does not possess disulfide bonds to stabilize its structure as extracellular serine proteases[87]. A Zn^{2+} ion together with its binding site formed by Cys[97], Cys[99], Cys[145] and His[149] stabilizes NS3 protease, activates NS3 hydrolysis and facilitates NS2 processing at the NS2-NS3 junction. Binding of NS4A further stabilizes NS3 by restructuring the N-terminus of NS3 protease through interaction with the central hydrophobic portion of NS4A, increases catalytic efficiency by influencing the spatial configuration of the catalytic triad and directs the cellular membrane localization because of the high hydrophobicity of the N-terminal transmembrane α-helix of NS4A. In addition, the C-terminal acidic portion of NS4A plays a role in regulating HCV genome replication and virus assembly by interacting with other viral proteins in the replication complex[15,87-89]. NS4A also regulates HCV replication by modulating NS5A hyperphosphorylation[90].

Liefhebber *et al*[91] has shown that NS3 might get phosphorylated in subgenomic HCV replicon cells through phosphospecific staining and dephosphorylation assay. However, phosphorylation efficiency is low and the phosphorylation sites are hard to identify. In addition, N-terminal acetylation of NS3 was identified by this research group through mass spectrometric analysis. The role of NS3 phosphorylation and acetylation in HCV life cycle needs to be further investigated.

It has been reported that protein arginine methyltransferases (PRMTs) can irreversibly and post-translationally methylate arginine residues in the arginine-glycine (RG)-rich region of many RNA-binding proteins[92,93]. Since NS3 protein can bind to RNA through its RNA helicase domain and contains seven RG motifs including two RG motifs in the helicase domain, it is a potential methylation target for PRMTs. Full-length NS3 and NS3 helicase domain are shown to be methylated at Arg[1493] in the [1486]Gln-Arg-Arg-Gly-Arg-Thr-Gly-Arg-Gly[1494] motif by PRMT1, but no methylation is found in NS3 protease domain[94]. Mutation analysis has demonstrated that Arg[1490] and Arg[1493] are determinants for the helicase activity[95]. Methylation of NS3 at Arg[1493] inactivates the helicase by inhibiting unwinding of double-stranded DNA[96]. The reason that arginine methylation is involved in protein-nucleic acid interaction is that methyl modification may affect the binding affinity, protein stability, transcription and signal transduction[92]. Negative regulation of PRMT1 by protein phosphatase 2A (PP2A) increases NS3 helicase activity and enhances HCV RNA replication, therefore PP2A is considered a potential target for HCV drug development[96].

The cofactor activity of NS4A is mediated by its central region and especially the hydrophobic Ile[25] and Ile[29] residues, since an I25A/I29A double mutant cannot form a complex with NS3[97]. To reactivate NS4A cofactor activity, the double mutant requires biotinylation at the N-terminus by biotin-aminohexanoic acid (ahx). However, the N-terminal biotin fusion alone without ahx or C-terminal biotin-ahx fusion cannot restore NS4A cofactor activity. On the other hand, N-biotinylation of wild-type NS4A by biotin-ahx can dramatically promote cofactor activity. Based on these data and the crystal structure, it is predicted that N-biotinylation by biotin-ahx resembles a hydrophobic environment that enhances the stabilization of NS3-4A complex and C-biotinylation may sterically interfere with the substrate binding pocket[98].

NS4B

HCV NS4B is relatively poorly understood compared to other HCV proteins. The liberation of NS4B happens at last during HCV polyprotein precursor processing in a strictly defined position[99]. It is a highly hydrophobic integral membrane protein that induces the formation of the membranous web around ER membrane where HCV genome replication takes place and functions by anchoring the HCV replication complex through an unknown mechanism[100]. It has been reported that NS4B can interact with other viral proteins such as NS5A, binds viral RNA and has NTPase activity. It is involved in RNA replication, virus assembly and release[101,102]. The multifunctional NS4B is also shown to activate ER stress pathways, contributes to steatosis by altering lipid metabolism and escape from innate immune system by inhibiting interferon[99]. Moreover, its anti-apoptosis function might be associated with HCC development[103].

There are two amphipathic helices (AH1 and AH2) located in the N-terminal region of NS4B with their hydrophobic sides facing the cytoplasmic side of ER membrane. AH2 is a membrane interacting domain that is essential for membrane trafficking, HCV genome replication and protein oligomerization. NS4B oligomerization is critical for replication complex formation[104-106]. The highly hydrophobic central core region of NS4B contains four transmembrane domains and the highly conserved C-terminus is a membrane binding domain that consists of two α-helical elements and plays a role in NS4B self-interaction, thus being important for replication complex formation[107,108].

There are three common lipid modifications of protein located in lipid raft, including palmitoylation, N-terminal myristoylation and palmitoylation, and glycosylphosphatidylinositol modification[109]. So far, only palmitoylation is detected in NS4B at Cys^{257} and Cys^{261} in the C-terminus and these two sites are relatively conserved among HCV genotypes. Site-directed mutagenesis confirmed that Cys^{261} palmitoylation is more crucial for protein-protein interaction and replication complex formation. Palmitoylation can enhance the polymerization activity of NS4B through its N-terminus[110].

NS5A

HCV NS5A is a phosphorylated zinc-metalloprotein without any enzymatic activity, but required for RNA replication and virion morphogenesis[111]. However, the precise mechanism of how NS5A functions is not clear. It is demonstrated that NS5A can bind to HCV RNA, other HCV proteins such as NS5B and cellular proteins such as human vesicle-associated membrane-associated protein of 33 kDa (hVAP-33), thus contributing to replication complex formation[8]. Several other functions of NS5A include interferon resistance, transcriptional activation and signaling pathway regulation[112,113].

NS5A is composed of three domains. Domain I contains a zinc-binding motif and is the determinant for HCV RNA replication. It is a nucleic acid-binding domain that binds to the 3' G/C rich sequence in HCV RNA. It also functions in LD association. Domain II may play a role in evading innate immune response as well as RNA replication. Domain III participates in virus assembly and core protein interaction[83,86,114]. In addition, there is an amphipathic α-helix in the N-terminal region responsible for ER membrane anchoring[111].

NS5A is a phosphoprotein that exists in two forms, a basally phosphorylated form (56 kDa) and a hyperphosphorylated form (58 kDa), which is conserved among HCV genotypes[115]. The basally phosphorylated sites are mainly serine residues and the minority are threonine residues located in the central and C-terminal region. Major hyperphosphorylated sites are identified in a serine-rich region in the central portion of NS5A[112,115]. The basally phosphorylated form may be affected by NS2 and NS4A, whereas hyperphosphorylation of NS5A requires

NS3, NS4A and NS4B. Cellular protein kinases in the CMGC kinase family are involved in NS5A phosphorylation, including cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3) and casein kinase Ⅱ (CKⅡ)[90,112,116-118]. Since the subcellular distributions of both NS5A forms are similar, the degree of phosphorylation does not affect NS5A localization to the ER membrane[119]. However, the degradation of NS5A is enhanced by increased degree of phosphorylation[115]. Mutation analysis revealed that reduced NS5A hyperphosphorylation promotes HCV RNA replication, whereas reduced basal phosphorylation does not have an effect on HCV RNA replication in a replicon system. This suggested that the ratio of these two NS5A phosphorylation forms may be important for viral RNA replication[120,121]. NS5A is also involved in virion production through its interaction with core protein, which requires basal phosphorylation of NS5A[122].

NS5B

HCV NS5B is a conserved RNA-dependent RNA polymerase (RdRp) that initiates complementary negative-strand RNA synthesis and then synthesizes positive-strand RNA using the newly synthetic negative-strand RNA as template. Due to the lack of proofreading of RdRp, HCV replication is error-prone[83,86]. NS5B can interact with other viral proteins such as NS3, NS4A and NS5A, and cellular proteins like hVAP-33, which facilitates the formation of the viral RNA replication complex[123,124]. Furthermore, it can form a complex with the retinoblastoma tumor suppressor protein (pRb) and promote pRb degradation in an ubiquitin dependent manner, therefore contributing to HCC development[125].

Like other polymerases, the crystal structure of NS5B reveals that it resembles the configuration of a right hand. The finger, thumb and palm domains compose a unique shape. The active site located in the palm domain has a highly conserved GDD motif. There are four allosteric sites within the thumb and palm domains which serve as targets for antiviral development[126-128]. Besides, NS5B is a tail-anchored protein with its C-terminal hydrophobic tail associated to the ER membrane[86].

The function of many cellular enzymes for DNA and RNA metabolism and viral RdRps is often regulated by phosphorylation[129]. Hwang *et al*[130] demonstrated that NS5B is a phosphoprotein in insect cells. Kim *et al*[129] discovered that the protein kinase C-related kinase 2 (PRK2) is the specific enzyme for NS5B phosphorylation within the N-terminal finger domain. Knock-down and over-expression of PRK2 demonstrated PRK2 up-regulates HCV RNA replication in HCV subgenomic replicon cells, suggesting that NS5B phosphorylation can enhance HCV replication.

Gao *et al*[131] identified an interaction between ubiquitin-like protein hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton) and NS5B. Since hPLIC1 interacts with both proteasome and

E3 ubiquitin protein ligases E6AP and βTrCP, the ubiquitination modification of NS5B through hPLIC1 binding promotes ubiquitin-dependent proteasome degradation, resulting in decreased level of NS5B. NS5B mainly functions in RNA replication, so decreased NS5B leads to HCV genome RNA reduction[131,132]. Although the ubiquitination sites within NS5B and the detailed mechanism of hPLIC1-induced NS5B degradation are still not clear, up-regulating NS5B ubiquitination may represent a target for anti-viral development.

SUMMARY AND PERSPECTIVES

PTMs of HCV viral proteins include phosphorylation, glycosylation, disulfide bridging, methylation, palmitoylation, acetylation, and ubiquitination. These protein modifications ensure proper protein functions by regulating protein activity, subcellular localization, protein-nucleic acid interaction, and protein-protein interactions. Among the already identified PTMs of HCV proteins, some are essential for HCV virion production such as sequential proteolytic cleavage of core protein, whereas others have regulatory roles in virus replication such as phosphorylation of NS5A and ubiquitination of NS5B. PTM sites and PTM pathways are potential pharmacological targets for antiviral drug development. However, much work remains to be done to unveil the precise PTM sites and the underlying mechanisms.

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TOPIC HIGHLIGHT
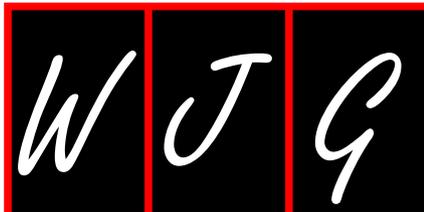
WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Hepatitis C virus protease inhibitor-resistance mutations: Our experience and review

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Abstract

Direct-acting antiviral agents (DAAs) for hepatitis C virus (HCV) infection are one of the major advances in its medical treatment. The HCV protease inhibitors boceprevir and telaprevir were the first approved DAAs in the United States, Europe, and Japan. When combined with peginterferon plus ribavirin, these agents increase sustained virologic response rates to 70%-80% in treatment-naïve patients and previous-treatment relapsers with chronic HCV genotype 1 infection. Without peginterferon plus ribavirin, DAA monotherapies increased DAA-resistance mutations. Several new DAAs for HCV are now in clinical development and are likely to be approved in the near future. However, it has been reported that the use of these drugs also led to the emergence of DAA-resistance mutations in certain cases. Furthermore, these mutations exhibit cross-resistance to multiple drugs. The prevalence of DAA-resistance mutations in HCV-infected patients who were not treated with DAAs is unknown, and it is as yet uncertain whether such variants are sensitive to DAAs. We performed a population sequence analysis to assess the frequency of such variants in the sera of HCV genotype 1-infected patients not treated with HCV protease inhibitors. Here, we reviewed the literature on resistance variants of HCV protease inhibitors in treatment naïve patients with chronic HCV genotype 1, as well as our experience.

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Key words: Direct-acting antiviral agent; Hepatitis C virus; Protease inhibitor; Resistance mutation; Sequence analysis

Core tip: The standard of care for the treatment of hepatitis C virus (HCV) infection was peginterferon plus ribavirin until the recent approval of telaprevir- and boceprevir-containing combination therapies. These HCV protease inhibitors occasionally cause HCV variants with resistance mutations. We reviewed the literature reports of resistance variants of HCV protease inhibitors in treatment-naïve patients with chronic HCV genotype 1, as well as our experience. Even in treatment-naïve patients with chronic HCV genotype 1, naturally occurring HCV protease inhibitor-resistance mutations exist in some cases. The combination of direct-acting antiviral agents against regions other than HCV NS3/4A could eradicate HCV with these resistance variants.

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INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single-stranded RNA virus, approximately 9600 nt in length, that belongs to the *Flaviridae* family. Globally, HCV infects 170 million people and approximately 120-140 million chronic HCV carriers exist[1,2]. HCV infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[3,4]. HCV is classified into six major genotypes and > 100 subtypes[5]. HCV genotype 1 (subgenotypes 1a and 1b) is the most common genotype in western countries and Japan[5]. Treatment of HCV is complicated by the existence of different HCV genotypes. The standard of care was peginterferon plus ribavirin until the recent approval of telaprevir- and boceprevir-containing combination therapies[6-14]. Combination of peginterferon plus ribavirin results in sustained virological response (SVR) in nearly 70%-80% of patients with HCV genotype 2 or 3, but in only approximately 50% of those with HCV genotype 1[15,16]. Thus, treatment response is dependent on HCV genotypes and viral loads[17], viral sequence[18-21], host factors such as IL28B genotypes[22-35], drug adherence[36], and adverse events induced by therapeutic drugs[36].

Pharmaceutical companies are actively investigating and developing direct-acting anti-viral agents (DAAs) against HCV, which directly target specific HCV proteins such as NS3/4A protease[6-14], NS5A protein[37-39], and NS5B polymerase[40], which are important for HCV replication in hepatocytes. Two first-generation HCV protease inhibitors, boceprevir and telaprevir, were approved in combination with peginterferon plus ribavirin for treatment of chronic HCV genotype 1 in 2011[6-14]. Both protease inhibitors combined with peginterferon plus ribavirin increased SVR rates up to 70%-80% in treatment-naïve patients and previous-treatment relapsers with chronic HCV genotype 1 infection[6-14]. Next-generation HCV protease inhibitors will be available in clinics in the near future (Table 1)[41]. For example, simeprevir[42,43], faldaprevir[44,45], and vaniprevir[46-48] are currently in phase 3 trials. HCV protease inhibitors primarily are specific agents for HCV genotype 1. However, studies have demonstrated that simeprevir is fairly active against most HCV genotypes with the exception of HCV genotype 3a[42], and recently, in a phase 2 trial, the novel protease inhibitor MK-5172 showed even broader activity across HCV genotypes compared to simeprevir[49].

The low fidelity of HCV NS5B polymerase, high replication rate, and strong selective pressures on this virus lead to emergence of viral quasispecies. The quasispecies nature exists in a mixed population of viruses, with the fittest viruses being the predominant viral populations, as observed by sequence analysis[50,51]. In addition, new populations with every potential substitution are likely created and lost each day, some of which convey various degrees of resistance to DAAs[52-54]. Due to the high sequence diversity of HCV, naturally occurring pre-existing resistance mutations have been found at a low prevalence in HCV-infected, treatment-naïve patients[55,56].

In a previous study[56], 9% of the HCV genotype 1a-infected patients who were not treated with HCV protease inhibitors had at least one pre-existing dominant protease inhibitor-resistant variant, as observed by population sequencing. In another report[57], although the number of patients was small, the prevalence of protease inhibitor-resistance mutations was high (28%) in 53 genotype 1a samples, while no mutations were found in only 5 patients infected with HCV genotype 1b. In HCV genotype 1b treatment-naïve patients, the percentage of naturally occurring pre-existing resistance mutations appears to be lower[58]. Recently, next generation sequencing technology, with a detection limit as low as < 0.1%, have shown the ability to detect most resistance variants (including high resistance variants, *i.e.*, 155, 156 and 168) in patients infected with protease inhibitor-untreated HCV genotype 1[59]. Thus, the prevalence of naturally occurring pre-existing resistance mutations in patients infected with HCV genotype 1 who were not treated with HCV protease inhibitors remains unclear.

NATURALLY OCCURRING PREEXISTING RESISTANCE MUTATIONS IN PATIENTS INFECTED WITH HCV GENOTYPE 1B

We estimated a 98%-99% prevalence of HCV subgenotype 1b among patients infected with HCV genotype 1 in Japan[60]. Sera from 88 Japanese patients infected with HCV genotype 1b who were not treated with HCV protease inhibitors were examined. Some patients had been included in a previous study[60]. We investigated the naturally occurring pre-existing resistance mutations in these patients by using a direct-sequencing method. The study protocol was approved by the Ethics Committee of Chiba University School of Medicine.

The clinical background of the 88 patients is shown in Table 2. All but one patient had high viral loads. Population sequencing of the HCV NS3 region was performed in these 88 patients, and then the amino acid sequences were compared to the HCV NS3 amino acid sequence corresponding to the HCV genotype 1b Con1 strain. The prevalence of pre-existing variations in the HCV genotype 1b samples was 39% (34/88). Among these mutations, the resistance mutations T54S and D168N were found in 6.8% (6/88) and 1.1% (1/88) of the patients, respectively. Other resistance mutations Q80L, V170Y, V170N and V170L were found in 22% (19/88), 4.5% (4/88), 3.4% (3/88) and 1.1% (1/88) of the patients, respectively. In 5.7% (5/88) of the patients, more than one mutation was identified: four patients had T54S and Q80L, and one patient had T54S, Q80L and

Table 1 Overview of representative clinical trials of hepatitis C virus NS3/4A protease inhibitors

Name of drug (other name)	G	Trial phase	Features of clinical trials (ClinicalTrials.gov Identifier)
Telaprevir (VX-950)	1	FDA approved	Telaprevir, PEG-IFN alpha-2a, RBV
	1b	3	Telaprevir, Daclatasvir (NS5A inhibitor), PEG-IFN alpha-2a, RBV (COMMAND-3) (NCT01492426)
	1	3	Telaprevir, PEG-IFN lambda-1a, RBV (NCT01598090)
	4	2	Telaprevir, PEG-IFN alpha-2a, RBV (NCT0050801)
Boceprevir	1	FDA approved	Boceprevir, PEG-IFN alpha-2a, RBV
(SCH 503034)	1	3	Simeprevir, PEG-IFN alpha-2a, RBV (NCT01290731)
Simeprevir (TMC435)	1b/4	2	Simeprevir, IDEX719 (NS5A inhibitor), RBV (NCT01852604)
Faldaprevir (BI201335)	1	3	Faldaprevir, PEG-IFN alpha-2a, RBV
	1a	2	Faldaprevir, PPI-668 (NS5A inhibitor), BI207127 (non-nucleoside NS5B inhibitor), (+ RBV) (NCT01859962)
	1b	2	Faldaprevir, BI207127, RBV (NCT01858961)
Danoprevir (ITMN-191)	1	2	Danoprevir, PEG-IFN alpha-2a, RBV (NCT00963885)
	1/4	2	Danoprevir, Ritonavir, PEG-IFN alpha-2a, RBV (NCT01220947)
	1	2	Danoprevir, Ritonavir, RO5024048 (NS5B inhibitor), RBV, (± PEG-IFN alpha-2a) (NCT01331850)
Vaniprevir (MK-7900)	1	3	Vaniprevir, PEG-IFN alpha-2b, RBV (NCT01405937)
Asunaprevir (BMS-650032)	1	3	Asunaprevir, Daclatasvir (NCT01497834)
	1	2	Asunaprevir, PEG-IFN lambda, RBV (NCT01309932)
	1/4	2	Asunaprevir, PEG-IFN alpha-2a, RBV (NCT01030432)
	1a/1b/4	2	Asunaprevir, Daclatasvir, BMS-791325 (NS5B inhibitor) (NCT01455090)

Data from http://www.clinicaltrials.gov accessed on September 8, 2013. FDA: United States Food and Drug Administration; G: Genotype; HCV: Hepatitis C virus; PEG-IFN: Peginterferon; RBV: Ribavirin.

Table 2 Clinical characteristics of hepatitis C virus genotype 1b-infected patients in sequence analysis study of the hepatitis C virus NS3 region

No. of patients (men/women)	88 (43/45)
Age (yr)	55 ± 14
HCV RNA levels (low/high)	1/87
ALT (IU/L)	67 ± 44
WBC (x 10^3/mcL)	5.2 ± 1.5
Hemoglobin (g/dL)	14 ± 1.2
Platelet counts (x 10^4/mcL)	20 ± 18
IL28B rs8099917, TT/TG/GG/unknown	45/29/0/14

HCV RNA levels, low: less than 5 log IU/mL; HCV RNA levels, high: equal to or more than 5 log IU/mL; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; WBC: White blood cell.

V170N (Table 3). We did not identify high resistance variants at 155 and 156 in protease inhibitor-untreated HCV genotype 1b-infected patients (Table 3). Suzuki *et al*[58] reported that amino acid substitutions conferring resistance to protease inhibitors (V36A, T54S, Q80H, and D168E) were detected in 15 of 307 (4.9%) patients infected with HCV genotype 1b who had not received DAAs previously, and T54S (3.3%) predominated over V36A (0.3%), Q80R (0.7%) and D168E (0.7%), similar to our results. Leggewie *et al*[61] measured the prevalence of natural resistance polymorphisms in 38 acutely human immunodeficiency virus (HIV)-HCV co-infected treatment-naïve patients by using direct and deep sequencing. They found that 26% of patients (10/38) had a majority variant resistance mutation (in order of frequency: Q80K-16%, V36M-5%, T54S-3%, V55A-3% and D168A-3%). Low-frequency mutations were detected in all samples.

RESISTANCE MUTATIONS AND VIROLOGIC FAILURE

Despite extensive efforts to develop more potent next-generation protease inhibitors, the long-term efficacy of this drug class is challenged by the rapid emergence of resistance[62,63], which could result in treatment failures such as viral breakthrough or relapse. Our identified mutations associated with resistance to protease inhibitors are shown in Figure 1. In the Protease Inhibitor for Viral Evaluation (PROVE) 1 and 2 clinical trials[8,9] of telaprevir in combination with peginterferon plus ribavirin, viral breakthrough occurred in approximately 7% of patients with HCV genotype 1a infection, compared with about 2% of those with HCV genotype 1b infection; approximately 10% of patients with either subgenotype 1a or 1b suffered a relapse after cessation of HCV protease inhibitor-treatment. In both ADVANCE and Illustrating the Effects of Combination Therapy with Telaprevir (ILLUMINATE) trials[11,13], about 60% of patients treated with telaprevir-based triple therapy achieved an extended rapid virologic response (eRVR), with no virus detected at weeks 4 and 12.

HCV variants associated with on-treatment virologic failure or relapse were evaluated by using site-directed mutagenesis in HCV replicon assay[62,64]. Variants V36A/M, T54A/S, R155K/T, and A156S conferred lower levels of *in vitro* resistance to telaprevir (three- to 25-fold increase in telaprevir IC50), and A156V/T and V36M + R155K variants conferred higher levels of *in vitro* resistance to telaprevir (> 25-fold increase in telaprevir IC50). HCV replicon variants generated from patient-derived

Table 3 Naturally occurring pre-existing resistance amino acid mutations in the hepatitis C virus NS3 regions of 28 protease inhibitor-naive patients infected with hepatitis C virus genotype 1

Patient No.	V36	T54	V55	Q80	R155	A156	D168	V170Y/N/L		
	A/M	S	A	L	K/T/Q	S/T/V	N	Y	N	L
31		S		L						
95				L						
15				L						
17				L						
24				L						
26		S		L						
29		S								
81		S		L						
61								Y		
72								Y		
11				L						
12				L						
2								Y		
53				L						
55				L						
66				L						
84				L						
85				L						
110				L						
112								Y		
114		S		L						
100				L						
101									N	
107							N			
111									N	
99										L
92				L						
97		S		L					N	

sequences showed similar results. The *in vitro* replication capacity of telaprevir-resistant variants was lower than that of wild-type virus in the HCV genotype 1b Con1 replicon system[64-67]. When telaprevir-resistant variants were tested for cross-resistance against representative protease inhibitors in the HCV replicon system, HCV replicons with single substitutions at position 155 or 156 and double variants with substitutions at residues 36 and 155 showed cross-resistance to all protease inhibitors tested with a wide range of sensitivities. All telaprevir-resistant variants studied remained fully sensitive to interferon-alpha, ribavirin, and representative HCV nucleoside and non-nucleoside polymerase inhibitors in the replicon system. There are limited clinical data regarding re-treating patients who have failed an HCV NS3-4A protease inhibitor-based therapy such as telaprevir monotherapy, suggesting that re-treatment with triple therapy might be useful for certain patients.

In the boceprevir Serine Protease Inhibitor Therapy 2 (SPRINT-2) trial[6], patients showing a decrease in HCV viral load ≥ 1 log10 IU/mL during the four-week lead-in period of peginterferon plus ribavirin therapy had very low rates of emergence of boceprevir-resistant mutants (4%-6%) during subsequent triple therapy, whereas those with a < 1 log10 IU/mL decrease in HCV RNA had higher rates (40%-52%) of boceprevir-resistance-associated variants (genotypic mutations of the protease

conferring reduced sensitivity to boceprevir). The majority of boceprevir-treated subjects not achieving SVR had one or more specific treatment-emergent NS3 amino acid substitutions, most of which were previously shown to reduce the anti-HCV activity of boceprevir. These substitutions included V36A, V36M, T54A, T54S, V55A, V107I, R155K, A156S, A156T, A156V, V158I, D168N, I/V170A, and I/V170T. Detection of these substitutions was most common among subjects who experienced virologic breakthrough or incomplete virologic response[68].

COMBINATIONS OF DAAS FOR HCV STRAINS WITH RESISTANCE MUTATIONS

Protease inhibitors are used in combination with peginterferon plus ribavirin because monotherapy with protease inhibitors results in the early emergence of drug-resistance mutations[62,63]. As peginterferon plus ribavirin treatment is frequently associated with serious adverse events, an interferon-free DAA combination therapy such as protease inhibitors with an NS5A inhibitor and/or NS5B inhibitor would offer an ideal treatment option for patients with chronic HCV infection. However, combinations of DAA-resistant variants both in a single target protein and across multiple targets have been reported following failure of single and combination DAA regimens[55,69-71]. HCV population sequences of the complete HCV NS3 and 4A regions obtained from 2,111 HCV subgenotype 1a and HCV subgenotype 1b DAA-naïve patients were analyzed by Bartels *et al*[72]. It was reported that the strongest association was the combination of variants at NS3 V55, with lower-level resistance to boceprevir, and NS3 T54, with lower-level resistance to boceprevir and telaprevir[73]. The complete HCV NS3 study dataset showed that 69% (33/48) of patients with HCV NS3 V55I also had T54S. An association was also observed between HCV NS3 positions 54 and 155, with 17% (3/18) of the patients with the HCV NS3 T54S substitution also having R155K. The HCV NS3 T54S and R155K combination appeared in boceprevir and telaprevir trials. The study[73] also reported that treatment-naïve patients with viral populations containing the telaprevir-resistant variants HCV NS3 V36M, T54S or R155K at baseline achieved a 74% SVR rate with DAAs, similar to that (76%) in patients without resistant variants detected prior to treatment. Further studies are needed to confirm these findings.

DIFFERENCES IN RESISTANCE MUTATION SELECTION BETWEEN HCV GENOTYPE 1A AND HCV GENOTYPE 1B

It is possible that a different pattern of nucleotide changes is required for the resistance amino acid mutations between HCV genotypes 1a and 1b[67]. Substitutions at
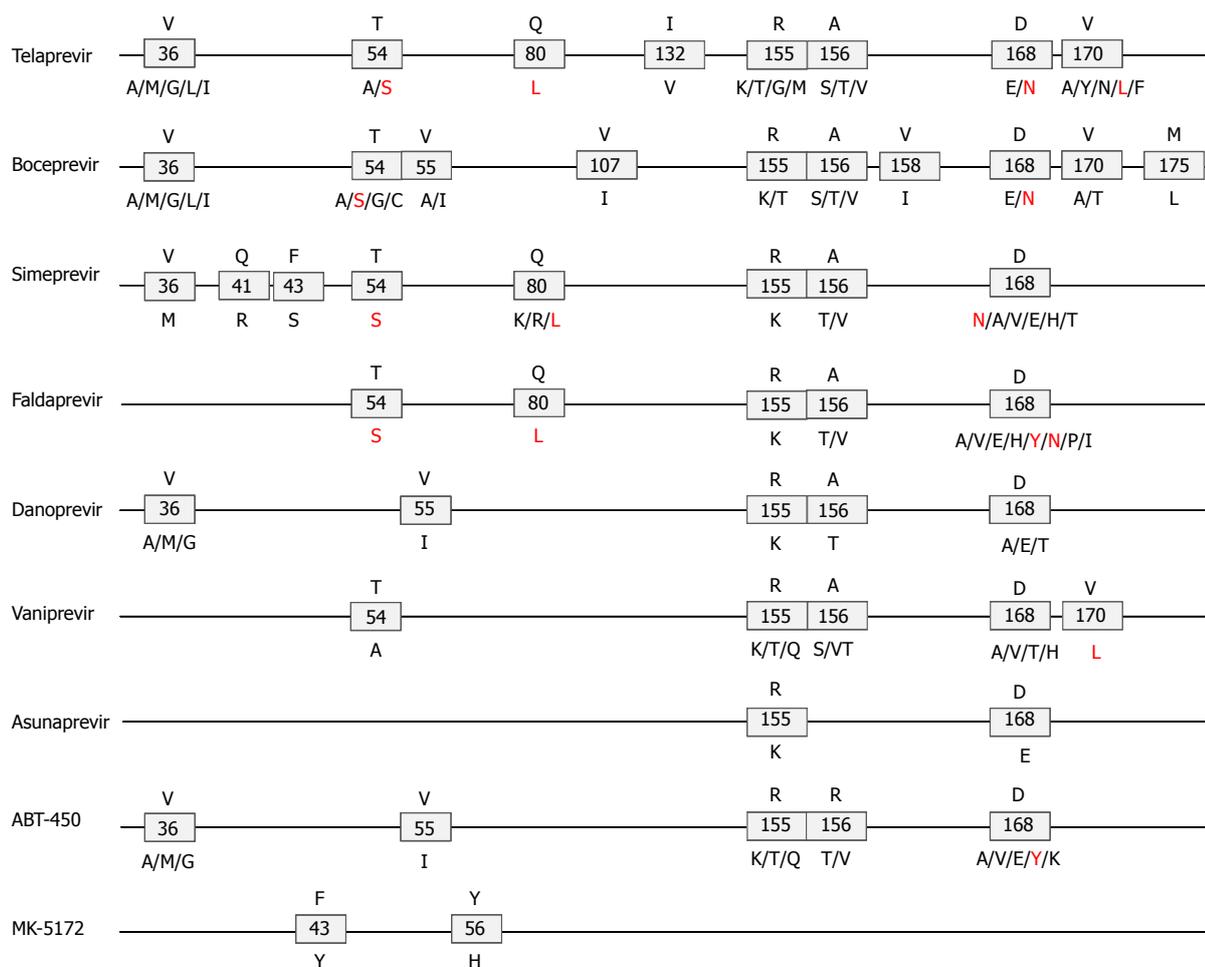
Figure 1 Mutations in hepatitis C virus NS3/4A serine protease that impact susceptibility to hepatitis C virus drugs approved by the United States Food and Drug Administration and investigated in phase 2 or 3 clinical trials. The numbers indicate the positions of the amino acids of the hepatitis C virus genotype 1 Con 1 strain. The amino acids above and below the numbers indicate wild-type amino acids and their substitutions, respectively. The red color indicates the mutations detected among the population using sequencing in the present study.

Table 4 Nucleotide changes were required for amino acid substitutions at position 155 of hepatitis C virus NS3 among hepatitis C virus genotype 1 samples

Amino acid at position 155	HCV genotype 1a	HCV genotype 1b
R	AGG	CGG
K	AAG	**AAG**
T	ACG	**ACG**
S	AGC	**AGC**
I	ATC	**ATC**

Bold-faced nucleotides nucleotides were required for amino acid substitutions at position 155. HCV: Hepatitis C virus.

A156 (A156S, A156T or A156V) require only a one-nucleotide change in HCV genotype 1a and HCV genotype 1b. In contrast, substitutions at R155 with K, T, S, M or I require a two-nucleotide substitution in HCV genotype 1b isolates. However, R155K/T/S/M/I substitutions require a one-nucleotide substitution in HCV genotype 1a isolates. The need for a two-nucleotide change for substitution R155 in HCV genotype 1b could be one of the reasons that HCV genotype 1a is more resistant to pro-

tease inhibitors than HCV genotype 1b (Table 4). In the ELECTRON study[74] of NS5B inhibitor sofosbuvir, no differential resistance was observed between genotypes 1a and 1b despite 89% of the subjects being in the HCV genotype 1a population, suggesting that combination DAAs targeting other HCV regions with next-generation HCV protease inhibitors could overcome the challenges of resistance mutations. In the near future, although mutation analysis was previously performed with population sequencing using Sanger methods, ultra-deep sequencing technology should provide new information[60,75-78]. Ligand bioactive conformation also plays a critical role in the design of HCV NS3 protease inhibitors and may allow for a large variety of HCV protease drug candidates to be designed[79].

CONCLUSION

In summary, we reviewed the literature reports of resistance variants of HCV protease inhibitors in treatment naïve patients with chronic HCV genotype 1, as well as our experience. Even in treatment-naïve patients with

chronic HCV genotype 1, naturally occurring HCV protease inhibitor-resistance mutations exist in some. Monotherapy with HCV protease inhibitors should be absolutely avoided. Regarding HCV protease inhibitor-resistance mutations, attention should also be paid to DAA-treatment-experienced patients, who previously used HCV protease inhibitor-monotherapies or combination therapies with HCV protease inhibitors. HCV genotype 1a is more resistant to protease inhibitors than HCV genotype 1b, and it is easier for HCV genotype 1a strains to be resistant to the currently available HCV protease inhibitors. At present, patients should be treated according to the recommendations of several HCV clinical practice guidelines[80-86]. However, it may also be possible that the combination of DAAs against regions other than HCV NS3/4A could eradicate HCV with these resistance variants.

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TOPIC HIGHLIGHT
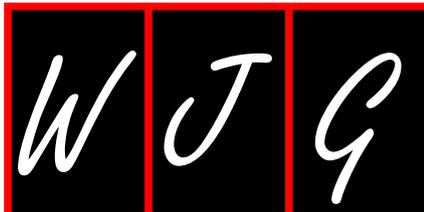
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Prospects for nucleic acid-based therapeutics against hepatitis C virus

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Abstract

In this review, we discuss recent advances in nucleic acid-based therapeutic technologies that target hepatitis C virus (HCV) infection. Because the HCV genome is present exclusively in RNA form during replication, various nucleic acid-based therapeutic approaches targeting the HCV genome, such as ribozymes, aptamers, siRNAs, and antisense oligonucleotides, have been suggested as potential tools against HCV. Nucleic acids are potentially immunogenic and typically require a delivery tool to be utilized as therapeutics. These limitations have hampered the clinical development of nucleic acid-based therapeutics. However, despite these limitations, nucleic acid-based therapeutics has clinical value due to their great specificity, easy and large-scale synthesis with chemical methods, and pharmaceutical flexibility. Moreover, nucleic acid therapeutics are expected to broaden the range of targetable molecules essential for the HCV replication cycle, and therefore they may prove to be more effective than existing therapeutics, such as interferon-α and ribavirin combination therapy. This review focuses on the current status and future prospects of ribozymes, aptamers, siRNAs, and antisense oligonucleotides as therapeutic reagents against HCV.

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Key words: Hepatitis C virus; Nucleic acid-based therapeutics; Ribozyme; Aptamer; siRNA; Antisense oligonucleotide

Core tip: Nucleic acids have emerged as new anti-hepatitis C virus (HCV) agents due to their great specificity, chemical synthesizability, pharmaceutical amenability, and broad targeting ability. Clinical applications of nucleic acids have been delayed due to their potential immunogenicity and toxicity, low efficacy, possible off-target effects, and lack of efficient delivery vehicles. However, recent advances in delivery carriers and chemical modification methods have improved the efficacy and bioavailability of nucleic acid-based agents. Hence, nucleic acids may be attractive anti-HCV options. In this report, the current status and future prospects of ribozymes, aptamers, siRNAs, and antisense oligonucleotides as anti-HCV regimens will be discussed.

INTRODUCTION

Hepatitis C Virus (HCV) infection is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular

carcinoma[1,2]. Nearly 170 million people are chronically infected worldwide by HCV, and approximately 27% of all cases of liver cirrhosis and approximately 25% of hepatocellular carcinoma cases may be related to HCV infection[3]. Given this obvious therapeutic need, international efforts to develop new antiviral drugs and vaccines that are effective against all HCV genotypes have been prompted. However, HCV has seven major genotypes with numerous subtypes[4] and exists as a variable quasispecies because HCV NS5B displays an error-prone RNA-dependent RNA polymerase activity that lacks proofreading functions[5]. Unfortunately, this high variability in HCV genomic RNA hampers the development of prophylactic and therapeutic vaccines and antiviral drugs[5,6]. Until recently, the usual treatment option for HCV infection has been a combination of pegylated interferon-α (PEG-IFNα) and ribavirin. This treatment clears infections by genotypes 2 and 3 in up to approximately 85% of cases. However, in infections with genotype 1, approximately 45% of cases are able to support a sustained viral response after the combination treatment[7]. Moreover, this treatment is associated with many side effects including flu-like symptoms, severe depression and hemolytic anemia[8]. Recent approval of two direct-acting antivirals (DAA) targeting the HCV NS3 protease, telaprevir (VX-950) and boceprevir, gives hope for the treatment of HCV infection. However, these drugs, given in combination with PEG-IFNα and ribavirin, are prone to selecting for drug-resistant viruses[9,10]. Therefore, DAAs that are more specific, effective, and safer are required. Over the last three decades, nucleic acids have been developed as potential antiviral therapeutic agents. Nucleic acid-based drugs are theoretically capable of targeting many types of molecules such as DNA, RNA, protein, lipid and even small molecules[11]. This property could overcome the limitations of the current therapeutics, which target only a limited number of proteins. Nucleic acid-based agents bind to target molecules through sequence complementarity (antisense oligonucleotide, siRNA, ribozyme, and antimiR) or on the basis of three dimensional structure (aptamer) (Figure 1 and Table 1)[12]. For example, aptamers bind to target molecules and function as decoys and/or inhibitors, whereas siRNAs and miRNAs make use of the RNA-induced gene silencing complex (RISC), which induces target RNA cleavage or translation inhibition[13]. AntimiRs block miRNA activity and thus induce expression of miRNA target genes[14]. Antisense oligonucleotides bind to complementary RNAs and suppress access to the cellular machinery, thereby inhibiting expression or function of the targeted RNAs[12]. Ribozymes are catalytic RNAs that cleave target RNAs (for example, hairpin ribozyme and hammerhead ribozyme) or selectively replace target RNAs with desirable RNAs (*trans*-splicing ribozyme)[15]. These variable modes of action provide many opportunities and options for the treatment of intractable diseases including genetic disorders, cancers, and infectious diseases. Despite their great potential, only a few nucleic acid-based therapeutics have

been approved; these include fomivirsen (an antisense oligonucleotide drug for the treatment of cytomegalovirus retinitis in patients with AIDS), pegaptanib (an aptamer for combating wet age-related macular degeneration), and mipomersen (an antisense oligonucleotide drug for the treatment of homozygous familial hypercholesterolemia)[16-18]. The problems involved in the application of RNA therapeutic agents include potential immunogenicity, inherent unstable nature, and the requirement for a delivery tool[11]. However, recent technological advances, such as the improvement of synthetic delivery carriers and the chemical modifications of nucleic acids, may help to overcome these obstacles. Recently, a phase Ⅱ clinical trial with SPC3649 (formerly Miravirsen), an LNA-modified antimiR-122, was completed for the treatment of HCV[19]. Many other nucleic acid-based anti-HCV therapeutics are in the pre-clinical and clinical stage. In this review, we summarize the current status of nucleic acid-based therapeutics that target the HCV RNA genome or HCV-encoded proteins. Moreover, we summarize their mechanisms of action and discuss the prospects for their future application to the treatment of HCV infections.

RIBOZYMES

A ribozyme is a catalytic RNA that cleaves or reprograms a target RNA sequence specifically, thus inhibiting the target RNA's expression or inducing new therapeutic gene expression only when the target RNA exists[20]. Since HCV has an RNA genome that replicates and exists exclusively in the cytoplasm, ribozymes are an attractive therapeutic option for HCV RNA clearance in infected cells. As HCV NS5B is an error-prone RNA-dependent RNA polymerase that lacks proofreading functions, viral replication is accompanied by the occurrence of mutations[5]. Therefore, sequence-specific therapeutics may induce escape mutant viruses. To overcome this obstacle, most ribozymes against the HCV genome have been designed to specifically target the HCV 5'- or 3'-untranslated regions (UTRs), which are highly conserved among all HCV genotypes and are essential for viral replication[21]. Promising *in vitro* results were obtained in the 1990s, using ribozymes directed against the HCV 5'- and 3'-UTRs[22-25]. Typically, naturally occurring ribozymes can be categorized into two groups depending on their mechanism of action: cleaving ribozymes (RNase P, hairpin ribozyme, and hammerhead ribozyme) and splicing ribozymes (group Ⅰ and Ⅱ introns)[20]. For therapeutic tools against HCV infection, researchers have modified and optimized these naturally occurring ribozymes or have engineered synthetic ribozymes to target the HCV 5'- or 3'-UTRs.

Cleaving ribozymes

Cleaving ribozymes are divided into two subgroups according to their natural traits: self-cleaving or *trans*-cleaving[20]. Hairpin, hepatitis delta virus and hammerhead ribozymes are all naturally self-cleaving ribozymes required
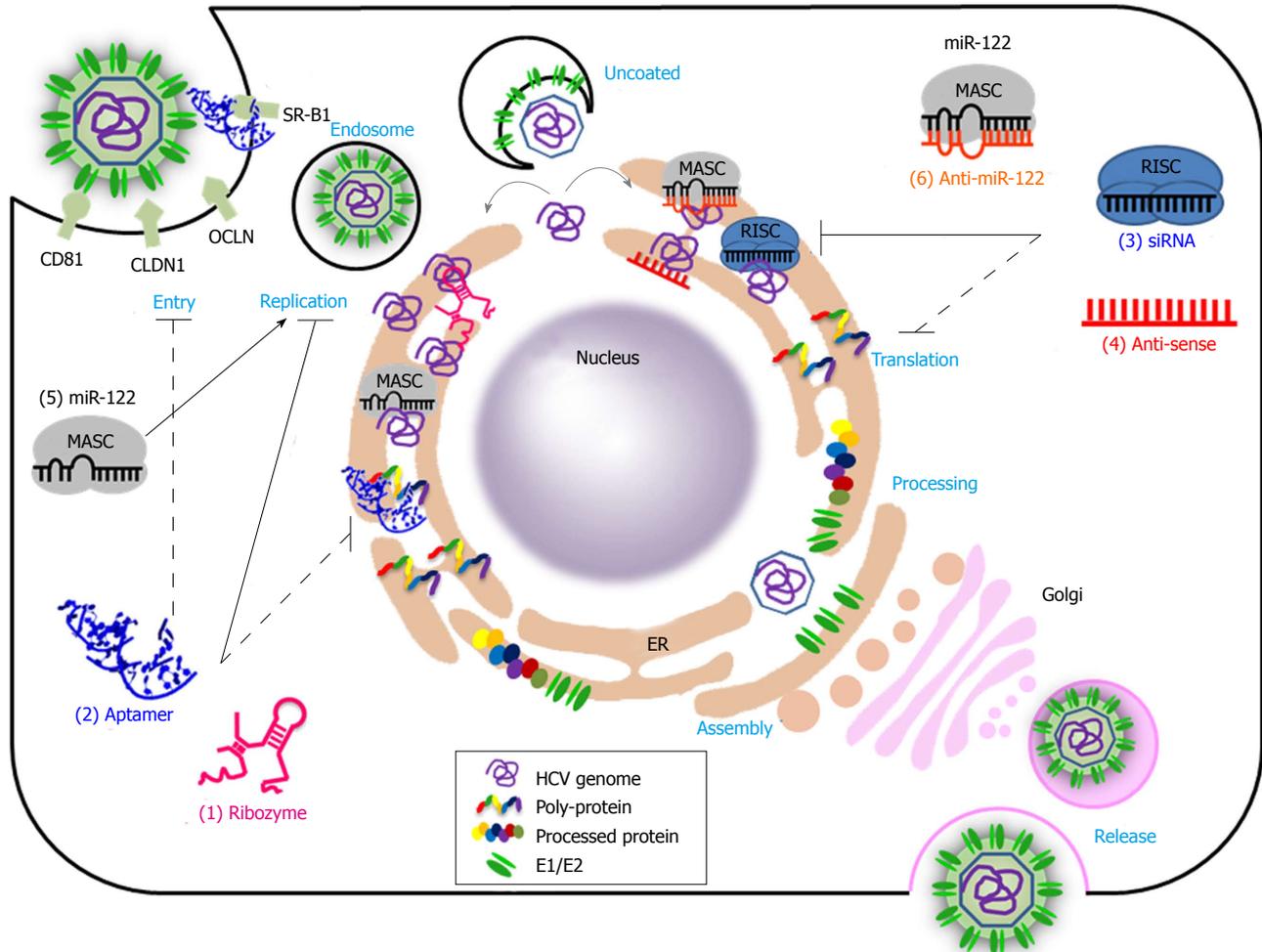
Figure 1 Overview of hepatitis C virus life cycle and antiviral target. The hepatitis C virus (HCV) life cycle includes entry, un-coating, replication, translation, processing of poly-proteins, assembly, and release. HCV has an RNA genome which replicates and is translated in the cytoplasm. Various nucleic acid-based thera-peutics target viral or host factors during the HCV infection as follows: (1) Ribozymes cleave or reprogram HCV RNA, sequence-specifically, thus inhibiting HCV RNA expression or inducing new therapeutic gene expression; (2) Aptamers can target to receptors (CD81, CLDN1, OCNL, SR-B1), which are needed for HCV entry, or to HCV regulatory proteins. Therefore, aptamers function as decoys during HCV entry or during replication to inhibit the viral life cycle; (3) siRNAs target the HCV genome as well as host factors, and can cleave and/or suppress translation of target RNA, sequence-specifically, through the RNAi induced silencing complex (RISC); (4) Antisense oligonucleotides induce inhibition of HCV gene expression through RNase H-dependent degradation of hybridized HCV RNA or by blocking access to cellular machinery necessary for the HCV translation; (5) MiR-122 is a host factor which regulates HCV replication. MiR-122 is incorporated into microRNA associated stabilizing complex (MASC) and increases HCV replication through binding to the HCV 5' IRES; and (6) Anti-miR-122 down-regulates miR-122, inhibiting HCV replica-tion. Lines represent the following: inhibition of replication (solid line), translation (short dashed line) and entry (long dashed line). An arrow indicates augmentation (black arrow) or the direction (gray arrow) of replication. Nucleic acid-based therapeutic molecules are shown as follows: ribozyme (pink), aptamer (blue), siRNA (sky blue), anti-sense (red) and anti-miR-122 (orange). CD81: Cluster of differentiation 81; CLDN1: Claudin-1; OCLN: Occludin; SR-BI: Scavenger receptor BI; ER: Endoplasmic reticulum.

for the replication process of RNA genomes. RNase P is an essential enzyme in the biosynthesis of tRNAs that specifically cleaves the pre-tRNAs, releasing 5'-sequences and mature tRNAs. Except for the plant chloroplast and Trypanosomatid enzymes, all known RNase P enzymes are ribonucleoproteins that contain an RNA subunit es-sential for the catalysis[26].

Hairpin ribozymes

Hairpin ribozymes consist of four helical domains and five loops. The cleavage site is flanked by the two sub-strate-binding sequences formed between the target RNA and the ribozyme, allowing the design of *trans*-acting ribozymes for target RNA sequence-specific cleavage[27].

The first effort to utilize engineered *trans*-cleaving hairpin ribozymes occurred in the 1990s[24]. *Trans*-cleaving hair-pin ribozymes targeting HCV RNA 5'-UTR and capsid gene regions were generated and shown to inhibit the ex-pression of a cotransfected reporter gene containing the HCV RNA target sequences. However, these ribozymes were not tested using an HCV cell culture replication sys-tem, such as the subgenomic replicon or the virus parti-cle-producing JFH-1 system[6], due to the unavailability of those systems at that time. Therefore, the effects of these ribozymes on HCV replication are unknown. Recently, other hairpin ribozymes targeting the HCV 5'- or 3'-UTRs were reported[28]. In Huh-7 cells that stably express sub-genomic HCV construct, I389/hyg-ubi/NS3-3' 5.1, the

Table 1 Nucleic acid-based anti-hepatitis C virus therapeutics

Class	Mode of action	Target	Status
Hairpin ribozyme	Cleave target RNAs	5'-UTR[24,28], 3'-UTR[28], and core region[24]	Tested in *in vitro*[24] and in cell culture model[28]
HDV ribozyme		5'-UTR[30]	Tested in *in vitro*
Hammerhead ribozyme (Hepatozyme)		5'-UTR[33-35]	Completion of phase Ⅱ
DNAzyme		5'-UTR[41], core and NS5B region[39,40]	Tested in *in vitro* and in
RNase P		5'-UTR[45-47]	cell culture model
Splicing ribozyme	Selectively replace target RNAs with desirable RNAs	5'-UTR[51]	
Allosteric ribozyme	Inhibit HCV replication[54] or cleave HCV RNA[31] through recognizing ligands	miR-122[54] and 5'-UTR[31]	
Aptamer	Bind to target molecule and function as decoys and/or inhibitors	NS3[68-70] NS5B[75-78] E1E2[79] Viral RNA[80,81]	
RNAi	Target RNA cleavage or translation inhibition	5'-UTR and 3'-UTR[93-96,100-102] Protein coding regions[103-109]	
Antisense oligonucleotide	Bind to complementary RNAs and suppress the access to cellular machinery, thereby inhibiting expression or function of the targeted RNAs	5'-UTR[134-137]	Completion of Phase Ⅱ
AntimiR	Block miRNA activity	miR-122[19,138]	Completion of Phase Ⅱ

inhibitory efficacy of these ribozymes on HCV replication was minimal (approximately 30%-40%). However, the inhibitory effects of these ribozymes were increased when combined with an HCV 3'-UTR targeting ribozyme (rather than HCV 5'-UTR targeting) and an HCV 5'-UTR targeting siRNA. This approach reduced HCV RNA and NS5B protein levels by 80%-90%. This result offers a promising combinatorial strategy for silencing HCV replication.

Hepatitis delta virus ribozyme

Among the different ribozymes, the hepatitis delta virus (HDV) ribozyme is the only catalytic cleaving RNA enzyme that has been discovered in humans[29]. The HDV ribozyme appears to be well adapted to the human cell environment, and hence, is a potential candidate for the development of anti-HCV therapeutics. The HDV ribozyme has been modified and designed to be able to cleave any specific RNA targets in *trans* possessing a complementary sequence to the recognition sequence of the ribozyme[20]. Unfortunately, the HDV ribozyme has not been developed extensively as an anti-HCV reagent; thus, its catalytic activity has been tested only in an *in vitro trans*-cleavage assay using the HCV 5'-UTR as a substrate[30]. Recently, an HDV ribozyme possessing a specific on/off adapter (SOFA), named SOFA-HDV ribozyme, was reported[31]. This SOFA-HDV ribozyme is discussed further in the allosteric ribozyme section.

Hammerhead ribozyme

The hammerhead ribozyme is one of the smallest ribozymes and likely the most widely studied ribozyme[26]. Due to its small size, specificity, and catalytic efficacy, the hammerhead ribozyme is the most commonly used ribozyme as a therapeutic agent for human diseases. Many attempts have been made to develop hammerhead ribozymes that

can efficiently cleave the HCV RNA genome and inhibit HCV translation and replication[22,23,32]. For example, a nuclease-resistant synthetic ribozyme with modified nucleotides and phosphorothioate linkages that target the HCV 5'-UTR was developed by Ribozyme Pharmaceuticals (RPI) in collaboration with Eli Lilly. This hammerhead ribozyme, named Hepatozyme™, successfully inhibited viral replication in cell culture with a chimeric HCV-poliovirus in a dose-dependent manner, and this effect was potentiated by interferon[33,34]. In a phase Ⅰ trial, Hepatozyme, administered either by subcutaneous injection or intravenously, was found to be safe[35]. A subsequent phase Ⅱ trial assessing the safety and efficacy of this ribozyme in combination with IFNα has been initiated. While a reduction of HCV RNA in serum was observed in some patients, RPI opted to discontinue the development of this drug because of toxicology findings in primates[36].

Deoxyribozyme

Unfortunately, ribozymes have the disadvantages of being both short lived and prone to losing their biological activity when they encounter alternative base substitutions[37]. Deoxyribozyme (DNAzymes) can be an effective alternative because of their small size (30-40 bases), ease of synthesis, and increased resistance to chemical or nuclease degradation[38]. DNAzymes have been shown to efficiently cleave target RNAs at purine-pyrimidine junctions *in vitro*. Similar to the RNA-based ribozymes, DNAzymes were usually engineered to target highly conserved sequences in the HCV core and/or the NS5B protein coding region[39,40] and 5'-UTR[41]. Lee *et al*[42] constructed a pool of 10-23 DNAzymes that possessed randomized annealing arm sequences and then selected the most available site for DNAzyme cleavage. All reported DNAzymes targeting the HCV genome cleaved the tar-

get RNA and thus inhibited translation and replication of HCV in the cell culture system. However, the effects were minimal or not superior compared with those produced with RNA-based ribozymes. DNAzymes are yet in their infancy as therapeutics, and further improvements are needed.

Ribonuclease P

Ribonuclease P (RNase P) is a ubiquitous endoribonuclease and is one of the most abundant and efficient enzymes in the cell. RNase P is a ribonucleoprotein complex that specifically cleaves pre-tRNAs, releasing 5'-sequences and mature tRNAs[43]. RNase P requires a short complementary oligonucleotide called an external guide sequence for its activity to recognize and cleave target RNA[44]. As an anti-HCV therapeutic, RNase P has been shown to display cleavage activity against the HCV 5'-UTR *in vitro,* but has not yet been extensively studied in a cell culture system[45-47]. Therefore, further evidences of its efficacy and safety in cell culture systems are needed to develop RNase P as an anti-HCV drug.

Splicing ribozymes

The self-splicing group I intron from *Tetrahymena thermophila* has been previously demonstrated to *trans*-splice an exon attached to its 3' end onto a separate 5' exon RNA not only *in vitro*[48] but also in *Escherichia coli*[49] and mammalian cells[50]. A promising advantage of *trans*-splicing group I intron is the cleavage of target RNA and the simultaneous induction of new therapeutic gene expression only when target RNA exists. Thus, *trans*-splicing ribozymes could potentially be used for the selective induction of new antiviral gene activities only in HCV-infected cells while simultaneously destroying the viral RNAs. Our group developed a *trans*-splicing group I ribozyme targeting the HCV 5'-UTR with the diphtheria toxin A (DTA) gene as a 3' exon[51]. This *trans*-splicing ribozyme specifically cleaved the HCV 5'-UTR and ligated DTA RNA to the cleaved HCV 5'-UTR, thus inducing HCV RNA-specific cell death. To further improve the anti-HCV activities and the safety of the *trans*-splicing ribozyme, a more careful selection of an antiviral gene as 3' exon, such as interferon instead of DTA, may be required as DTA can cause extensive death of HCV-infected hepatocytes.

Allosteric ribozymes

An allosteric ribozyme is a ribozyme whose activity can be specifically regulated by ligands. Commonly, ribozymes recognize only a 7-15 nucleotide long target RNA, and thus, the possibility of nonspecific off-target side effects is significant. To overcome this limitation, specific sensing ligands, such as RNAs, proteins or small molecules, can be tagged to the ribozyme to specifically regulate its activity. The SOFA (a specific on/off adapter)-HDV ribozyme and aptazyme have been suggested as representative allosteric ribozymes for HCV therapeutics. The SOFA-HDV ribozyme can switch its cleavage activity from off to on solely in the presence of the desired RNA ligand. The SOFA module is composed of three domains: a blocker, a biosensor, and a stabilizer[52]. The blocker sequence inhibits the cleavage activity of the ribozyme by binding *in cis*. The biosensor must bind its complementary sequence on the substrate to unlock the SOFA module. This binding induces the folding of the catalytic core of the HDV ribozyme into the on conformation. Both the blocker and the biosensor increase the substrate specificity of the ribozyme's cleavage by several orders of magnitude, compared with the wild-type HDV ribozyme[53]. Lévesque *et al*[31] attempted to develop a SOFA-HDV ribozyme to target HCV. They screened and identified the most active SOFA-HDV ribozyme against HCV RNA strands of both polarities. Unfortunately, the inhibition of HCV replication through targeting of the HCV replicon system with the SOFA-HDV ribozymes was not very effective, even though the SOFA-HDV ribozymes were active in an *in vitro* cleavage assay. Further elucidation of the reasons why SOFA-HDV ribozymes were not active in the cell culture HCV model is needed to optimize their activities against HCV. An aptazyme is composed of three independent modules: aptamer, communication module, and ribozyme. An aptamer binding to its ligand results in conformational change in the communication module, which can induce the on or off status of ribozyme activity. Recently, our group developed a specific aptazyme that can silence miR-122 activity only in HCV-infected cells[54]. MiR-122 is a positive regulator of HCV translation and replication. Functional sequestration of miR-122 effectively reduces the abundance of viral RNA, implicating miR-122 as a potential target for anti-HCV therapeutics[19]. However, miR-122 can also regulate the expression of a large number of genes involved in cellular physiological functions such as lipid metabolism and tumor suppression[55-60]. To overcome any possible nonspecific side effects due to miR-122 silencing in the normal liver, we created a hammerhead ribozyme-based aptazyme that can release anti-miR-122 through self-cleavage activity, depending on the presence of the HCV NS5B protein[54]. This HCV NS5B-dependent antimiR-122 releasing aptazyme specifically inhibited miR-122 function only in the HCV-infected cells. Moreover, this aptazyme more efficaciously hampered HCV replication than the miRNA silencing approach did, as it contains an aptamer domain that can specifically bind and sequester the HCV NS5B protein. Through the combination of selective miR-122 silencing and specific sequestering of HCV NS5B, this aptazyme approach could be a promising anti-HCV therapeutic treatment.

APTAMERS

Aptamers are small structured single-stranded nucleic acid sequences that have emerged as attractive and feasible alternatives to small molecule and antibody-based therapy, due to their great specificity, high affinity, easy and large-scale synthesis with a chemical method, phar-

maceutically flexibility, and poor immunogenicity[61,62]. Aptamers can be evolved using systematic evolution of ligands by exponential enrichment, an iterative selection method, and can bind target proteins with high affinity and specificity[63,64] through formation of well-defined complementary three-dimensional structures[65]. The first aptamer drug, known as pegaptanib (Macugen), was approved for the treatment of wet age-related macular degeneration by the United States FDA[18]. Other aptamer drug candidates now in the clinical development phase include transcription factor decoys and aptamers against thrombin, factor IXa, and nucleolin[62]. Establishment of a robust HCV cell culture system[66,67] has allowed the identification and biochemical characterization of two viral enzymes, NS3-4A and NS5B, that are major targets for antiviral therapeutics. NS3-4A and NS5B are essential proteins for the HCV replication cycle, and therefore, most of the aptamers have been developed against these two viral proteins to clear HCV infection.

NS3 targeting aptamers

The HCV NS3 is a multifunctional protein with three known enzymatic activities. The serine protease activity (in conjunction with cofactor NS4A) is present within the first 180 N-terminal amino acids, while the nucleoside triphosphatase (NTPase) and helicase activities are in the carboxy-terminal region[6]. These three activities are important to HCV replication. Most DAAs, including the FDA-approved VX-950 and boceprevir, target NS3 protease activity, as DAAs targeting the NS3 helicase domain have met with limited success[6]. In contrast, aptamers have been developed to target not only the protease domain[68] but also the helicase[69,70] and NTPase domains. Moreover, simultaneous targeting of protease and helicase activities through conjugation of protease and helicase aptamers is possible[71]. So far, among the NS3 aptamers, only the helicase-specific aptamer developed by our group has been tested for its ability to inhibit HCV replication in an HCV cell culture system[70]. As the NS3 region of the HCV genome may be a hot spot for mutations that are not deleterious to HCV replication, and due to the similarity of the NS3 helicase to cellular RNA helicases[72,73], a more careful examination is required to develop NS3 protease and helicase targeting aptamers as anti-HCV drugs.

NS5B targeting aptamers

The RNA dependent RNA polymerase (RdRp) NS5B is the key enzyme in HCV RNA replication. Due to its essential role in the HCV life cycle, the NS5B protein is an attractive target for the development of specific anti-HCV drugs. Many nucleoside analogues and nonnucleoside inhibitors have been shown to inhibit RdRp activity *in vitro*, as well as in the replicon cell culture system[74]. Jones et al[75] developed a DNA aptamer against the HCV genotype 3a NS5B protein. They confirmed that selected DNA aptamers specifically inhibited the NS5B polymerase activity of genotype 3a, but not of genotypes

1a and 1b. The therapeutic effectiveness of such aptamers should be carefully considered, as the most prevalent HCV genotype throughout the world is genotype 1. Moreover, their inhibitory effects against HCV should be carefully tested using the genotype 3a cell culture system that has been recently developed[76]. Bellecave et al[77] also reported DNA aptamers against HCV NS5B, and their aptamers inhibited HCV JFH-1 replication and viral particle formation in the cell culture system. However, those aptamers were not examined with regard to cell toxicity profiles, distribution in animals, or side effects during long-term treatment. Therefore, concerns about safety and the possibility of escape mutant virus appearance during repeated treatment cannot be excluded with these DNA aptamers. Recently, our group reported two types of RNA aptamers against the HCV NS5B protein composed of 2'-hydroxyl ribonucleotides (2'-OH) or 2'-fluoro pyrimidine ribonucleotides (2'-F)[78]. Both aptamers avidly bound to the HCV NS5B replicases of genotypes 1b and 2a and efficiently inhibited HCV replication of both genotypes in cells without inducing the generation of escape mutant viruses, innate immunity, or cellular toxicity. In addition, therapeutically amenable quantities of 2'-F aptamer conjugated with galactose-PEG moiety were efficiently distributed in the mouse liver tissue. These results suggest that RNA aptamers against HCV NS5B have a potential as a new therapeutic tool and are a potentially feasible alternative or additive to the current HCV therapeutics.

Viral RNA or HCV structural protein targeting aptamers

In addition to aptamers against the HCV regulatory proteins, a DNA aptamer targeting the HCV E1E2 structural protein was recently reported[79]. The DNA aptamer exerted its antiviral effects through inhibition of virus binding to the host cell receptors and thus inhibited the viral life cycle. Other aptamers have been reported that target viral RNA to inhibit either HCV translation[80] or replication[81]. Efficacy of the aptamers was confirmed in the HCV cell culture system. However, issues about cell toxicity profiles, distribution in animals, escape mutant appearance or side effects during the long-term treatment were not addressed.

RNA INTERFERENCE

RNA interference (RNAi) is a sequence-specific cellular post-transcriptional gene silencing (PTGS) pathway that regulates gene expression and is considered as a defense mechanism against invading viral pathogens and transposable elements in multiple organisms from worms to plants to mammals[82,83]. RNAi is initiated by double-stranded RNA (dsRNA) that is processed in the cytoplasm by the RNase III enzyme Dicer to form 21-22 nucleotide (nt)-long small interfering RNA (siRNA) with 5' phosphate groups and two nt 3' overhangs[84,85]. siRNA is then incorporated into an Argonaut-containing RISC (RNA-induced silencing complex), which unwinds the

siRNA into the sense (passenger) strand and the antisense (guide) strand. The passenger strand is then cleaved and removed, while the guide strand brings RISC to the mRNA, which has a sequence that is complementary to the guide strand[86,87]. The degree of complementarity between the target mRNA and the guide strand determines the extent to which RISC silences the expression of the target mRNA. If there is perfect complementarity of the guide strand with the target mRNA, RISC mediates site-specific cleavage that degrades the target mRNA. In contrast, partial complementarity represses translation of the target mRNA[88,89]. RNAi in mammalian cells was first described in 2001[90], and triggering of the RNAi pathway with synthetic (exogenous) siRNA has become the most powerful and essential tool for drug development against various human diseases such as viral infections, tumors, and metabolic disorders, due to its high knockdown efficacy and sequence specificity[91,92]. Because HCV has a single positive-stranded RNA genome that replicates in the cytoplasm, RNAi is an attractive therapeutic option for the treatment of HCV infection. Many attempts have been made to target HCV RNA with siRNA or with short hairpin RNA (shRNA) as an RNAi trigger.

RNAi against HCV 5'- and 3'-UTR sequences
Because siRNAs display high sequence specificity (up to a single nucleotide resolution), any mismatches between the siRNA and target RNA affect the activity of siRNA[91,92]. The 5'- and 3'-UTRs are the most highly conserved regions of HCV RNA and are also essential for HCV translation and replication. Therefore, both 5'- and 3'-UTRs are ideal regions for targeting with siRNAs[93,94]. Several groups have reported potent siRNA activity against HCV 5'-UTR in the subgenomic replicon system[93-95]. These reports demonstrated that siRNA targeting the HCV 5'-UTR resulted in 80%-90% inhibition of HCV. Prabhu *et al*[96] showed that siRNA that targets the highly conserved stem loop Ⅱ region of the HCV IRES efficiently inhibited translation and replication of infectious full-length clones of HCV 1a and 1b strains. Moreover, this siRNA effectively mediated degradation of the HCV IRES RNA and inhibited GFP expression that was controlled by the IRES sequences of six different HCV genotypes. Compared with synthetic 21-22 nt siRNAs, expressed shRNAs can induce long term stable knockdown of their target RNAs as long as transcription of the shRNAs occurs[97,98]. Moreover, shRNAs can act as substrates for Dicer, which increases the incorporation rates of siRNAs into RISC. This process enhances RNAi potency and efficacy[99]. For these reasons, two groups have utilized HCV 5'-UTR-targeting vector-derived shRNAs instead of 21-22 nt siRNA[100,101]. In both cases, the shRNAs inhibited replication and decreased titers of HCV genotypes 1a and 2a. Ray *et al*[102] also reported that shRNA targeting the 5'-UTR suppressed the replication of different HCV genotypes in the replicon cell culture systems.

RNAi against HCV coding regions
Because HCV RNA replicates in the cytoplasm, and its genome acts like mRNA, any region of the HCV genome is theoretically targetable with RNAi. A number of groups have demonstrated siRNAs or shRNAs that target the protein coding regions of HCV. Three different groups have shown that siRNA against the HCV core region reduced HCV RNA and protein expression[103-106]. Ansar *et al*[106] showed that siRNAs against the HCV core region showed a 70% reduction in viral titers, while siRNAs against E1 and E2 caused viral titers to drop by as much as 93% in HCV-infected liver cells. Moreover, Kim *et al*[105] demonstrated that siRNAs against the NS3, NS4A, and NS4B regions of HCV effectively inhibited HCV replication and translation. Ali Ashfaq *et al*[107] showed an 88% reduction in HCV replication with siRNA directed against HCV NS3 and a greater than 90% inhibition with siRNAs directed against the NS4B and NS5B regions. Two other studies also demonstrated that siRNAs against HCV coding regions significantly inhibited HCV RNA replication[108,109]. For example, Wilson *et al*[109] showed that siRNAs against the NS5A and NS5B regions dramatically reduced HCV replicon RNA levels by up to 99% and 94%, respectively.

RNAi against host factors
Host genes that modulate HCV infection and replication have been identified[110-113], and, unlike HCV itself, these genes are not prone to mutations. Therefore, these genes could be important targets for anti-HCV therapeutics. Several studies have shown that siRNAs against HCV entry receptors, such as CD81, SRBI, Claudin Ⅰ, or occludin, markedly decreased the susceptibility of human hepatoma cells to HCV infection[114-116]. In addition, cellular proteins with enzymatic functions have also been targeted by siRNA as an anti-HCV therapy[117-120]. Importantly, a combination of siRNAs directed against cellular HCV cofactors and the HCV genome had more pronounced effects on suppressing HCV replication than either treatment alone[116,118,121]. The instances of siRNAs targeting cellular factors for antiviral therapy against HCV has been more extensively reviewed in the literature[122,123].

RNAi with multiple siRNA
Because HCV has an error prone RNA-dependent RNA polymerase, the occurrence of drug-resistant escape mutant viruses is one of the major concerns for the development of antiviral therapies against HCV. Because RNAi has a high sequence specificity, prolonged treatment with siRNA could result in the appearance of escape mutant viruses that cannot be targetable by the siRNA. Wilson *et al*[124] reported that continuous treatment with one siRNA to an HCV replicon could induce the emergence of multiple point mutations within the target sequence region. One strategy to prevent the formation of escape mutant viruses is to use multiple siRNAs targeting multiple regions of the HCV genome combined with siRNAs

against cellular HCV cofactors[116,118,121]. Long shRNA can be processed by the host cell machinery into two or more siRNAs. A vector that directs expression of three shRNAs targeting the 5'-UTR and two NS5B regions of the HCV genome showed sequence-specific antiviral activity in the HCV replicon and in infectious virus systems[125,126]. When using a mutant virus with a genome containing an escape mutation against one siRNA, the remaining two siRNAs that could target the mutant virus displayed fully active and effective anti-HCV effects. Yang *et al*[127] designed a vector-derived shRNA that could be processed into multiple siRNAs, using the endogenous miRNA-17-92 cluster as scaffolds. These authors did this because a previous study had demonstrated that overexpression of exogenously introduced shRNA competed with endogenous miRNA and thus led to saturation of the endogenous miRNA pathway, resulting in serious toxicity in mouse liver, and in some instances, death[128]. This vector-derived shRNA consisted of five siRNAs targeted against HCV RNA; three target sequences in the 5'-UTR and two others in the core and NS5B regions of HCV. This vector-derived shRNA inhibited HCV RNA replication and translation up to between 93%-98% in the infectious virus systems without inducing toxicity.

Current limitations and future prospects of RNAi

RNAi-based antiviral therapeutics has a number of advantages. However, some limitations exist, such as the inherently unstable nature of RNA, the requirement of a delivery vehicle, off-target effects, potential immunogenicity, and toxicity resulting from interference with the endogenous miRNA machinery[11,119,128]. These barriers may be overcome with improved chemical modification of siRNAs and synthetic and viral delivery tools. Recent advances in chemical modification methods have increased the stability and efficiency and reduced the off-target effects, immunogenicity, and toxicity of siRNAs. The properties of chemically modified siRNAs have been extensively described in recent reviews[11,12]. Delivery methods are also important to consider when contemplating the use of an siRNA as an antiviral therapy. As vector-derived shRNAs are difficult to modify chemically, many researchers have manipulated the shRNA structure[127] and expression strategies by using tissue specific or inducible promoter to improve their usefulness as antivirals[129]. To test siRNA as an anti-HCV therapeutic in animal models, viral delivery systems have been employed[125-127,129]. Sakamoto *et al*[130] used adenovirus to deliver an shRNA expression vector into the livers of transgenic mice that could be induced to express HCV structural proteins by the Cre/loxP switching system. These authors showed that intravenous injection of the adenovirus expressing shRNA resulted in the specific suppression of virus protein synthesis in the liver. In other studies, adeno-associated virus (AAV) was used as a delivery vehicle[126,127]. Suhy *et al*[126] described an AAV serotype 8-based viral vector that expresses three shRNAs simultaneously. A single intravenous injection

of AAV8 expressing the shRNAs showed comprehensive transduction into hepatocytes in a nonhuman primate model. In addition to viral delivery systems, Chandra *et al*[131] have demonstrated the efficacy of a nanosome (lipid nanoparticles)-based siRNA delivery system. Multiple siRNAs directed against the 5'-UTR of HCV and encapsulated into nanosomes efficiently inhibited HCV replication in a liver tumor-xenotransplanted mouse model. Recent advances in nanobiotechnology will increase the available repertoire of synthetic delivery carriers for siRNAs directed against HCV RNA.

ANTISENSE OLIGONUCLEOTIDE

Antisense oligonucleotide (ASO) refers to a short DNA or RNA molecule that is designed to base pair with a specific target gene sequence in a sequence-specific manner. Most ASOs are synthetic single-stranded DNA or modified derivatives. Therefore, sequence-specific hybridization of ASOs to the target mRNA induces inhibition of target gene expression through RNase H-dependent degradation of the hybridized mRNA or through steric hindrance that blocks the access of the cellular machinery necessary for mRNA processing or translation[12,132]. Various modifications have improved the efficacy of ASOs through enhancement of nuclease resistance, increase in tissue half-life, affinity, and potency, and reduction of non-sequence-specific toxicity[133]. To improve resistance against nuclease degradation, a phosphorothioate-modified backbone was used (first-generation ASO). In addition, to further enhance nuclease resistance and increase binding affinity, 2'-O-Methyl (2'-OME) and 2'-O-Methoxyethyl (2'-MOE) modifications were developed (second-generation ASO). Peptide nucleic acid (PNA), locked nucleic acid (LNA), and phosphoramidate morpholino oligomer (PMO) have been recently developed as third-generation ASOs to further improve target affinity, nuclease resistance, biostability, and pharmacokinetics[132,133]. The United States FDA has approved ASOs Fomivirsen (ISIS 2922: Isis Pharmaceuticals) and, more recently, Mipomersen (ISIS301012: Isis Pharmaceuticals) for the treatment of cytomegalovirus retinitis in patients with AIDS and in patients with homozygous familial hypercholesterolemia, respectively[16,17]. In addition, a number of other ASOs are also undergoing clinical trials[133]. Several ASOs have been reported to inhibit HCV replication and translation. A phase Ⅱ clinical trial with ISIS 14803 (Isis Pharmaceuticals), a phosphorothioate oligodeoxynucleotide against the HCV 5'-UTR IRES, was completed in 2007, although results were not announced[134,135]. McCaffrey *et al*[136] demonstrated that morpholino phosphoramidate antisense oligonucleotides (morpholinos) complementary to the HCV 5'-UTR specifically inhibited HCV IRES-dependent luciferase translation by up to 95% for at least 6 d in mouse liver. Moreover, an adenoviral vector-expressing an RNA ASO has been reported to block HCV replication in the HCV replicon and in the infectious HCV JFH-1 cell culture

system by up to 40% and 76%, respectively[137]. Recently, a very promising ASO against HCV was reported with LNA-modified Miravirsen (SPC3649; Santaris Pharmaceuticals), which is directed against microRNA 122 (miR-122)[57]. MiR-122 has been reported to promote HCV replication through an increase in either stability or translation of HCV RNA by interacting with the 5'-UTR of the viral genome[138-140]. Therefore, silencing of miR-122 is a new plausible approach for anti-HCV therapeutics. LNA-modified ASO (SPC3649) caused a long-lasting suppression of HCV viremia in chronically HCV infected chimpanzees[141]. Moreover, a phase Ⅱ clinical trial showed that SPC3649 treatment resulted in a dose-dependent prolonged reduction of up to 2-3 logs of HCV RNA in patients chronically infected with HCV genotype 1[19]. More studies are needed regarding the long-term suppression of miR-122, as miR-122 functions as a tumor suppressor miRNA[58-60], and HCV escape variants resistant to SPC3649 could potentially occur[142].

CONCLUSION

For almost two decades, major endeavors to develop nucleic acid-based therapeutics against hepatitis C virus have been undertaken. Compounds such as ribozymes, aptamers, siRNAs, and antisense oligonucleotides have been shown to perturb various steps in the HCV life cycle (Figure 1 and Table 1). However, clinical application of these nucleic acid-based therapeutics has been hampered by their low efficiency, off-target effects, toxicity, inefficient delivery, and the lack of cell culture and animal models. These limitations have been gradually overcome with recently improved delivery carriers (viral and synthetic) and chemical modifications of nucleic acids that can ameliorate the efficiency and bioavailability, while also reducing the toxicity and off-target effects[11,12]. Moreover, great efforts have been made to establish HCV cell culture systems[66,67,76] and small animal models[143,144], which are highly useful for the evaluation of anti-viral efficacy, and thus, for the realization of effective nucleic acid-based anti-HCV drugs in the future.

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TOPIC HIGHLIGHT
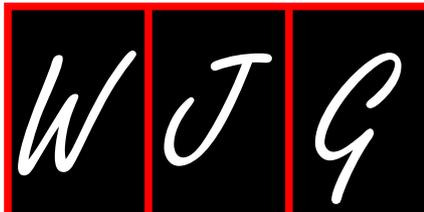
WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Antiviral treatment of hepatitis C virus infection and factors affecting efficacy

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Abstract

Hepatitis C virus (HCV) infection is the leading cause of chronic liver-related diseases, including cirrhosis, liver failure, and hepatocellular carcinoma. Currently, no effective vaccine is available for HCV infection. Polyethylene glycol interferon-α (PegIFN-α) in combination with ribavirin (RBV) is the standard of care (SOC) for chronic hepatitis C. However, the efficacy of PegIFN-α and RBV combination therapy is less than 50% for genotype 1 HCV, which is the dominant virus in humans. In addition, IFN and RBV have several severe side effects. Therefore, strategies to improve sustained virological response (SVR) rates have been an important focus for clinical physicians. The serine protease inhibitors telaprevir and boceprevir were approved by the United States Food and Drug Administration in 2011. The addition of HCV protease inhibitors to the SOC has significantly improved the efficacy of treatments for HCV infection. Several direct-acting antiviral drugs currently in late-stage clinical trials, both with and without peg-IFN and RBV, have several advantages over the previous SOC, including higher specificity and efficacy, fewer side effects, and the ability to be administered orally, and might be optimal regimens in the future. Factors affecting the efficacy of anti-HCV treatments based on IFN-α include the HCV genotype, baseline viral load, virological response during treatment, host *IL28B* gene polymorphisms and hepatic steatosis. However, determining the effect of the above factors on DAA therapy is necessary. In this review, we summarize the development of anti-HCV agents and assess the main factors affecting the efficacy of antiviral treatments.

Core tip: Understanding the effectiveness and affecting factors of antiviral regimens are critical for making informed treatment decisions for hepatitis C virus (HCV) infection. In this review, we have summarized the history of anti-HCV agents from interferon to the direct-acting antiviral drugs (DAAs) without polyethylene glycol interferon-α therapies and the affecting factors of antiviral treatment, focusing on investigating the optimal combination of antiviral therapies to achieve higher efficacy and better medication compliance. Although the efficacy of DAAs is significantly improved, many unmet needs and questions remain, such as avoidance of cross-resistance, the remaining high incidence of side effects, the role of IL28B status as well as the management of patients who do not respond to therapy.

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INTRODUCTION

Hepatitis C virus (HCV) infection, a worldwide public health problem affecting 170 million patients, is likely the cause of chronic hepatitis, liver cirrhosis, liver failure, and hepatocellular carcinoma[1]. Of the patients with chronic HCV infection, 40%-75% still exhibit extrahepatic manifestations including metabolic, hematological, vascular and rheumatological diseases[2-5]. Until recently, however, there have been no effective vaccines available. In the early 2000s, polyethylene glycol interferon-α (PegIFN-α) combined with ribavirin (RBV) became the standard of care (SOC) regimen for HCV, which showed a SVR that was mainly associated with its genotype. For example, patients with genotype 1 achieved a sustained virological response (SVR) of less than 50%. Additionally, this treatment regimen has several side effects, including granulocytopenia, anemia, and depression, and it is associated with a long treatment duration and increased cost. In 2011, the first direct-acting antiviral drugs (DAAs), telaprevir and boceprevir, were approved by the United States Food and Drug Administration (FDA). Combined with PegIFN-α and RBV, these DAAs resulted in a higher SVR rate in patients with HCV genotype 1. Thus, this treatment regimen became the SOC regimen for such patients. Soon afterward, other DAAs in the pre-clinical or pilot phase also achieved good treatment results. Current studies are focusing on investigating the optimal combination of antiviral therapies to achieve higher efficacy, shorter treatment duration, more simple administration, and better medication compliance. In response to an approved DAA, an evaluation of multiple factors (HCV genotype, baseline viral load, virological response during the treatment, and *IL28B* gene polymorphisms) affecting anti-HCV treatment therapy based on IFN is necessary.

ADVANCES IN ANTIVIRAL TREATMENT

Interferon

PegIFN-α: When administered as a once-a-week injection, PegIFN-α increased the SVR rate and compliance in patients by delaying renal clearance to extend the *in vivo* half-life by cross-linking polyethylene glycol and interferon-α. Currently, treatment combining PegIFN-α and RBV is still the most widely used SOC regimen.

Many clinical studies have compared the SVR rates in patients receiving different PegIFN-α (*e.g.*, IFN-α-2a and IFN-α-2b), dosages, and treatment durations. The results suggested that the patients given a standard dose (180 μg) of PegIFN-α-2a had higher SVR rates than those given a weight-based dose (1.5 μg/kg) of PegIFN-α-2b[6-8]. The IDEAL study, which included 3070 patients with hepatitis C, showed that the SVR rate in patients with HCV genotype 1 infection given different doses of PegIFN-α-2b (1.0 or 1.5 μg/kg) was not different from that in patients given PegIFN-α-2a (180 μg)[9]. In patients with HCV genotype 2/3 infections, those given a standard dose of PegIFN-α-2b (1.5 μg/kg) had a higher SVR rate than those given a low dose[10-12]. Meanwhile,

the SVR rates in patients receiving a high dose of RBV (1000-1400 mg/d) were higher than those in patients receiving a low dose (800 mg/d) of the PegIFN-α-related treatments[13,14]. The difference was especially obvious in the patients with a genotype 1 infection. Some studies investigated the antiviral therapy administered to patients with a genotype 2/3 infection. Although the overall SVR rate decreased after shortening the duration, a 12- or 16-wk treatment period was recommended for patients who achieved rapid virological responses (RVR)[10,15,16].

The IDEAL study results showed that regardless of which PegIFN-α was chosen to treat hepatitis C, the type and frequency of the adverse responses appeared similar (serious adverse responses, approximately 4%; headaches, 46%; myalgia, 40%; neutropenia, 5%; hemoglobin less than 86 g/L, approximately 3%), with a higher incidence of depression but lower incidence of skin rash associated with PegIFN-α-2b compared with PegIFN-α-2a[9].

Human serum albumin IFN-α fusion: Albinterferon is a genetic fusion protein used for the treatment of chronic hepatitis C (CHC), which takes advantage of the long half-life of human albumin to provide a new treatment approach that enables albinterferon administration at 2- or 4-wk intervals in individuals with CHC. Studies have demonstrated that the SVR rate resulting from the combined treatment of albinterferon and RBV was equivalent to that resulting from the SOC treatments, and the incidence rates of adverse drug reactions were also similar[17,18]. However, albinterferon is associated with the risk of reduced lung function, particularly in patients being treated for more than 6 wk[19].

PegIFN-λ-1a: IFN-λ is a class Ⅲ interferon and has completely different receptors from those of IFN-alpha *in vivo*. Its receptors are mainly distributed in the liver, which means that the extrahepatic adverse reaction from IFN-λ is significantly reduced compared with that from IFN-α. In recent years, PegIFN-λ-1 has been confirmed to have anti-HCV activity and mild adverse reactions[20]. One clinical trial assessed the efficacy and safety of PegIFN-λ-1a plus RBV compared to the SOC for the treatment of naive patients with HCV genotypes 2/3. The results showed that the curative effects of the two treatments were similar but that the viral load in the PegIFN-λ-1a group decreased faster and that the adverse reactions were significantly reduced[21].

DAAs

NS3 protease inhibitors: The unique structure and function of NS3 protease in the HCV life cycle makes it a new target for anti-HCV drug development. In addition to cleaving the polyprotein and generating the NS3, NS4A, NS4B, NS5A, and NS5B proteins, NS3 protease acts as an antagonist of the host innate immune system by cleaving signaling molecules that mediate a cellular antiviral response and resulting in the suppression of interferon production. The two NS3 protease inhibitors

discussed herein are telaprevir and boceprevir.

Telaprevir, as the first approved DAA, has a recommended dose of 750 mg tid, combined with PegIFN-α and RBV treatment (triple therapy), for a duration of 48 wk for naive or previous treatment failure HCV genotype 1 patients. The disadvantage of this medication is the need to ingest it with greasy food, which may cause an incredible weight increase during treatment. Six randomized clinical trials assessed the efficacy of the triple therapy compared with the SOC in naive HCV genotype 1 patients[22-27]. All patients were treated with telaprevir, PegIFN-α, and RBV for 8 or 12 wk, followed by the combined therapy of PegIFN-α and RBV. The results showed that the telaprevir triple therapy for 24 wk yielded a higher SVR rate than the SOC[22,24-26]. Even when the duration was shortened to 12 wk, the SVR rates were equivalent to those of the SOC[24], but prolonged duration did not improve its efficacy for those who achieved rapid virological response (RVR) and early virological response (EVR)[26,27]. The administration frequency of telaprevir (750 mg tid or 1125 mg bid) and PegIFN type (α-2b or α-2a) in patients with RVR and EVR had no effect on the SVR rate[23]. Among the previous treatment failure patients, the telaprevir triple therapy group had a higher SVR rate than that of the SOC group, but the overall effect was poor, especially for non-responders, with an SVR rate of 29%-33%[28]. Common adverse reactions to telaprevir include anemia, rash, nausea, hemorrhoids and itching. Because telaprevir treatment can lead to resistant mutants over the short term, the long-term use of the drug should be limited. Drug resistant mutants have been found to exhibit the following changes: V36A/M, T54A/S, R155K/T, and A156S/T.

Boceprevir is another NS3 protease inhibitor approved at the same time as telaprevir. The recommended dose of boceprevir is 800 mg tid, combined with PegIFN-α and RBV therapy, for a duration of 48 wk for naive or previous treatment failure HCV genotype 1 patients. Unlike telaprevir, boceprevir is started at week 4 of treatment, following a 4-wk lead-in period of treatment with peg-IFN and RBV, and RBV is required to enhance the efficacy of boceprevir[29]. Studies showed that the boceprevir triple regimen among naive patients for 48 wk increased the SVR rate associated with the SOC treatment to 16%-37%[30,31], whereas the SVR rates in the previous treatment failure patients were significantly higher (59%-66% *vs* 21%)[32]. Boceprevir-related adverse effects include fatigue, anemia, nausea, headache, dry mouth, granulocyte decreases, taste disorders, and thrombocytopenia. The long-term use of this drug can also lead to resistance mutations, including V36A/M, T54A/S, V55A, R155K/T and A156/S/T/V.

Simeprevir is a second-generation NS3 protease inhibitor and a competitive reversible macrocyclic, noncovalent inhibitor of NS3/4A protease[33]. Phase Ⅱ clinical trials compared the efficacy of simeprevir, PegIFN-α and RBV with the SOC treatment for naive or previous treatment failure HCV genotype 1 patients. Among the naive patients, those treated with the triple therapy with different doses of simeprevir (75 or 150 mg) once a day (qd) for 12 or 24 wk and then with PegIFN-α and RBV, for a total treatment course of 24 or 48 wk, obtained a higher SVR ratio compared with that of patients treated with the SOC (74.7%-86.1% *vs* 64.9%). In addition, for the majority of patients, the duration can be shortened to 24 wk[34]. The phase Ⅱb ASPIRE study demonstrated that simeprevir is a highly potent, efficacious, and well-tolerated once-daily PI for the majority of prior null or partial responders and relapsers compared to IFN-based therapy. Simeprevir has entered a phase Ⅲ clinical study. The most common adverse reactions are nausea, fatigue and hyperbilirubinemia, which are generally mild and reversible. The resistance mutations include Q8K and R155K.

Faldaprevir is a second-generation HCV NS3/4A protease inhibitor. Phase Ⅱ clinical trials have compared the efficacy of the SOC with that of the combined treatment with faldaprevir, PegIFN-α, and RBV in treatment-naive or treatment-experienced patients with chronic hepatitis C genotype 1 infection. The SVR rate in the treatment-naive patients who underwent 24-wk triple therapy including faldaprevir 240 mg qd with no lead-in was the highest, at up to 84%, whereas the group receiving the same drug dose with lead-in during the early phase of treatment or receiving a half dose of faldaprevir had a 72% SVR; in contrast, the other group (SOC regimen) had a SVR of only 56%[35]. Similar results were obtained for the treatment-experienced patients. The group receiving triple therapy with faldaprevir 240 mg qd for 48 wk with no lead-in had the highest SVR rate (50% in prior partial responders and 35% in prior null responders); the SVR rate in the lead-in treatment group that received the same dose was the lowest[36]. The adverse responses of faldaprevir include jaundice, skin changes (*e.g.*, rash), photosensitivity, pruritus, nausea, vomiting, diarrhea, and drying. The incidence of side effects is associated with the dosage. To date, the resistance mutations R155K and D168V/E have been observed.

Danoprevir is another second-generation NS3 protease inhibitor used for the treatment in naive or experienced HCV genotype 1 patients, and it is expected to eliminate the use of IFN-based drugs. One clinical trial compared the efficacy of the SOC with that of the combined treatment with danoprevir, PegIFN-α and RBV in treatment-naive patients with HCV genotype 1 infection[37]. The SVR rate in the group given danoprevir 600 mg q12h was the highest at up to 85%, whereas the group receiving the SOC had a SVR rate of 42%. Even when the duration among patients given danoprevir who had an extended rapid virological response (eRVR4-20: HCV RNA < 15 IU/mL during weeks 4-20) was shortened to 24 wk, 96% had an SVR. The INFORM-1 study evaluated the combination of danoprevir and mericitabine. Combination therapy was administered for up to 2 wk, resulting in a reduction in viral load and undetectable HCV RNA levels at the end of dosing in 63% of

treatment-naive patients[38]. Relevant evidence indicates that ritonavir can inhibit the metabolism of danoprevir *in vivo*, reduce the side effects, and improve the SVR rate, providing the possibility for IFN-free combination therapy. The INFORM-SVR study provided SVR data for the combination of mericitabine and danoprevir/ritonavir with or without RBV for 12-24 wk. SVR rates in HCV genotype 1a and genotype 1b patients were 26% and 71% in treatment arms including RBV, respectively, but significantly lower SVR rates were found in all RBV-free treatment groups[39]. The adverse reactions of danoprevir mainly include anemia, neutropenia, and rash. The resistance mutations R155K and D168T/E have been observed.

ABT-450 is a potent, specific protease inhibitor of HCV NS3. Ritonavir is used to increase the plasma concentration of ABT-450, prolong its half-life, and reduce the risk of drug resistance, enabling an ABT-450 dose regimen of once daily[40,41]. Fifty HCV genotype 1 patients including naive, prior partial or null responders participated in an open-label, multiple-center phase Ⅱ ABT-450 clinical trial. In the application of the combined treatment of ABT-333 [non-nucleoside inhibitors (NNI)], RBV, and ritonavir, the curative effects of different doses of ABT-450 over 12 wk were assessed. The results suggested that the SVR rates were higher than 90% in treatment-naive patients and 47% in prior partial or null responders[40]. The common adverse responses of ABT-450 include fatigue, pain, hyperbilirubinemia, and vomiting.

There are many other NS3 protease inhibitors in clinical studies, such as asunaprevir (BMS 650032), vaniprevir (MK-7009), narlaprevir (SCH 900518), VX 985, and MK-5172. Some of these NS3 protease inhibitors are expected to be approved for anti-HCV therapy in the near future.

NS5A inhibitors: NS5A is an essential viral component of the membrane-associated HCV replication complex and plays an important role in the formation of HCV infectious particles. Daclatasvir (BMS) 790052 was the first-in-class NS5A-specific targeted molecular inhibitor to be developed. Preclinical studies have shown that this NS5A inhibitor has broad genotype antiviral activity, but the associated mechanism is unclear. A phase Ⅱa study compared the efficacy of the combination of daclatasvir and asunaprevir (two-drug treatment) with or without the addition of PegIFN-α and RBV for the treatment of HCV genotype 1 prior null responders over a 24-wk duration. The results showed that the sustained virological response at post-treatment week 14 (SVR24) of the two-drug treatment was 36% and that the sustained virological response at post-treatment week 12 (SVR12) and SVR24 of the four-drug treatment were 100% and 90%, respectively[42]. High virological response rates were obtained in 90 treatment-naive patients administered the combination of daclatasvir with sofosbuvir, with or without RBV, for 24 wk. In HCV genotype 1 patients, RVR and SVR rates were 100% and 100%, while in HCV

genotype 2 and genotype 3 patients they were 100% and 91%, respectively[43]. However, it is notable that all failures were relapses after therapy. Analyses of resistance *in vivo* and *in vitro* showed mutations in the amino acid residues L31V/M and Y93H/N.

Several other NS5A inhibitors have also entered clinical trials, including ABT-267, ledipasvir (GS-5885), ACH-2928, and IDX791. Some of these inhibitors may be approved to become anti-HCV drugs.

NS5B polymerase inhibitors: NS5B is an RNA-dependent RNA polymerase (RdRp) in the HCV replication complex that catalyzes the synthesis of positive- and negative-stranded viral RNAs. Because mammals lack RdRp, new drugs to act as HCV NS5B polymerase inhibitors will be highly specific. NS5B enzyme activity can be inhibited by two different types of compounds: nucleoside/nucleotide derivative inhibitors (NIs) and NNIs. NIs can competitively bind to RdRp active sites, whereas NNIs target allosteric enzyme binding sites. Therefore, because both classes of drugs affect RdRp at different sites, cross-resistance is not easily produced.

NIs can simulate natural polymerase nucleotide substrates and act as a terminator that can be incorporated into RNA. The highly conserved HCV RdRp activation center showed that NIs have a similar efficacy on different HCV genotypes, as well as a high barrier to and low incidence of resistance genes.

Sofosbuvir can be used for the treatment of non-genotype 1 HCV infection[44,45]. A randomized, double-blind phase Ⅱ clinical trial showed that treatment with sofosbuvir, PegIFN-α, and RBV for 12 wk, followed by subsequent treatment with PegIFN-α and RBV for 12 or 36 wk, resulted in a SVR12 rate of 90% in HCV genotype 1 patients, which was similar to that in genotype 2/3 patients (92%)[44]. Another clinical trial showed that the 12-wk treatment of HCV genotype 1 naive patients with sofosbuvir, PegIFN-α and RBV was safe and effective. In addition, extended duration did not improve the efficacy, although these results need to be further confirmed by phase Ⅲ clinical trials[45]. It is notable that no viral breakthrough or resistance development during therapy has been described. Because of the absence of cross-resistance with the other DAAs, including NS5A inhibitors, sofosbuvir can be used for salvage therapy.

Mericitabine is a nucleoside analog polymerase inhibitor of HCV. Phase Ⅱ clinical study data showed that the treatment with mericitabine combined with PegIFN-α and RBV was safe and well tolerated. In the triple regimen for 24 wk, the SVR rate in HCV genotype 1/4 treatment-naive patients was higher than the SOC group[46]. The phase Ⅱ MATTERHORN study showed that for genotype 1a/1b prior null and partial responders after the combined treatment with ritonavir, danoprevir, mericitabine, PegIFN-α and RBV, the sustained virological response at post-treatment week 4 (SVR4) reached 83% and 100%, respectively. Currently, resistance mutants have not been found.

The design of NNIs involves targeting one of at

least five non-contiguous sites of RdRp allosteric enzymes, resulting in conformational changes that inhibit the enzyme activity, which have limitations on the genotype compared with NIs. A low genetic barrier may soon induce virus mutations. In phase I and II clinical studies, the results showed that BI 207127 and VX-222, regardless of whether they were combined with PegIFN-α treatment, can both improve the genotype 1 HCV infection RVR or EVR rate and demonstrate good tolerance. However, reducing the treatment with PegIFN-α resulted in a relatively high proportion of virological breakthroughs[47-49].

Cyclosporine - a cyclophilin inhibitor: Cyclophilins are a family of cell isomerases, including cyclophilins A, B, and C. The importance of human cyclophilins in HCV replication was confirmed by the anti-HCV activity of cyclosporine A. The mechanism of action of cyclosporine A involves NS5A and/or NS5B. Alisporivir (Debio-025) is a derivative of cyclosporine A, which removed the immunosuppressive activity but retained the potent antiviral activity against a wide range of HCV genotypes. All cyclophilin inhibitors have a high barrier to resistance. In vitro studies have shown a lack of significant cross-resistance with NS3/4A or other protease inhibitors. Moreover, there is an additive effect when cyclophilin inhibitors are combined with PEGIFN-α. Thus, in addition to having the advantage of once-daily administration, these agents are promising host-directed antivirals[50,51].

Supplementation therapy: *In vitro*, vitamin B12 acts as a natural inhibitor of HCV replication. A study assessed the effect of vitamin B12 on the virological response in antiviral therapy-naive patients with chronic HCV infection. The SVR rate was significantly higher in the SOC plus B12 group than in the SOC group[52]. At present, it is also believed that vitamin D has an anti-HCV activity *in vitro* that is mediated through its active metabolite, calcitriol[53]. The SVR of treatment-naive patients with chronic HCV genotype 1 or 2/3 infection is significantly improved by adding vitamin D to conventional PegIFN-α and ribavirin therapy[54,55]. However, given the very small number of available studies, additional studies are needed to assess potential differences in the associations between vitamin B12/vitamin D and SVR for HCV.

The hematologic adverse events of PegIFN-α combined with RBV therapy include anemia, thrombocytopenia, and leukopenia, which most frequently lead to drug discontinuation or dose modifications. L-Carnitine is a necessary nutrient factor in energy production and has been proposed as a potential adjuvant treatment to improve anemia, thrombocytopenia, and leukopenia. A study comparing the PEGIFN-α plus RBV plus an L-carnitine group versus the PEGIFN-α plus RBV group observed a significant improvement in SVR for 50% *vs* 25% of patients[56]. This finding suggests that L-carnitine supplementation may be useful in patients treated for HCV. Other supplementations including erythropoietin, zinc and probiotics have been assessed in clinical studies, but the effects of those on SVR are still not clear.

FACTORS AFFECTING THE EFFICACY OF HCV ANTIVIRAL THERAPY

The main factors influencing the efficacy of HCV antiviral treatments are divided into two categories: viral and host-related. The viral category includes the HCV genotype, baseline viral load, and virological response during treatment, and the host category includes age, gender, race, drinking habits, obesity, degree of liver fibrosis, and *IL28B* gene polymorphisms. In particular, *IL28B* gene polymorphisms are associated with the SVR. With approved DAAs on the market, more clinical treatment choices have been provided. The efficient and reliable prediction of the efficacy is essential to create individual antivirus solutions, improve the efficacy, reduce the side effects, and lower the treatment cost.

Viral factors

HCV genotype: Genotype plays an important role in predicting the response to the SOC treatments and determining the appropriate antiviral treatment. The response of patients with HCV genotype 1/4/5/6 infection is worse than that of patients with genotype 2/3 infection. DAAs are mainly used for the treatment of HCV genotype 1 infection. Although the effects of partial drugs on non-type 1 infection have been evaluated, there have been no sufficient data to clarify the relationship between the genotype and the effect of DAAs. Short-term data from a study on sofosbuvir indicated that the treatment with sofosbuvir combined with the SOC regimen resulted in a SVR12 of 91% in genotype 1 treatment-naive patients and 92% in patients with genotype 2/3. Another study showed that sofosbuvir combined with RBV resulted in a SVR rate of 84% in genotype 1 treatment-naive patients and 100% in patients with genotype 2/3[57]. Whether the HCV genotype affects the efficacy of DAA treatment remains to be confirmed by further studies.

Baseline viral load: Many studies have demonstrated that, regardless of the HCV genotype, a low baseline viral load (before treatment, HCV RNA < 600000-800000 IU/mL) was an independent predictive factor of the SVR[14,58,59]. In this range, the impact of the changes in the HCV RNA concentration on the SVR was not linear; when the HCV RNA was lower than 400000 IU/mL, an increase in the amount of virus decreases the SVR rate. However, an HCV RNA concentration higher than 400000 IU/mL results in a relatively stable SVR rate[51,60]. In 2011, the European guidelines for the prevention and treatment of hepatitis C suggested that if the baseline viral load was less than 400000-800000 IU/mL, the course of treatment for genotype 1/4 naive patients who received RVR can be shortened to 24 wk and that for patients with genotype 2/3 may be shortened to 12-16 wk[52,61].

Virological response during treatment: Using different patterns of response such as RVR, EVR, and delayed virological response (DVR: not having achieved RVR and EVR but testing negative for HCV RNA before the 24th wk) to predict the efficacy, determine the duration, and tailor the program can maximize benefits, rationalize the course of treatment, and minimize the recurrence rate. In the 2011 European guidelines[61] for the prevention and treatment of hepatitis C, the following adjustments are made. For the genotype 1/4 patients, if the baseline viral load was low before treatment and RVR was acquired after treatment, the duration could be reduced to 24 wk. If the patient acquired DVR, the duration should be prolonged to 72 wk to reduce the recurrence rate. For the genotype 2/3 patients, if the baseline viral load was low and RVR was acquired, the duration could be shortened to 12-16 wk. For patients who did not acquire RVR and EVR or only acquired DVR or exhibit combined effects from other factors (such as obesity and insulin resistance), as long as the viral load was undetectable at the 24th wk, the duration could be extended to 48 or 72 wk. Regardless of the genotype, if the viral load decreased to less than 2log IU/mL at the 12th wk and HCV RNA can still be detected at the 24th wk, the treatment could be discontinued. RGT principles are also applied to NS3 protease inhibitors. For HCV genotype 1 naive patients, using telaprevir or boceprevir combined with SOC and having acquired RVR and EVR, shortening the duration can be considered, but for patients with liver cirrhosis, a recommended treatment for 48 wk would be appropriate. The simeprevir results show that, according to the RGT principle, the treatment duration in HCV genotype 1 naive patients can be shortened to 24 wk, but further research is needed to confirm this recommendation[34]. The existing faldaprevir data show that extending the duration from 24 wk to 48 wk did not increase the SVR rate in HCV genotype 1 naive patients who achieved RVR and EVR, but for the previous treatment failure patients, a 48-wk course should be considered[35,36].

Host factors
Polymorphisms of the *IL28B* gene: In 2009, three genome-wide association studies (GWAS) found that single nucleotide polymorphisms (SNPs) in the *IL-28B* gene, located on chromosome 19, are associated with hepatitis C treatment efficacy[62-64]. In patients with HCV type 1 infection, Ge *et al*[62] found that rs12979860 (3 kilobases upstream of the *IL28B* gene encoding the type Ⅲ interferon IFN-l3) showed a strong correlation with the treatment response. The SVR rate of SOC in CHC patients carrying the CC genotype was 2-3 times higher than that in patients not carrying the genotype. A Japanese study showed that rs8099917 was correlated with the HCV treatment response and was one of the most important predictors of non-response after the logistic regression analysis[65]. The frequency difference in different populations with the rs12979860 CC genotype is very large, with East Asians having the highest frequency of the CC

genotype[62], followed by Europeans, and with Africans having the lowest frequency[63]. In a multivariate regression model, the IL28B polymorphism was the best predictor of treatment response, being better than the ethnic background, baseline viral load, degree of liver fibrosis, fasting glucose level, BMI, and other predictors[66]. Halfon *et al*[67] analyzed the predictive values of rs12979860 and rs8099917 in 198 patients with HCV genotype 1 with respect to their response to treatment and showed that rs12979860 seemed to be sufficient for clinical decisions. EASL guidelines showed that IL28B polymorphisms can be used to predict treatment response but have a low predictive value[61]. In contrast, AASLD argues that for determining the treatment regimen (SOC regimen combined with or without DAA), the IL28B polymorphism is a very strong predictor[68].

The predictive value of IL28B polymorphisms is not only limited to SOC regimen but has also been demonstrated in a study from Japan in patients receiving triple therapy with telaprevir. The study showed that rs12979860 and rs8099917 were associated with SVR, and the univariate and multivariate analyses confirmed that rs8099917 can be used as an independent predictor of the SVR[69]. Similar results were also found in other studies on the SOC treatments combined with DAAs[70-73]. An IFN-free study of mericitabine as a monotherapy or in combination with danoprevir showed that the rs12979860 CC genotype was related to faster and earlier viral decline[74].

Thus, the *IL28B* gene has a better predictive value with respect to not only the SOC but also DAAs. However, further research is still needed to confirm these observations.

Hepatic steatosis and other negative predictors: The value of steatosis as a negative predictor of response to anti-HCV therapy was confirmed in two large clinical trials. In one study, 574 HCV patients treated with the SOC were evaluated, and the results showed that the presence of steatosis reduces the likelihood of achieving EVR and SVR in genotype-1 infected patients[75]. In another study, 231 HCV patients treated with the SOC were evaluated[76]. The results showed that steatosis negatively affected SVR in HCV genotype non-3-infected patients. In the last year, new data showing that steatosis is also an independent predictor of relapse in genotype 3 have been published[77]. Steatosis has been associated with significantly higher rates of relapse, irrespective of viral load, in patients infected with HCV genotype 3 who had a rapid virological response (RVR)[78]. Several studies[59,78] have shown that RVR consistently remains an important determinant of SVR in patients with HCV genotype 2 or 3. Recent studies have confirmed that RVR is a good indicator for SVR in genotype 2, but not in genotype 3, in which steatosis is a predictor of relapse. This suggests that the underlying pathogenic mechanisms of steatosis differ between genotype 3 and other genotypes and may influence response to IFN-based therapy. These data sug-

gest that new therapeutic strategies are necessary for this subgroup of HCV genotype 3[59,78].

Other adverse predictive factors affecting the efficacy of HCV treatment include liver cirrhosis[79], age ≥ 40 years old[80], insulin resistance[81,82] and metabolic syndrome[83,84]. In patients with these factors, either the treatment duration may need to be extended or the dose may need to be increased.

CONCLUSION

PegIFN-α combined with RBV is currently the most classic and widely used standard treatment; however, its limited efficacy and significant side effects, as well as the absence of an HCV vaccine, promoted the development of new drugs. In recent years, the development of HCV antiviral drugs has progressed. Two HCV NS3 protease inhibitors, telaprevir and boceprevir, were approved by the United States FDA in 2011, and their combined treatment with the SOC not only significantly improved the SVR rate in HCV naive patients but also showed good efficacy in patients with previous treatment failure. Many other HCV NS3 protease inhibitors, NS5A inhibitors, and NS5B RdRp inhibitors are in the final stage of clinical trials and are likely to soon be approved as anti-HCV drugs. DAAs have shown a trend toward a gradual replacement of the SOC scheme. Although the efficacy of DAAs is significantly improved, the incidence of treatment-related side effects appears to be high, and because of the direct-acting antiviral effect, resistance mutations appear to be more likely to appear. Therefore, the implementation of personalized treatment approaches is very important. The application of many HCV antiviral drugs provides clinicians with more effective treatment choices for CHC. Host genetic factors guide individualized treatment strategies and aid in determining the best treatment plan for each patient. Polymorphisms in the *IL28B* gene have been used in clinical practice to help determine anti-HCV treatment strategies. Genetic markers need further verification, which can be performed in the preclinical testing stage. At the same time, accurately predicting the success of treatment and the progression of the disease will enhance the treatment compliance of patients, which will aid in maximizing the treatment effect.

Although DAAs show good potential, it is difficult to completely overcome the associated drug toxicity and occurrence of drug resistance; thus, not all patients can be cured by antiviral therapy. Therefore, determining how to prevent infection with HCV is an important research direction. Over the years, HCV vaccine development strategies are mostly based on the viral genome, unable to overcome HCV high variability, and starting from the human genome to explore other ways to prevent HCV infection may open up a new era in infection prevention

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REVIEW
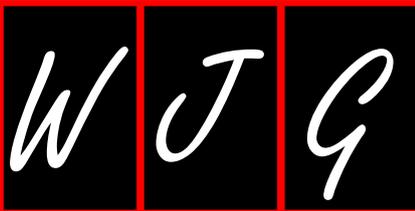
Extra-intestinal and long term consequences of *Giardia duodenalis* infections

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Abstract

Giardiasis is the most common waterborne parasitic infection of the human intestine worldwide. The etiological agent, *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*), is a flagellated, binucleated protozoan parasite which infects a wide array of mammalian hosts. Human giardiasis is a true cosmopolitan pathogen, with highest prevalence in developing countries. Giardiasis can present with a broad range of clinical manifestations from asymptomatic, to acute or chronic diarrheal disease associated with abdominal pain and nausea. Most infections are self-limiting, although re-infection and chronic infection can occur. Recent evidence indicating that *Giardia* may cause chronic post-infectious gastrointestinal complications have made it a topic of intense research. The causes of the post-infectious clinical manifestations due to *Giardia*, even after complete elimination of the parasite, remain obscure. This review offers a state-of-the-art discussion on the long-term consequences of *Giardia* infections, from extra-intestinal manifestations, growth and cognitive deficiencies, to post-infectious irritable bowel syndrome. The discussion also sheds light on some of the novel mechanisms recently implicated in the production of these post-infectious manifestations.

Core tip: This review offers a state-of-the-art discussion on the long-term consequences of *Giardia* infections, the most common waterborne parasitic infection of the human intestine worldwide, from extra-intestinal manifestations, growth and cognitive deficiencies, to post-infectious irritable bowel syndrome. The discussion also sheds light on some of the novel mechanisms recently implicated in the production of these post-infectious manifestations.

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INTRODUCTION

Gardia duodenalis (*G. duodenalis*) (syn. *Giardia lamblia*, *Giardia intestinalis*) is an intestinal flagellated protozoan parasite of the upper small intestine. Very common worldwide, *Giardia* was recently included in the World Health Organisation's Neglected Disease Initiative[1,2]. *Giardia* is

Table 1 Main pathophysiological effects of *Giardia duodenalis* and their mechanisms

Giardia-induced pathophysiological responses	Mechanisms involved or hypothesized to be involved	Selected references
Intestinal epithelial cell apoptosis	Induction of pro-apoptotic factors: Caspase-3, 8 and 9, Inhibition of anti-apoptotic factors: poly (ADP-ribose) polymerase (PARP) cleavage	[10,18,19,21,23]
Halt of enterocyte cell-cycle progression	Nutrient competition (arginine), up-regulation of cell-cycle genes	[25]
Intestinal barrier dysfunction	Disruption of claudin-1 and alpha-actinin by unknown mechanisms, caspase-3 mediated disruption of zonula-occludens (ZO)-1, myosin light chain kinase-mediated disruption of F-actin, and ZO-1	[10,17,19,21,26,27,29,30]
Small intestinal hypermotility	Adaptive immunity, neuronal nitric oxide, mast cell degranulation	[118-120]
Diffuse shortening of brush border microvilli	CD8+ lymphocytes - mediated *via* parasite secretory/excretory products	[13,16,21,31]
Crypt hyperplasia	Alteration villus/crypt ratio	[21,62,99]
Microbiota composition	Microbiota from infected host may become pathogenic	[14,33]
Increased mucus production	Increased mucus secretion in response to the parasite	[66]
Brush border enzyme activity deficiencies	Loss of surface area (microvilli and villi)	[16,21,98,121,122]
Disaccharidases deficiencies	Loss of surface area (microvilli and villi)	[10,16,99,122]
Electrolyte/nutrient/water malabsorption	Loss of surface area (microvilli and villi)	[10,19,21,62,99,123]
Anion hypersecretion	Unknown mechanisms	[10,19,99]

PARP : Poly adenosine diphosphate ribose polymerase.

transmitted through the ingestion of cysts in contaminated food or water, or directly *via* the fecal/oral route. Ingestion of cysts results in giardiasis, a disease causing intestinal malabsorption and diarrhea in a wide variety of species including humans. In developing countries, the prevalence of human giardiasis commonly ranges from 20% to 30% of the population, with reports of 100% prevalence in some populations; in developed countries, prevalence ranges from 3% to 7%[3,4]. The classification of *G. duodenalis* is a topic of debate and at present, the species is divided into eight distinct genetic assemblages, *i.e.*, assemblages A to H. Only the assemblages A and B are considered to be pathogenic in humans. Although parasites with assemblage A or B can infect non-human mammalian species, other genotypes appear to have a more restricted host range; for example assemblages C and D are commonly found in dogs[5], while assemblage E is common in cattle[6]. Ongoing research suggests that giardiasis is often due to anthroponotic spread, but zoonotic transmission can occur[7-9]. A striking feature of giardiasis is the spectrum of clinical symptoms that occur in infected individuals. The clinical manifestations can range from asymptomatic, to acute or chronic diarrheal disease. When present, the clinical signs of infection may include diarrhea, nausea, weight loss, bloating and abdominal pain[3,10]. In giardiasis, the acute pathophysiology occurs without invasion of the small intestinal tissues by the trophozoites, and in the absence of overt inflammatory cell infiltration, with the exception of a modest increase in intraepithelial lymphocytes[11-13]. Multiple factors have been proposed to account for the disease variability, including the state of the host immune system, host age and nutritional status, strain genotype, infectious dose and, possibly co-infections[3,8,10]. The pathophysiological consequences of *Giardia* infection are clearly multifactorial, and involve both host and parasite factors, as well as immunological and non-immunological mucosal processes. Recent observations suggest a role for disruptions of the host intestinal microbiota during the acute infection stage in the production of chronic symptoms, and further research is warranted to corroborate these findings[14]. The pathophysiology of giardiasis, and key aspects of the host response to *Giardia* remains incompletely understood.

PATHOPHYSIOLOGY OF GIARDIASIS

Central features of the pathophysiology of giardiasis are briefly outlined below, as these mechanisms may be key to our understanding of the complications discussed further (Table 1). While the *Giardia* genotype has been proposed to play a role in the induction of symptoms, there is currently no consensus concerning the connection between genotype and virulence[15].

After cyst ingestion in contaminated water or food, excystation occurs liberating two or four trophozoites, which adhere to the epithelial surface of the intestine *via* a ventral adhesive disk. This tight attachment between *Giardia* trophozoites and intestinal epithelial cells, as well as the production of yet incompletely characterized parasitic products, culminate in the production of diarrhea. Pathophysiology is believed to involve heightened rated of enterocytes apoptosis, intestinal barrier dysfunction, activation of host lymphocytes, shortening of brush border microvilli with or without coinciding villous atrophy, disaccharidase deficiencies, small intestinal malabsorption, anion hypersecretion and increased intestinal transit rates[10,13,16-22].

As it is the case with other enteropathogens, induction of apoptosis in enterocytes by *Giardia* represents a key component in the pathogenesis of the infection[3,18,19,22-24]. Enterocytes apoptosis during giardiasis is caspase-3 and -9 dependent[18,23]. While both host and parasite factors may modulate intestinal epithelial cell apoptosis, the products responsible for its activation during giardiasis have yet to be identified. In addition to promoting increased rates of enterocyte apoptosis, *Giardia* trophozoites may also halt enterocyte cell-cycle progression *via*

consumption of arginine, and up-regulation of cell-cycle inhibitory genes[25].

Findings from studies on giardiasis *in vivo* demonstrate that the most severe intestinal permeability and macromolecular uptake coincides with the peak of trophozoites colonization[17,26-28]. The effects of the infection on gut barrier function following host parasite clearance require further investigation. *Giardia*-mediated increases in intestinal permeability result from alterations to the apical tight junctional complexes, including disruption of F-actin, zonula-occludens-1, claudin-1 and alpha-actinin, a component of the actomyosin ring that regulates paracellular flow[19,26,28-30]. The role of *Giardia* proteinases in these effects is a topic of ongoing research.

Giardia-induced diffuse shortening of epithelial brush border microvilli represents a key factor in the production of diarrhoeal disease *via* malabsorption and maldigestion[13,16,31]. Whether or not the diffuse loss of microvillous border surface area associated with giardiasis is related to the release of a "toxin" by the parasite, a phenomenon similar to the release of proteases in the bacterial overgrowth syndrome[32], remains poorly understood. Regardless, *G. duodenalis* infection causes microvillous shortening in a lymphocyte-mediated manner which in turns impairs activities of disaccharidases[13].

Bacterial components of the intestinal microbiota from *Giardia*-infected hosts may act as stimulatory factors for protozoan pathogenicity[33]. Indeed, micro-organisms isolated from the duodenal microbiota of patients with symptomatic giardiasis can stimulate the pathogenicity of *G. duodenalis* in a gnotobiotic animal model[33]. The biological basis of this phenomenon remains unclear.

Giardia infections tend to be self-limiting in individuals with competent immune systems. A recent study in Brazilian children suggests that symptoms are less severe during re-infection, consistent with the hypothesis that if previous exposure does not always protect against future infections, it does at least reduce the severity of pathology[34]. Patients with common variable immunodeficiency and Bruton's X-linked agammaglobulinemia are prone to chronic giardiasis[35,36], which underscores the necessity of antibodies to fully control giardiasis.

In addition to its acute symptoms, giardiasis may also cause anorexia and failure to thrive. Indeed, *Giardia* infections may have detrimental effects on nutritional status, growth status and cognitive function in humans[37-41]. *Giardia* infections may also have detrimental effects on body weight in food-producing animals making this a serious concern for the agricultural industry[42-45].

LONG-TERM CONSEQUENCES OF GIARDIASIS

Extra-intestinal consequences of Giardia infections

Until recently, the scientific literature rarely reported extra-intestinal manifestations in giardiasis. However, a recent study estimated that 1/3 of the patients infected with this parasite will express long-term extra-intestinal symptoms, suggesting that this phenomenon is not as uncommon as previously thought[46].

Ocular pathologies: The first description of ocular complications in patients with giardiasis was made by Barraquer[47], who reported cases of iridocyclitis, choroiditis, and retinal hemorrhages in patients that presented diarrhea linked to the presence of *Giardia*. More recent observations described a "salt and pepper" degeneration (punctuate areas of normal hyperpigmentation on a light yellow pink-retina) involving the retinal pigmented epithelium in children suffering from giardiasis[48]. The same complication was described in children with past giardiasis, indicating that the ocular changes observed did not require the concurrent presence of the parasite in the gut[49]. Small children appear to be more susceptible to ocular lesions during giardiasis, and the lesions are thought to be caused by damage to the cells of the retina, accompanied by the release of pigment granules in retinal layers, where they can be seen as blackish dots[49]. The mechanisms linking ocular lesions with giardiasis remain obscure, but they exclude the possibility of direct invasion by the parasite. It has been speculated that the pigmented degeneration may result from toxic metabolites produced by the parasites, which has yet to be proven[48]. The role of increased intestinal permeability in the ocular complications seen in giardiasis needs to be elucidated.

Arthritis: Reactive arthritis is classically seen following infection with enteric pathogens such as *Yersinia* sp.*, Salmonella* sp.*, Campylobacter jejuni* and *Shigella* sp., but inflammatory arthritis has also been described following enteric infections with other organisms such as *Clostridium difficile, Brucella* sp. and *Giardia* sp.[50]. The interval between the preceding infection and the manifestation of arthritis is 2 to 4 wk[50]. Post-infectious arthritis has a predilection for joints of the lower limbs particularly the knee and ankle[51]. Post-infectious reactive arthritis has been classified as a classical spondyloarthropathy associated with human leukocyte antigen (HLA)-B27, an allele of the major histocompatibility complex class I present in 50% of the cases of patients with enteric-infection-related arthritis[51,52]. However, inflammatory arthritis following infection with *Clostridium* sp. or *G. duodenalis* does not fit classical spondyloarthropathy, as it fails to show association with HLA-B27[50]. Therefore, these are referred to enteric-infection-related-*arthritides*. Although *G. duodenalis* infections account for a significant proportion of enteric infections worldwide, reports of an association with post-infectious arthritis are relatively few. Little is known of the pathogenesis of arthritis in these conditions. Unlike post-enteric reactive arthritis, these arthritides are characteristically responsive to antibiotic therapies[52]. The variable degrees of host immune responses, and the lack of a robust systemic inflammatory response, may account for the infrequency of post-giardiasis arthritis despite the high prevalence rate of the infection[50]. Antigens from enteric bacteria have been isolated from the synovial fluid

Table 2 International reports of post-giardiasis metabolic consequences

Post-giardiasis effects	Country	Selected references
Lower cognitive function Lower intellectual quotient Lower social quotient	India, Peru, Turkey	[40,67,68,76,77]
Lower weight Lower height Stunting	Brazil, Columbia, Ecuadora, Guatemala, Iran, Israel, Mexico, Rwanda, Turkey, United States	[37-39,63,64,66-68,72,77,78,124-129]
Failure to Thrive	Columbia, Ecuadora, United States	[64,66,127]
Nutrient deficiencies	Iran, Mexico, Tanzania	[38,69,78,81]

of affected joints[52]. In a case of *Yersinia pseudotuberculosis* reactive arthritis, evidence of viable bacteria within the joint was demonstrated over a year later[53]. Here again, a possible role for increased intestinal permeability in enteric-infection-related-arthritis warrants further investigation.

Allergies: Concomitant presence of *G. duodenalis*, cutaneous allergic manifestations, and gastrointestinal symptoms have been described, which may explain why complete symptom resolution can be achieved with metronidazole and corticosteroids[54]. Significant anecdotal evidence suggests a causative link between giardiasis and the development of urticaria. In a recent study in children, giardiasis was associated with an increase in total serum immunoglobulin E (IgE) levels, and an enhanced IgE antibody response to common allergens[55]. These patients also demonstrated IgE reactivity to cow's milk and *Giardia* antigens. These observations suggests that alteration in antigen uptake from the small intestine during giardiasis, perhaps in association with connective tissue mast cell proliferation, may contribute to the development of allergic disease[56-58]. Dysfunction of the intestinal barrier during giardiasis may facilitate the translocation of food macromolecules and in turn prime the host for sensitization[55].

Muscular complications: Hypokalemic myopathy has been associated with celiac disease, radiation enteropathy, immunosuppressive drugs, and various infectious diseases. In the patient, this presents as marked proximal muscular weakness in all four limbs and the neck[59]. Analyses of muscular biopsies reveal an abnormal size of the muscular fiber due to the presence of numerous rounded atrophic and hypertrophic fibers, proliferation of myonuclei, and necrotic fibers[60]. The findings are consistent with impairment of muscle excitability and denervation due to muscle necrosis. Analysis of these fiber components showed that glycogen and lipid levels, as well as the inter-myofibrillar network pattern, are normal[60]. Several cases of myopathy following hypokalemia induced by giardiasis have been reported in both immunocompetent and immunocompromised patients[59,60]. This suggests

that *G. duodenalis* infections can trigger muscular manifestations independently of the immune status of the host. During giardiasis, potassium loss is closely related to the number of bouts of diarrhea per day[60]. Loss of potassium result in hypokalemia which can trigger a severe and transient myopathy[60]. In fact, muscular symptoms can improve with increased levels of potassium and recovery from diarrhea[59]. However, *G. duodenalis* diarrhea as a cause of myopathy due to hypokalemia is rare. It seems that the duration of symptoms is crucial for development of hypokalemic myopathy[60]. Giardiasis-associated hypokalemia occurs more often in elderly people, particularly women, who are hospitalized for giardiasis[61]. The causes, and the clinical consequences, of *Giardia*-associated hypokalemia remain unclear. It has been suggested that giardiasis-induced impairment of nutrient and electrolyte absorption may contribute at least in part to hypokalemia and hyponatremia[62].

"Metabolic" consequences of Giardia infection

Nutritional consequences: In developing countries of the world, because of infectious diseases and lack of food, 206 million children under 5 years of age suffer from stunting, 50 million from chronic wasting disease, and 167 million are grossly underweight[63]. Growth failure, reflected in stunting, wasting and underweight conditions, is assessed by anthropometric indices of height-for-age, weight-for-age, and weight-for-height[64]. Optimum health for children has long been linked to physical, socio-cultural, economic and environmental factors. In developing countries, the incidence of giardiasis is often over four times higher than the incidence reported in industrialized countries[65]. Children between 6 mo and 5 years of age are the most susceptible[66]. In combination with diarrhea, *G. duodenalis* infection can cause iron deficiency anemia, micronutrient deficiencies, protein-energy malnutrition, growth and cognitive retardation, and malabsorption[63,67]. Studies conducted on children from Brazil and Peru found that diarrheal disease occurring in the first 2 years of life negatively correlates with cognitive function, verbal fluency, and physical fitness, and may lead to long-term growth faltering[40,68]. These studies demonstrate that the effects of early childhood diarrhea are more far-reaching than merely causing dehydration. Diarrhea caused by *Giardia* sp. or *Cryptosporidium* sp. has frequently been associated with stunting and lower cognitive function[40,68] (Table 2). Intriguingly, a recent study observed that in a cohort of Tanzanian children infected with *Giardia,* infection had a protective role against diarrhea, and that this protection was lost with multi-nutrient supplementation[69]. Research needs to determine whether these interesting findings reflect a negative regulation by *Giardia* sp. of other enteric pathogenic processes that may occur in these children.

Failure to thrive: Childhood and adolescence are the period of most rapid skeletal growth. Failure to thrive (FTT) is a term generally used when a child presents

with a rate of weight gain that is significantly below that expected of similar children of the same sex, age and ethnicity. Failure to thrive is a common problem, that may be present at any time during the childhood, but is usually prevalent within the first 1-2 years of life. Long-term sequelae involving all areas of growth, behaviour and development may be seen in children suffering from FTT[70]. Causes for FTT usually include: (1) inadequate food intake; (2) reduced absorption or digestion of nutrients or excessive loss of nutrients; and (3) excessive utilisation of energy. There is a strong association between *Giardia* infection and malnutrition, wasting and stunting[38,63-65,69,71]. Malabsorption, maldigestion and malnutrition due to giardiasis have been shown to affect anthropomorphic factors as well as the calorie intake during childhood, most commonly in the second year of life in infected children[37-39,63,72]. Duration of the infection episodes, and their association with diarrhea, appeared to be the key factors associated with growth disturbance and failure to thrive[37]. While several studies have established a strong link between *Giardia* infection during the first two years of life, FTT and development impairment, more research is needed to unravel the mechanisms and the potential implications of polyparasitism in these phenomena.

Malnutrition, a common feature of numerous intestinal diseases, has been associated with an increase in macromolecular uptake due to heightened intestinal permeability[73], two phenomena known to occur during giardiasis[19,56]. *Giardia* infection can reduce food intake, and produce steatorrhea, maldigestion and malabsorption of carbohydrates and vitamins (including vitamin A, B3, B5, B6, B12, E, and folacin)[3,21,64,74]. Together, these effects may contribute at least in part to failure to thrive in giardiasis (Table 2).

Stunting: Growth failure due to malnutrition and chronic infections like giardiasis is associated with increased morbidity and mortality in children from developing countries[37,63,64]. More specifically, significant impairments in weight-for-age and weight-for-height scores have been associated with *G. duodenalis* infection during the first two years of life[72]. Indeed, the relative odds of low height-for-age may be 7.7 times higher among children with giardiasis[63]. In a number of developing countries, diarrhea caused by enteric parasitic Protozoa in early childhood represents predictors of stunting[64]. Given the high prevalence of asymptomatic infection in this study population (78.8%), children may appear to have normal weight-for-age and weight-for-height early on, but, present with growth retardation at a later age. This phenomenon is known as "homeorhesis", and it is probable that the high prevalence of asymptomatic *Giardia* infection among children may play a key role in it. Similarly, *Giardia* infection has been associated with decreased weight gain and impaired feed conversion efficiency in lambs and cattle, illustrating that growth retardation associated with *Giardia* infections also poses an important problem to the

agriculture industry[42-45]. Overall, human giardiasis combines with other factors, including low nutritional status, as well as sanitary and socioeconomic conditions, to lead to stunting[64]. However, findings from numerous studies, to date, indicate that the well established loss of intestinal surface area, maldigestion, and malabsorption caused by giardiasis may contribute to growth retardation following *Giardia* infection (Table 2).

Impaired cognitive function: Cognitive function in children can be affected by environmental and health related factors[75]. Risk factors that interfere with cognitive function are especially important during infancy because the first two years of life are an essential period of rapid growth and development, that is marked by rapid brain growth and maturation, by neuronal arborisation, myelinisation and emergence of brain networks. Thus the development of cognitive function in early life depends on the hierarchical maturation of neocortical association areas, as well as interactions with the environment. Nutrition, infection, and environment, have been found to affect neuroplasticity and to have long lasting effects in developing children[76]. Many of the hazards to early brain development are well known, and include head injury, newborn asphyxia, infections of the brain *in utero* and in the first year of life, genetic defects, lead poisoning and malnutrition. Micronutrient deficiencies (*e.g.,* Iodine) and iron deficiencies have also been found to impair cognitive development[76]. Studies have attempted to link possible long-term cognitive deficits with severe diarrhea in early childhood[40,41,68]. The complex interrelation among malnutrition, diarrheal disease and environmental factors such as socioeconomic status and education make it difficult to determine the unique contribution of either malnutrition or diarrheal disease to cognitive development. However, chronic malnutrition and stunting during infancy secondary to *G. duodenalis* infections, has been associated with poor cognitive function[40,41,77]. Moreover, diarrhea during early childhood was also found to impair visual-motor coordination, auditory short-term memory, information processing, and cortical cognitive function[68,76].

Interestingly, poor language cognition and impaired psycho-motor development appear to be associated with *Giardia* sp. more so than with other enteropathogenic parasites such as *Entamoeba histolytica, Ascaris lumbricoides, Enterobius vermicularis,* or *Trichuris trichiura*[63]. These studies have suggested a role for nutrient malabsorption and micronutrient deficiencies, such as zinc, iron or vitamins (A and B-12) in humans as well as in animals[63,74,78,79]. Indeed, significantly lower levels of iron and ferritin, known to affect psychomotor development, have been detected in patients with giardiasis[64]. Similarly, diarrhea due to giardiasis was linked to poor cognitive function by causing zinc and iron micronutrient deficiencies, as well as defects in the anti-oxidant system, which may all affect neuroplasticity[76]. Indeed, perinatal iron deficiency in rats reduces neuronal metabolic activity, specifically targeting

areas of the brain involved in memory processing[80]. Zinc supplementation was recently found to reduce the rate of diarrhea caused by giardiasis[81]. The complexity of these profound effects on functional impairments requires further investigation. More research is also needed to determine whether and how these effects can be reversed with targeted antimicrobials, with micronutrient and/or oral rehydration, or nutrition therapy[68] (Table 2).

Chronic fatigue syndrome: Viral, bacterial, as well as parasitic pathogens can trigger chronic fatigue syndrome (CFS), and are responsible for work-related disability reflected in long-term sickness, absence from studies and employment[82]. Although the biological basis of CFS is unknown, it is generally thought that post-infectious fatigue develops shortly after acute infection. CFS has been described following Q-fever, Epstein-Barr virus infection, Ross river virus infection, brucellosis, Lyme disease, viral meningitis and Dengue fever[83]. Recent studies have reported a high prevalence of post-infectious fatigue following a giardiasis outbreak in Bergen, Norway, in 2004[15,82-86]. Fatigue was reported in 41% of the people in Bergen 2 years after the *Giardia* outbreak, compared to 22% in the general population[82]. In this population, old age and female gender were a significantly higher risk factor for post-infectious fatigue[84,87].

Although *Giardia* is a non-invasive parasite, post-giardiasis CFS is likely to include immunologic components[82]. Studies have implicated differences in activation and function of peripheral blood lymphocytes subsets in post-giardiasis CFS, including altered natural killer-cell levels and lowered CD4:CD8 ratios[87,88]. The exact roles of immune factors in co-morbidities associated with gastrointestinal disorders and CFS need to be further explored. Fatigue is a frequent symptom in patients with functional gastrointestinal disorders (FGID), especially irritable bowel syndrome (IBS)[89].

Chronic gastrointestinal consequences of Giardia infections

FGID: FGID represent a group of disorders characterized by recurring or chronic gastrointestinal symptoms without an identifiable disease process. IBS and functional dyspepsia (FD) are the best described FGID. Post-infectious-IBS (PI-IBS) has been reported following acute gastroenteritis due to bacteria such as *Salmonella* sp., *Shigella* sp. and *Campylobacter jejuni*[90,91]. Recent evidence now indicates that a proportion of patients diagnosed with *Giardia duodenalis* will also develop PI-IBS symptoms in the absence of detectable parasitic loads[92,93].

Post-infectious irritable bowel syndrome: IBS is the most common functional gastrointestinal disorder diagnosed by gastroenterologists today. It is characterized by abdominal discomfort and altered bowel habit, with no abnormality on routine diagnostic tests. Several mechanisms have been considered in the pathogenesis of IBS including genetic, psychological and environmental factors as well as intestinal motor and sensory func-

tions associated with brain-gut interactions[94]. In some patients, IBS symptoms seem to arise *de novo* following acute gastroenteritis (GE). This PI-IBS denotes the persistence of abdominal discomfort, bloating and diarrhea, despite clearance of the inciting pathogen. Recent meta-analyses demonstrated that the risk of developing IBS increases six-fold after gastrointestinal infection and remains elevated for at least 2-3 years post-infection, and it is estimated that 7%-31% of patients with infectious GE go on to develop PI-IBS[90,91]. Higher risk factors include longer duration of symptoms, young age and female gender. The current conceptual framework regarding the pathophysiological mechanisms for PI-IBS suggests that it is associated with increased intestinal permeability and motility, increased numbers of enterochromaffin cells and persistent intestinal inflammation, characterized by increased numbers of T-lymphocytes and mast cells, and increased expression of pro-inflammatory cytokines[95-97]. Possible mechanisms for PI-IBS include genetic predisposition, motility dysfunction, such as accelerated colonic transit and smooth muscle hyper-reactivity to acetylcholine, continuous antigenic exposure (bacterial, parasitic or dietary), or molecular mimicry of foreign antigens[98].

Early reports indicated that *Giardia* may cause prolonged symptoms, including secondary lactose intolerance, for several weeks after successful treatment[99]. Chronic giardiasis resembles IBS, and symptomatic infection may exacerbate existing IBS[100]. *Giardia* infection has been diagnosed in 5%-10% of patients with IBS[101,102], and it was recently demonstrated that *G. duodenalis* may indeed cause IBS and functional dyspepsia[93]. High frequency of microscopic duodenal inflammation was found in patients post-giardiasis when the infection lasted 2-4 mo, further supporting the hypothesis that longer duration of infection is a risk factor for PI-IBS[103]. Early diagnosis of *Giardia* infection and treatment may shorten the duration of the infection and hence may help reduce the risk for such complications[83].

Interactions between the host and gastrointestinal microbiota may play a key role in the pathogenesis of IBS. Fecal microbiota are altered in patients with IBS, and patients with diarrhea-predominant IBS appear to host more *Proteobacteria*, and fewer *Bacteroidetes* compared to asymptomatic patients[104,105]. The mechanisms by which altered fecal flora may induce disease are poorly understood. Abnormalities in short chain fatty acids have been reported in patients with diarrhea-predominant IBS[105]. Whether these alterations may result from abnormalities in the host microbiota requires further investigation[14].

Historically, IBS was considered as a psychosomatic disorder, with an emphasis on psychiatric comorbidity[106,107]. During the past decades, GE and low grade inflammation as mechanisms underlying gastrointestinal (GI) dysfunction have been involved in IBS symptoms[106,108]. It is now well established that there is a relationship between the neural and immunological networks within the gut, and that the central nervous system and the gut are engaged in constant bi-directional communication, often related to as the brain-gut axis (BGA).

Table 3 Extra-intestinal and long-term consequences of giardiasis

Post-infectious consequences	Speculated mechanisms involved	References
Ocular pathologies	Speculated involvement of toxic metabolites produced by the parasite	[47-49]
Arthritis	Bacterial antigens in synovial fluids possibly due to increased intestinal permeability	[50-52]
Allergies	Alteration in antigen uptake	[54-57]
	Dysfunction of the intestinal barrier	
Hypokalemic myopathy	Loss of potassium related to diarrhea, impaired nutrient and electrolyte absorption	[59-62]
Failure to thrive	Inadequate food intake, Reduced nutrients absorption, excessive utilisation of energy, Steatorrhea, maldigestion, malabsorption	[38,39,63-65,69,71,118]
Stunting	Nutritional status, sanitary, socio-economic conditions, loss of intestinal surface area, maldigestion, malabsorption	[37,63,64,67,77,121]
Impaired cognitive functions	Chronic malnutrition and stunting following *G. duodenalis* infection	[40,41,63,64,68,76-78]
	Nutrient malabsorption and micronutrient deficiencies	
Chronic fatigue syndrome	Altered natural killer-cell levels	[15,82-87,89]
	Lower ratio CD4:CD8	
Post-infectious irritable bowel syndrome	Microscopic duodenal inflammation	[14,84,93,100-105]
	Interaction host - gastrointestinal microbiota	
	Increased T-cells and Mast-cells	
Cancer	No cause-to-effect established	[113-116]

Among the pathophysiological mechanisms of IBS, disorder of the BGA has been associated[106,108]. Recently, more evidence of emerging dysbiosis in IBS patients have been made[104], suggesting an important role of the microbiota-gut-brain axis[106-111]. Nevertheless, our understanding of the mechanisms of the bi-directional interactions between microbiota and GI physiology and its association with behavior needs to be explored with focus on the contributions of immunological and neural components to the microbiota-BGA relationship. Insights into the interactions between enteric pathogens, the host epithelia, and the intestinal microflora are needed to improve our understanding of disease processes that may initiate IBS or even exacerbate intestinal inflammation in patients with IBD[112]. Studies on giardiasis offer a powerful model to investigate these mechanisms.

Cancer

A few reports have described *Giardia* trophozoites in the tumoral mass of pancreatic tissue and gallbladder. While *G. duodenalis* trophozoites are generally localized to the proximal small bowel, they may also be identified in the stomach, distal small bowel, or caecum, and studies have reported pancreatic infection with *Giardia*[113-115]. Although the relationship between pancreatic giardiasis and pancreatic cancer is presently unknown, the coexistence of these 2 diseases may prompt exploration into mechanisms of carcinogenesis in giardiasis. In another study, following cholecystectomy with liver bed resection and lymph node dissection, intra-operative cytological examination of the patient's bile juice revealed the presence *G. duodenalis* trophozoites, and pathological examination revealed gallbladder cancer[116]. However, no cause-to-effect has yet been established between the presence of *Giardia* and the development of cancer.

CONCLUSION

Infections with *Giardia duodenalis* may remain asymp-

tomatic, or cause acute or chronic diarrheal disease. The observations discussed herein also demonstrate that, in addition to its classical intestinal presentation, giardiasis may cause ocular complications, arthritis, skin allergies or myopathy. Moreover, giardiasis is now a well established cause of failure to thrive, stunting and growth retardation in human and animals, diminished cognitive functions, and chronic fatigue. Finally, *Giardia* may lead to post-infectious functional gastrointestinal disorders such as irritable bowel syndrome and functional dyspepsia. A few cases of *Giardia* trophozoites associated with tumoral masses have also been reported, but cause-to-effect relationships between giardiasis and cancer have yet to be established (Table 3).

Long-term complications of giardiasis may present 2 to 3 years following the infection. In some cases, they may last for a few weeks, and may be eliminated with anti-parasitic treatment, observations that have been reported for example in cases of myopathy and skin allergies. In other cases, long-term consequences may be present for several years, in the form of failure to thrive, stunting, IBS, and chronic fatigue syndrome, in the absence of any parasite.

The mechanisms responsible for post-infectious and extra-intestinal manifestations in giardiasis remain obscure. Both parasitic and host factors have been implicated, indicative of a multifactorial pathogenic process. However, as anti-microbial treatment often leads to recovery, the infection itself represents a key element in these complications. As giardiasis can be asymptomatic, the complex processes leading to extra-intestinal and post-infectious manifestations represent a challenging topic for further research (Table 3).

Given the high prevalence of giardiasis in young children in developing countries, its significant effects on stunting and wasting, and the lasting effects of early childhood diarrhea and malnutrition, giardiasis is of considerable public-health importance. Even though the consequences of giardiasis are variable, school health

programs and health education for children and parents aimed at reducing the prevalence of intestinal parasitic infection in children may have beneficial effects on child growth and development. Improved diagnostic methods, particularly in asymptomatic patients, as well as more-effective treatment and control strategies, are sorely needed to help reduce the detrimental impact of the infection on human societies as well as in the agriculture industry.

In the recent few years, and particularly since the 2004 giardiasis outbreak in Bergen, Norway, *G. duodenalis* infections have been linked to post-infectious IBS and functional dyspepsia *via* mechanisms that are unclear. Findings from several studies indicate roles of specific pro-inflammatory cytokines, and hyperlplasia of enterochromaffin cells, mast cells, and lymphocytes, perhaps causing motility dysfunction and visceral hypersensitivity; but much controversy remains on the topic[117]. Together, the data strongly suggest that the appearance of post-infectious complications in giardiasis are multifactorial. A number of the post-infectious complications seen after giardiasis are shared with those caused by common bacterial enteropathogens like *C. jejuni*, *E. coli*, or *Salmonella* sp. One area of growing interest in this field is the role played by disruptions of the host microbiota during the acute stage of the infection in the initiation of delayed immune-mediated pathophysiology. Therefore, a better understanding of the mechanisms responsible for the extra-intestinal and post-infectious manifestations of giardiasis will help unravel common pathways leading to these phenomena.

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REVIEW

Non-microbial approach for *Helicobacter pylori* as faster track to prevent gastric cancer than simple eradication

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Abstract

Although the International Agency for Research on Cancer declared *Helicobacter pylori* (*H. pylori*) as a definite human carcinogen in 1994, the Japanese Society for Helicobacter Research only recently (February 2013) adopted the position that *H. pylori* infection should be considered as an indication for either amelioration of chronic gastritis or for decreasing gastric cancer mortality. Japanese researchers have found that *H. pylori* eradication halts progressive mucosal damage and that successful eradication in patients with non-atrophic gastritis most likely prevents subsequent development of gastric cancer. However, those who have already developed atrophic gastritis/gastric atrophy retain potential risk factors for gastric cancer. Because chronic perpetuated progression of *H. pylori*-associated gastric inflammation is associated with increased morbidity culminating in gastric carcinogenesis, a non-microbial approach to treatment that provides long-term control of gastric inflammation through nutrients and other interventions may be an effective way to decrease this morbidity. This non-microbial approach might represent a new form of prerequisite "rescue" therapy that provides a quicker path to the prevention of gastric cancer as compared to simple eradication.

Core tip: Gastric cancer is a multi-factorial and multi-step disease associated with various risk factors including environmental and pathogenic microbial chronic inflammation. Pharmaceutical intervention and the eradication strategy can provide rapid relief of acute inflammation but fails to correct the underlying cause of chronic inflammation. A non-microbial approach for modulating *Helicobacter pylori* associated gastric inflammation may be an attractive and rapid alternative to optimize cancer prevention strategies and minimize adverse side effects associated with therapeutic regimens.

INTRODUCTION

Helicobacter pylori (*H. pylori*), a Gram-negative bacterial pathogen that infects approximately 50% of the world's population, provokes chronic gastric inflammation which is considered a major risk factor for the development of gastric and duodenal ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma[1]. In 1994, *H. pylori* was classified as a type I (definite) carcinogen

by the International Agency for Research on Cancer[2]. Although the relationship between *H. pylori* and gastric cancer has been acknowledged by diverse forms of clinical evidence, it is still debatable as to whether eradication can lead to the prevention of gastric cancer[3-5]. Traditionally, treatment for *H. pylori* has focused primarily on eradicating the bacteria from the stomach using a combination of antibiotics, such as amoxicillin and clarithromycin, with a proton-pump inhibitor[6-10]. The eradication rate, however, has been declining due to the increasing prevalence of antibiotic resistance, especially clarithromycin resistance[11-15]. This increase in the prevalence of antibiotic resistance has diminished enthusiasm for the use of many popular *H. pylori* eradication therapies. To overcome this decline in the use of first-line treatment options, bismuth-containing quadruple and sequential therapies are emerging as second-line treatments for *H. pylori* infection[16-21]. Although newer treatments for eradicating *H. pylori* continue to be introduced, research on even more effective eradication regimens continues to be conducted. Unfortunately, literature from all over the world continues to document increases in *H. pylori* resistance to antibiotics and this major obstacle has prompted the introduction of new drugs and treatment schemes. It is also important to note that although removal or amelioration of gastric inflammation has been implicated in the prevention of gastric carcinogenesis, the persistent gastric inflammation observed in *H. pylori*-associated gastric carcinogenesis is not always amelioration by *H. pylori* eradication alone.

Because gastric cancer is a multi-step and multi-factorial disease, not all individuals infected with *H. pylori* will develop gastric cancer. In fact, the multi-factorial processes associated with the development of gastric cancer can give hope to some susceptible individuals that it may be prevented through the eradication of *H. pylori*. Conversely, in cases where chronic inflammation is caused by other environmental factors such as diet, eradication of *H. pylori* may only delay the development of gastric cancer rather prevent it. Importantly, there is no overt biomarker supporting the rationale of *H. pylori* eradication in clinic, although endoscopic findings might be recommended (Figure 1). Moreover, the nationwide cost associated with eradicating *H. pylori* in order to prevent gastric cancer would be prohibitive and represent a burden to socioeconomically challenged people in developing countries. Therefore, the strategy of cancer prevention through chemopreventive agents may be the most efficacious way to reduce the global burden of cancer.

Cancer chemoprevention was established by Dr. M. Sporn in 1976 and was defined as "the use of natural, synthetic, or nontoxic chemical substances to reverse, suppress, delay or prevent carcinogenic progression" by Dr. M. Sporn and Dr. W. K. Hong[22,23]. The results of several preclinical and clinical studies have indicated that diverse chemoprevention strategies can decrease gastrointestinal (GI) cancer incidence and mortality rates[24]. Essentially, the chemoprevention strategy involves inter-

ventions during all three stages of carcinogenesis, (initiation, promotion, and progression) using chemopreventive agents in order to interfere with tumor promotion or progression and reduce the risk of various cancers. All GI cancers have a unique etiology but share common mechanisms including oxidative stress-induced damage of genomic DNA, modification of cellular proteins and lipids, altered cell signaling, and persistent local tissue inflammation. Therefore, the combination of *H. pylori* eradication, anti-oxidant interventions, interventions to normalize aberrant cell signaling, and anti-inflammatory interventions may be an essential and anticipatory strategy for prevention of gastric cancer. Recently, numerous studies have investigated the potential therapeutic benefits of probiotics, phytochemicals, and antioxidant or vitamin supplementation as chemopreventive agents as well as adjuncts to increase the eradication rates of *H. pylori* infection. In this article, we discuss what is known currently about non-microbial preventive strategies for chronic infection with *H. pylori* which may represent a faster option for cancer prevention *via* enhancement of host adoptive responses as well as removal of inflammation responsible for mutagenesis (Figure 2).

ANTICIPATING NON-MICROBIAL APPROACHES FOR PREVENTING *H. PYLORI*-ASSOCIATED GASTRIC CARCINOGENESIS

Cyclooxygenase and 5-lipoxygenase inhibition

H. pylori-induced inflammatory responses have been associated with high concentrations of phospholipase A2 (PLA2) which is an essential enzyme for the release of arachidonic acid (AA). AA metabolites, prostaglandins (PGs) or hydroxyl fatty acids (HETEs), are key mediators in inflammatory responses and are metabolized by cyclooxygenase (COX) and lipoxygenase (LOX)[25]. COX-1 and COX-2 are responsible for the production of inflammatory PGs and 5-LOX increases the release of gastrotoxic leukotrienes (LTs)[26]. Conversely, findings demonstrating that inflammatory responses were decreased by inhibiting COX and 5-LOX led research on COX and 5-LOX inhibitors as attractive medications for anti-inflammatory effects[27-29]. *H. pylori* infection induces higher levels of COX-2 expression, overexpression of which has been detected in various cancers including gastric cancer[30-36]. In this regard, nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for preventing cancers as well as reducing pain and inflammation by inhibiting both COX-1 and COX-2 or COX-2 only[37-39]. Long-term use of NSAIDs attenuated gastric mucosal chronic inflammation induced by *H. pylori* infection suggesting that NSAIDs may be preventive agents of the gastric carcinogenesis associated with *H. pylori* infection[40]. However, the adverse effects associated with the use of NSAIDs may present an obstacle to their use as chemopreventive agents. Traditional NSAIDs non-selectively inhibit both
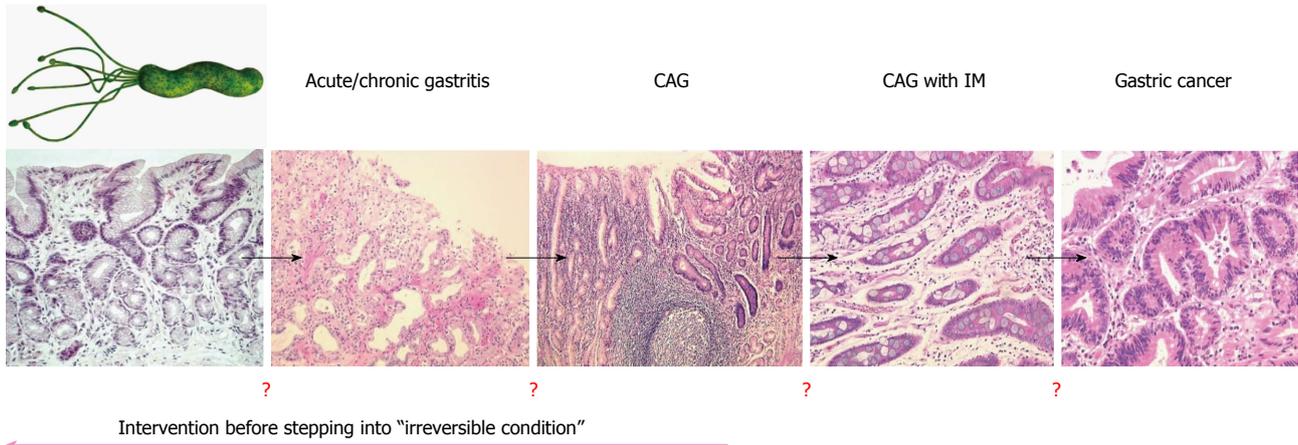
Figure 1 **Point of no return in *Helicobacter pylori* infection.** *Helicobacter pylori* (*H. pylori*) infection is responsible for acute and chronic gastritis, chronic atrophic gastritis, and intestinal metaplasia. The results of a few studies have shown that the eradication of *H. pylori* significantly reverted these gastric pathologies and promoted restoration of gastric function. *H. pylori* is also implicated in several extragastric manifestations including idiopathic thrombocytopenic purpura, iron deficiency anemia, atherosclerosis, and chronic urticaria. Because there are no biomarkers suggestive of a point of no return, the results of several large scale cohort studies continue to provide support for the strategy of *H. pylori* eradication in gastric cancer prevention.
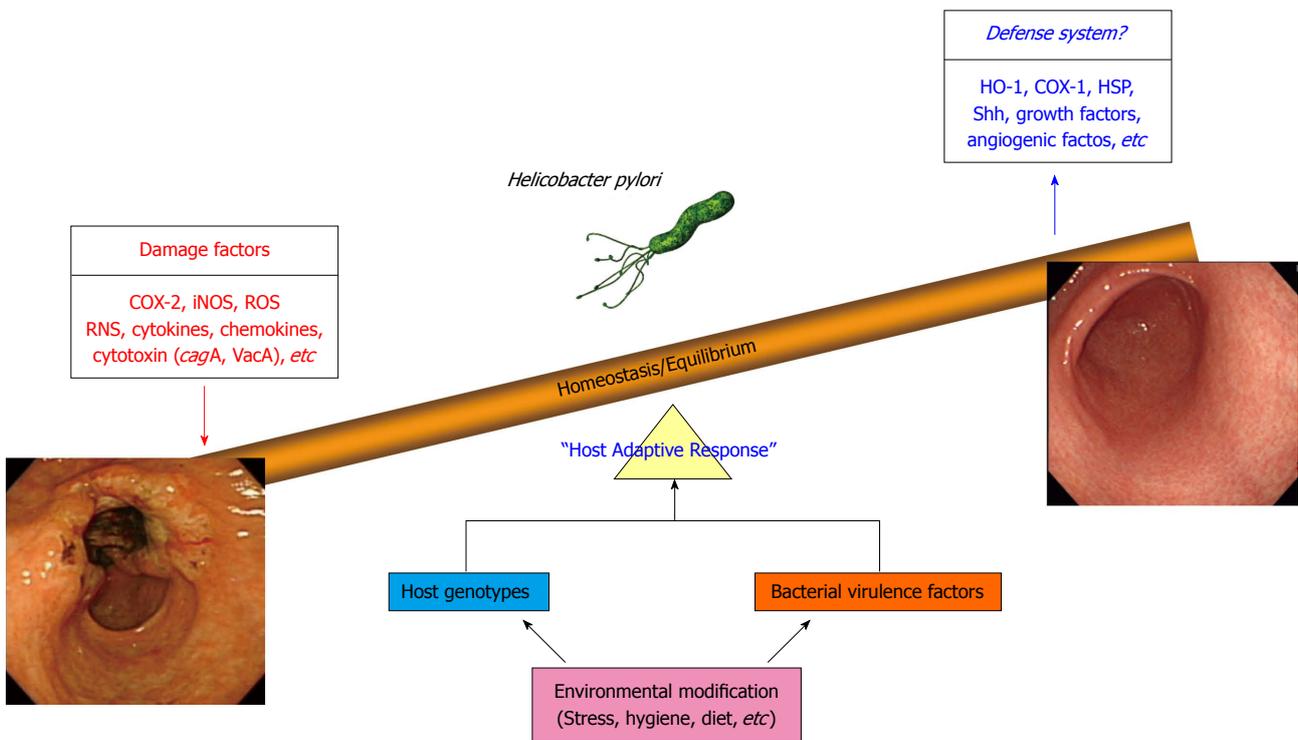
Figure 2 **Host adoptive response through a non-microbial approach as the core defensive mechanism against *Helicobacter pylori* infection, especially gastric cancer prevention.** Even though host genotype, environmental risk, and bacterial virulence factor are all implicated in *Helicobacter pylori* (*H. pylori*) infection, a non-microbial approach may provide the fastest means of cancer prevention as well as amelioration of *H. pylori*-associated gastric pathologies.

COX-1 and COX-2 and may cause GI toxicity, as COX-1 is a house keeping enzyme involved in the cytoprotection of gastric mucosa. Though selective COX-2 inhibitors (*coxib*s), such as celecoxib, rofecoxib, and valdecoxib, have been developed to improve the GI safety[41], *coxib*s also carry the risk of thromboembolic or cardiovascular complications[42-44]. 5-LOX inhibitors also suppressed *H. pylori*-induced proinflammatory mediators such as interleukin-8 and tumor necrosis factor-α in *H. pylori*-infected gastric

epithelial cells, indicating that 5-LOX inhibitors can be preventive agents against *H. pylori*-associated gastric inflammation and carcinogenesis by inhibiting the 5-LOX signaling pathway and suppressing its expression[25].

Phytochemicals and phytonutrients

The results of several recent studies have shown that dietary phytochemicals can modulate key molecular signaling cascades by interacting with small molecules in

cancer cells[45] and that phytochemicals present in foods can inhibit *H. pylori*-induced inflammation. Therefore, the combination of *H. pylori* eradication and the suppression of *H. pylori*-induced inflammation may represent a promising strategy for gastric cancer prevention. For instance, curcumin (diferuloylmethane), the yellow pigment of turmeric (*Curcuma longa* L.) possesses strong anti-inflammatory activities and has shown diverse suppressive actions against various cancers including gastric cancer. It has been reported that curcumin inhibits *H. pylori*-induced nuclear factor (NF)-κB activation, pro-inflammatory cytokines such as interleukin 8, matrix metalloproteinase-3 and -9, and the *H. pylori*-induced motogenic response[46,47]. In addition to these anti-inflammatory and anti-mutagenic actions, curcumin has showed anti-microbial effects in *H. pylori*-infected C57BL/6 mice as well as restorative actions following *H. pylori*-induced gastric damage[48]. Furthermore, curcumin inhibited the proliferation and invasion of gastric cancer cells by suppressing PAK1 activity and cyclin D1 expression[49]. Collectively, the results of these studies suggest that curcumin has potential as an antimicrobial compound and chemopreventive agent against *H. pylori* infection. The results of one recent study suggested that curcumin may prevent cancer therapy-induced oral mucositis due to its antibacterial and anti-inflammatory kinetics[50]. Further research has shown that foods such as broccoli sprouts and oils posses anti-*H. pylori*-associated inflammatory effects mediated by reducing the release of pro-inflammatory cytokines and suppressing the NF-κB pathway[51,52]. Additionally, daily intake of sulforaphane-rich broccoli sprouts was associated with anti-*H. pylori* activity and protection of the gastric mucosa against *H. pylori*-induced oxidative stress[53]. Broccoli sprouts contain high levels of glucoraphanin, a glucosinolate precursor of the isothiocynate sulforaphan known to suppress interleukin (IL)-8 *via* the NF-κB pathway[51,54]. Because *H. pylori*-induced inflammation has been associated with the expression IL-8, a potent neutrophil-attracting chemokine, *via* activation of the NF-κB pathway[55,56], reduction or disruption of this cascade or levels of this cytokine may be an appropriate strategy to intervene in *H. pylori*-induced inflammation.

Omega-3 polyunsaturated fatty acids

There is growing evidence that the diverse biological roles of n-3 polyunsaturated fatty acids (PUFAs) may contribute to their protective actions against chronic inflammatory disease[57]. In bacteria, n-3 PUFAs cause cell lysis, while in other cell types, n-3 PUFAs can be incorporated into membrane phospholipids that can cause a loss of membrane fluidity and may be associated with lipid raft assembly and function[58]. These lipid rafts are cholesterol-rich microdomains at the host cell surface and are required for NF-κB-dependent responses to *H. pylori*[59]. Recently, the results of several studies have suggested that n-3 PUFAs can be converted into bioactive mediators, including resolvins, that have inflammation-resolving properties *via* counter-regulation of lipid mediators such

as pro-inflammatory LTs and PGs[52,57]. Correia *et al*[60] conducted experiments that showed that docosahexaenoic acid (DHA) significantly inhibited *H. pylori* growth both *in vitro* and *in vivo* in a dose-dependent manner and decreased mouse gastric mucosa inflammation. These results suggested that DHA could be used as an adjunct agent in *H. pylori* eradication treatment. In contrast, Meier *et al*[61] showed that an n-3 PUFA-containing eradication regimen failed to show any benefit when compared to a conventional eradication regimen. Thus, our group investigated the long-term treatment of n-3 PUFAs in an *H. pylori*-infected animal model and found that long-term administration of n-3 PUFAs ameliorated *H. pylori*-induced gastric inflammation, atrophied gastritis, and attenuated the incidence of *H. pylori*-associated gastric carcinogenesis. Kuriki *et al*[62] conducted a clinical investigation of the association between gastric cancer risk and the erythrocyte composition of DHA using 179 incident gastric cancer cases and 357 non-cancer controls (matched by age, sex, and season of sample collection). The study authors found that the erythrocyte composition of DHA was negatively associated with the risk of gastric cancer, especially of well-differentiated adenocarcinoma. Detailed, randomized, controlled trials should be conducted to obtain strong evidence for the incorporation of nutraceuticals, including n-3 PUFAs, into the therapeutic armamentarium in near future, as their use as therapeutic agents for GI disorders is moving rapidly into clinical settings and scientific studies are providing mechanisms of action to explain the therapeutic effects.

Probiotics and microbiota

Probiotics such as non-pathogenic microbial feed or food supplements are already being widely studied in the treatment of GI diseases including irritable bowel syndrome, inflammatory bowel disease, sever acute pancreatitis, and chronic liver diseases[63-66]. The use of probiotics in the treatment of GI infections is gaining traction as an alternative or complement to antibiotics due to their potential to decrease the use of antibiotics or reduce their side effects[67]. Results of clinical trials combining the use of agents for first-line eradication and adjunctive probiotics have been reported to increase the *H. pylori* eradication rate[68-70]. Moreover, emerging evidence shows that probiotics attenuate *H. pylori* infection rates and associated inflammation. The results of several *in vitro* studies have shown that *Lactobacillus* can ameliorate *H. pylori*-induced inflammation by modulating cytokine induction, activating suppressor of cytokine signaling (SOCS) expression, and inactivating the JAK2, Smad7 and NF-κB signaling pathways[71-73]. Twelve human studies have investigated the efficacy of combinations of antibiotics and probiotics, whereas 16 studies used probiotics alone as an alternative to antibiotics for the treatment of *H. pylori* infection. Most of the studies showed an improvement of *H. pylori* gastritis and decreases in *H. pylori* colonization after probiotic administration. None of the studies, however, could demonstrate complete eradication of *H. pylori* in-

fection by probiotic treatment[67,74]. It should be noted, however, that one of the well-documented advantages of probiotic combinations was a reduction in adverse effects induced by *H. pylori* eradication treatment[75]. Since long-term intake of products containing probiotic strains may have a favorable effect on *H. pylori* infection in humans, particularly by reducing the risk of developing disorders associated with high degrees of gastric inflammation, it is possible that they contributed ultimately to chemoprevention. Recent advances in high throughput analysis technology have highlighted the importance of probiotics in *H. pylori* infection as well as other GI diseases involving "*microbiota*" as key controllers of *H. pylori* infection. The human organism is colonized by a large number of microorganisms that play important roles in several biochemical reactions. The microorganisms that colonize the human GI tract are collectively described as *microbiota* and a typical human may carry over 40×10^3 bacterial species in the intestinal microbiome[76]. The microbiota of the human stomach and its influence on *H. pylori* colonization has been characterized. Most phylotypes belong to the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Fusobacteria*. *Lactobacillus* species are acid-resistant and commensal and their concentrations in the normal human stomach vary between 0 and 10^3 mL^{-1}. The human microbiome co-evolved with mankind, is part of human physiology, and contributes to homeostasis. Although microbiota–host interactions through metabolic exchange and co-metabolism of substrates, or metabolome–metabolome interactions are still poorly understood, they may be implicated in the etiology of many human diseases including *H. pylori* infection. Therefore, the advantages attributed to probiotics in *H. pylori* infection, such as augmentation of the eradication rate, attenuation of side effects associated with eradication drugs, and some direct anti-inflammatory action, may represent only a small part of their involvement. Extensive investigation of the *microbiota* relevant to *H. pylori* infection will be required to elucidate additional mechanisms and relationships.

Mesenchymal stem cells

Although gastric epithelial stem cells have been localized, little is known about their molecular biology. Recent reports described the use of inducible Cre recombinase activity to indelibly label candidate stem cells and their progeny in the distal stomach[77]. *H. pylori*-induced chronic inflammation affects differentiation and promotes metaplasias, in which cellular and molecular mechanisms in spasmolytic polypeptide-expressing TFF2 pseudo-pyloric metaplasia predominates. The identification of signaling pathways and events that take place during embryonic development that eventually establish adult stem cells to maintain the specific features and functions of the stomach mucosa have elucidated how gastric epithelial stem cells contribute to either good regeneration, such as healing or rejuvenation, or bad regeneration, such as carcinogenesis. For example, because bone marrow-derived mesenchymal stem cells [BM-Mesenchymal stem

cells (MSCs)] are known to play an important role in *H. pylori*-induced gastric carcinogenesis, Lin *et al*[78] transplanted BM-MSCs into the stomach of mice with a 44 wk mouse-adapted *H. pylori* infection. Study results revealed that transplantation of BM-MSCs into a chronic *H. pylori*-infected mouse led to an immunosuppressive environment such that stem cells fostered an environment compatible with the development of *H. pylori*-induced gastric cancer. Similarly, recent investigations into gastric stem cell or progenitor cell biology have uncovered valuable information for understanding gastric gland renewal and maintenance of homeostasis relevant to *H. pylori* infection. Ding and Zheng[79] provided clues for further defining the mechanisms by which gastric cancer may originate and progress. Using Lgr5, villin-promoter, TFF2-mRNA, and Mist, all of which are factors identified as gastric stem/progenitor cell markers, they explored how *H. pylori* or chronic inflammation affected gastric stem cells or their progenitors which give rise to mucus-, acid-, pepsinogen-, and hormone-secreting cell lineages. From their study results, they concluded that *H. pylori* infection induced oncogenic transformation and propagation into tumors based on the tumor microenvironment. In his recent publication, Peek stated that chronic *H. pylori* infection led to DNA damaged stem cells, a condition which could have severe negative consequences[80]. In detail, *H. pylori*-infected rodents that developed dysplasia harbored a subset of gastric epithelial cells in which levels of spermidine oxidase (SMO) production and DNA damage were high, but which were resistant to apoptosis, thereby representing a cellular population poised for neoplastic transformation targeted for gastric stem cells. In contrast to the results of these harmful interventions using gastric stem cells in *H. pylori*-associated gastric carcinogenesis, we found that exogenous stem cells could provide options for cancer prevention and intervention, as MSCs were able to rejuvenate atrophic gastritis into non-atropic condition and significantly ameliorate *H. pylori*-induced gastritis. Because gastric stem cells can have positive or negative effects dependent upon how they are used, further experimentation will be necessary to advance our understanding of stem cell properties in *H. pylori* infection, as well as the potential for rejuvenation of *H. pylori*-infection-associated chronic atrophic gastritis with or without intestinal metaplasia.

Antioxidants

H. pylori leads to chronic inflammation which in turn leads to oxidative stress derived from immune cells and gastric epithelial cells and is one of the main contributors to DNA damage associated with apoptosis and neoplastic transformation[81]. Both pathogen and host factors contribute directly to oxidative stress, including *H. pylori* virulence factors, and pathways involving DNA damage and repair, polyamine synthesis and metabolism, and oxidative stress responses. As previously mentioned, polyamine oxidation by SMO causes H_2O_2 release, DNA damage and apoptosis, and subsequent gastric transfor-
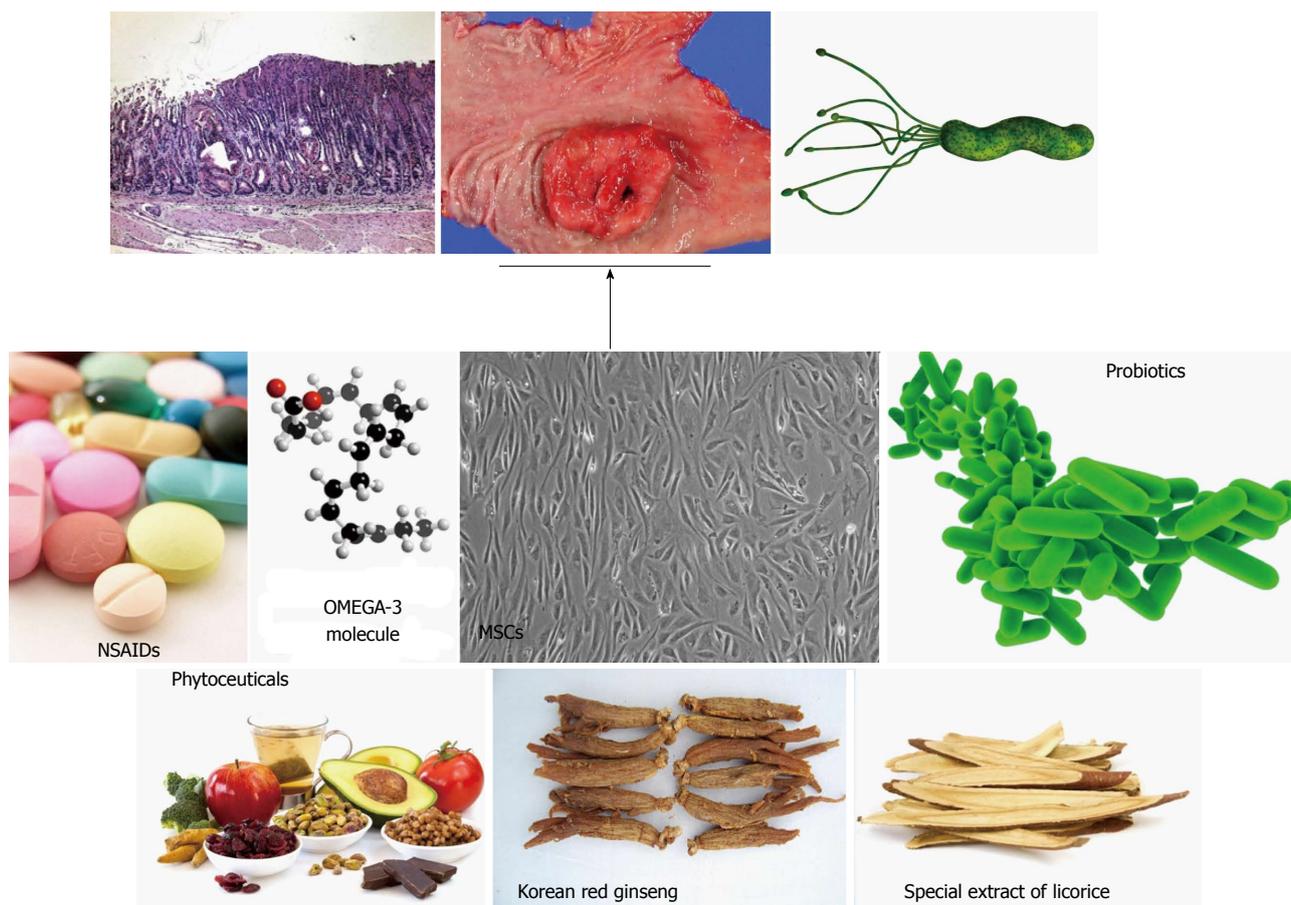
Figure 3 A non-microbial approach for *Helicobacter pylori*-associated gastritis as well as gastric cancer. Simply removing *Helicobacter pylori* (*H. pylori*) can contribute to gastric cancer prevention in some patients. For example, *H. pylori* eradication suppressed the metachronous occurrence of gastric cancer in patients who underwent endoscopic submucosal dissection, whereas insignificant outcomes were noted in general eradication. Supplementation or treatment with long-term phytoceuticals or other agents were proven to be very efficacious in the prevention of *H. pylori*-associated gastric carcinogenesis. These treatment strategies are supported by the clear mechanisms of anti-inflammation, anti-oxidation, and anti-mutagenesis associated with their use.

mation[82,83]. Since many studies reporting the potential contribution of oxidative stress and chronic inflammation to *H. pylori*-associated gastric carcinogenesis, antioxidants can provide enough hope for cancer prevention. *H. pylori*-associated inflammation can induce DNA damage due to oxygen radicals by persistent inflammatory cell infiltrations in the gastric mucosa, which may lead to alterations of the gene and result in the development of diffuse-type carcinoma. In order to elucidate the influence of *H. pylori* on changes in inflammation-related DNA damage, Hahm *et al*[84] measured the sequential changes of the 8-hydroxydeoxyguanosine (8-OHdG) content of DNA and changes of two biomarkers, inducible nitric oxide synthase (iNOS) and apoptosis, from human gastric mucosa according to the status of *H. pylori*. The increased levels of oxidative DNA damage, increased occurrences of apoptosis, and increased expressions of iNOS seemed to provide the mechanistic links between *H. pylori* infection and gastric carcinogenesis. In a subsequent study, we treated *H. pylori*-associated chronic atrophic gastritis with an antioxidative drug, rebamipide, and found that it contributed to either augmentation of the eradication rate or a significant decrement of 8-OHdG content[85]. Diseases

associated with free radical overproduction are provoked by "blazed reactive oxygen species productions" far beyond the host's capacity to quench. Free radicals have been implicated in the pathogenesis of diverse GI diseases including gastroesophageal reflux disease, gastritis, enteritis, colitis, and associated cancers, as well as pancreatitis and liver cirrhosis[86]. Antioxidants administered in a nutritional way or *via* pills will surely contribute to the amelioration of *H. pylori*-associated gastric catcinogenesis. However, additional proof of concept evidence is required.

CONCLUSION

Gastric cancer is a multi-factorial and multi-step disease associated with a variety of risk factors including environmental and pathogenic microbial chronic inflammation. In addition to life-style factors, especially diet, infection with the pathogenic microorganism *H. pylori* is a major concern for gastroenterologists because *H. pylori* infection causes chronic atrophic gastritis and peptic ulcer with an inflammatory response. Unfortunately, modern medicine cannot completely prevent gastric cancer

and even eradication of *H. pylori* is problematic due to expense and antibiotic resistance, as well as insufficient evidence supporting a rationale for eradication. However, the Japanese government decided to take on the great challenge of *H. pylori*-associated chronic gastritis by including its eradication in their guideline this year in an attempt to decrease gastric cancer incidence and mortality. Until such time as proof emerges supporting the concept that *H. pylori* eradication is the fastest means of preventing gastric cancer, the attenuation or intervention of *H. pylori*-induced chronic inflammation may be alternative or complementary methods to achieve the prevention of gastric cancer. As shown in this review, the inhibitors of COX and LOX, a number of natural phytochemicals, including curcumin and broccoli sprouts (sulforaphane), oils such as omgega-3 PUFAs, probiotics, and stem cells have been shown to have anti-inflammatory and antimicrobial activities by targeting small molecules or regulating signaling cascades (Figure 3). Pharmacotherapy and the eradication strategy can provide rapid relief of acute inflammation but cannot correct the underlying cause of chronic inflammation. However, a non-microbial approach for modulating *H. pylori*-associated gastric inflammation may be an attractive and fast way to optimize cancer preventive strategies and minimize adverse side effects associated with therapeutic regimens.

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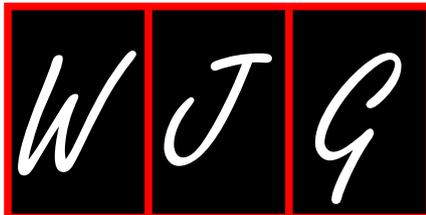
Digestive cancer surgery in the era of sentinel node and epithelial-mesenchymal transition

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Abstract

Lymph node involvement is one of the most important prognostic indicators of carcinoma of the digestive tract. Although the therapeutic impact of lymphadenectomy has not been proven and the number of retrieved nodes cannot be considered a measure of successful cancer surgery, an adequate lymph node count should be guaranteed to accurately assess the N-stage through the number of involved nodes, lymph node ratio, number of negative nodes, ratio of negative to positive nodes, and log odds, *i.e.*, the log of the ratio between the number of positive lymph nodes and the number of negative lymph nodes in digestive carcinomas. As lymphadenectomy is not without complications, sentinel node mapping has been used as the rational procedure to select patients with early digestive carcinoma in whom nodal dissection may be omitted or a more limited nodal dissection may be preferred. However, due to anatomical and technical issues, sentinel node mapping and nodal basin dissection are not yet the standard of care in early digestive cancer. Moreover, in light of the biological, prognostic and therapeutic impact of tumor budding and tumor deposits, two epithelial-mesenchymal transition-related phenomena that are involved in tumor progression, the role of staging and surgical procedures in digestive carcinomas could be redefined.

Core tip: We summarize the current knowledge on the assessment of nodal status and nodal staging in digestive carcinomas and highlight the prognostic impact of two epithelial-mesenchymal transition-related phenomena, tumor budding and tumor deposits, that are involved in tumor progression. In light of the biological, prognostic and therapeutic impact of these phenomena, the role of staging and surgical procedures in digestive carcinoma could be reevaluated and redefined.

INTRODUCTION

Lymph node involvement is one of the most important prognostic indicators of carcinoma of the digestive tract. In contrast to Eastern countries, in Western countries lymph node involvement is not considered to be a prognostic "governor" and the therapeutic impact of lymphadenectomy is not acknowledged. Recent advances in minimally invasive treatment procedures for cancer have promoted their application for the assessment of lymph node status (positive/negative), *i.e.*, sentinel node mapping and biopsy. In addition, other prognostic factors related to epithelial-mesenchymal transition (EMT) that are involved in tumor progression, such as tumor budding and tumor deposits, have been gaining ground.

Here, we review the current knowledge on these issues and highlight the need for a redefinition of the role of surgical and staging procedures in digestive cancer surgery in light of recent advances in our understanding of the biology of tumor progression.

NUMBER OF EXAMINED NODES, LYMPH NODE RATIO, LOG ODDS

Several studies have shown an association between the number of excised nodes and overall survival, providing evidence that examination of an insufficient number of lymph nodes (LNs) may have a detrimental effect on survival in patients with gastrointestinal carcinoma[1,2]. However, much of this appears to be the effect of stage migration, which impacts the stage-specific survival without affecting overall survival[3]. Variations in patient demographics, tumor location and tumor biology raise questions regarding the evidence for a minimum LN harvest[1,4]. In gastric cancer, the stage migration effect is most striking when fewer than 10 LNs are assessed, but it is still present with a greater number of examined LNs[5,6]. Therefore, although current guidelines support the assessment of a minimum of 16 LNs, examination of more LNs is necessary to reduce the stage migration effect[1]. In colorectal cancer, the aim should be to collect as many LNs as possible to improve staging and increase survival. In fact, particularly following neo-adjuvant treatment for rectal cancer, downstaging with fewer LNs implies a positive treatment response and a more favorable prognosis[4].

An association between better postoperative long-term survival and a greater number of dissected nodes has also been reported in patients with several N0 digestive malignancies, including esophageal[7], gastric[8], colorectal[9], and pancreatic carcinomas[10,11]. This may be due to a not negligible rate of nodal micrometastasis, and the probability of missing a positive LN decreases as the number of examined LNs increases, i.e., the Will Rogers phenomenon[7,9,12]. In patients with node-negative gastric cancer, a prophylactic D2 lymphadenectomy[8,13] with almost 16 LNs examined[12] seems to be effective, although retrieval of more than 25 nodes has been suggested[14]. The removal of at least 18 LNs during an esophagectomy with curative intent results in improved survival in esophageal cancer, particularly in patients with adenocarcinoma[7]. In N0 pancreatic carcinoma, examination of more than 10 LNs has been associated with improved survival[10]. In stage II (T3-4N0) colorectal cancer, current guidelines consider a number of harvested LNs of less than 12 an indication to perform adjuvant chemotherapy; harvesting of less or more than 12 LNs allows a better prognostic stratification of stage IIa (T3N0) patients for postoperative treatment[9,15]. On the basis of statistical considerations, the current recommended goal of 12-15 recovered lymph nodes without evidence of metastatic disease provides approximately 80% negative predictive value for colorectal carcinoma metastasis[16].

However, the clinical significance of micrometastasis [pN1(mi), i.e., tumor cell clusters of > 0.2 mm but ⩽ 2 mm] and isolated tumor cells [pN0(i), i.e., single tumor cells or small clusters of cells of ⩽ 0.2 mm at their greatest extent that can be detected by routine hematoxylin and eosin (HE) stains or immunohistochemistry (IHC) or clusters of ⩽ 200 cells in a single histological cross-section][17] in gastrointestinal carcinoma remains unclear[18]. In early and advanced pN0 gastric cancer, the occurrence of nodal micrometastasis was shown to have no impact on prognosis[19]; however, other studies showed that LN micrometastasis was one of the most important prognostic factors in multivariate survival analysis of pT1N0[20], and the prognosis was significantly poorer in patients with isolated tumor cells than in those without them[21]. A recent systematic review and meta-analysis reported that molecular detection of tumor cells (isolated tumor cells and/or micrometastasis) in regional lymph nodes is associated with an increased risk of disease recurrence and poor survival in patients with N0 colorectal cancer[22].

In N+ digestive carcinomas, lymph node ratio (LNR) is a better prognostic factor than number of metastatic nodes (pN), and it may minimize the stage migration effect[23-28] because it is assumed to be constant regardless of the number of examined nodes[29]. However, LNR stages can be more accurately differentiated with a large number (> 15) of examined nodes[11,30-32]. Negative node count has been proposed as a prognostic indicator in patients with gastric cancer based on the assumption that nodal metastasis and micrometastasis cannot be prevented without adequate negative node dissection[33,34]. A negative lymph node count has been associated with improved survival in colorectal cancer patients, independent of patient, pathologic and molecular characteristics; however, the beneficial effects of a negative count are stronger in stage I-II patients than in stage III-IV patients[35]. Moreover, a straight ratio between negative and positive lymph nodes (RNPL), which provides direct information on nodal metastasis, micrometastasis, and the immune condition of the patient, could be more accurate than LNR for the prognostic evaluation of curatively resected gastric cancer[36]. At the same time, the log odds of positive lymph nodes (LODDS), i.e., the log of the ratio between the number of positive LNs and the number of negative LNs, is superior to the pN+ and LNR classifications for prognostic assessment in gastric and colorectal carcinoma[37,38]. In effect, LODDS is a function of the number of negative LNs, whereas LNR is a function of the total number of LNs[39]. Moreover, LNR is not applicable to pN0 patients, whereas LODDS is a useful lymph node classification for pN0 patients because it can discriminate between subgroups with different survival rates[38]. With respect to the pN and LNR classifications, LODDS has shown more power for minimizing the stage migration phenomenon caused by an insufficient number of retrieved nodes[38,40].

The prognostic power of the number of involved nodes in patients with digestive carcinomas is limited.

Furthermore, although the therapeutic impact of lymphadenectomy has not been proven and the number of retrieved nodes cannot be considered a measure of successful cancer surgery, an adequate LN count should be guaranteed to accurately assess the N-stage through the number of involved nodes, LNR, number of negative nodes, ratio of negative to positive nodes, and LODDS in digestive carcinomas[4,41]. In fact, in Western countries, D2 lymphadenectomy is gradually becoming the recommended surgical approach for patients with resectable gastric cancer[11,42,43], and total mesorectal excision (TME) is the recommended procedure for extraperitoneal rectal carcinoma. However, because lymphadenectomy is not without complications and institutional screening programs leading to the detection of cancer at an early stage have increased the prevalence rate of clinical N0 tumors, sentinel node (SLN) mapping has been used as the rational procedure to select patients in whom nodal dissection may be omitted or a more limited nodal dissection may be preferred.

Sentinel node mapping and biopsy

Recent meta-analyses have shown acceptable SLN detection rates and accurate determination of lymph node status in gastric cancer[44,45]. However, SLN mapping and nodal basin dissection are not yet the standard of care in early gastric cancer because of several unsolved anatomical (skip metastasis, multidirectional lymphatic drainage patterns) and technical (dye method, radio-colloid method or combination of the dye method and radio-colloid method) issues that may impact the detection rates and false negative rates. Moreover, there is another problem regarding the pathological diagnosis of SLN metastasis, including micrometastasis. Pathologic examination of SLNs has not been standardized in gastric cancers[46]. Serial sectioning results in a more accurate evaluation of metastases; however it is time-consuming. HE staining and IHC have been used in combination with serial sections of frozen and paraffin-embedded specimens for the detection of micrometastatic disease in SLNs[47]. Occult metastasis in SLN has been detected in 4% of pN0 gastric cancer patients using IHC in the 5-μm-thick serial step sections at 85-μm intervals of whole formalin-fixed paraffin-embedded tissues of all resected SLN[48]. The highly sensitive real-time reverse transcription polymerase chain reaction (RT-PCR) system, which enables rapid analysis to detect the mRNA of CK19, CK20 and carcinoembryonic antigen[49], and the one-step nucleic acid amplification (OSNA) assay[50] are promising tools for intraoperative diagnosis of SLN involvement in gastric cancer. In rectal carcinoma, the "in vivo" procedure of sentinel node mapping and biopsy entails breaking the mesorectal fascia intraoperatively to search for and dissect the SLNs. However, from a surgical point of view, the preservation of the integrity of the mesorectal fascia

during rectal excision is necessary to minimize the risk of both residual tumor and relapses, and this assumption is the basis of the TME technique. The aim of the currently adopted SLN mapping procedure in colorectal carcinoma is not to avoid extended nodal dissection and therefore related morbidities, but rather to improve the sensitivity of the histopathological evaluation through the selective application of serial step sectioning, immunohistochemistry, and/or RT-PCR techniques, and "ex vivo" techniques of sentinel node mapping have been developed for this goal[51]. We observed that this ex vivo sentinel node procedure is an effective method for improving nodal staging in clinically node-negative colorectal carcinoma by immunohistochemical detection of micrometastasis in SLNs. However, it is not useful for the detection of satellites (i.e., the presence of macroscopic or microscopic tumor deposits in pericolorectal adipose tissue), which should be assessed by TNM staging of colorectal cancers[52]. Moreover, the "in vivo" and "ex vivo" procedures are associated with a identification rate of 90% and a sensitivity of less than 70%[53]. Advances in imaging technologies could allow a more accurate preoperative detection of SLNs than the current dye- or radio-guided methods. Moreover, new dye-guided intraoperative technologies might revolutionize the SLN mapping procedure in gastrointestinal cancers. Indocyanine green (ICG) infrared or fluorescence imaging may identify a higher number of SLNs than radio-guided methods because the particle size of dyes is smaller than that of radioactive colloids. In gastric cancer, ICG infrared imaging is a useful tool in laparoscopic detection of SLNs. ICG fluorescence imaging is feasible even by preoperative ICG injection at, for instance, 1 or 3 d before surgery; it is also feasible in laparoscopy-assisted gastrectomy via a small laparotomy[47]. There is only limited experience with the application of ICG fluorescence-guided SLN mapping in colon cancer. The method has been shown as feasible and safe but further analyses in larger series are necessary to determine its definitive role in colon cancer patients[54].

The rationale for performing SLN mapping and biopsy is to determine the N status in tumors in which the N status may impact the prognosis, thus potentially avoiding unnecessary lymphadenectomy. This is possible if the determination of N status is accurate, i.e., when the SLN procedure has acceptable false-negative rates. Actually, in pN0 cases, a greater number of retrieved nodes have a beneficial impact on outcome, and a false-negative rate of SLN determination is common in gastrointestinal carcinomas. Moreover, apart from anatomical, technical, surgical and pathological issues, in light of the latest knowledge about the biology of tumor progression, determination of N status by the sentinel node mapping procedure, leaving out of consideration currently emerging progression-related phenomena, may not be sufficient for prognostic evaluation.

EPITHELIAL-MESENCHYMAL TRANSITION-RELATED PHENOMENA OF TUMOR PROGRESSION: TUMOR BUDDING AND TUMOR DEPOSITS

Two EMT-related phenomena involved in cancer progression have been recently shown to have prognostic impact: tumor budding (TB), which is the presence of de-differentiated, isolated single cells or small cell clusters (up to five cells) scattered in the stroma at the invasive front of the tumor[55]; and the formation of tumor deposits (TDs, satellites), which are macroscopic or microscopic nests or nodules found in the lymph drainage area of a primary carcinoma without evidence of residual lymph nodes in the nodule. TDs may represent discontinuous spread, venous invasion or a totally replaced lymph node[17].

The EMT process allows an epithelial cell to assume a more mesenchymal phenotype with increased migratory capacity, invasiveness, resistance to apoptosis and production of extracellular matrix molecules[56]. Loss of E-cadherin, a transmembrane glycoprotein localized in the adherens junction of epithelial cells, is a key event in EMT, enabling tumor cells to migrate, invade and metastasize[57]. Interestingly, the first step in a tumor bud's life seems to be its detachment from the main tumor body by loss of membranous expression of the adhesion molecule E-cadherin[58]. TB has been observed in gastrointestinal carcinomas including colorectal, esophageal, gastric, ampullary and pancreatic carcinomas[55,59-65]. Although the definition of "high-grade budding" (i.e., 10 buds in a 25 × field) by Ueno et al[55] is the most widely applied, there are no well-defined, evidence-based criteria for quantitative (i.e., optimal cut-off and field diameter) and qualitative assessment of TB[66]. In colorectal carcinoma, TB is an independent predictor of tumor progression and outcome, especially in stage II (T1-3 N0) tumors, in which high TB may be used as a high-risk criterion to select patients for adjuvant therapy[66,67]. In pancreatic carcinoma, high grade TB has been identified as an independent and highly unfavorable prognostic factor. Moreover, TB is associated with more aggressive phenotypes such as advanced pT classification and lymphatic invasion[65]. In esophageal squamous cell carcinoma, TB is a significant prognostic factor for patients who have undergone surgery alone[61], and high grade TB has been reported to be the most important predictor of poor prognosis in patients who received chemotherapy followed by surgery[62]. Moreover, tumor buds could be used as a potential target for new therapeutic approaches[58,65].

TDs have been detected in various types of carcinomas other than colorectal carcinoma, including gastric, pancreatic, gallbladder and bile duct carcinomas[68]. The latest TNM classification of colorectal carcinoma has categorized TDs as N1c[17]. However, the nature of TDs as well as their histopathological definition and prognostic classification regarding primitive tumor (T), regional nodal (N), or distant metastasis (M) categories are debated[69-71]. Several authors support the inclusion of TDs in the staging of gastric cancer[70-72]. Snail and Twist are transcriptional repressors of E-cadherin and EMT inducers. In colorectal cancer, overexpression of Twist enhances TD formation, and upregulation of Snail expression contributes to lymph node metastasis through two different molecular pathways, both involving EMT, by repression of the membranous expression of E-cadherin: Twist-EMT-TDs and Snail-EMT-LN metastasis[73]. Overexpression of Snail and Twist has been shown in pancreatic carcinoma[74].

Therefore, the occurrence of TB and formation of TDs seem to be the result of different steps in tumor progression promoted by EMT. Although the precise involvement of the EMT process in tumor progression is not well understood, the existence of other progression-related phenomena with biological, prognostic and therapeutic impact between the T, N and M is undeniable. In digestive cancers, the role of staging and surgical procedures could be re-evaluated and redefined from the perspective of the biological, prognostic and therapeutic impact of these tumor progression-related phenomena.

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Pancreatic trauma: A concise review

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Abstract

Traumatic injury to the pancreas is rare and difficult to diagnose. In contrast, traumatic injuries to the liver, spleen and kidney are common and are usually identified with ease by imaging modalities. Pancreatic injuries are usually subtle to identify by different diagnostic imaging modalities, and these injuries are often overlooked in cases with extensive multiorgan trauma. The most evident findings of pancreatic injury are post-traumatic pancreatitis with blood, edema, and soft tissue infiltration of the anterior pararenal space. The alterations of post-traumatic pancreatitis may not be visualized within several hours following trauma as they are time dependent. Delayed diagnoses of traumatic pancreatic injuries are associated with high morbidity and mortality. Imaging plays an important role in diagnosis of pancreatic injuries because early recognition of the disruption of the main pancreatic duct is important. We reviewed our experience with the use of various imaging modalities for diagnosis of blunt pancreatic trauma.

Core tip: The pancreas is a relatively uncommon organ to be injured in abdominal trauma and difficult to diagnose. Pancreatic injuries are usually subtle to identify by different diagnostic imaging modalities and these injuries are often overlooked in cases with extensive multiorgan trauma. They are associated with considerably high morbidity and mortality in cases of delayed diagnosis, incorrect classification of the injury, or delays in treatment. This review provides an overall concise update on pancreatic trauma and highlights the findings of pancreatic trauma on various imaging modalities.

INTRODUCTION

The pancreas is a relatively uncommon organ to be injured in trauma, occurring in less than 2% of blunt trauma cases, and this injury is associated with considerably high morbidity and mortality in cases of delayed diagnosis, incorrect classification of the injury, or delays in treat-

ment[1,2]. Mortality for pancreatic injuries ranges from 9% to 34%; however, only 5% of the pancreatic injuries are directly related to the fatal outcome. Physical examination is usually not reliable in the setting of acute pancreatic trauma[3]. Early and accurate diagnosis can decrease morbidity and mortality, and various imaging modalities play a key role in recognition of pancreatic injuries[4,5].

Knowledge about the mechanisms of pancreatic injury, the presence of coexisting injuries, the time to diagnosis, the presence or absence of major ductal injury, and the roles of various imaging modalities is essential for prompt, early and accurate diagnosis. Early detection of disruption of the main pancreatic duct is of paramount importance because such disruption is the main cause of delayed complications like pseudopancreatic cyst[6]. The most common site of traumatic pancreatic injury is at the junction of the body and tail. Significant pancreatic injury may occur in the absence of abnormality on various imaging modalities.

Pancreatic trauma occurs commonly in connection with multiple injuries after motor vehicle accidents in adults and bicycle handlebar injuries in children[7]. Conservative management is mainly advocated for pancreatic trauma without ductal injuries. Computed tomography (CT) is routinely used as the first-line imaging modality in acute abdominal trauma cases and is helpful in recognizing injuries to the pancreas and other organs and their associated complications[8]. Ultrasonography (US) is useful in cases of pancreatic ascites and pseudocyst formation, which are more likely to occur in cases with traumatic pancreatitis[3,9]. Magnetic resonance cholangiopancreatography (MRCP) allows direct imaging of the pancreatic duct and its disruption[10]. The purpose of this paper is to review the findings of pancreatic trauma on various imaging modalities.

ANATOMIC CONSIDERATIONS

The pancreas is a long J-shaped, soft, lobulated retroperitoneal organ. It is situated transversely across the posterior abdominal wall, at the back of the epigastric and left hypochondriac regions at level of lumbar (L1-2) spine (Figure 1). In adults, the pancreas is about 15-20 cm long, 1.0-1.5 cm thick and weighs approximately 90-100 g[11]. The main pancreatic duct of Wirsung traverses the entire length of the gland. The superior pancreaticoduodenal artery from the gastroduodenal artery and the inferior pancreaticoduodenal artery from the superior mesenteric artery run in the concave contour of the second part of the duodenum to supply the head of the pancreas. The pancreatic branches of the splenic artery supply the neck, body and tail of the pancreas. The body and neck of the pancreas drain into the splenic vein, whereas the head drains into the superior mesenteric and portal veins. The lymphatic drainage of the pancreas is *via* the splenic, celiac and superior mesenteric lymph nodes. The proximity of many larger vessels such as the inferior vena cava (IVC), portal vein and abdominal aorta makes injuries to the pancreas difficult to manage because of the risk of

exsanguinating hemorrhage, which is a frequent cause of death in patients with a pancreatic injury. The splenic artery and splenic vein run superior and posterior to the body and tail of the pancreas and are relatively easier to expose and control compared to the IVC and portal vein. The vascular anatomy causes problems in repairing the injuries to the head of the pancreas whereas injuries to the body and tail are easier to manage[11,12].

PATHOPHYSIOLOGY OF INJURY

Injuries to the pancreas most commonly result from penetrating trauma caused by gunshot or stab wounds and occur in approximately 20%-30% of all patients with penetrating traumas. The penetrating injury caused by firearms results in the highest frequency of pancreatic trauma. The relatively protected retroperitoneal location of the pancreas protects it from most instances of blunt abdominal trauma. Blunt trauma to the pancreas is, in most instances, caused by a sudden localized force to the upper abdomen that compresses the pancreas against the vertebral column (*e.g.*, steering wheel injury in a motor vehicle accident in adults and from bicycle handlebar injury or direct blow from a kick or fall in children)[8]. Blunt pancreatic injury is more common in children and young adults because they have a thinner or absent mantle of protective fat, which surrounds the pancreas in older adults[10]. In order of frequency, injuries to the pancreas involve the body, head and tail. Pancreatic injury is rarely a solitary injury, and in the majority of instances there is at least one coexistent injury; 60% are duodenopancreatic lesions, while 90% involve at least one other abdominal organ[1]. Therefore, multiple organ injuries are a red flag suggesting the possibility of coexistent pancreatic injury.

CLINICAL PRESENTATIONS

Patients with pancreatic trauma present usually with features of acute pancreatitis. The typical clinical triad of pancreatic trauma is upper abdominal pain, leukocytosis, and elevated serum amylase level, that may, however, be absent in adults during the first 24 h and even for several days[12,13]. Pancreatic trauma is difficult to recognize because of coexisting injuries to other intra-abdominal organs and its retroperitoneal location, which makes signs and symptoms less marked, and consequently this trauma ends up causing higher morbidity and mortality rates than observed in injuries to other intra-abdominal organs[14,15]. Symptoms of injury to other intra-abdominal organs or structures commonly mask or supersede that of pancreatic injury, both early and late in the course of trauma. Therefore, a high degree of suspicion is required to ensure that pancreatic injuries are not overlooked or missed either early or late in their course.

LABORATORY FINDINGS

Raised amylase in serum or diagnostic peritoneal lavage (DPL) fluid can be useful in diagnosis, but there is
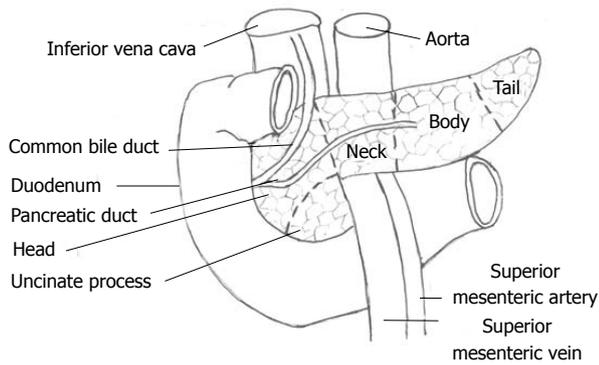
Figure 1 Gross anatomy of the pancreas.

Figure 2 Ultrasound image. Axial ultrasound image shows localized traumatic enlargement of the pancreas with diffuse edema. Transection of distal body of pancreas communicating with large fluid collection anterior to pancreas (white arrow).

poor correlation between raised amylase and pancreatic trauma because amylase may be elevated in injuries of the salivary gland, in duodenal trauma, hepatic trauma, and injuries to the head and face, and in an intoxicated patient[16-18]. A raised amylase level after blunt pancreatic trauma is time dependent, and a persistently elevated or a rising amylase level is a more reliable indicator of pancreatic trauma, but it does not indicate the severity of the injury[14]. Amylase detected in DPL fluid is a much more sensitive and specific indicator of pancreatic injury than blood or serum amylase estimations. Serum lipase activity is also not specific for pancreatic injury[12].

RADIOLOGIC STUDIES

Diagnostic imaging plays an important role in the recognition, evaluation, and follow-up of traumatic pancreatic injuries. The imaging findings in patients with pancreatic trauma are nonspecific and often indistinguishable from those of inflammatory pancreatitis.

Conventional radiography

A plain X-ray of the abdomen in patients with pancreatic trauma is nonspecific and none of the radiologic abnormalities on plain films can be used for specific diagnostic purposes. Conventional radiography can be valuable in detecting penetrating trauma by visualizing and localizing foreign bodies such as bullet fragments and projectile-induced bony injury, as well as pulmonary parenchymal injury, gastric dilatation and pneumoperitoneum.

Findings are often indistinguishable from those of inflammatory pancreatitis. Pancreatic hemorrhage and edema widen the duodenal sweep with distension of the duodenum. Dissection along the transverse mesocolon results in gaseous distension of the colon, which may terminate abruptly usually at the splenic flexure to produce the "colon-cutoff sign". A sentinel loop representing localized ileus may be seen in the mid-abdomen.

US

Although US is easy to perform, portable and cost-effective, pancreatic injuries are difficult to diagnose in spite of technically adequate sonograms[19]. However, it is reliable in the follow-up of complications such as pseudocysts. Real-time contrast-enhanced US is an effective technique in emergency imaging, but its role should not be considered as a replacement for CT[20].

US may show localized traumatic enlargement of the pancreas or diffuse edema simulating inflammatory pancreatitis. In trauma patients, peripancreatic fluids may be a sign of pancreatic contusion[21]. A traumatic pseudocyst of the pancreas may be detected by US and monitored on serial examinations. Since complications of trauma are most likely to occur from rupture or stenosis of the main pancreatic duct, it is important to try to delineate this structure in all cases of pancreatic injury. Transection throughout the pancreas parenchyma is suggestive of ductal injury (Figure 2).

CT

CT is the simplest and least invasive diagnostic modality currently available for evaluating suspected pancreatic trauma and its complications, because of the subtlety of the US findings. However, this study is only rarely useful in acute penetrating injury. Computed tomography is the radiographic examination of choice for hemodynamically stable patients with abdominal trauma as it provides the safest and most comprehensive means of diagnosis of traumatic pancreatic injury[10].

The pancreas may appear normal in 20%-40% of patients when CT is performed within 12 h after trauma because pancreatic injuries may produce little change in the density which may not be detectable on CT scan[1,22]. In addition, there may be minimal separation of lacerated pancreatic fragments (Figure 3A). Currently, multidetector-row CT scanners are used for evaluation of abdominal trauma cases as they are faster to scan, which greatly reduces bowel artifacts and resolves many previous technical problems[8]. Lacerations tend to occur at the junction of the body and tail due to shearing injuries with compression against the spine (Figure 3A).

Direct signs of pancreatic injury include laceration, transection, focal pancreatic enlargement and inhomo-
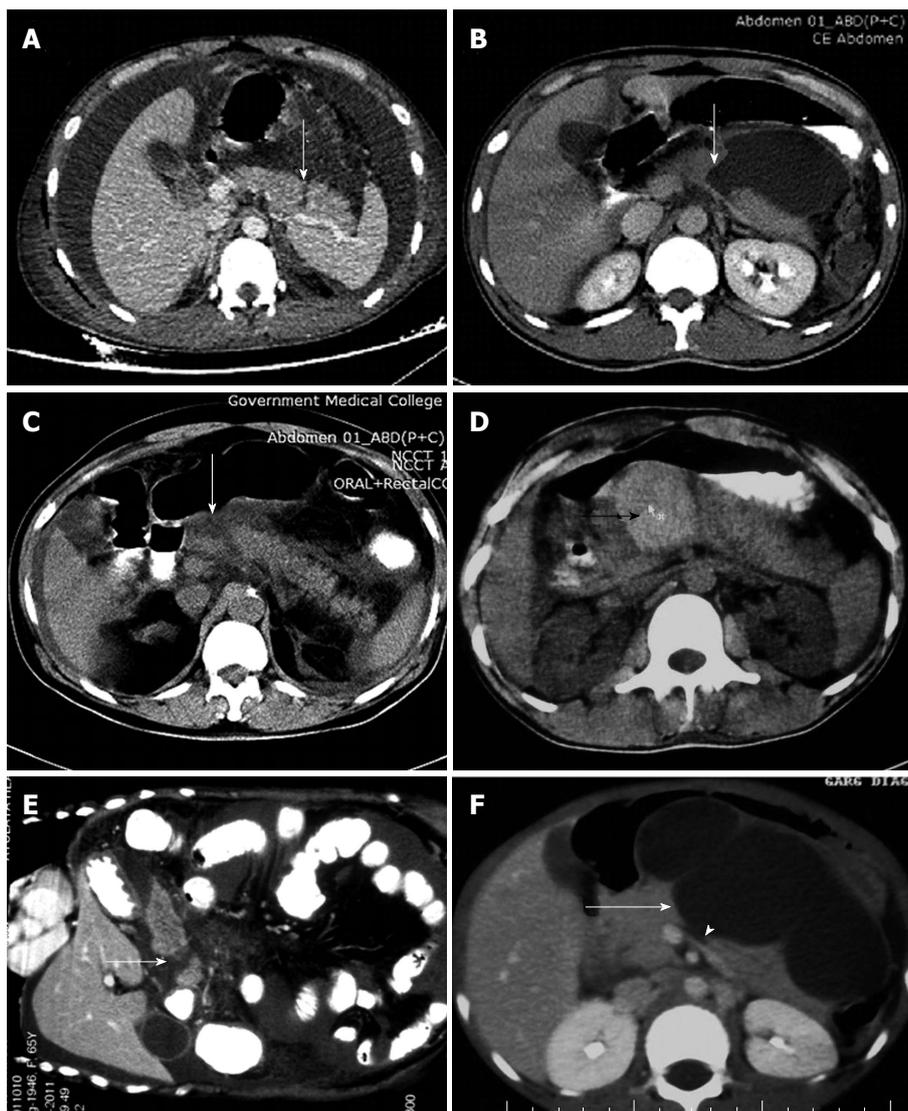
Figure 3 Computed tomography images. A, B: Axial contrast-enhanced computed tomography shows a heterogeneous appearance of the body and tail of pancreas with a linear laceration (white arrow) across the distal body of the pancreas. There is also fluid in the lesser sac, perihepatic space, perisplenic space and hemoperitoneum. There is free air into chest wall muscles on right side in a case of blunt pancreatic trauma (A), and transection throughout extent of pancreatic parenchyma in proximal body region (suggestive of ductal injury) with a large fluid collection (white arrow) anterior to pancreas communication with the transection in another case of blunt injury to upper abdomen (B); C: Contrast-enhanced computed tomography demonstrating mild diffuse hypodensity of the body of pancreas. Contusions of the head and neck also demonstrated (white arrow) with secondary signs of traumatic pancreatitis, *i.e.*, increased density of the peripancreatic fat, thickening of left anterior pararenal fascia, fluid in the lesser sac and hemoperitoneum; D: Plain axial computed tomography section at the level of pancreas shows a large hyperdense hematoma (black arrow) in proximal body of pancreas suggestive of pancreatic injury. E: Multiplanar reconstruction image of contrast-enhanced computed tomography demonstrating a pancreatic fracture (white arrow) in neck region with separation of pancreatic fragments; F: Contrast-enhanced axial computed tomography scan in a child with bicycle handlebar injury more than a month old shows a large lobulated pseudocyst anterior to pancreas communicating with pancreatic laceration in the neck of pancreas representing ductal injury. There is fluid between posterior pancreas and the splenic vein (arrow heads).

geneous enhancement. Fluid collections like hematoma and pseudocyst are usually seen communicating with the pancreas at the site of laceration or transection (Figure 3B). Secondary signs include peripancreatic fat stranding, peripancreatic fluid collections, fluid between the splenic vein and pancreas, hemorrhage, thickening of the left anterior pararenal fascia and associated injuries to adjacent structures[10] (Figure 3C, Table 1).

Contusion appears as focal or diffuse low attenuation areas and laceration is seen as a linear hypodense line perpendicular to the long axis of the pancreas[6,23,24]. Pancreatic fracture on CT is diagnosed if there is a clear separation of fragments across the long axis of the pancreas[25]. Intrapancreatic hematoma is a very specific sign of pancreatic injury[26] (Figure 3D). Fluid between the splenic vein and pancreas is a very non-specific sign but it may suggest pancreatic injury if associated with history of blunt abdominal trauma[27]. Pseudocysts are more likely to occur in patients with traumatic pancreatitis[28]. The risk of abscess or fistula formation in patients with disruption of the pancreatic duct approaches 25% and 50%, respectively, in comparison with 10% without duct injuries[7]. So it is important that imaging focuses on the integrity of the duct or findings that suggest damage to the pancreatic duct. The accuracy of detecting a major ductal injury by CT has been reported to be as low as 43%[10,17,29-31].

Computed tomography may not always directly demonstrate the ductal disruption; injury to the duct can be suggested based on the degree of parenchymal injury and can only be inferred following visualization of a through and through laceration of the pancreas (Figure 3E). A computed tomography grading scheme has been devised (Table 2), which parallels the surgical classification of Moore[10,32]. Grade A injuries with laceration involving < 50% pancreas are usually seen with an intact pancreatic duct by surgical grading, whereas grade B and C injuries correlate with duct disruption, especially when CT shows deep lacerations or pancreatic transection[32]. Overestimation on CT can occur in grade C I and C II injuries if merely deep lacerations or "single scan" transections are identified at the pancreatic head. However, urgent endoscopic retrograde cholangiopancreatography (ERCP) may be quite valuable in such patients with strong clinical

Table 1 Computed tomographic signs of pancreatic injury

Specific signs	Fracture of the pancreas
	Pancreatic laceration
	Focal or diffuse pancreatic enlargement/edema
	Pancreatic hematoma
	Active bleeding/extravasation of intravenous contrast
	Fluid separating the splenic vein from posterior aspect of pancreas
Non-specific signs	Inflammatory changes in peripancreatic fat and mesentery
	Fluid surrounding the superior mesenteric artery
	Thickening of the left anterior renal fascia
	Pancreatic ductal dilatation
	Acute pseudocyst formation/peripancreatic fluid collection
	Fluid in the anterior and posterior pararenal spaces
	Fluid in transverse mesocolon and lesser sac
	Hemorrhage into peripancreatic fat, mesocolon and mesentery
	Extraperitoneal fluid
	Intraperitoneal fluid

Table 2 Computed tomographic grading of blunt pancreatic injuries

CT grading	CT findings of blunt pancreatic injury
Grade A	Pancreatitis and/or superficial lacerations at any site
Grade B	
B I	Deep laceration at distal pancreas
B II	Transections at distal pancreas
Grade C	
C I	Deep lacerations at proximal pancreas
C II	Transections at proximal pancreas

Reproduced from Wong *et al*[32]. CT: Computed tomography.

evidence of pancreatic injury and an equivocal CT scan, to establish the final diagnosis[10,32]. A patient with a post-traumatic pseudocyst should be considered to have a ductal leak until proven otherwise[1] (Figure 3F).

MRCP

Since the outcome of pancreatic trauma patients largely depends upon the integrity of the pancreatic duct, evaluation of the duct is essential. In the past, ERCP was the only method available for evaluating pancreatic duct integrity. More recently, MRCP has emerged as an attractive alternative non-invasive diagnostic tool for direct imaging of the pancreatic duct and it is being used more frequently to assess injury to the ductal components[33]. Dynamic secretin-stimulated (DSS) MRCP is a variation on standard MRCP and may compete with ERCP in diagnostic accuracy. Like ERCP, DSS MRCP provides dynamic information as to whether there is continuing leakage from an injured main pancreatic duct. The advantages of DSS MRCP include it being noninvasive, faster and more readily available than ERCP, and it can illustrate the entire pancreatic parenchymal and ductal anatomy, as well as pathologic fluid collections and ductal disruptions[34]. The main pancreatic duct (MPD) can be identified by MRCP within the pancreatic head

Table 3 Classification of pancreatic injuries by endoscopic retrograde cholangiopancreatography

Grade	Description
I	Normal main pancreatic duct on ERCP
II a	Injury to branches of main pancreatic duct on ERCP with contrast extravasation inside the parenchyma
II b	Injury to branches of main pancreatic duct on ERCP with contrast extravasation into the retroperitoneal space
III a	Injury to the main pancreatic duct on ERCP at the body or tail of the pancreas
III b	Injury to the main pancreatic duct on ERCP at the head the pancreas

Reproduced from Takishima *et al*[38]. ERCP: Endoscopic retrograde cholangiopancreatography.

in up to 97% of cases and within the pancreatic tail in up to 83%[35]. In addition, MRCP may demonstrate abnormalities not visible at ERCP, such as fluid collections upstream of the site of duct transection (Figure 4A), and is helpful in assessing parenchymal injury[36]. For assessing the parenchyma, fat-suppressed T1- and T2-weighted sequences are performed. Magnetic resonance pancreatograms are acquired by using heavily T2-weighted breath-hold or non-breath-hold sequences. Fast spin-echo (two-dimensional or three-dimensional) and rapid acquisition with relaxation enhancement sequences performed in the coronal and axial planes are usually sufficient[10].

ERCP

ERCP is increasingly being used to help in both early and in delayed diagnosis of pancreatic ductal injuries in patients with strong clinical evidence of pancreatic injury and an equivocal CT scan. Endoscopic retrograde cholangiopancreatography is the most accurate investigation for diagnosing the site and extent of ductal injury by demonstrating extravasation or a cutoff, especially in patients with delayed presentations[37]. It can be performed preoperatively, intraoperatively or postoperatively in patients with pancreatic injury. Although ERCP is the most useful procedure for the diagnosis of pancreatic ductal injury in stable patients, surgery should be considered in hemodynamically unstable patients. A classification of pancreatic injuries (Table 3) has been devised according to the findings on ERCP[38]. Although MRCP (Figure 4B) has become the noninvasive imaging method of choice when evaluating for pancreatic duct injury, ERCP remains important because of its potential to direct image-guided therapy (Figure 5). Endoscopic retrograde cholangiopancreatography in selected patients allows non-operative treatment in the absence of ductal injury and earlier operative treatment or primary therapy as stent placement in the presence of ductal injury[39]. It also aids the treatment of late complications of pancreatic duct injuries such as pseudocysts and pancreatic fistulae. Both endoscopic transpapillary and transmural drainage are effective options for managing delayed local complications of pancreatic trauma. The endoscopist must be skilled
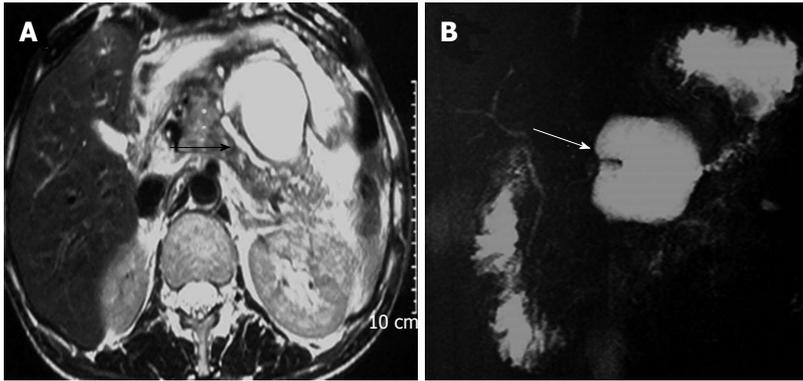
Figure 4 **Magnetic resonance images.** T2 weighted axial image (A) and magnetic resonance cholangio-pancreatography (B) in a case of traumatic pancreatitis show heterogenous signal intensity of pancreas with peripancreatic stranding. Main pancreatic duct is dilated in the body and tail region (black arrow). A lobulated pseudopancreatic cyst is seen in lesser sac anterior aspect of body of pancreas (white arrow) demonstrated in magnetic resonance cholangiopancreatography.

Figure 5 **Endoscopic retrograde cholangiopancreatography image.** Another case of traumatic pancreatitis. Fluoroscopic image showing main pancreatic duct disruptions during endoscopic retrograde cholangiopancreatography with multiple contrast filled outpouching is seen, suggestive of pseudocysts (white arrow). Multiple contrast filled tracts are also visualized (black arrowhead). Few tracts are seen in retroperitoneum and one of the tracts is reaching into mediastinum (black arrow). Endoscope is visible.

Table 4 American Association for the surgery of trauma classification of pancreatic trauma

Grade	Injury	Description
I	Hematoma	Minor contusion without ductal injury
	Laceration	Superficial laceration without ductal injury
II	Hematoma	Major contusion without ductal injury or tissue loss
	Laceration	Major laceration without ductal injury or tissue loss
III	Laceration	Distal transection or pancreatic parenchymal injury with ductal injury
IV	Laceration	Proximal transection or pancreatic parenchymal injury involving the ampulla
V	Laceration	Massive disruption of the pancreatic head

Reproduced from Campbell *et al*[42].

and experienced in its use as this procedure has potential complications that can limit its usefulness in patients with pancreatic trauma.

COMPLICATIONS OF PANCREATIC TRAUMA

Early diagnosis and treatment are associated with better overall outcomes in traumatic pancreatic injury patients. Mortality associated with pancreatic injuries approximates 20% and results primarily from hemorrhage caused by injuries to other intra-abdominal organs and from sepsis[40,41]. There is an increase in infectious complications in patients who have pancreatic wounds co-associated with injury to small and large intestine. Blunt pancreatic injuries without ductal leak usually resolve with mere conservative management. On the other hand, damage to the ductal system, if inadequately treated or untreated, can result in prolonged morbidity. Complications of traumatic pancreatic injury are manifold and range from minor pancreatitis to death[40,42]. Fistula formation is the most frequently observed complication. Traumatic pancreatitis, pseudocyst formation, abscesses and duct stricture are common complications. Other less frequent complications include peritonitis, intestinal obstruction, gastrointestinal bleeding, endocrine or exocrine insufficiency, splenic artery pseudoaneurysm formation or rupture and splenic vein thrombosis[6,24].

CLASSIFICATION AND GRADING OF PANCREATIC INJURIES

Pancreatic injuries are classified and graded according to the damage to the pancreatic parenchyma and the ductal system. Grading of pancreatic injuries enables an exact description of injuries, can influence management, and allows a comparison of outcomes and effective quality control of treatment[12]. There are several classification systems of traumatic pancreatic injuries[32,38] (Tables 2 and 3) but the pancreatic organ injury scale (OIS) proposed by the American Association for the Surgery of Trauma (AAST) fulfills most of these criteria and at present is the universally accepted classification scheme[43]. This OIS scale involves five grades, which concedes the significance of more complex injuries to the pancreas, and particularly those injuries affecting the pancreatic duct and the pancreatic head (Table 4). This classification scheme can also be correlated with other organ injury scales, as well as integrated into more complex scoring systems, such as injury severity score or trauma score - injury severity score from which probability of survival of an individual case is determined.

Blunt pancreatic injury

Hemodynamically stable patient
Helical multislice CT of abdomen

AAST - OIS grade

Low-grade blunt pancreatic injuries
Grade Ⅰ
Grade Ⅱ

High-grade blunt pancreatic injuries
Grade Ⅲ
Grade Ⅳ
Grade Ⅴ

ERCP or MRCP

Surgical intervention

Ductal injury

Yes

No

Non-operative management

Surgical intervention
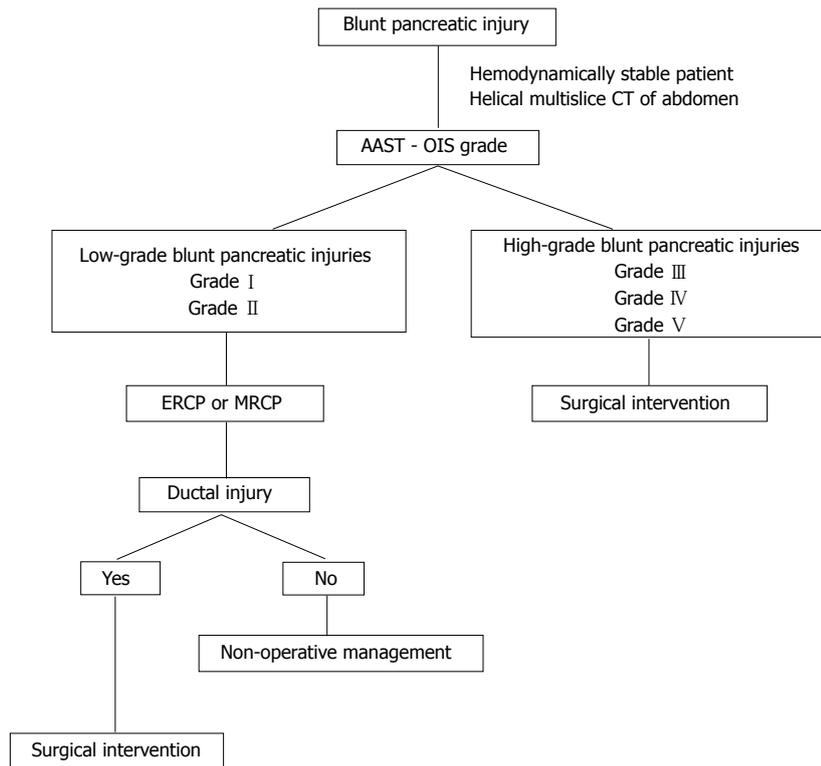
Figure 6 Management algorithm for traumatic pancreatic injury patients. Reproduced from Ilahi *et al*[14]. ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.

MANAGEMENT OF PANCREATIC INJURIES

Many patients with pancreatic injuries have multiple associated injuries including vascular and other intra-abdominal organs injury; priority must be given to stabilizing the patient before any definitive management of the pancreatic injury. The initial priorities include control of hemorrhage and spillage of intestinal contents. The decision regarding therapeutic approach of the traumatic pancreatic injury, either with a conservative approach or a surgical approach, depends upon the integrity of the MPD, extent of pancreatic parenchymal damage, anatomical location of the injury, stability of the patient and degree of associated organ damage (Figure 6)[44]. In patients with an isolated pancreatic contusion or superficial lacerations without ductal disruption, conservative management may be warranted. Treatment of traumatic pancreatitis consists of bowel rest, nasogastric suction, and nutritional support[29]. ERCP-guided stent placement to the MPD injury has been indicated in select cases[45]. Endoscopic transpapillary drainage has been successfully used to heal duct disruptions in the early phase of pancreatic trauma and in the delayed phase to treat the complications of pancreatic duct injuries. However, in patients with major ductal injury in blunt pancreatic trauma cases, morbidity and mortality greatly increase unless surgery is undertaken within the first 24 h. By using the pancreatic OIS grading system of the AAST to help to guide the appropriate surgical management, the morbidity and mortality in blunt pancreatic injury are decreased[46]. Grades Ⅰ and Ⅱ are treated with non-operative management techniques or simple drainage, whereas

grade Ⅲ or higher injuries often require resection with possible reconstruction and/or drainage procedures[47]. There are a number of alternative procedures that can be used for the management of high-grade blunt pancreatic injury, such as duodenal diversion, pyloric exclusion, the Whipple procedure or simple drainage, with the choice dependent on the patient's hemodynamic status and the presence or absence of associated duodenal injury[48,49]. Sometimes, the decision to perform a pancreaticoduodenectomy is unavoidable in select cases. If the patient is hemodynamically unstable, pancreaticoduodenectomy should be performed as a two-step procedure. After the initial damage control surgery, anastomoses are completed at a second surgery when the patient is stable[50].

The standard of care in penetrating injuries is a surgical approach depending upon the location of the injury and associated abdominal injuries. Damage control surgery in hemodynamically unstable patients with massive injury to the pancreas and associated intra-abdominal organs reduces morbidity and mortality.

CONCLUSION

Pancreatic injury is uncommon and usually difficult to diagnose. Because of the subtlety of the ultrasound findings, computed tomography is the preferred method for evaluating suspected pancreatic trauma; however, pancreatic duct injury may not be detected on computed tomography scan except when there is through and through laceration. In select situations, including minor injuries, a conservative approach may be successful. With modern imaging modalities and expertise in endoscopic retrograde cholangiopancreatography, isolated pancreatic

duct injury can be successfully managed. A surgical approach is appropriate with major pancreatic injury that necessitates urgent surgical intervention.

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ORIGINAL ARTICLE

Clinically detected gastroenteropancreatic neuroendocrine tumors are on the rise: Epidemiological changes in Germany

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Abstract

AIM: To study the epidemiologic changes of gastroenteropancreatic neuroendocrine tumors (GEP-NET) in Germany, we analyzed two time periods 1976-1988 and 1998-2006.

METHODS: We evaluated epidemiological data of GEP-NET from the former East German National Cancer Registry (DDR Krebsregister, 1976-1988) and its successor, the Joint Cancer Registry (GKR, 1998-2006), which was founded after German reunification. Due to a particularly substantial database the epidemiological data from the federal states of Mecklenburg-Western Pomerania, Saxony, Brandenburg and Thuringia, covering a population of more than 10.8 million people, were analyzed. Survival probabilities were calculated using life table analysis. In addition, GEP-NET patients were evaluated for one or more second (non-GEP-NET) primary malignancies.

RESULTS: A total of 2821 GEP neuroendocrine neoplasms were identified in the two registries. The overall incidence increased significantly between 1976 and 2006 from 0.31 (per 100.000 inhabitants per year) to 2.27 for men and from 0.57 to 2.38 for women. In the later period studied (2004-2006), the small intestine was the most common site. Neuroendocrine (NE) neoplasms of the small intestine showed the largest absolute increase in incidence, while rectal NE neoplasms exhibited the greatest relative increase. Only the incidence of appendiceal NET in women showed little change between 1976 and 2006. Overall survival of patients varied for sex, tumor site and the two periods studied but improved significantly over time. Interestingly, about 20% of the GEP-NET patients developed one or more second malignancies. Their most common location was the gastrointestinal tract. GEP-NET patients without second malignancies fared better than those with one or more of them.

CONCLUSION: The number of detected GEP-NET increased about 5-fold in Germany between 1976 and 2006. At the same time, their anatomic distribution changed, and the survival of GEP-NET patients improved significantly. Second malignancies are common and influence the overall survival of GEP-NET patients. Thus, GEP-NET warrant our attention as well as intensive research on their tumorigenesis.

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Core tip: Modern endoscopic and radiological tumor imaging have been implicated in the rise of the incidence of detected neuroendocrine tumors (NET) in Western countries. The particularities of German history, which resulted in two German states with two different health care systems from 1949-1989, allowed to study the epidemiological changes of NET in Germany on the background of two health care systems in 1949-1989. The number of detected gastroenteropancreatic-NET increased about 5-fold between 1976 and 2006. Most likely, the general availability of endoscopy after German reunification contributed to the major rise in frequency of detected rectal, gastric and duodenal NET in the new federal states of reunified Germany.

INTRODUCTION

Gastroenteropancreatic neuroendocrine tumors (GEP-NET) are infrequent, constituting only 1%-2% of all neoplasms. Most commonly they present as indolent, slow-growing tumors[1-4]. Their anatomic distribution reflects that of the neuroendocrine cells from which they derive: up to 65% in the gastrointestinal tract, about 25% in the bronchopulmonary system, and the remaining 10% at other sites[1,5].

In Western countries, pancreatic neuroendocrine tumors are diagnosed in 0.5-1 per 100000 inhabitants and represent 1%-2% of all clinically manifest pancreatic neoplasms[6-8]. GEP-NET occur in approximately 2.0-2.5 per 100000, carcinoid syndrome being most frequently associated with NET of the jejunum and ileum[9-13].

Previous and current WHO classifications distinguish between well-differentiated and poorly differentiated neoplasms. All well-differentiated neoplasms, whether benign or metastatic, are now called neuroendocrine (NE) and are graded as G1 (Ki-67 $\leqslant$ 2%) or G2 (Ki-67: 3%-20%). The poorly differentiated neoplasms are called neuroendocrine carcinomas and are graded as G3 (Ki-67 > 20%). The term "carcinoid" is now synonymous with G1 well-differentiated neuroendocrine tumor.

Population-based data from the Third National Cancer Survey and the United States Surveillance Epidemiology and End Results (SEER) Program, covering 10%-14% of the United States population, show a steady increase in the incidence of NET throughout the 35-year period between 1969 and 2004[14-17]. The overall incidence of GEP-NET increased two- to three-fold during this period, and there were significant changes in the anatomic distribution. Thus, the proportion of GEP-NET located in the appendix decreased from 43% to 4% with corresponding increases in the stomach (from 2% to 9%), small intestine (from 31% to 42%), and rectum (from 15% to 27%)[8,16]. The observed changes may in part reflect the increased number of asymptomatic GEP-NET incidentally identified thanks to increased availability of modern endoscopic and radiological imaging[3,8,18].

There have been few studies on GEP-NET epidemiology in Germany[17], and no comprehensive and comparative epidemiological studies have as yet been published on GEP-NET at various locations.

Therefore we evaluated epidemiological data from the former East German National Cancer Registry (DDR Krebsregister) for 1976-1988 and from its successor, the Joint Cancer Registry (GKR) for 1998-2006. After German reunification, the East German registry was renamed and thus became the GKR of the new federal states of Germany and Berlin. After an interruption of several years, it continued to collect epidemiological data. Thanks to a particularly substantial database we analyzed epidemiological data from the federal states of Mecklenburg-Western Pomerania, Saxony, Brandenburg and Thuringia, covering a population of over 10.8 million people.

MATERIALS AND METHODS

The official population statistics for Germany as reported by the German government were used to estimate the incidence rate of GEP-NET. Absolute numbers were used to estimate the crude incidence rate both in former East Germany and - after German reunification - in the new federal states, including Berlin. Since other federal states, including Berlin, had a less extensive database, their data were not used in the analysis presented here.

The study included all persons (living in Mecklenburg-Western Pomerania, Saxony, Brandenburg or Thuringia) diagnosed with GEP-NET between 1976 and 1988 or between 1998 and 2006. Mortality data from 1976 to 1990 and from 1998 to 2009 were used. We included all patients with NE tumors at the following tumor sites according to ICD10: C15-C25, D37.1-D37.5 (esophagus, stomach, small intestine, large intestine, appendix, rectosigmoid, rectum, anus and pancreas), C26.0, C26.8-9, D37.7, D37.9 (unspecified location in the digestive tract) and an NE morphology code according to ICD-O-3: 8150-8153, 8155-8157, 8240-8246, 8249 or 8574.

Annual incidence rates were calculated for the periods 1976-1988 and 1998-2006. Incidence rates are presented for each tumor site, sex and age group. Trends in incidence are presented as overall change throughout the two periods and between two representative 2-year periods, *i.e.*, 1976-1978 and 2004-2006. The absolute number of GEP-NET reported per 100.000 inhabitants per year is

Table 1 Incidence of gastroenteropancreatic neuroendocrine tumors according to anatomic site, sex, and the time periods 1976-1988 and 1998-2006

Period	Stomach (M/F)[1]	Small intestine (M/F)[1]	Pancreas (M/F)[1]	Appendix (M/F)[1]	Colon (M/F)[1]	Rectum (M/F)[1]
1976-1978	0.02/0.00	0.11/0.15	0.03/0.08	0.20/0.35	0.04/0.05	0.01/0.01
1979-1981	0.04/0.03	0.10/0.11	0.07/0.014	0.15/0.34	0.04/0.07	0.01/0.03
1982-1984	0.04/0.04	0.16/0.12	0.11/0.08	0.15/0.35	0.04/0.07	0.03/0.02
1985-1988	0.05/0.04	0.20/0.18	0.10/0.10	0.24/0.35	0.08/0.15	0.03/0.07
1989-1997						
1998-2000	0.16/0.12	0.39/0.31	0.22/0.18	0.13/0.28	0.19/0.19	0.09/0.10
2001-2003	0.18/0.18	0.44/0.40	0.25/0.25	0.23/0.46	0.22/0.23	0.16/0.15
2004-2006	0.27/0.23	0.51/0.52	0.25/0.25	0.31/0.39	0.20/0.28	0.26/0.24

[1]Male/female (M/F) patients. Incidence rates were age-adjusted to the German standard population of 1987. The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for the years 1976-1988 or from its successor, the Joint Cancer Registry for the time period 1998-2006. No valid data are available for the period between 1989 and 1997.
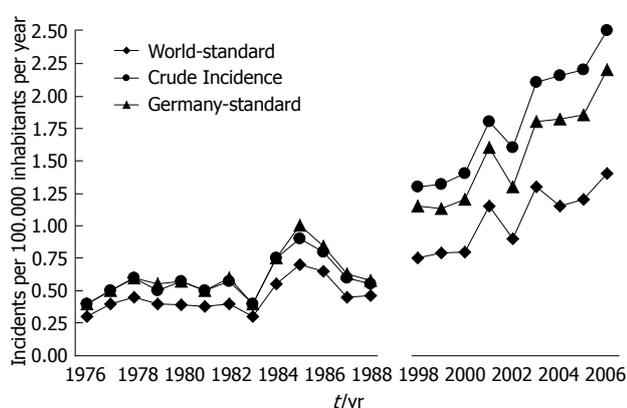
Figure 1 The incidence of gastroenteropancreatic neuroendocrine tumors is shown over time (1976-1988 and 1998-2006). It is presented either as crude incidence or as the number of tumors (per 100.000) age-adjusted to the 1966 world standard population (World standard) or to the 1987 German standard population (German standard). The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for 1976-1988 or from its successor, the Joint Cancer Registry, for 1998-2006.

referred to as the "crude incidence rate".

Age-adjusted incidence rates were calculated using the World Standard Population published in 1966[5] and the 1987 German standard population.

Survival probabilities were calculated using life table analysis. Since data were available only on the time but not the cause of death, only the overall survival of the registered GEP-NET patients could be determined. Tumor-specific survival could not be analyzed. The Wilcoxon-Gehan test was used to determine significance when comparing the survival rates.

In addition, all GEP-NET patients were evaluated for one or more second primary malignancies. Second (non-GEP-NET) neoplasms were analyzed for location of the second primary.

RESULTS

Frequency
From 1976 to 1988 and from 1998 to 2006, a total of 2821 cases of GEP-NET were registered in Mecklen-burg-Western Pomerania, Saxony, Brandenburg and Thuringia - 1001 cases from 1976 to 1988 and 1820 cases from 1998 to 2006. The total patient population comprised 1291 men (45.8%) and 1530 women (54.2%).

In 2006, a total of 10837539 persons lived in Mecklenburg-Western Pomerania, Saxony, Brandenburg and Thuringia - 5329539 men (49.2%) and 5508000 women (50.8%).

Incidence rates
The crude incidence rate of GEP-NET (per year and 100.000 population) rose from 0.45 in 1976 to 2.53 in 2006, which corresponds to a 462% increase. The incidence rate increased by 342% when age-adjusted to the 1966 world population and by 270% when adjusted to the 1987 population of Germany (for details, Figure 1).

The crude incidence rate increased more prominently in men (from 0.31 in 1976 to 2.7 in 2006) than in women (from 0.57 in 1976 to 2.38 in 2006).

Tumor site
The age-adjusted (population of Germany in 1987) incidence is given in Table 1 for the periods 1976-1988 and 1998-2006. Both age-adjusted and crude incidence rates increased at all tumor sites. When comparing representative time intervals such as 1976-1978 and 2004-2006, the largest increment in absolute numbers was found for the small intestine; the absolute increase in crude incidence was 0.56 in men and 0.48 in women. Focusing on relative changes revealed the largest increase for rectal NE neoplasms; the crude incidence rose by about 6000% in men and by more than 2700% in women. In contrast, hardly any change was observed for appendiceal NE neoplasms in the female population between 1976 and 2006 (see Figures 2 and 3).

Survival
Overall survival increased significantly between the time periods 1976-1988 and 1998-2006 (*P* < 0.001). The 1-, 5- and 10-year overall survival rates were 59%, 50% and 47% for the earlier period and 79%, 63% and 50% for the later period (Figure 4).
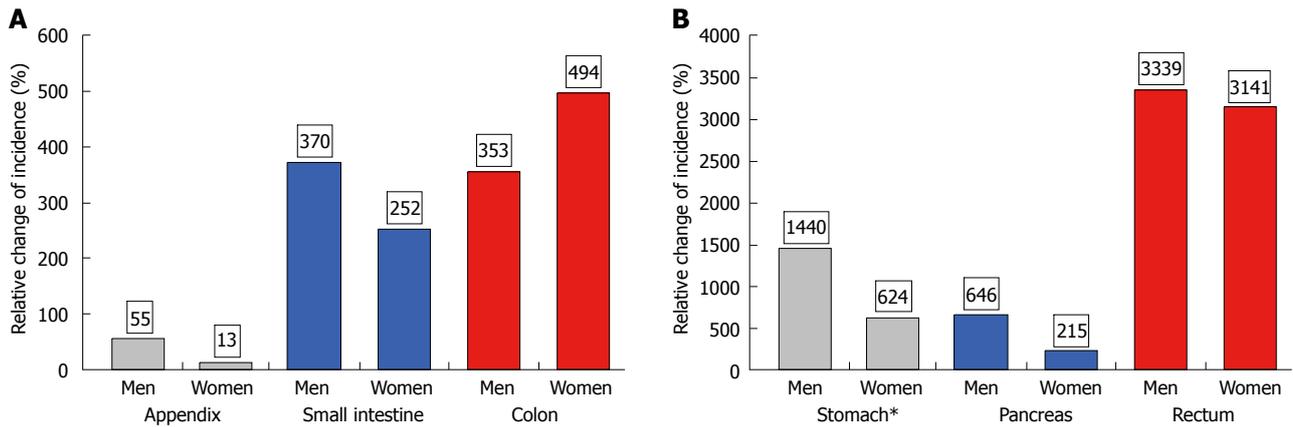
Figure 2 Relative changes in the incidence of gastroenteropancreatic neuroendocrine tumor are depicted according to tumor site and sex. A: Changes were calculated for the reference periods 1976-1978 and 2004-2006*; the intervals 1979-1981 and 2004-2006 were chosen for the stomach, since no gastric NE neoplasms were registered in women before 1979; B: Changes in incidence are presented as relative increments age-adjusted to the 1987 German standard population. The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for the years 1976-1978 or from its successor, the Joint Cancer Registry, for the time period 2004-2006. NE: Neuroendocrine.
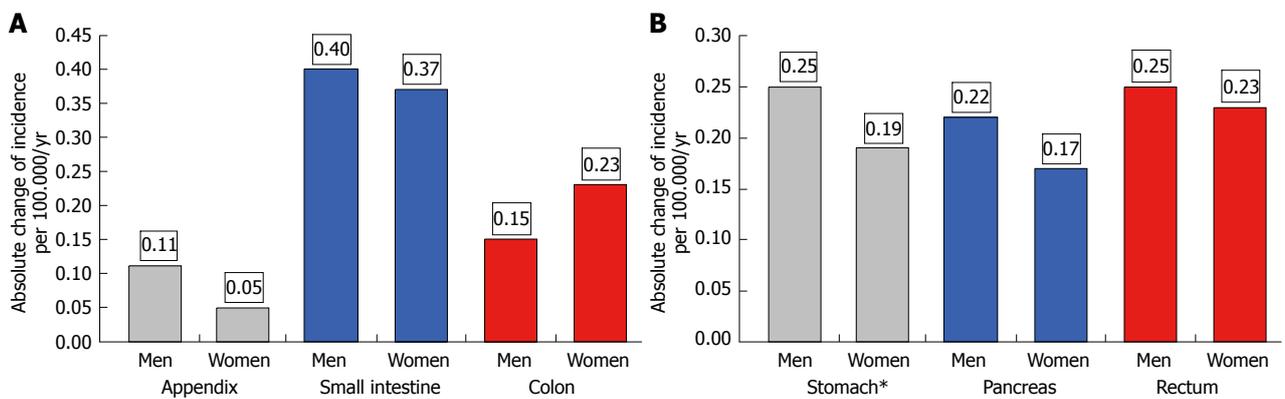
Figure 3 Absolute changes in the incidence of gastroenteropancreatic neuroendocrine tumor are shown according to tumor site and sex. A: Changes were calculated for the reference periods 1976-1978 and 2004-2006*; the periods 1979-1981 and 2004-2006 were chosen for the stomach, since no registered gastric NE neoplasms were registered in women before 1979; B: Changes in incidence are presented as absolute increments age-adjusted to the 1987 German standard population. The data shown originate from the former East German National Cancer Registry (DDR Krebsregister) for the years 1976-1978 or from its successor, the Joint Cancer Registry, for the time period 2004-2006. NE: Neuroendocrine.

Overall survival differed significantly ($P < 0.001$) between men and women. In the earlier period (1976-1988), 51% of men and 64% of women were alive after 1 year; 43% of men and 55% of women stayed alive after 5 years, and 41% of men and 51% of women were alive after 10 years. In the later period (1998-2006), 1-year survival was 75% for men and 83% for women; 5-year survival had increased to 57% for men and 68% for women, and 10-year survival was 42% for men and 58% for women. Overall survival differed not only for sex but also for the primary tumor site. Table 2 shows significant differences in survival for various anatomic locations of the primary as well as for the two time periods (1976-1988 and 1998-2006).

Second primary neoplasms

Of the 2821 NE tumor patients diagnosed between 1976-1988 and 1998-2006, 472 developed 533 second malignancies (Figure 5). The 533 second malignancies

were diagnosed as pre-existing, synchronous or metachronous malignancies during the same time intervals as the GEP-NET (1976-1988 or 1998-2006); 16.7% of the GEP-NET patients suffered from second neoplasms.

GEP-NET patients without second malignancies fared better than those with one or more of them. An analysis of all 2821 GEP-NET patients showed significantly better 5- and 10-year overall survival for those without than for those with one or more second malignancies (5-year survival of 60% *vs* 53%; 10-year survival of 52% *vs* 42%, $P < 0.05$).

The main locations of second malignancies were the digestive tract (28%), the female genital organs (12%), the skin (12%), the breasts (7%) and the male genital organs (7%).

DISCUSSION

Analysis of DDR Krebsregister for the period 1976-1988

Survival probability for GEP-NET-patients
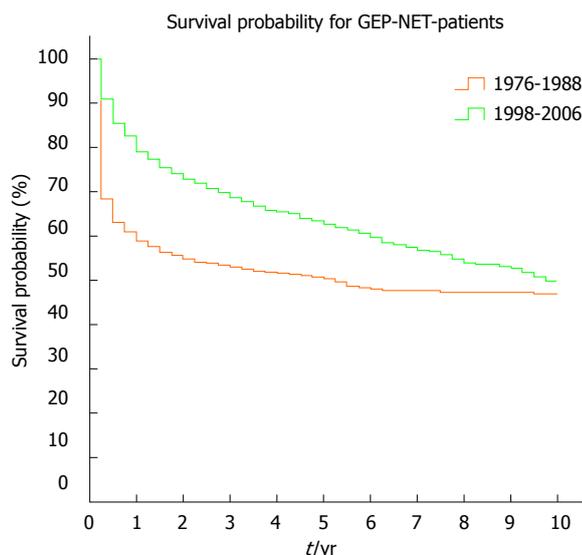
Figure 4 Overall survival of patients with gastroenteropancreatic neuroendocrine tumor. Two periods of time are compared (1976-1988 and 1998-2006). GEP-NET: Gastroenteropancreatic neuroendocrine tumor.

A Absolute number of GEP-NET patients with second primary malignancies

B Relative proportion of GEP-NEN patients with second primary malignancies

Figure 5 Second malignancies occurred in 472 of 2821 patients with gastroenteropancreatic neuroendocrine tumor. A: The absolute number; B: The percentage of patients with one or more second (non-gastroenteropancreatic neuroendocrine tumor, non-GEP-NET) neoplasms. 0 = No second neoplasm. GEP-NET: Gastroenteropancreatic neuroendocrine tumor.

and its successor, the GKR, for the period 1998-2006 revealed major increases in the incidence of detected GEP-NET in Germany. Our findings are in line with a recent report from the United States American SEER registry[8,16]. Recent epidemiological data from England and Norway also showed increases[8,14]. The current incidence of GEP-NET in Germany compares well with the incidence rates found in the United States and Australia as well as in several other European countries, i.e., 1.33 to 3.8 per 100.000[8,14,16]. Despite these similarities, there are a number of important differences.

The nomenclature and classifications of NE neoplasms have not been uniform in the last 35 years[17]. Thus the low incidence rates from 1976-1988 may in part reflect differences between the classification and nomenclature used by East German pathologists (of the DDR Krebsregister) and those applied elsewhere. On the other hand, recent improvements in the general awareness and immunohistological diagnosis of NE neoplasms may have contributed to increased incidences in the last 20 years.

Moreover, colorectal cancer screening and the general availability of high-resolution endoscopy and radiological imaging may well have facilitated the detection of early asymptomatic GEP-NET. The hypothesis is supported by studies demonstrating that the incidence and anatomic distribution differ significantly between tumors detected post mortem and those diagnosed clinically[1,7,8]. These studies suggest that most small (≤ 1 cm) GEP-NET remain asymptomatic and were generally not diagnosed in the era before the widespread availability of high-resolution endoscopy and computed tomography (CT) imaging.

The question arises whether the increased detection of early (asymptomatic) NET disease has contributed to recent epidemiological trends. In recent years, localized
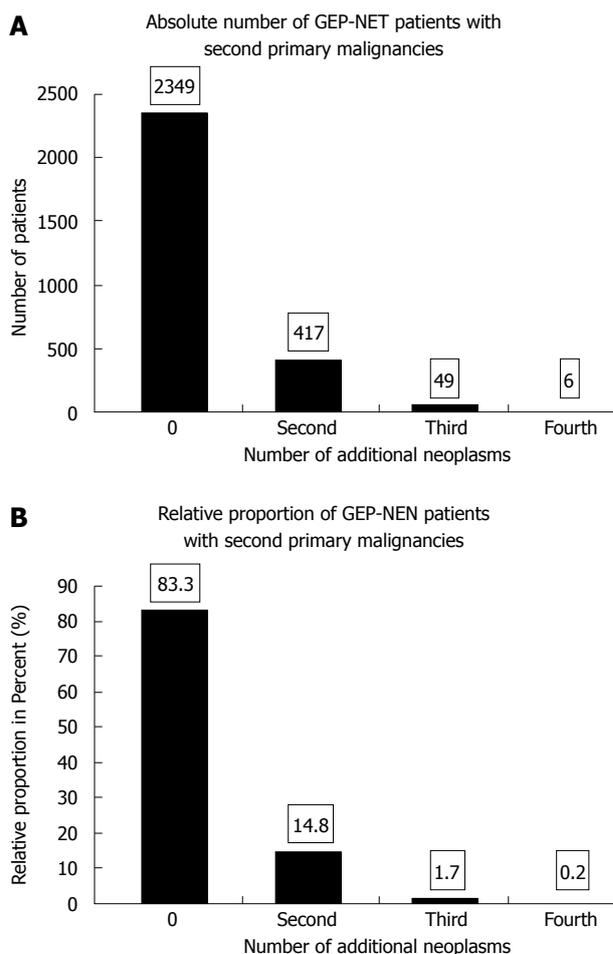
NET constitute by far the largest subgroup in the SEER registry and are largely responsible for the increased incidence of GEP-NET[3,18]. Consistent with this notion, Japanese, South Korean and Polish endoscopy screening programs detected rectal NET in 50-70 of 100000 persons screened[19-21]. The vast majority of rectal NE neoplasms detected by screening are 1 cm or smaller in diameter. Comparison with historical registries shows that screening is associated with a shift to smaller-sized rectal NET[3,18]. A national program of endoscopic colorectal cancer screening was introduced in Germany in October 2002. Screening colonoscopy is now offered free of charge to any person aged 55 years or older. Both colonoscopy and esophagogastroduodenoscopy are now readily available in Germany. In former East Germany (up to 1989), on the other hand, gastrointestinal endoscopy was available only at 3-4 centers.

The now widespread availability of endoscopy and radiological imaging may well have contributed to the observed increases in gastroduodenal, rectal and pancreatic NET[18,22-26]. On the other hand, the incidence of appendiceal NET remained quite stable in the Eastern

Table 2 1-, 5- and 10-year overall survival of patients with gastroenteropancreatic neuroendocrine tumors

Overall survival/period	Stomach	Small intestine	Pancreas	Appendix	Colon	Rectum
1-yr/1976-1988	22%	30%	26%	95%	35%	50%
5-yr/1976-1988	11%	18%	11%	92%	16%	37%
10-yr/1976-1988	5%	10%	8%	90%	13%	37%
1-yr/1998-2006	71%	85%	74%	95%	68%	74%
5-yr/1998-2006	53%	68%	52%	86%	48%	65%
10-yr/1998-2006	43%	53%	35%	81%	34%	50%

Data are given for different primary tumor sites and two periods of time (1976-1988 and 1998-2006).

parts of Germany from 1976 to 2006. Even today, they are generally diagnosed postoperatively in patients who undergo appendectomy for suspected appendicitis. Endoscopy and radiological imaging probably do not have much impact on their early detection ($\leq$ 1 cm). Instead, they are found incidentally in one out of 200-300 appendectomy specimens. Appendectomies are among the most common surgical procedures performed in Germany. They account for 135.000 procedures per year. This contributes to the frequent detection of early appendiceal NET[27].

In line with recent reports from England[28] and Austria[27], we observed a large increase in the incidence of gastric NET. In the current analysis, however, the nature of the epidemiological data does not enable an examination of underlying causes. Noteworthy is the fact that the incidence rates of both gastric and rectal carcinoids are most likely underestimated in DDR Krebsregister as well as in its successor registry, the GKR of the new federal states, including Berlin. This is due to the fact that only malignant NE neoplasms had to be reported to either registry. Thus, well-differentiated small (< 1-2 cm) carcinoids of the stomach or rectum were probably not consistently documented in the past. Recent prospective data from Austria identify the stomach and colorectum as the most common sites of GEP-NET[27]. The Austrian observation has been confirmed by a retrospective study including 150 consecutive GEP-NET patients diagnosed at the Vivantes Hospitals in Berlin between 2005 and 2009. The stomach was the most frequent site of GEP-NET in the Vivantes Hospitals, closely followed by the small intestine and colorectum (data not shown).

Overall survival of GEP-NET patients has improved significantly in Germany during the last 35 years. This applies to both sexes and all examined anatomic sites except the appendix. In the latter location, overall survival decreased in women and remained unchanged in men. But even in the period 1998-2006, the 5- and 10-year survival reached 86% and 81%. The significant improvement in overall survival of GEP-NET patients can probably be attributed to earlier diagnosis, the greater effectiveness of modern multimodal treatment strategies, and the general increase in life expectancy.

Only a few studies have reported on the frequency of one or more second malignancies in GEP-NET patients. Second primaries were found in 16.7% of our GEP-NET patients. This is consistent with data from Florida (23.6%), a meta-analysis from 13 studies including more than 5000 GEP-NET patients (17%), and the SEER registry (22.4%)[15,29-31]. At 28%, the incidence of these second primaries was highest in the gastrointestinal tract and much lower at 12% in both the female genital tract and the skin. These data on second malignancies are consistent with the observations reported in the studies mentioned above.

GEP-NET patients appear to have an increased risk of second malignancies, although there is an ongoing debate. In their review, Habal *et al*[29] summarize several theories regarding the influence of NET on the emergence and growth of second malignancies. They estimated that the risk of developing a second tumor is twice as high for patients with GEP-NET than for those with other neoplasms[29]. Several studies have examined amines, peptides, growth hormones and other compounds secreted by NET for their relation to the formation and growth of neoplasms in the breast or gastrointestinal tract[31-33].

Due to the high prevalence of second neoplasms, screening for other malignancies seems advisable in GEP-NET patients. Remarkably, Zar *et al*[34] observed that many GEP-NET patients died of their second malignancies but not of their GEP-NET. Consistent with the data of Zar *et al*[34], our GEP-NET patients with one or more second malignancies did not fare as well as those without them.

We conclude that both the frequency of detected GEP-NET and the overall survival of GEP-NET patients have increased significantly in Germany between 1976 and 2006. These epidemiological changes warrant our attention. Future research efforts will focus on the carcinogenesis of GEP-NET.

COMMENTS

Background
Neuroendocrine tumors belong to the three malignancies that increase most in frequency in Western countries.

Research frontiers
The genetic footprints of neuroendocrine neoplasias are about to be unravelled.

Innovations and breakthroughs

The frequency of second malignancies in neuroendocrine tumors (NET) patients is highlighted in this paper. The power of screening endoscopy in detecting small neuroendocrine tumors (*e.g.*, of the rectum) is evidenced by its availability in former East Germany after German reunification in 1989.

Applications

The general availability of modern endoscopy enables the (early) detection of small neuroendocrine tumors of the stomach, duodenum and rectum. Detection of small asymptomatic neuroendocrine tumors appears to have contributed to their epidemiological rise.

Peer review

The authors describe an increase of gastroenteropancreatic-NET (GEP-NET) for the time period 1977-1988 to 1998-2006 by five-fold. However, they quite clearly demonstrated in the discussion section that this increase is mainly due to different reasons: Nomenclature has been changed; improvement of general awareness and immunological diagnoses; availability of the German National Programme of Colorectal Cancer Screening since October 2002; better imaging diagnoses. An important finding of the project is that almost 17 percent of GEP-NEN patients showed second primary malignancies and therefore screening for other malignancies in those patients should be important for the future.

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ORIGINAL ARTICLE

Nrf2 and Snail-1 in the prevention of experimental liver fibrosis by caffeine

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Abstract

AIM: To determine the molecular mechanisms involved in experimental hepatic fibrosis prevention by caffeine (CFA).

METHODS: Liver fibrosis was induced in Wistar rats by intraperitoneal thioacetamide or bile duct ligation and they were concomitantly treated with CFA (15 mg/kg per day). Fibrosis and inflammatory cell infiltrate were evaluated and classified by Knodell index. Inflammatory infiltrate was quantified by immunohistochemistry (anti-CD11b). Gene expression was analyzed by quantitative reverse transcription-polymerase chain reaction for collagen Ⅰ (Col-1), connective tissue growth factor (CTGF), transforming growth factor β1 (TGF-β1), tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, superoxide dismutase (SOD) and catalase (CAT). Activation of Nrf2 and Snail-1 was analyzed by Western-blot. TNF-α expression was proved by enzyme-linked immunosorbant assay, CAT activity was performed by zymography.

RESULTS: CFA treatment diminished fibrosis index in treated animals. The Knodell index showed both lower fibrosis and necroinflammation. Expression of profibrogenic genes *CTGF*, *Col-1* and *TGF-β1* and proinflammatory genes *TNF-α*, *IL-6* and *IL-1* was substantially diminished with CFA treatment with less CD11b positive areas. Significantly lower values of transcriptional factor Snail-1 were detected in CFA treated rats compared with cirrhotic rats without treatment; in contrast Nrf2 was increased in the presence of CFA. Expression of SOD and CAT was greater in animals treated with CFA showing a strong correlation between mRNA expression and enzyme activity.

CONCLUSION: Our results suggest that CFA inhibits the transcriptional factor Snail-1, down-regulating profibrogenic genes, and activates Nrf2 inducing antioxidant enzymes system, preventing inflammation and fibrosis.

Core tip: This paper shows the protective effect of caffeine in the liver to the constant aggressiveness of a hepatotoxic. Here we present evidence not published before of some molecular mechanisms like inhibition of Snail-1 and activation of Nrf2 that could be involved in this beneficial effect down-regulating pro-fibrogenic genes and up-regulating antioxidant molecules.

Gordillo-Bastidas D, Oceguera-Contreras E, Salazar-Montes A, González-Cuevas J, Hernández-Ortega LD, Armendáriz-Borunda J. Nrf2 and Snail-1 in the prevention of experimental liver fibrosis by caffeine. *World J Gastroenterol* 2013; 19(47): 9020-9033 Available from: URL: http://www.wjgnet.com/1007-9327/full/

INTRODUCTION

The liver performs essential functions in the body[1]. Hepatic stellate cells (HSC) are key in the fibrogenic process[2]. After stimulation of liver damage, HSC undergo a process called "activation"; characterized by synthesis of type I and III collagens[3-5]. This state of activation is maintained by growth factors such as transforming growth factor β1 (TGF-β1)[6], connective tissue growth factor (CTGF)[7], and pro-inflammatory molecules such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin 6 (IL-6)[8] and reactive oxygen species (ROS).

Epidemiological studies had associated caffeine (CFA) consumption with protection against development of chronic liver disease or reduction of disease severity[9-11]. *In vitro* studies have shown beneficial effects of CFA, that can be useful in preventing HSC activation and perpetuation of this state[12,13]. Among CFA effects observed *in vitro* are: inhibition of expression of CTGF[14-16]; reduction of pro-inflammatory cytokines expression such as TNF-α, IL-1 and IL-6 by mechanisms not yet defined[8], and CFA antioxidant effect[17-21].

On the other hand, a potent natural antioxidant, quercetin increases the transcriptional and translational activity of the transcriptional factor Nrf2 which has potent antioxidant activity[22].

Activation of HSC is a complex process where the transcriptional factor Snail-1 has an important role. Several authors have reported the overexpression of Snail-1 in pathological conditions associated with extracellular matrix (ECM) deposition[23,24]. Snail-1 expression has been shown in cholangiocytes and hepatocytes of fibrotic livers[14,16], and recently, Snail-1 has been published as a central transcription factor on the activation of HSC demonstrating its essential role in regulating the liver fibrosis process[25].

According to our findings, CFA-mediated molecular mechanisms comprise in part down-regulation of pro-fibrogenic genes, diminishing of inflammatory cell infiltrate, down-regulation of pro-inflammatory cytokines, and up-regulation of antioxidant enzymes. Our results suggest that these events could be mediated, at least in part, by Nrf2 activation and inhibition of Snail-1 which are key factors in the development of this process.

MATERIALS AND METHODS

Materials

CFA was acquired from Sigma Aldrich Co., (St Louis Missouri). Thioacetamide (TAA) was purchased from Merck Company, (Darmstadt, Germany). CD11b antibody was obtained from Biolegend (San Diego, CA, United States). Biotinylated secondary antibody and avidin-conjugated peroxidase were obtained from Vector Laboratories (Burlingame, CA, United States).

DuoSet enzyme-linked immunosorbant assay (ELISA) Development kit was acquired from R and D Systems, (Minneapolis, United States). Primers and probes to design real time polymerase chain reaction (PCR) were acquired from Applied Biosystems (Hammonton, NJ, United States). Poly vinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules CA, United States). Nrf2, Snail-1 and secondary antibodies were purchased from Avcam Inc (Cambridge MA, United States).

Animals and experimental design

Wistar rats used in this study were obtained from Charles Rivers (Boston, MA, United States) and housed according to the Animal Care protocol established by University of Guadalajara. Thirty male Wistar rats, weighing 250-280 g were divided into three groups (10 rats in each group) as follows: (1) healthy ($n = 10$); (2) TAA ($n = 10$), rats with intraperitoneal TAA to develop liver fibrosis; and (3) bile duct ligation (BDL) ($n = 20$), rats that underwent a laparotomy and BDL. Finally 5 rats of each group were treated with CFA and other 5 rats received vehicle only (fibrotic rats).

CFA administration in TAA-intoxicated and BDL rats

Two *in vivo* models were intended to assess fibrosis prevention *via* CFA administration, TAA and BDL. TAA-induced fibrosis was achieved using a dose of 200 mg/kg administrated intraperitoneally 3 times a week for 7 wk, as described previously[26-28]. BDL-induced fibrosis was achieved under general anesthesia and laparotomy was made, the common bile duct was localized, doubly ligated and cut between these two ligatures[29]. CFA administration was carried out concomitantly with BDL and TAA intoxication regimen once a day with a dose of 15 mg/kg by the orogastric route. Rats sacrifice was performed at the seventh week for the TAA model, and at the fourth week for the BDL model. Representative liver sections were excised and either fixed with 4% buffered paraformaldehyde for histological examination, or frozen for RNA and protein extraction.

Biochemical assays

Blood was obtained from animals immediately before sacrifice, and serum transaminases, alanine transaminase (ALT) and aspartate transaminase (AST), were determined in automated Vitros DT 60 equipment (Johnson and Johnson, New Jersey, United States).

Histological examination of liver sections

For histological studies, livers were removed and fixed by immersion in 4% paraformaldehyde diluted in PBS, dehydrated in graded ethylic alcohol, and embedded in paraffin.

Assessment of liver inflammatory activity and fibrosis: The Modified Histological Activity Index of Knodell was used to grade the severity of the necroinflammatory

process (0-18 scale) and fibrosis (0-6 scale), and was performed blindly by two experienced pathologists[30-32]. Additionally, liver fibrosis was also quantitatively assessed by Masson's trichromic staining in 4-m liver sections by light microscopy as described previously[33,34] using a computer-assisted morphometric analyzer (Image-ProPlus 6.0; Media Cybernetics, Inc., Bethesda, MD, United States) by analyzing ten random fields per slide and calculating the ratio of connective tissue to the whole liver area, expressed as fibrosis percentage.

Immunohistochemical determination of CD11b: Hepatic tissue sections were deparaffinized and rehydrated with xylene and decreasing graded ethanol. Slides were incubated in 3% H_2O_2 for 30 min, followed by incubation with polyclonal anti-rat against purified CD11b/c (Biolegend, Cat. No. 201801, San Diego, CA, United States) diluted in PBS (1:100).

The primary antibody was incubated at 4 ℃ overnight, followed by incubation with biotinylated secondary antibody (Vectastain, Universal Quick Kit, Cat. No. PK-8800). Secondary antibodies were complexed individually with avidin-conjugated peroxidase Vectastain ABC-Elite reagent (Vector Laboratories, Burlingame, CA, United States) and resulting peroxidase activity was detected with 3,30-diaminobenzidine in sections that were briefly counterstained with hematoxylin. Positive areas were analyzed in 20 random fields of pericentral, mid-zonal and periportal areas. Counting was carried out using automated software (Image-Pro plus Analyzer, Qwin-Leica, United States). Results were expressed as a percentage of the positive area.

ELISA assay for TNF-α
Liver tissue was homogenized with Polytron (Janke Kunkel IKA-WERK, Staufen im Breisgau, Germany) and centrifugated at 4 ℃ for 4 min at 12000 *g* in lysis buffer with protease inhibitors [50 mmol/L Tris (hydroxymethyl) aminomethane-HCI buffer, pH 7.4, containing 0.02% sodium azide, 150 mmol/L NaCl, 0.1% Tween-20, 150 mmol/L NaCl, 10 g/mL aprotinin, 5 g/mL pepstatin, 5 g/mL leupeptin, 1 mmol/L phenyl-methylsulfonyl fluoride and 25 g/mL E64][35].

Protein concentration of cleared tissue lysates were determined by Bradford method. After quantitation samples were stored at -80 ℃ until analysis.

We used the kit DuoSet for ELISA for rat TNF-α/TNFSF1A (DuoSet ELISA Development kit, rat TNF-α Cat. No. DY510, R and D Systems, Minneapolis, United States), following the protocol provided by the manufacturer. Finally, the reaction was stopped and the optical density of each well was determined at 450 nm.

Quantitative real-time reverse transcriptase-PCR
RNA was isolated from the liver from different groups of rats with Trizol reagent (Invitrogen, Carlsbad, CA, United States)[36]. Retrotranscription using 2 g of total RNA was achieved using moloney-murine leukemia virus reverse transcriptase (Invitrogen). Then, 2 μL of cDNAs were subjected to real-time PCR using a Rotor Gene Termocycler under the following conditions: 2 min at 50 ℃, 5 min at 94 ℃, and 45 cycles of 30 s at 94 ℃ and 40 s at 60 ℃. Specific primers and probes designed to align in collagen α1 (Ⅰ), CTGF, TGF-β1, TNF-α, IL-1, IL-6, superoxide dismutase (SOD) and catalase (CAT) rat RNAs were acquired from Applied Biosystems (Hammonton, NJ, United States). Gene amplification was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Relative quantification by the $2^{-\Delta\Delta CT}$ method was realized by comparing to control groups as an internal calibrator[37,38]. Expression gene levels are shown as expression relative units.

Catalase activity
Reported CAT activity was determined according to the zymographic method by Gennady P Manchenko (1994). The method is based on the starch-iodine reaction. Thiosulfate in the staining solution is inactivated by hydrogen peroxide except at the sites of CAT activity, where hydrogen peroxide is destroyed enzymatically. The iodide is oxidized by hydrogen peroxide to iodine which forms a chromatophore with the starch and sites of CAT localization remain achromatic[29].

Western-blot assays
Western blot assays of tissue homogenates were performed to analyze the activation of Nrf-2 and Snail-1. Proteins were extracted from 100 mg of liver tissue using lysis buffer (50 mmol/L Tris-HCl pH 8.0, 150 nmol/L NaCl, 0.02% NaN_3). After centrifugation at 13000 rpm/5 min/4 ℃, supernatant was collected and quantified by Bradford assay. Briefly, 30 μg of total proteins were separated by 10% sodium dodecyl sulfate psulfate polyacrylamide gel electrophoresis under reducing conditions and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules CA, United States). Blocking was carried out using 3% dry milk for 2 h; primary antibody dilution was 1:500 for GAPDH (loading control) and 1:800 for Nrf2 and Snail-1 antibodies. (Abcam Biotechnology, Santa Cruz CA, United States). Antibody binding was revealed with a secondary anti-antibody diluted 1:5000-1:6000 using BM Chemiluminiscence kit (Roche Diagnostics, Indianapolis IN, United States). Densitometric analysis was realized with a Kodak 1D 3.5 Image analyzer (Eastman Kodak Co., Rochester NY) GAPDH was used as a cell fractionation control.

Statistical analysis
Normally distributed data were analyzed using *t* test, where statistical significance was $P < 0.05$. Data are shown as the mean ± SD. For real-time PCR experiments, results are shown as the $2^{-\Delta\Delta CT}$ value (mean ± SD), where the standard deviation was calculated as: $s = [s_{(GAPDH)}^2 + s_{(target\ gene)}^2]^{1/2}$, according to user bulletin 2 from Applied Biosystems.

Table 1 Weight at the beginning of the treatment and after caffeine treatment, serum markers enzymes in bile duct ligation and thioacetamide-intoxicated rats

Group	Healthy	TAA	TAA + CFA	BDL	BDL + CFA
Rats weight at the beginning of the treatment (g)	296.0 ± 9.73	284.0 ± 22.1	282.2 ± 9.1	280.1 ± 18.3	275.0 ± 19.0
Rats weight after CFA treatment (g)	321.0 ± 8.5	223.1 ± 14.0[b]	253.7 ± 5.7	219.4 ± 15.3	221.0 ± 17.5
AST (U/L)	226.0 ± 61.0	379.7 ± 179.8	318.2 ± 144.3	576.7 ± 70.0	329.5 ± 41.4[c]
ALT (U/L)	63.0 ± 1.0	132.7 ± 7.5	98.2 ± 28.7	214.7 ± 37.0	76.0 ± 10.6[d]

Treatment duration: 7 wk for thioacetamide (TAA) and 4 wk for bile duct ligation (BDL). [b]$P < 0.01$ *vs* TAA group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* BDL group. Data are shown as the mean ± SD (n = 10). CFA: Caffeine; AST: Aspartate transaminase; ALT: Alanine transaminase.

RESULTS

CFA prevents weight loss in TAA-intoxicated rats

Basal weight and weight at the end of treatment were registered in all groups. As shown in Table 1, CFA prevented weight loss of rats in the TAA model, which suggested that CFA had an effect in improving the nutritional status of rats measured solely by weight.

CFA dosed groups had less hepatocellular damage

AST and ALT levels were higher in TAA-intoxicated (1.7- and 2.1-fold respectively) and BDL (2.6- and 3.4-fold respectively) groups compared with the healthy rats group. The BDL + CFA group showed lower levels in AST compared to the BDL group (1.8-fold) ($P < 0.05$). The TAA + CFA group only showed a tendency to lower levels. Similarly, ALT levels in the BDL + CFA group were lower (2.8-fold) when compared against the BDL group ($P < 0.01$) (Table 1).

CFA treatment reduced both BDL and TAA-induced liver fibrosis

To test the antifibrogenic effect of CFA, morphological analysis of liver sections stained with Masson's was performed. Looking at the histology of the healthy group, we observed a normal morphology, with scarce ECM and hepatocytes arranged in a radial pattern. Histology of TAA and BDL groups showed an altered morphology, with thick collagen bundles, much more noticeable in the BDL group. In contrast, the treated groups TAA + CFA and BDL + CFA showed lower ECM content (Figure 1A). Quantification of ECM demonstrated a potent antifibrogenic effect of CFA. In the TAA + CFA group, fibrosis was lower by 80% compared to the TAA group ($P < 0.0001$). Likewise, in the BDL + CFA group fibrosis was lower by 38% compared to the BDL group ($P < 0.0001$) (Figure 1B).

The Knodell score indicated lower fibrosis in the TAA + CFA and BDL + CFA groups (3 ± 0.5 and 4 ± 0.5 points respectively) compared with the TAA and BDL cirrhotic groups (6 ± 0 and 6 ± 0 points respectively) (both $P < 0.05$) (Figure 1C).

Fewer inflammatory cells infiltrate in CFA groups

It was noted that CFA groups had a low amount of inflammatory infiltrate. TAA and BDL groups had a large number of inflammatory cells, especially the BDL group.

In contrast, both cirrhotic groups treated with CFA had a lower amount of inflammatory infiltrate, more evident in the TAA + CFA group (Figure 1D). The Knodell score resulted in lower necroinflammation in the treated groups, TAA + CFA and BDL + CFA (8 ± 0.5 and 9 ± 0.5 points), compared with the cirrhotic groups, TAA and BDL (16 ± 0 and 18 ± 0 points) (both $P < 0.001$) (Figure 1E).

Fibrogenic genes expression decrease with CFA treatment even in the continuous presence of liver fibrosis inducers

In addition to the histologic analysis we analyzed expression of the fibrogenic genes.

As expected, cirrhotic groups showed an increase in fibrogenic genes expression. In the TAA group there was a 5.3-fold increase for CTGF ($P < 0.01$), a 10.5-fold increase for collagen I (Col-1) ($P < 0.01$) and and a 4.3-fold increase for TGF-α1 ($P < 0.05$). In the BDL group fibrogenic genes expression also showed an increase; this increase was 11.6-fold for CTGF ($P < 0.01$), 21.5-fold for Col-1 and 3.5-fold for TGF-α1 ($P < 0.05$), compared with healthy rat group levels (Figure 2A-C). Treatment with CFA also induced a lower expression of fibrogenic genes; in the TAA + CFA group this was 3.5-fold lower for CTGF ($P < 0.01$), 3.5-fold lower for Col-1 ($P < 0.05$) and 3.1-fold lower for TGF-β1 ($P < 0.01$) compared with the TAA group. In the BDL+CFA group the reduction in gene expression was 5.0-fold lower for CTGF ($P < 0.01$), 3.0-fold lower for Col-1 ($P < 0.01$), and 1.5-fold lower for TGF-β1, indicating only a declining trend but no statistical significance, compared with BDL group (Figure 2A-C).

CFA limits pro-inflammatory genes expression in experimental liver fibrosis models

We performed an immunohistochemical determination of CD11b in hepatic tissue sections. We observed that CD11b positive areas in the TAA + CFA versus the TAA group were lower by 65.5% ($P < 0.01$), and the BDL group treated with CFA versus the BDL group were lower by 60.8% ($P < 0.05$) (Figure 3A). In addition to testing the anti-inflammatory effect of CFA at the protein level, we analyzed TNF-α expression by ELISA. Both liver cirrhotic groups showed an increase in TNF-α levels, 560.2 ± 67.8 pg/mL ($P < 0.0001$) for the TAA group,
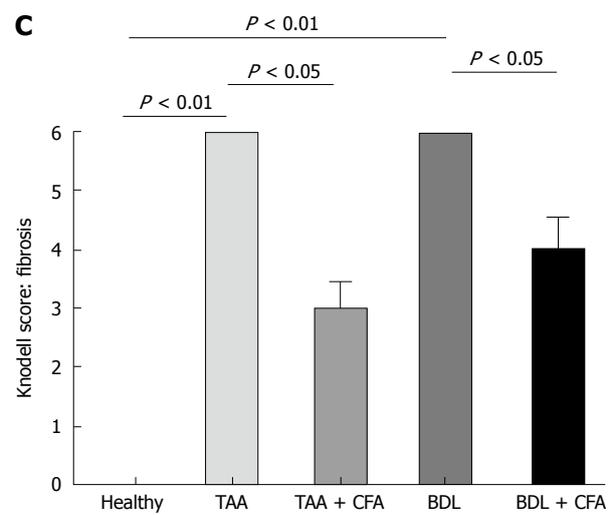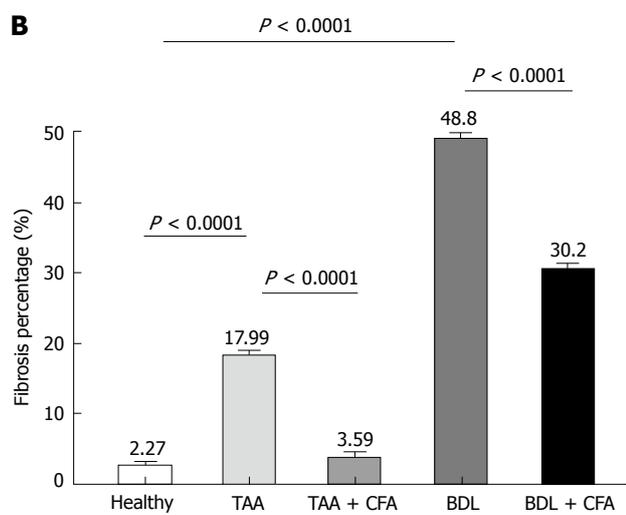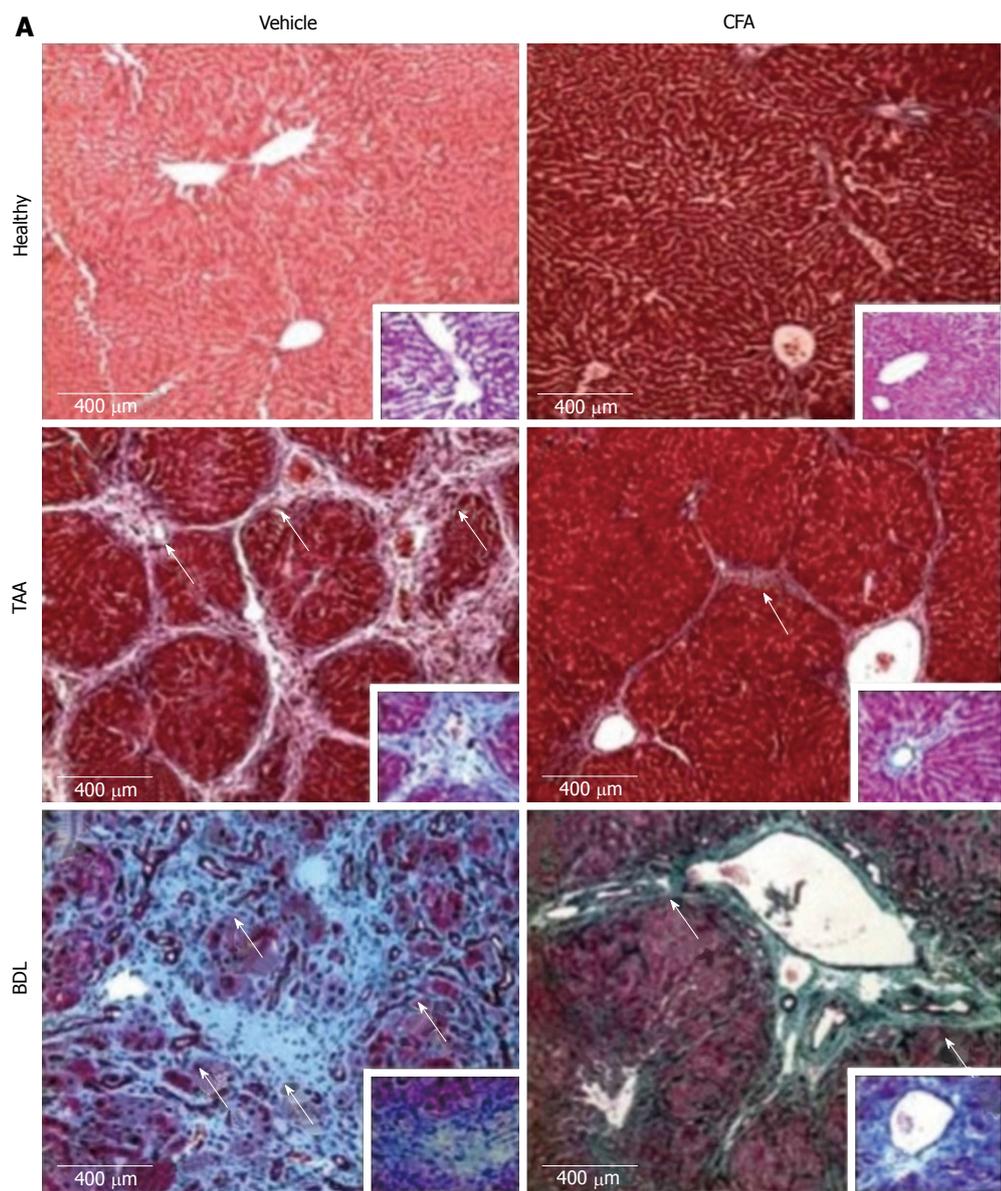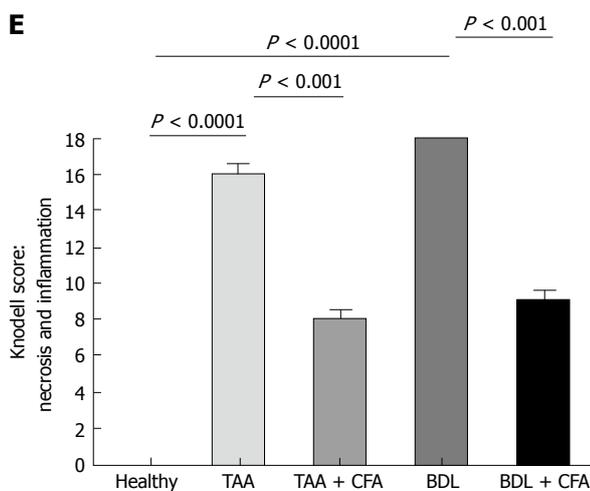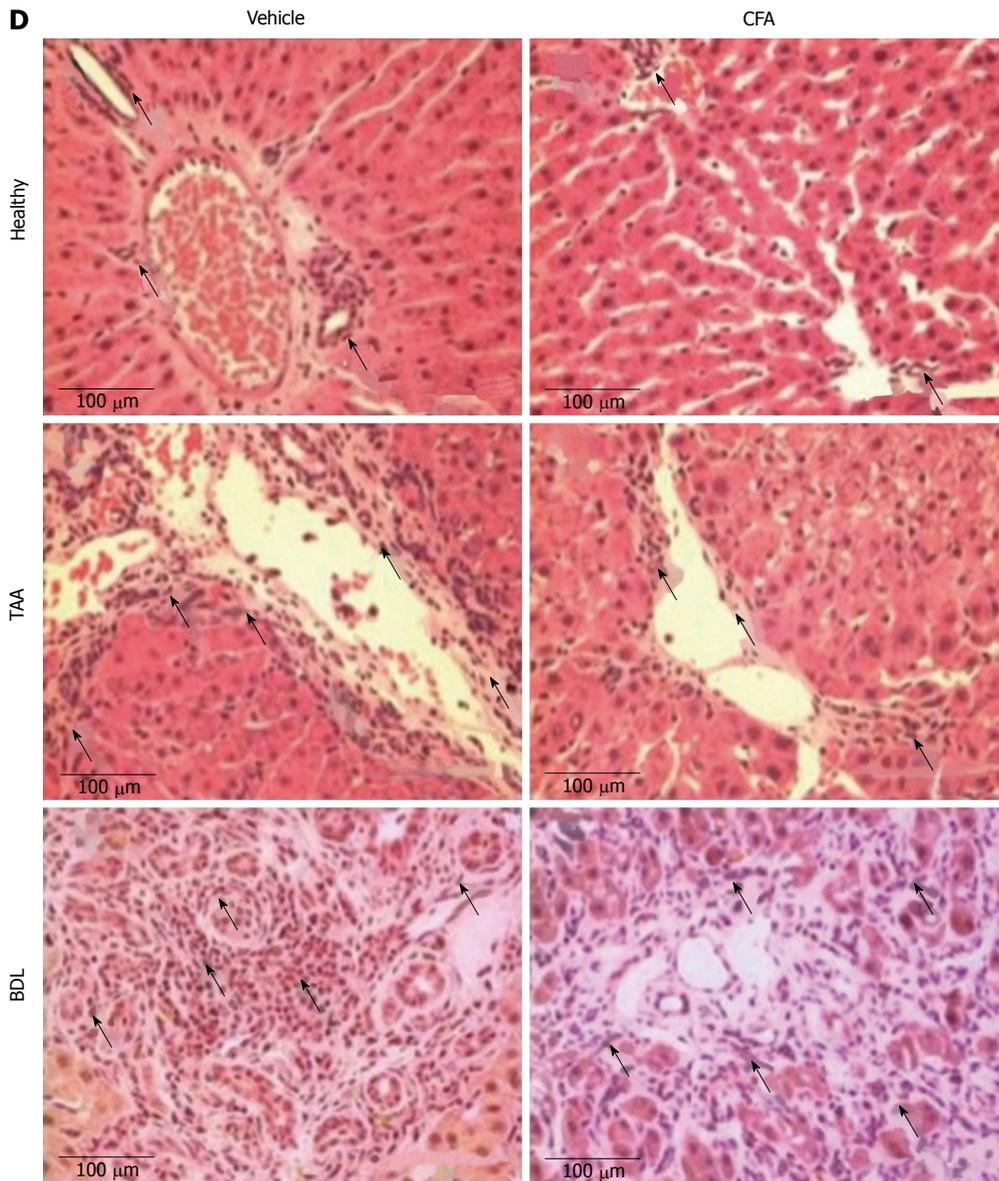
Figure 1 Macroscopic and histological differences and fibrosis index with caffeine treatment. A: Liver fibrosis percentage of healthy rats, caffeine (CFA)-treated, thioacetamide (TAA)-intoxicated, TAA-intoxicated treated with CFA, bile duct ligation (BDL) rats, BDL rats treated with CFA. Sections, 4 μm thick, stained with Masson's trichrome, × 10. White arrows show the extracellular matrix (ECM) (fibrosis); B: Fibrosis quantification. Fibrosis percentages are shown, they were obtained by computer-assisted morphometric analysis (Software Image pro plus 6.3); C: Knodell Index for fibrosis, sections 4 μm thick, stained with Masson's trichrome, × 10; D: Inflammatory infiltrate amount. Sections 4 μm thick, stained with hematoxylin and eosin, × 40. Black arrows show inflammatory cells; E: Knodell Index for fibrosis, sections 4 μm thick, stained with hematoxylin and eosin, × 40.

A

mRNA CTGF/GAPDH
$2^{-\Delta\Delta CT} \pm SD$

B

mRNA Col-1/GAPDH
$2^{-\Delta\Delta CT} \pm SD$

C

mRNA TGF-β1/GAPDH
$2^{-\Delta\Delta CT} \pm SD$

D

mRNA SOD/GAPDH
$2^{-\Delta\Delta CT} \pm SD$

E

mRNA CAT/GAPDH
$2^{-\Delta\Delta CT} \pm SD$

F

Antioxidant CAT activity

G
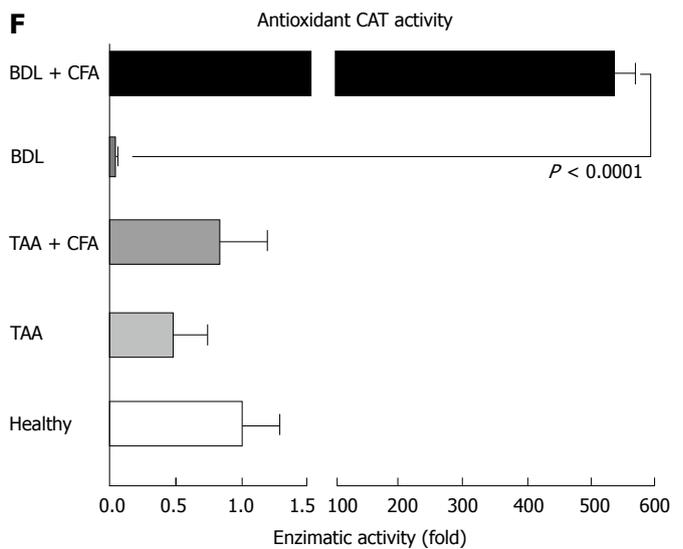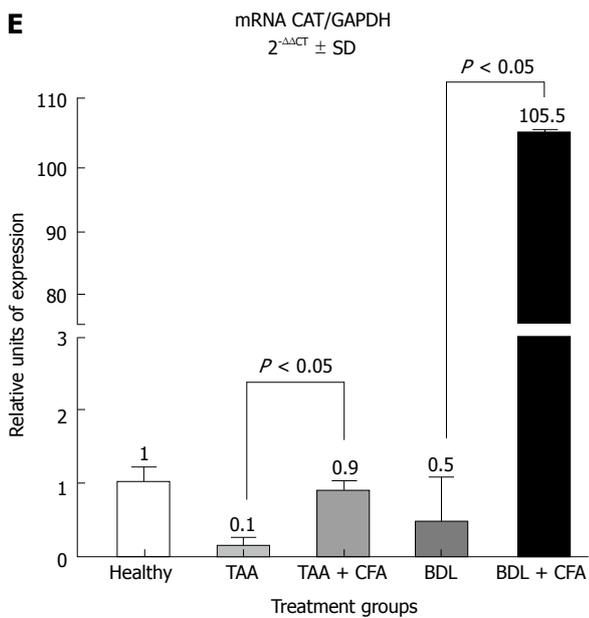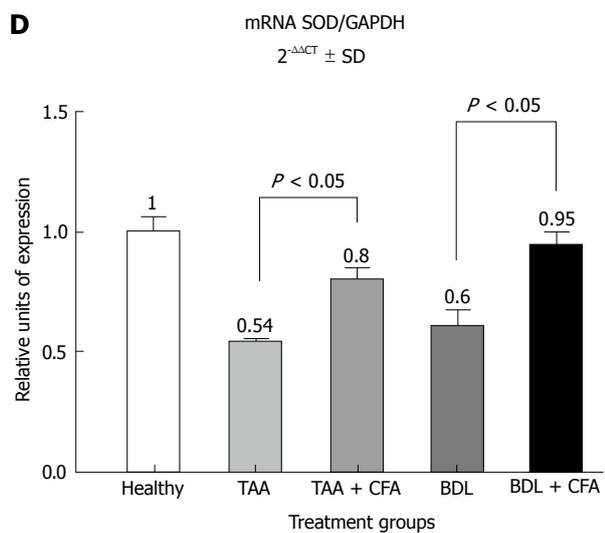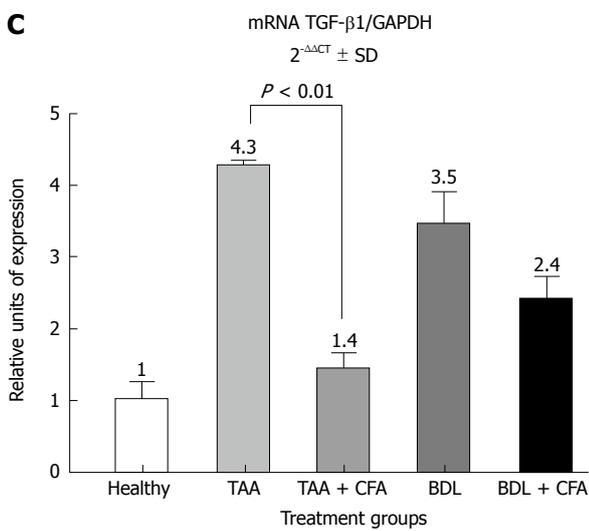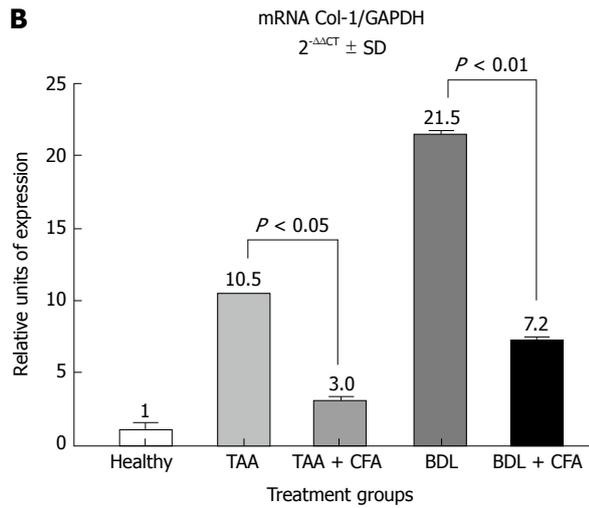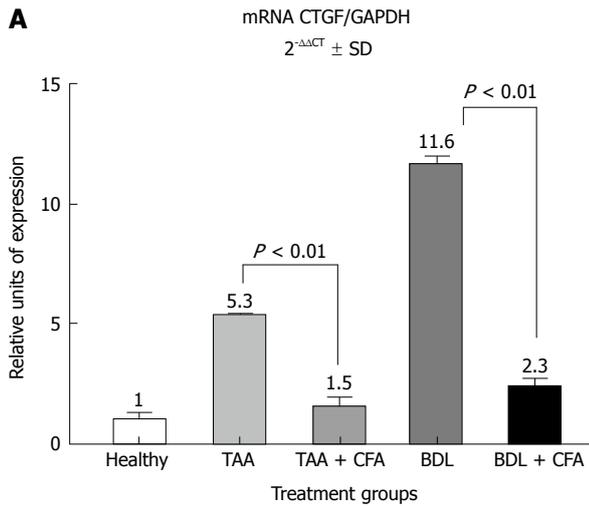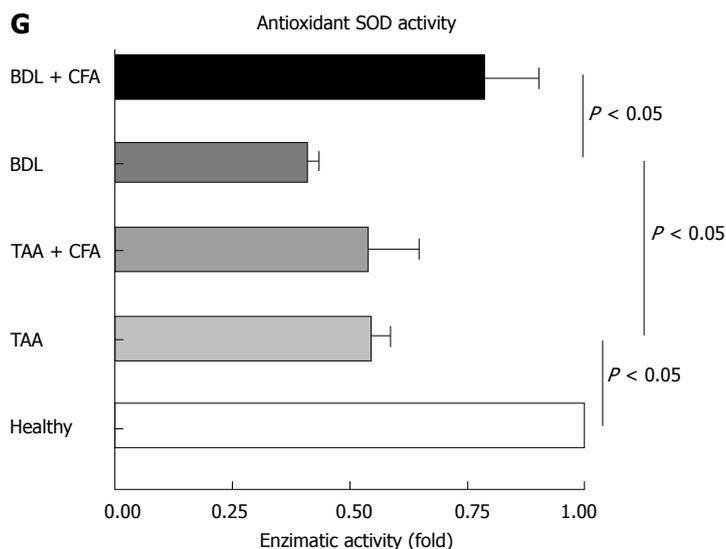
Figure 2 **Expression of fibrogenic and antioxidant genes in liver.** Reverse transcription-polymerase chain reactions were performed for connective tissue growth factor (CTGF) (A), collagen I (Col-1) (B), and transforming growth factor β1 (TGF-β1) (C), superoxide dismutase (SOD) (D) and catalase (CAT) (E). Gene amplification was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. CAT (F) and SOD antioxidant activity (G) were analyzed by zymography in acrylamide gels. TAA: Thioacetamide; BDL: Bile duct ligation; CFA: Caffeine.

and 590.3 ± 71.3 pg/mL ($P < 0.0001$) for the BDL group, compared with healthy rat group levels (140.4 ± 3.4 pg/mL). We observed lower levels in the CFA treated groups; for the TAA + CFA group 313.1 ± 56.6 pg/mL ($P < 0.01$), and for the BDL + CFA group 420.6 ± 166.1 pg/mL (Figure 3B).

Then, we analyzed at the molecular level the proinflammatory genes expression of TNF-α, IL-1β and IL-6. Both cirrhotic groups showed a significant increase in all proinflammatory genes expression; in TAA group this was 1.8-fold for TNF-α ($P < 0.05$), 1.8-fold for IL-1 ($P < 0.05$) and 32.3-fold for IL-6 ($P < 0.001$). In the BDL group it was 6.6-fold for TNF-α ($P < 0.001$), 3.2-fold for IL-1β ($P < 0.01$) and 128.2-fold for IL-6 ($P < 0.0001$) compared with the healthy rats group (Figure 3C-E). In groups treated with CFA we observed a decrease of expression; in the TAA + CFA group this was 1.6-fold for TNF-α ($P < 0.05$), 9-fold for IL-1β ($P < 0.01$); and 6.1-fold for IL-6 ($P < 0.05$); and in the BDL + CFA group there was a decrease of 9.4-fold for TNF-α ($P < 0.001$), 1.1-fold for IL-1 and 5.1-fold for IL-6 ($P < 0.001$) (Figure 3C-E).

Antioxidant enzymes gene expression and activity is modified by CFA intake

It is known that both liver fibrosis models course with an oxidative stress state. Thus, antioxidant enzymes expression levels were analyzed. We noticed that hepatocellular expression of SOD increased 1.5 ($P < 0.05$) and 1.6 ($P < 0.05$)-fold in TAA and BDL models, respectively, when they received CFA (Figure 2D). Likewise, CAT enzyme expression was significantly increased, showing an increase of 1.5-fold ($P < 0.05$) in the TAA model, and an increase of 211-fold ($P < 0.05$) in the BDL model (Figure 2E). To explore this last effect we performed an assay to measure SOD and CAT antioxidant activities, where we

found a strong correlation between mRNA expression and enzyme activity; in the BDL + CFA group antioxidant CAT activity was significantly increased (535-fold) ($P < 0.0001$) (Figure 2F) and SOD activity increased twice compared with BDL group (Figure 2G).

Activity of Snai-1 and Nrf2 by Western blot

Protein levels of the antioxidant transcription factor Nrf2 were significantly higher in both animal models compared to healthy rats. Treatment with CFA in liver-injured rats increased these levels significantly in both BDL and TAA models (Figure 4A). This increase suggests that Nrf2 could be inducing SOD and CAT expression, thus preventing liver damage.

On the other hand, the pro-fibrogenic transcription factor Snail-1 reduced its protein levels when the animals were treated with CFA in both animal models. These values were 2.33 and 3.25 times higher than healthy animals for BDL and TAA respectively, where animals treated with CFA presented values only of 0.77 and 1.58 times higher with respect to healthy animals (Figure 4B).

DISCUSSION

There are epidemiological data indicating that consumption of CFA protects against development of chronic liver disease or reduces the severity of the disease[12-14]. *In vitro* studies have shown beneficial effects of CFA useful in preventing HSC activation and perpetuation of this state[15,16]. Although there is a recent preliminary report describing the effect of coffee on liver fibrosis[39], here we describe a more comprehensible mechanism for CFA action on the most important molecules implicated in liver fibrosis. Our experiments were designed to compare CFA effects in two experimental liver fibrosis models, BDL and chronic TAA intoxication, to test whether the

A

P < 0.05

P < 0.001

P < 0.01

P < 0.001

Percentage of area CD11b+

Healthy   TAA   TAA + CFA   BDL   BDL + CFA

B

BDL + CFA

BDL

TAA + CFA

TAA

Healthy

P < 0.0001

P < 0.01

P < 0.0001

TNF-α levels in liver (pg/mL)

C

mRNA TNF-α/GAPDH
$2^{-\Delta\Delta CT} \pm SD$

P < 0.001

P < 0.05

Relative units of expression

1   1.8   1.1   6.6   0.7

Healthy   TAA   TAA + CFA   BDL   BDL + CFA
Treatment groups
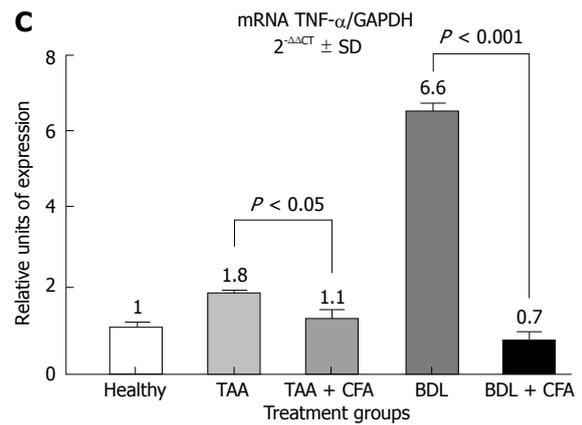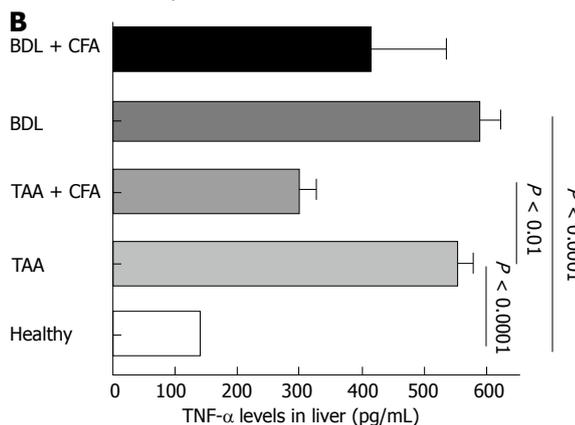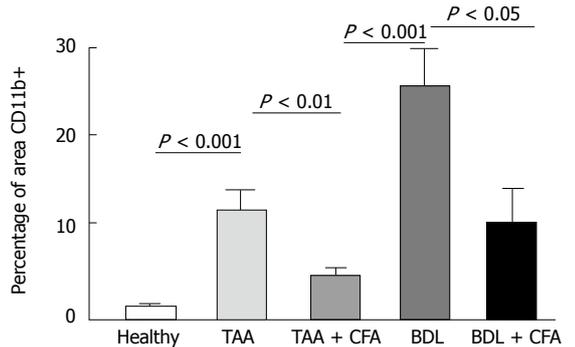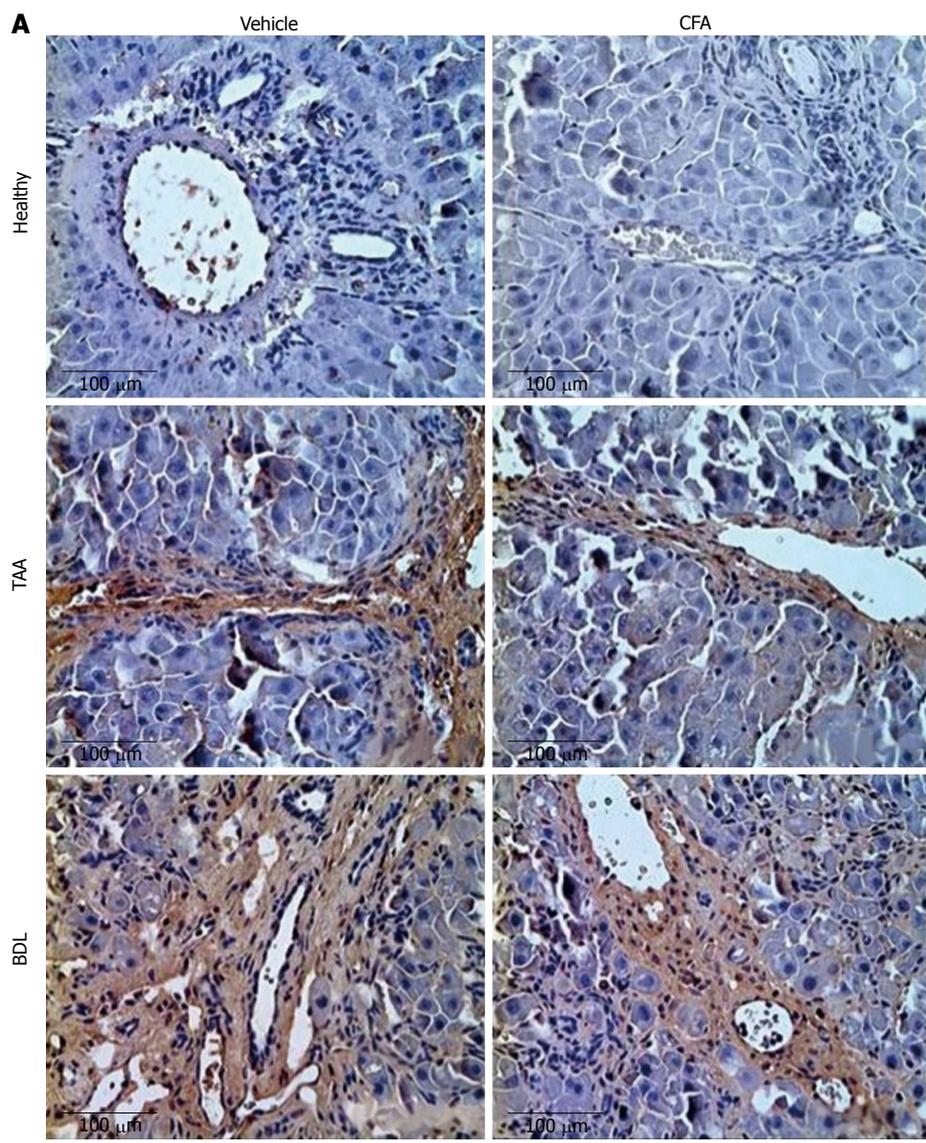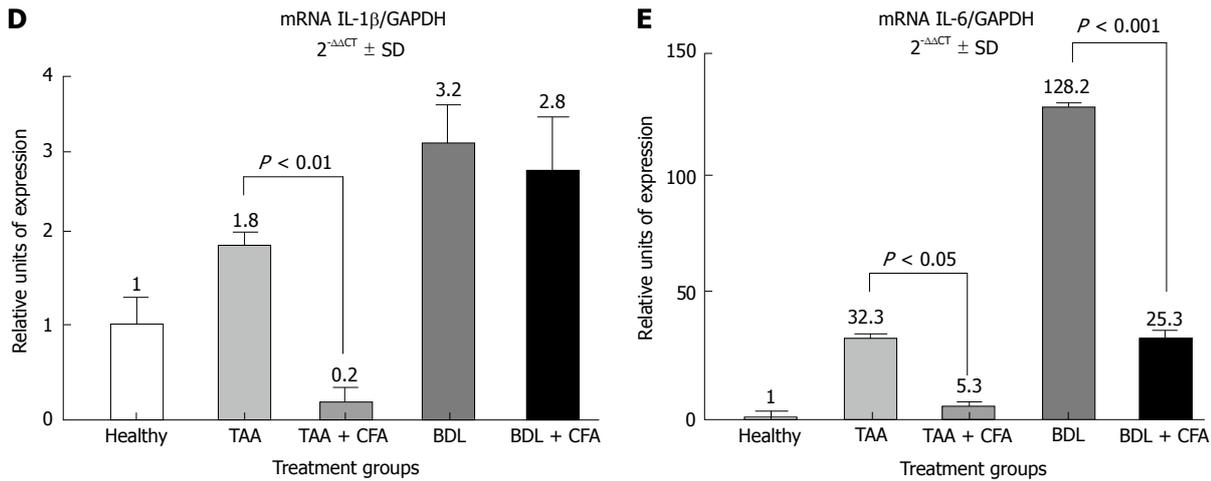
Figure 3 Expression of inflammatory genes in liver. A: Immunohistochemistry for CD11b, sections 4 μm thick, stained with Masson's trichrome, × 40. Results for CD11b positive area are shown as percentage; B: Tumor necrosis factor alpha (TNF-α) liver levels in different groups of treatment, performed by enzyme-linked immunosorbant assay; C-E: Reverse transcription-polymerase chain reaction were performed for TNF-α, interleukin-1β (IL-1β), IL-6. Gene amplification was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. TAA: Thioacetamide; BDL: Bile duct ligation. CFA: Caffeine.
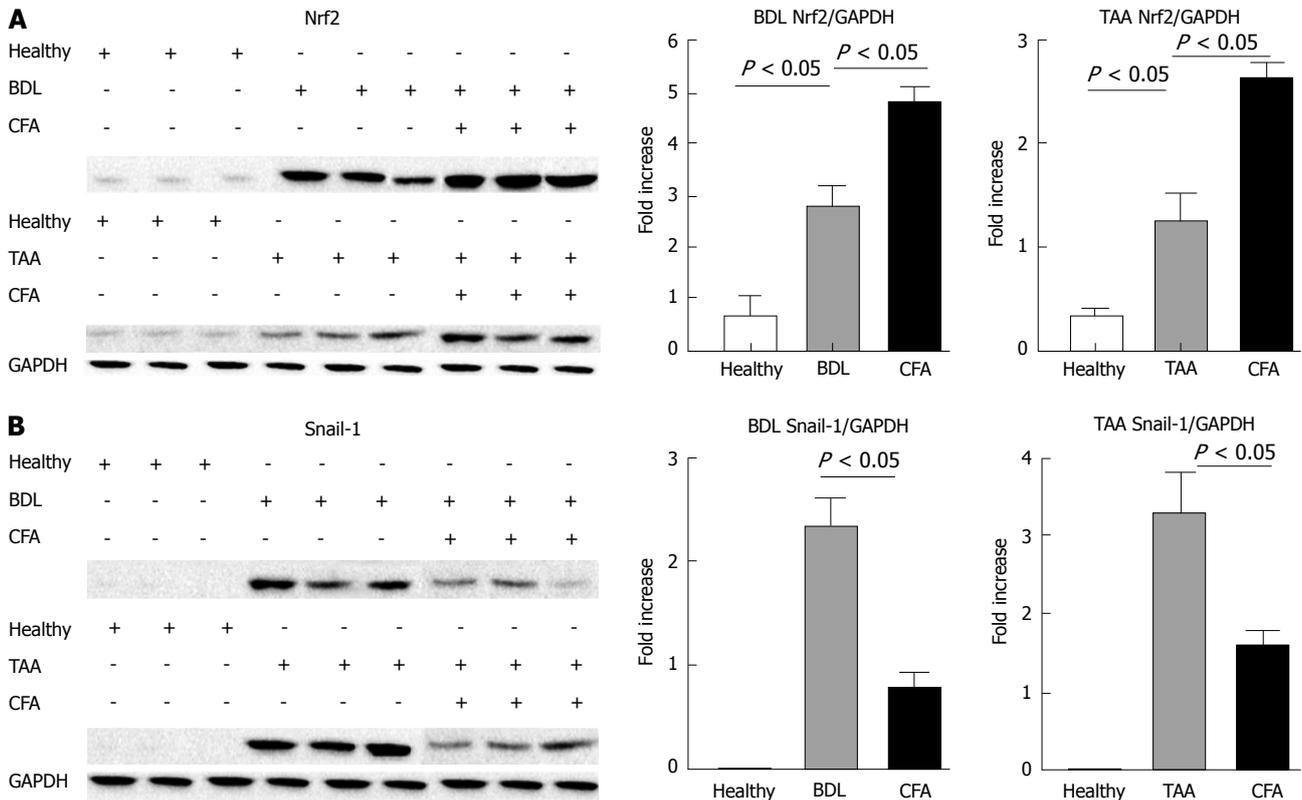
Figure 4 Expression of transcriptional factors. Western-blots were performed for the transcriptional factor Nrf2 (A) and Snail-1 (B). Densitometric values were normalized against the constitutive protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and represented like fold increase respect to healthy animals. TAA: Thioacetamide; BDL: Bile duct ligation. CFA: Caffeine.

preventive effects of CFA were independent of the etiology of liver damage. Dose was chosen based on positive effects of CFA regarding liver disease in epidemiological studies. These studies suggested a CFA consumption of 274 mg/d approximately in humans (2 cups of coffee)[12,40-43]. In our study we used a CFA dose of 15 mg/kg per day, because it is known that rat metabolism is ap-

proximately 10 times more accelerated than humans[42,43]. This animal dose is translated to a human dose using the body surface area (calculated with *Du Bois* formula)[44-47], and an adjustment of rat k_m (9) to human k_m (41), resulting in a human dose of 2.4 mg/kg (1.5 cups of coffee)[46], and expecting the same beneficial effects observed in the animal model and diminishing the probability of second-

ary effects.

It was observed that TAA-intoxicated rats treated with CFA had a higher body weight indicating overall improvement, probably because CFA reduces liver damage (as seen in histological analysis) and thus prevents the loss of appetite. Liver metabolic functions could be less altered, indicating that BDL and liver damage are the most important factors in weight loss.

In a previous experimental study of acute liver damage induced by a single dose of *D*-galactosamine/lipo-polysaccharide, CFA pretreatment correlated with lower levels of AST and ALT. Also, it has been reported in an animal model of liver damage with alcohol that transaminase levels are diminished by the effect of CFA. However these experimental models are different than the ones used in this communication[48,49].

Our results also show that CFA-treated groups had lower levels of AST and ALT. It was observed that levels of both enzymes were similar for both animal models (BDL + CFA and TAA + CFA), and comparing these levels with healthy rats, no significant differences were found. These results suggest that CFA treatment prevents hepatocellular damage resulting in normal levels of these enzymes with prevention of liver fibrosis.

A study in patients with chronic hepatitis C shows that a daily CFA consumption above 308 mg (approximately 2.25 cups of coffee) was significantly associated with reduced liver fibrosis, and the protective association persisted after controlling for age, sex, race, liver disease, body mass index and alcohol intake in all patients[12].

Our results presented in this report are similar; we showed that fibrosis was successfully prevented in the liver of rats treated with CFA, finding a strong effect of CFA on ECM content in rat liver, showing 80% reduction in the TAA + CFA group and 38% reduction in the BDL + CFA group. Both results show that CFA had a powerful preventive effect on the development of fibrosis. The Modified Histological Activity Index of Knodell resulted in significantly lower fibrosis in both treated groups.

In a previous *in vitro* study, it was found that CFA increases intracellular cAMP, resulting in inhibition of CTGF *via* Smads proteosomal degradation[18]. CTGF has similar effects to TGF-β1 as ECM production stimulation, chemotaxis, proliferation and integrin expression. Our *in vivo* data shows that CFA has a strong effect on hepatic CTGF expression, resulting in lower expression of profibrogenic and pro-inflammatory genes. TGF-β1 is a major fibrogenic mediator in which expression is increased in inflamed liver and it is considered the principal fibrogenic component[47]. It has been suggested that TGF-β1 up-regulates gene expression of connective tissue, and Col-1 in activated HSC[6,47]. Results obtained in CFA-treated groups are very interesting, since in both animal models Col-1 expression was significantly lower, a result that correlates with fibrosis percentage shown for each group with CFA.

It has been observed that liver fibrosis process development is accompanied by inflammation, in which pro-

inflammatory cytokines play an important role in the perpetuation of signaling pathways[47,50]. Furthermore, one report in alcoholic liver injury shows that CFA decreased serum and tissue inflammatory cytokines levels[48]. In this study we found that induction of both liver fibrosis models had a large amount of inflammatory cell infiltrate; in contrast, CFA-treated groups showed decreased number of inflammatory cells, necroinflammation, CD11b positive areas and TNF-α levels. These results at cellular and molecular levels match with serum and tissue inflammatory cytokines levels in other studies about CFA[48].

IL-1β expression was reduced in CFA-treated groups. ECM signaling is of great importance as it serves as a reservoir of various cytokines such as TGF-β1, TNF-α, platelet-derived growth factor (PDGF), IL-6 and IL-1β, protecting these factors for proteolysis and modulating its bioactivity and bioavailability. In this microenvironment, the cytokines might have a key role in the onset of fibrosis, and perpetuating inflammation[47], where CFA treatment could be useful to break this inflammatory circle, as demonstrated in our different experiments.

HSC have an important role in fibrosis and fibrosis development. HSC activation and proliferation, and collagen synthesis are influenced by factors derived from Kupffer cells (TGF-β1, TNF-α, IL-1β, IL-6 and IL-4), endothelial cells (PDGF) and hepatocytes (insulin-like growth factor). Also, TNF-α, IL-1β, TGF-β1, IL-6 and IL-4, and PDGF are regulated by NF-κB and this promotes inflammatory signaling pathway perpetuation[9-11]. We found that CFA promotes lower levels of pro-inflammatory cytokines expression. These findings could be due to the fact that CFA prevents HSC activation and ECM production.

ROS activate the NF-κB pathway. It is known that both liver fibrosis induction models used here, course with an oxidative stress state. Because of this, we measured antioxidant enzymes gene expression levels to monitor them with CFA treatment[51].

As expected, untreated groups showed lower expression of antioxidant enzymes SOD and CAT, indicating indirectly an oxidative stress state. CFA-treated rats showed higher levels of antioxidant enzymes, especially of CAT in the BDL + CFA group, that could be explained by the type of substrate metabolized (hydrogen peroxide). SOD catalyzes O_2^- dismutation into O_2 and H_2O_2. In contrast, CAT catalyzes decomposition of H_2O_2 into O_2 and H_2O[45]. Considering this, we assume that CAT was much higher in the BDL + CFA group, due to accumulation of H_2O_2 at 4 wk of treatment by SOD action. To verify this last effect we performed an assay to measure CAT antioxidant activity, where we found a strong correlation between mRNA expression and enzyme activity, especially in the BDL + CFA group; antioxidant CAT activity was significantly increased ($P <$ 0.0001) compared with BDL group.

Along with these results, the significant higher expression of transcription factor Nrf2 in the CFA treated groups supports the evidence of the potent antioxidant

effect of CFA acting as an important hepatoprotector agent in the presence of a chronic organ aggression. These results agree with the report by Boettler *et al*[52] where they found higher expression of Nrf2 in humans with consumption of coffee with respect to normal diet where Nrf2 expression was reduced. In response to oxidative stress Nrf2 is activated, translocates to the nucleus and binds to the promoter of its target genes such as CAT and SOD inducing their expression. Nrf2 half life is around 13-20 min. In oxidative stress and in the presence of antioxidant molecules like quercetin, the half-life is duplicated[53]. Thus, given that CFA is also an antioxidant molecule, we believe the same thing may be taking place, though it would require additional experiments to test this hypothesis. Nguyen *et al*[54] have suggested that in oxidative stress, Nrf2 diminishes its degradation accumulating in nucleus increasing its transcriptional activity.

On the other hand, activation of HSC is a complex process where the transcriptional factor Snail-1 has an important role.

In vertebrates Snail-1 is activated by a different signal pathway from ERK2, NF-κB and phosphatidylinositol 3-kinase[55-57]. All these pathways have been involved in activation of HSC. Several authors have reported the overexpression of Snail-1 in pathological conditions associated with ECM deposition[23,24]. *In vitro* studies showed that Snail-1 is expressed by HSC and its transcription is augmented *in vitro* and *in vivo* in activated HSC compared with quiescent HSC. At the protein level, the nuclear translocation of Snail-1 in activated HSC was observed[58].

Scarpa *et al*[25] reported that the use of an adenovector expressing Snail-1 small-interfering (sh) RNA to silence Snail expression in HSC isolated from mouse, dramatically reduced activation-related genes α-smooth muscle actin (α-SMA) and Col-1 and increased quiescence-related gene peroxisome proliferator-activated receptor, evidencing the important role of Snail-1 in HSC activation (Snail-1 transcription factor *Am J Physiol* 2011). However other studies suggest a multiple cell-type origin of cell source for Snail-1 in human liver fibrosis; thus, this fact should be analyzed. Indeed, it was reported that Snail-1 overexpression induces epithelial mesenchymal transition and siRNA against Snail-1 attenuated this epithelial mesenchymal transition. Immunostaining of fibrotic livers from mice treated with CCl4 revealed the presence of Snail-1^{+}, α-SMA^{+} cells as well as Snail-1^{+} α-SMA[-] and Snail-1-α-SMA^{+} cells along the fibrotic septa. This staining pattern could be explained by the epithelial mesenchymal transition process where hepatocytes transdifferentiate to mesenchymal cells resulting in new HSC[59-61].

In the same way, Dooley *et al*[62] observed a hepatic marker at the border of the inflamed region from human liver Snail-1^{+} cells lacking transferrin and they hypothesize that these cells are hepatocytes in a later stage of transition to mesechymal cells.

In our results, CFA treatment diminished Snail-1 expression in rats with chronic liver injury suggesting that CFA prevents HSC activation and suggesting its protector effect on fibrosis development. Our results together allow us to propose CFA use in pathologies with early chronic damage before the establishment of fibrosis.

The observed effect of CFA in this work on necrosis of hepatocytes and on HSC activation could be explained by an indirect effect of CFA. This might be taking place through a decrease of oxidative stress in the liver produced principally by Kupffer cells which secret cytokines activating HSC.

From the very beginning of its administration, CFA neutralizes free radicals and induces antioxidant molecules production which protect hepatocytes from CCl4 damage; this means there is less hepatocyte death, reflected in there being lower levels of ALT and AST found in CFA treatment groups. TGF-β and TNF-α production is decreased rendering a drop in HSC activation, and consequently, less fibrosis. On the other hand, ECM deposition and loss of microvilli on hepatocytes caused by CCl4 intoxication blocks the free flow of nutrients causing hepatocytes death. It was found in this paper that CFA treatment yields less fibrosis, less block of nutrients and less hepatocyte death. However, a direct effect of CFA on hepatocytes and HSC cannot be ruled out.

COMMENTS

Background
Hepatic stellate cells (HSCs) activation is a major hallmark in liver fibrosis, which is perpetuated by growth factors and pro-inflammatory molecules. Caffeine (CFA) modifies these events *in vitro*.

Research frontiers
CFA inhibits the transcriptional factor Snail-1, down-regulating profibrogenic genes, and activates the Nrf2 inducing antioxidant enzymes system, preventing inflammation and fibrosis.

Innovations and breakthroughs
CFA treatment diminished Snail-1 expression in rats with chronic liver injury suggesting that CFA prevents HSC activation and provides a protector effect on fibrosis development. Their results together allow the authors to propose CFA use in pathologies with early chronic damage before the establishment of fibrosis.

Peer review
The present manuscript provides a detailed study on the effect of CFA on experimental liver fibrosis in rats. Overall, this is a very interesting paper. The presented data is throughout of very good quality and the conclusions drawn are supported by sufficient data.

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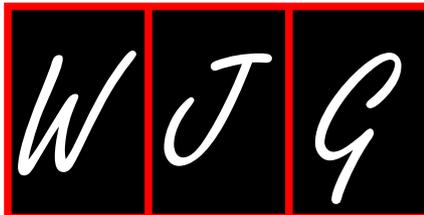
Superficial esophageal lesions detected by endoscopic ultrasound enhanced with submucosal edema

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Abstract

AIM: To determine if there is consistency between endoscopic ultrasound (EUS) findings and pathological results for detecting lesions of different depth in the esophageal mucosa.

METHODS: A canine (Beagle) model was established in which lesions of different depths were created in the esophageal mucosa by thermal burning. Seventy-two hours later, these lesions and adjacent tissue in the esophagus were examined by EUS. EUS findings including infiltrating depth, strength of echogenicity and homogeneity were recorded. Dogs were sacrificed and tissue specimens were obtained. We then compared the EUS findings with the pathology reports.

RESULTS: Thermal burns created at different power settings caused lesions of different depth in the esophageal mucosa. When the echo strength was shifted from high, medium, to low echogenicity, an increase in the infiltrating depth of the lesion was noted, which coincided with results of the pathology examination. Obvious submucosal edema visualized by EUS was also detected by pathology. Furthermore, because of the enhancement caused by the submucosal edema, the lesions invading into the submucosa were easily visualized by EUS.

CONCLUSION: There is consistency between EUS findings and pathological results of esophageal lesions with different depths. Submucosal edema can serve as an ultrasonic contrast agent.

Key words: Endoscopic ultrasound; Pathology; Lesion; Esophagus; Canine

Core tip: Nowadays, endoscopic ultrasound (EUS) is an optimal modality to detect early esophageal cancer (EC); however, it is still unknown whether there is correlation between EUS findings and pathological results.

In this animal study, superficial esophageal lesions with different infiltrating depth in dogs were created by thermal burning. There is consistency between EUS imaging and pathology. The accompanied submucosa edema can sever as an ultrasonic contrast agent.

INTRODUCTION

Treatment options for esophageal cancer (EC) differ according to the depth of the lesion[1-4]. According to a recent edition of the American Joint Committee On Cancer (AJCC) and the International Union Against Cancer (UICC) staging system and guidelines, patients with early EC confined solely to the mucosa (substaged as T1a or Tm) are able to undergo endoscopic mucosal resection; however, patients with lesions invading into the submucosa (sub-staged as T1b or Tsm) require esophagectomy[5,6]. Therefore, differentiating the depth or stage of the disease is of great importance preoperatively.

Depending on the frequency strength of the probe that is placed into the lumen of the esophagus, three to seven histological layers of the esophageal wall can be discerned[7-10]. In the clinic, endoscopic ultrasonography (EUS) is superior to other modalities such as computed tomography (CT) and positron emission tomography (PET) for distinguishing the tissue layers of the esophageal wall, and it has become the method of choice for determining the depth of esophageal lesions[11-18].

However, EUS has several limitations for detecting esophageal lesions. First, the mucosal layers (including squamous epithelium, lamina propria, and muscularis mucosa) have similar echoic characteristics, especially acoustic impedance. Thus, these layers have similar echogenicity, and it is very difficult to distinguish one from another. Second, because ultrasound propagates a similar speed through the different layers of the esophageal wall, it is difficult for EUS to detect minor differences in ultrasonic energy loss (also presenting as echoic gray scale) among the layers[19,20]. Other factors that may influence the efficacy of EUS are the size of the lumen in the esophagus (which prevents the ultrasonic probe from pressing close to the mucosa), the motility of the esophagus, and the experience of the endoscopist[21,22]. Therefore, the accuracy of EUS for determining the T stage of EC is poor and diverse in the literatures[5,6]. In addition, previous reports did not provide information on the accuracy of EUS for identifying the T1 sub-stage of EC, which is an important factor by which physicians determine treatment. Our team tried to sequentially combine

submucosal saline injection (SSI) with EUS to detect the T1 sub-stage of EC, and our preliminary data revealed that this technique is nearly 90% accurate. Therefore, SSI may enhance EUS for early EC diagnosis[23,24].

However, many questions about the efficacy of EUS for EC diagnosis remain[25,26]. For example, does the echo in the EUS reflect the actual structure or component of the esophageal wall? Is there consistency between EUS findings and the results of pathological examinations? What are the echoic characteristics of the water/liquid in the submucosa? Can the water/liquid enhance EUS, and if so, how? To answer these questions, we used different doses of thermal burns to create superficial lesions with different infiltrating depth in the esophageal mucosa, and EUS examinations were conducted to detect these lesions in a canine model.

MATERIALS AND METHODS

Animals and anesthesia

The experimental protocol used in this study was approved by the animal welfare and ethics committee of Sun Yat-sen University Cancer Center (approval number: GZR2012- 114). Male adult dogs (10 kg) were provided by the medical animal center in the north campus of Sun Yat-sen University. The flow diagram of this study is shown in Figure 1. The dogs were kept separately with an absolute diet for 8 h and dehydrated for 6 h before the experiment. Dogs were then injected intraperitoneally with 0.03 mg/kg pentobarbital sodium for premedication and then injected peritoneally with 0.03 mg/kg pentobarbital sodium per hour for maintenance.

Devices

EUS examinations were performed using an Olympus GF-UM2000 endoscopy system with a 12 MHz ultrasonic probe (Olympus Co. Ltd., Japan). An Endoscopic Electrosurgical Workstation was purchased from ERBE Co. Ltd., Germany, which included an argon plasma coagulation (APC) system and a high frequency electrocoagulation generator (HFE).

Canine model of superficial lesions with different infiltrating depths in the esophagus

Guided by endoscopy, esophageal lesions of variable infiltration depths were induced in anesthetized dogs using APC (40 W, 1.4 L/min, 2 s each time), short-time HFE (40 W, 2 s each time), medium-time HFE (40 W, 5 s each time), and long-time HFE (40 W, 10 s each time). Superficial round lesions of approximately 1 cm × 1 cm were formed, and the gap between lesions was approximately 5 cm. Seventy-two hours later, the dogs underwent EUS examination. The echoic characteristics of the lesions with different infiltrating depths (including the echogenicity of the lesions, leading and trailing edge, the echogenicity of each layer in the esophageal wall, *etc*.) and submucosal edema were recorded. Then, the dogs were sacrificed, and samples of the normal and abnor-

Male beagle dog

Canine model established

| Normal esophageal mucosa or structure | Lesion invaded into submucosa caused by mid-time HFE | Lesion involved in deep mucosa caused by short-time HFE | Lesion invaded into submucosa caused by mid-time HFE | Lesion invaded into muscularis caused by long-time HFE |

Endosonography and pathology

EUS examination three days later

Sacrifice Beagle dog and obtain pathology (H-E dyeing)

Analysis and hypothesis certified

Compare the EUS findings with pathological results

Repeat experiments three times

Certify consistency between ultrasonography and pathology as well as ultrasonic contrast role of submucosal edema in regard to esophageal lesions with different depths

Figure 1 Flow diagram of study protocol. EUS: Endoscopic ultrasonography; HFE: High frequency electro-coagulation generator.

mal esophagus containing superficial lesions with variable infiltrating depths were extracted and stored in 10% formaldehyde solution. The specimens were stained with hematoxylin and eosin (HE). In addition, we focused on submucosal edema and its role as an ultrasonic contrast agent. We compared the EUS findings with the corresponding pathological results to determine whether both were in agreement. Details regarding the creation of this canine are presented in Figure 2. Examinations were performed by an endoscopic expert with over 10 years of experience. Similarly, pathological examinations were performed by an expert with over 10 years of experience. The above experiment was repeated three times.

RESULTS

Thermal burns caused superficial lesions in the esophagus with different infiltrating depths

After exposure to APC and HFE at different power levels, lesions in the esophageal mucosa could be observed,

although the clarity was poor due to obvious congestion and edema in the adjacent mucosa. Seventy-two hours later, the edema in the surrounding mucosa decreased, and the lesions became more apparent. However, we only observed lesions in the lumen and could not confirm the exact burn depths by ordinary endoscopy. Therefore, we proceeded with EUS examination.

EUS examination of lesions and submucosal edema in the esophagus

Three layers of the normal esophagus can be visualized using EUS with a 10 MHz ultrasonic probe: the thickest, high echoic belt is revealed as the first layer, which corresponds to the mucosa and submucosa; a thick, low echoic belt corresponding to muscularis propria; and a narrow, high echoic belt that is thought to be the adventitia and other dense connective tissue (Figure 3A). In the canine model, different power levels can cause different burns with variable infiltration depths, as visualized by EUS. APC (Figure 4A), short-time HFE (Figure 4C),
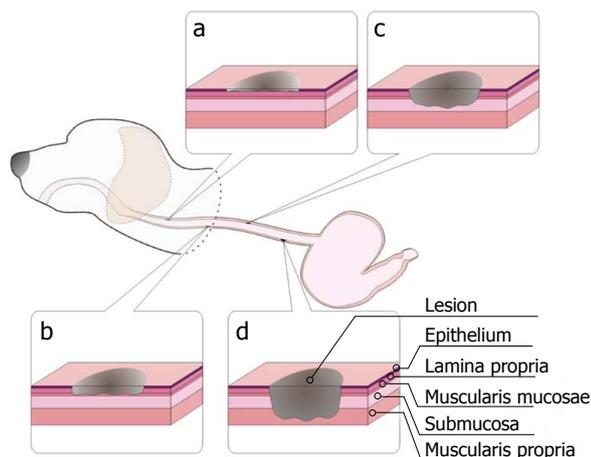
Figure 2 Schematic diagrams of superficial lesions in the esophagus with different depths in a canine model.

medium-time HFE (Figure 5A), and long-time HFE (Figure 5C) resulted in superficial mucosal injury, deep mucosa injury, injury involving the submucosa, and injury invading into the muscularis propria, respectively. The lesions presented as middle to low echogenicity, which were lower and more asymmetrical echoic compared to the normal mucosa on sonography, particularly in the case of lesions invading into the muscularis propria. Additionally, we found that the high echogenicity of the mucosa was related to the integrity of the squamous epithelium, especially the keratin pearl and intercellular bridge. Once these keratin pearls or intercellular bridges disappeared, the echo of the lesion decreased as shown in Figures 3B, 4B and 5B. Furthermore, a low echoic belt was the evidence of edema in the submucosa of the lesions, except in the lesions invading the muscularis propria. The edema was observed as a low echoic belt with diverse light spots, which separated the mucosa and submucosa, as shown in Figures 4A, C, and 5C. In addition, echo enhancement was observed in the trailing edge of the low echoic belt, confirming that the low echoic belt was water-filled tissue in the submucosa. Therefore, due to the contrast of the low echoic edema, the layer of muscularis propria was displayed as a smooth, middle echo belt. However, in the case of lesions involving the muscularis propria, there was no low echoic belt in the submucosa and no obvious boundary among the layers of the esophagus.

Pathological results

There were, in successive order, squamous epithelium, lamina propria, muscularis mucosa, submucosa, and muscularis propria and adventitia in the normal esophageal wall of dogs. The squamous mucosa was thick, with a distinct keratin pearl, intercellular bridges, and thin lamina propria and muscularis mucosa. The submucosa was also thick, and it was characterized by blood vessels, lymphatic and connective tissues. The muscularis propria contained a ring muscle layer (inner) and a longitudinal

muscle layer (outer), divided by thin connective tissue. The adventitia was composed of fibrous connective tissue (Figure 3B). Tissue sections of the lesions containing superficial mucosa showed partial epithelial degeneration with a complete squamous component and intact muscularis mucosa. Prominent submucosal edema was observed, and there were no obvious inflammatory cells in the submucosa (Figure 4B). Similarly, total degeneration was observed in the mucosa, with a partial squamous cell component and broken muscularis mucosa. Prominent submucosal edema was also found in the lesions containing deep mucosa. There were no obvious inflammatory cells in the submucosa (Figure 4D). In lesions that invaded into the submucosa, the squamous cell structure and muscularis mucosa disappeared with submucosal edema, and inflammatory cells were clearly present in the submucosa (Figure 5B). For lesions that invaded into the muscularis propria, the squamous cell structure and muscularis mucosa disappeared; however, this was not accompanied by submucosal edema and inflammatory cells in the submucosa (Figure 5D).

Consistency between EUS findings and pathology

In this study, echogenicity reflected characteristics of tissues propagated by ultrasound (Table 1). First, there was a correlation between the echoic belts or layers in the tissue sections. The esophageal mucosa and submucosa of dogs presented as a high echoic belt on sonography with a 10 MHz probe; the second low echoic belt corresponded to the muscularis propria; and the adventitia (mainly composed of dense fibrous connective tissue) was observed as a thin high echoic belt by EUS. Second, the ultrasonic echo decreased with increasing infiltration depth, and the inner echogenicity of the lesion changed from homogeneous to heterogeneous. The echoic changes (high-middle-low) corresponded to gradual changes of the lesions with intact squamous epithelium (complete, incomplete, and totally broken) and the different infiltrating depths of the lesions (located in the mucosa, involving the submucosa, and invading into the muscularis propria). Third, the identification of submucosal edema by sonography and pathology was highly correlated. Submucosal edema was obvious in lesions which were located in the mucosa, whereas the submucosal edema was narrow in lesions involving the submucosa. However, there was no submucosal edema in lesions involving the muscularis propria. With the help of submucosal edema, the infiltrating margin of the lesion was significantly distinguished. Therefore, we were able to easily judge whether a lesion invaded into the submucosa. After long-time HFE thermal burns, a lesion with a low echoic belt extending from the lumen to the second layer was difficult to distinguish from the adjacent tissue. Pathology revealed that the lesion already invaded into the muscularis propria, and it contained a complex composition of inflammatory cells and blood/lymphatic vessels. In addition, using the contrast of submucosal edema or low echoic belt, the layers of the esophagus

EUS image   **Schematic diagram**

Labels: Adventitia, Muscularis propria, Epithelium + lamina propria, Muscularis mucosae, Submucosa

Histopathology   **Schematic diagram**

Labels: Adventitia, Muscularis propria, Submucosa, Muscularis mucosae, Lamina propria, Epithelium
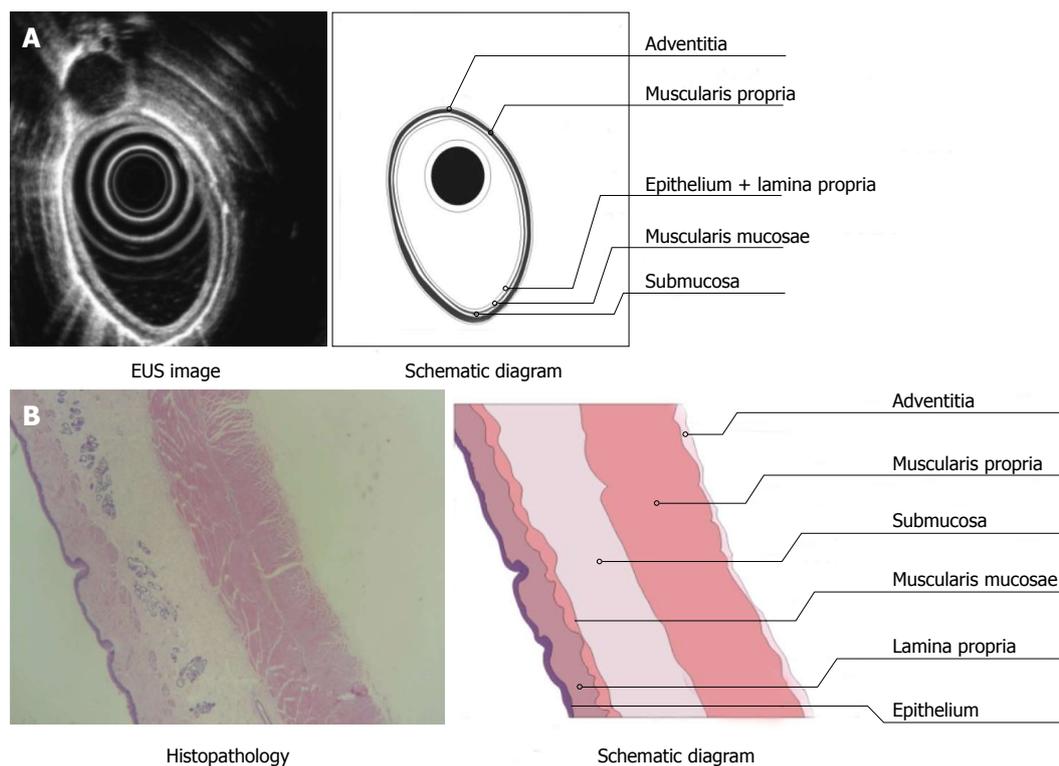
Figure 3 Endoscopic ultrasonography and tissue examination of the normal esophagus in a beagle dog. A: The three layers of a normal esophagus, as visualized by endoscopic ultrasonography (EUS); B: Tissue examination showed that the esophageal wall is composed of the mucosa (including squamous epithelium, lamina propria, and muscularis mucosa), submucosa, and muscularis propria and adventitia.

Table 1 Identification of esophageal lesions with different depths was consistent between the endoscopic ultrasonography findings and pathology results

	Tissue echogenicity					Echogenicity of submucosal edema		
	Echoic belts of esophagus	Echo strength of lesion	Involved layers	Homogeneity of lesion	Boundary among layers	Submucosal edema belt	Front edge of edema belt	Width of edema belt
EUS findings	H-L-H	/	/	/	Clear	/	/	/
Normal mucosa								
Superficial mucosa	H-L-H	H	1st	Homogeneous	Clear	YES	Smooth	Wide
Deep mucosa	H-L-H	H	1st	Homogeneous	Clear	YES	Less Smooth	Middle
Submucosa	M-L-H	M	1st	Heterogeneous	Clear	YES	Unsmooth	Narrow
Muscularis propria	L- H	L	1st-2nd	Chaotic	Dim	None	None	None
Pathology results								
Normal	Mu/SM-MS -AD	/	/	/	Clear	/	/	/
Superficial mucosa	Mu/SM-MS -AD	Mu	MU	Homogeneous	Clear	Yes	Smooth	Wide
Deep mucosa	Mu/SM-MS -AD	Mu	MU	Homogeneous	Clear	Yes	Less smooth	Middle
Submucosa	SM-MS -AD	SM	Mu-SM	Heterogeneous	Clear	Yes	Unsmooth	Narrow
Muscularis propria	MS-AD	MS	MU-SM-MS	Chaotic	Dim	None	None	None

MU: Mucosa; SM: Submucosa; MS: Muscularis propria; AD: Adventitia; H: High echogenicity; L: Low echogenicity; M: Middle echogenicity.

were apparent using both sonography and pathology.

Submucosal edema serves as an ultrasonic contrast agent

As stated above, thermal burns in the esophageal mucosa of dogs caused lesions with different depths and different degrees of submucosal edema (except in the case of lesions invading into the muscularis propria). Submucosal edema displayed as a smooth, low echoic belt between the lesions and the layer of muscularis propria. Because water or liquid is a good medium for ultrasound, with trivial loss in ultrasonic energy, the mucosa was easily distinguished from the submucosa under condition of submucosal edema. The smooth leading edge of the submucosal edema identified lesions that did not invade into the submucosa, whereas an irregular leading edge of the submucosal edema indicated that the lesion invaded into the submucosa.

A

EUS image   Schematic diagram

Muscularis propria
Edema submucosa
Mucosa
Lesion

B

Histopathology   Schematic diagram

Muscularis propria
Edema submucosa
Muscularis mucosae
Lesion

C

EUS image   Schematic diagram

Muscularis propria
Edema submucosa
Lesion
Mucosa

D

Histopathology   Schematic diagram

Lesion
Edema submucosa
Muscularis propria
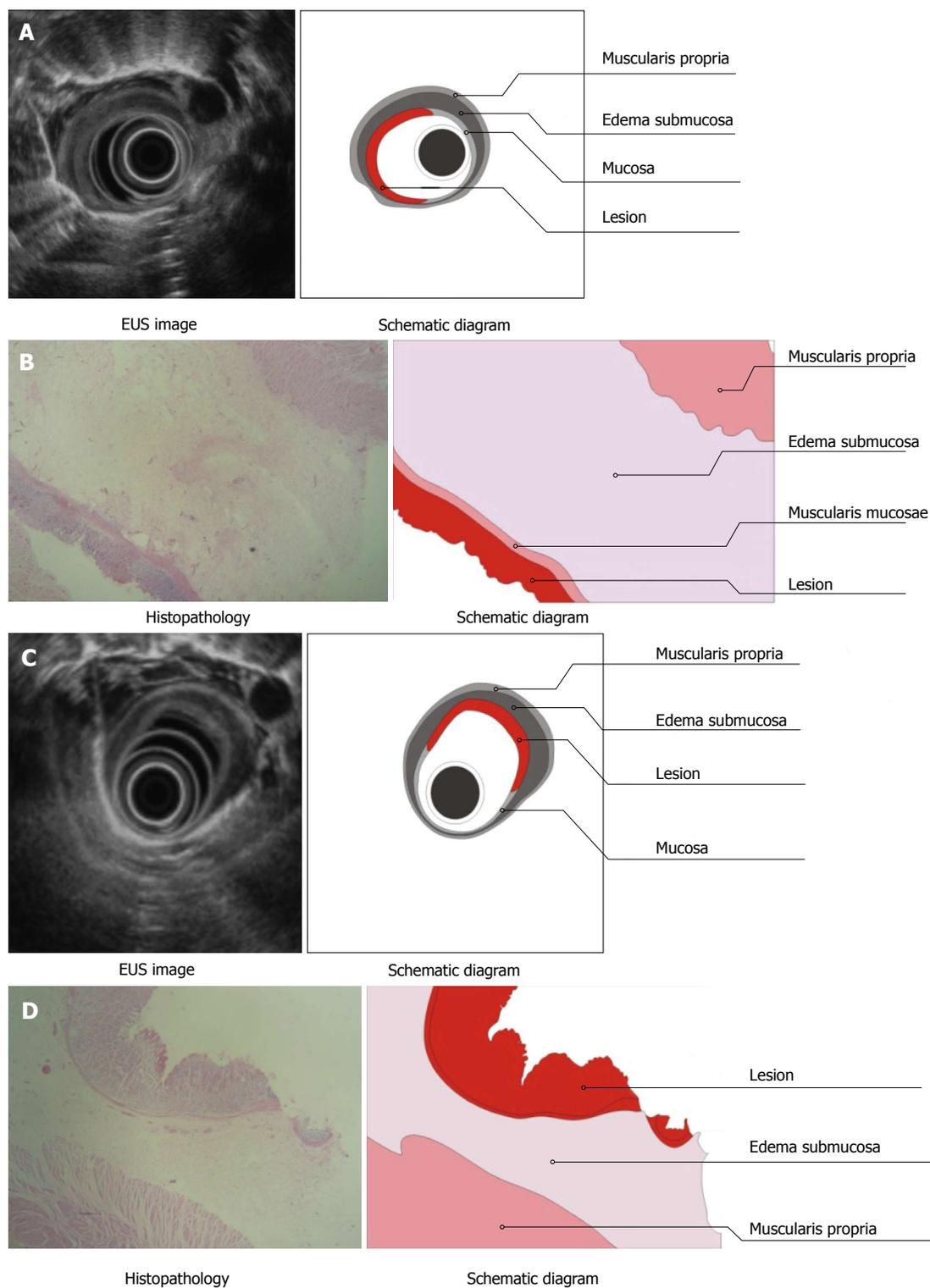
Figure 4 Endoscopic ultrasonography and tissue examination of esophageal lesions located in the superficial mucosa (A, B) and deep mucosa (C, D). A, C: Endoscopic ultrasonography (EUS) imaging: high echoic lesions located in the mucosa (A) and relatively high echoic lesion (C) located in the mucosa with obvious submucosal edema, as visualized by EUS; B, D: Pathology: tissue examination showed that the lesions were located in the mucosa with complete (B) and incomplete (D) squamous epithelium, intact muscularis mucosa, and obvious submucosal edema.

DISCUSSION

The strength of echogenicity depends on the energy of the reflection to the ultrasonic probe; when the reflection energy is higher, the gray scale of echogenicity is stronger[27,28]. In addition, the energy of the reflected ultrasound is correlated to the difference in acoustic impedance between the interfaces; when the difference is larger, the reflection is stronger and the echogenicity is higher. The difference of acoustic impedance depends

EUS image   Schematic diagram

Histopathology   Schematic diagram

EUS image   Schematic diagram

Histopathology   Schematic diagram
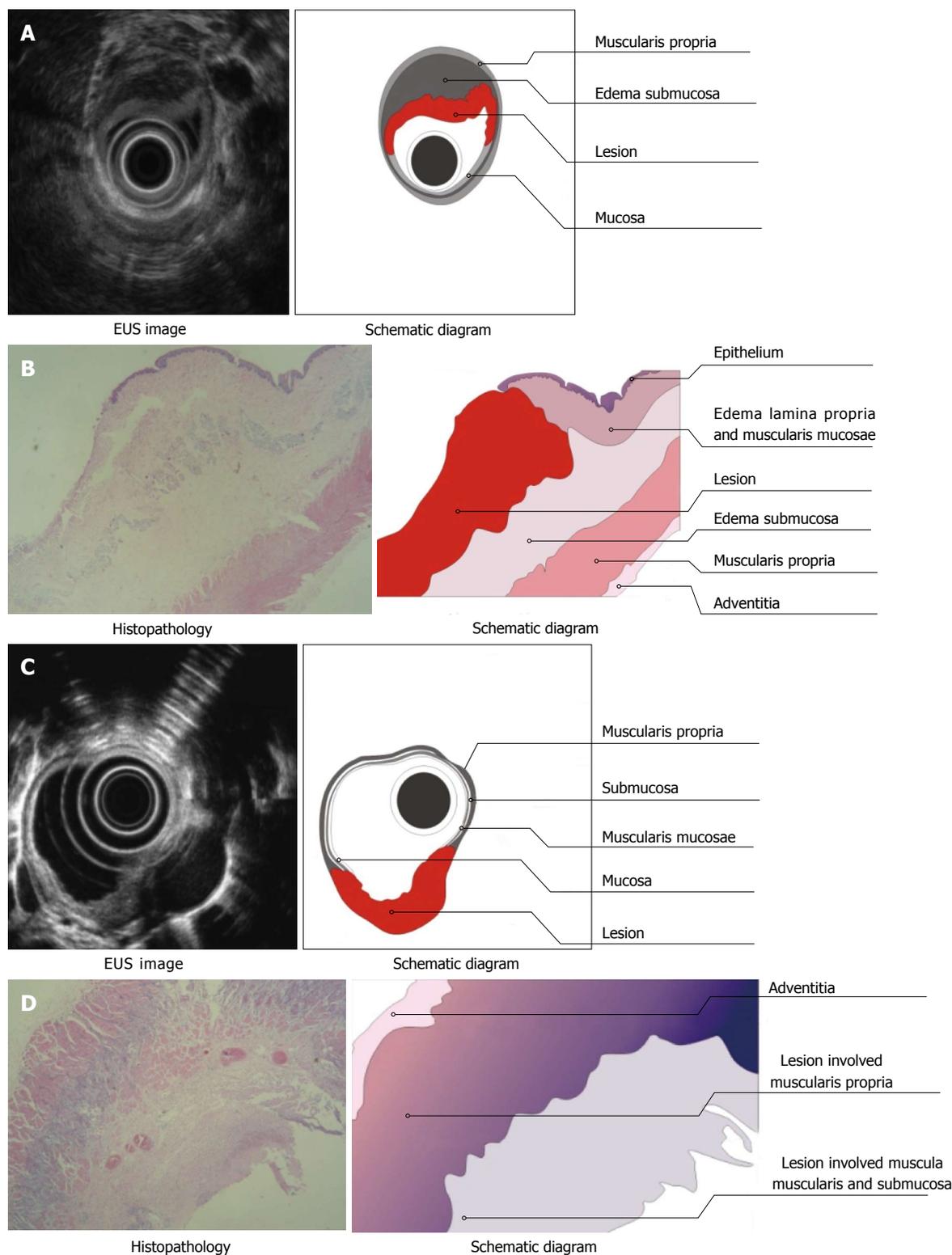
Figure 5 Endoscopy, endoscopic ultrasonography, and tissue examination of an esophageal lesion invading into the submucosa (A, B) and muscularis propria (C, D). A, C: Endoscopic ultrasonography (EUS) imaging: Middle echoic lesion (A) and low echoic lesion (C) invading the submucosa with obvious submucosal edema, as visualized by EUS; B, D: Pathology: Lesion invading into the submucosa (B) and muscularis propria (D) was characterized as squamous epithelium with disappearing muscularis mucosa and submucosal edema, as revealed by pathological examination.

on the tissue gradient. Generally, a homogeneous gradient in tissue reflects a small acoustic impedance difference, and the reflection of ultrasonic energy is small and the echoic gray scale is low. Alternatively, complicated tissue composition means that there are large acoustic impedance differences between interfaces, the ultrasonic energy is high and the echogenicity is strong[20,22]. It has been well established that the thickness of the

ultrasonic image is in direct proportion to ultrasonic propagation time. Under conditions of similar ultrasonic transmission speed in soft tissue, we can assume that the thickness of the ultrasonic image is equal to the actual thickness. In fact, the ultrasonic image corresponds to the histological characteristics of the tissue due to the pathway of ultrasound propagation, such as extracellular water, tissue density, histological type, vessels, adipose tissue, keratin pearls, and intercellular bridges in squamous cell epithelium[23].

Therefore, the high echo belt on sonography means that there are complicated gradients and a significant difference in acoustic impedance, thus corresponding to the mucosa (including the squamous cell epithelium with keratin pearl and intercellular bridges and muscularis mucosa, which is thin and has just one-fold muscular tissue) and the submucosal layer (composed of a complex gradient of vessels, lipid, and other soft connective tissue). The second low echoic belt indicates that the homogeneous tissue is mainly composed of muscular cells, with only a trivial difference in acoustic impedance and no significant difference between interfaces; thus, this low echoic echo corresponds to the muscularis propria. The thin, third strong echoic belt is indicative of dense tissue with a significant difference in the acoustic impedance between tissues; thus, it corresponds to the adventitia and other dense connective tissue[4].

The results of this study not only confirmed that thermal burns created at different energy levels caused superficial lesions with different infiltration depths but also that the EUS findings corresponded with pathological results in a canine model. Furthermore, our results indicate that submucosal edema separates the mucosa and submucosa, which caused drastic changes in acoustic impedance between the layers of the esophagus. Moreover, submucosal edema increased the thickness of the esophagus, allowing the layers to be definitively identified by sonography. Therefore, extracellular water or edema served as an ultrasonic contrast agent (negative role). Although the lesions caused by thermal burns in the canine model differ from actual EC, we can detect EC lesions using submucosal extracellular saline or fluid injection to enhance the accuracy of EUS. This is especially useful to distinguish T1a and T1b EC in the clinic. Our team tried to combine SSI with EUS examination to increase the accuracy of EUS for the staging and sub-staging of early esophageal squamous cell carcinoma preoperatively.

The esophagus of dogs has a thicker squamous cell epithelium and muscularis propria, as well as a thinner lamina propria and muscularis mucosa than that of human beings[25]. The large number of interfaces in the squamous epithelium, such as keratin pearls, extracellular bridges, vessels and lipid tissue in the submucosa, can cause strong ultrasonic reflection. Therefore, the first layer on sonography displayed as a high echoic belt with a 10 MHz ultrasonic probe; the mucosa and the submucosa were present as a high echoic belt, and they were difficult to distinguish from each other. Hence, the first low echoic belt includes the mucosa and submucosa in the normal esophagus of dogs. Once the lesion invades the submucosa, sonography cannot easily distinguish the lesion from the submucosa[15,16]. In fact, physicians are predominantly concerned with determining whether the lesions have already invaded into the submucosa or into deeper layers because EC patients with submucosa invasion are not eligible for endoscopic resection and must have esophagectomy[29]. With the contrast of fluid in the submucosa, lesions invading into the submucosa were easily identified by EUS.

In this study, we planned to perform SSI sequentially with EUS after thermal burning at different energy levels created superficial lesions of different infiltration depths. However, in pre-experiments, we found that lesions caused by thermal burning led to significant submucosal edema, so SSI was not needed to perform in order to enhance EUS. Furthermore, as the mucosa recovered (generally longer than two weeks), the submucosal edema gradually vanished. Therefore, performing SSI after the disappearance of submucosal edema was unnecessary because the mucosa had already recovered, with no remaining lesions. Furthermore, at 72 h post-thermal burning, the superficial lesions were obvious, whereas the mucosal edema had subsided, and the submucosal edema produced an effect similarly to the saline cushion caused by SSI.

The identification of esophageal lesions with different depths using ultrasonic technology is consistent with pathological results, demonstrating that the submucosal edema can serve as an ultrasonic contrast agent.

COMMENTS

Background
It is well known that treatment for esophageal cancer (EC) differs according to the depth of the lesion since the T1 stage or sub-stage of EC depends on the invading depth in early EC. Endoscopic ultrasonography (EUS) is the most common modality to stage early EC preoperatively.

Research frontiers
There are many questions about the effectiveness of EUS for EC diagnosis especially in consistency between sonographic and pathological results.

Innovations and breakthroughs
This study confirmed that there was consistency between EUS findings and pathological results of esophageal lesions with different depths. Submucosal edema can serve as an ultrasonic contrast agent.

Applications
Using submucosal saline as an ultrasonic contrast agent, the physicians may employ a novel technique-submucosal saline injection (SSI) to enhance the accuracy of EUS for staging or sub-staging early EC in clinic.

Terminology
EUS is a medical procedure in which endoscopy (insertion of a probe into a hollow organ) is combined with ultrasound to obtain images of the internal organs in the chest and abdomen. It can be used to visualize the walls of these organs, or to look at adjacent structures. Endoscopic ultrasonography is most commonly used in the upper digestive tract. SSI is a technique prior to endoscopic treatment for early EC to avoid damage to adjacent tissues.

Peer review
The authors present interesting data on the accuracy of EUS assessment of thermal esophageal burns, facilitated by submucosal edema in a canine model. Further studies will be required to determine the utility of submucosal fluid en-

hanced EUS examination of esophageal carcinoma.

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BRIEF ARTICLE

Small bowel tumors detected and missed during capsule endoscopy: Single center experience

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Abstract

AIM: To characterize small bowel (SB) tumors detected by capsule endoscopy (CE), and identify missed tumors.

METHODS: The study included 145 consecutive patients in whom 150 CEs were performed. Following CE, the medical records of the study population were reviewed. Results of double- or single-balloon enteroscopy performed after CE and the results of surgery in all patients operated on were retrieved. The patients were contacted through telephone interviews or postal mail. In addition, the national cancer registry and the polish clinical gastrointestinal stromal tumor (GIST) Registry were searched to identify missed neoplasms.

RESULTS: Indications for CE included overt and occult obscure gastrointestinal bleeding ($n = 81$, 53.7%), anemia ($n = 19$, 12.7%), malabsorption ($n = 18$, 12%), abnormal CB follow through ($n = 9$, 6%), abdominal pain ($n = 7$, 5%), celiac disease ($n = 5$, 3%), neuroendocrine tumor ($n = 3$, 2%), Crohn's disease ($n = 2$, < 2%), Peutz-Jeghers syndrome ($n = 2$, < 2%), other polyposes ($n = 2$, < 2%), and diarrhea ($n = 2$, < 2%). The capsule reached the colon in 115 (76.6%) examinations. In 150 investigations, CE identified 15 SB tumors (10%), 14 of which were operated on or treated endoscopically. Malignancies included metastatic melanoma ($n = 1$), adenocarcinoma ($n = 2$), and GIST ($n = 3$). Benign neoplasms included dysplastic Peutz-Jeghers polyps ($n = 4$). Non-neoplastic masses included venous malformation ($n = 1$), inflammatory tumors ($n = 2$), and a mass of unknown histology ($n = 1$). During the follow-up period, three additional SB tumors were found (2 GISTs and one mesenteric tumor of undefined nature). The National Cancer Registry and Polish Clinical GIST Registry revealed no additional SB neoplasms in the post-examination period (follow-up: range 4.2-102.5 mo, median 39 mo). The sensitivity of CE for tumor detection was 83.3%, and the negative predictive value was 97.6%. The specificity and positive predictive value were both 100%.

CONCLUSION: Neoplasms may be missed by CE, especially in the proximal SB. In overt obscure gastrointestinal bleeding, complementary endoscopic and/or radiologic diagnostic tests are indicated.

Core tip: The aims of this study were to characterize small bowel (SB) tumors detected by capsule endoscopy (CE) and identify SB tumors missed by CE. The study included 150 consecutive CE investigations. Following CE, the medical records of the study population were reviewed and the patients contacted by telephone or postal mail. National cancer registries were searched to identify missed neoplasms. CE detected 15 SB tumors (10%). During the follow-up period, three additional SB tumors were found. The sensitivity of CE for tumor detection was 83.3% and the negative predictive value 97.6%. The specificity and positive predictive value were both 100%.

INTRODUCTION

Capsule endoscopy (CE) has become a first-line diagnostic tool in obscure gastrointestinal bleeding (OGIB) when the small bowel (SB) is a suspected source. Compared with push enteroscopy (PE), which is performed to establish the source of bleeding, CE detects more than twice as many clinically-significant abnormalities (56% *vs* 26%), whereas any abnormalities are detected in 63% with CE *vs* 28% with PE[1]. Balloon-assisted enteroscopy (BAE), most often double-balloon enteroscopy (DBE), is performed following both a negative CE or as a complementary procedure guided by the CE findings. Initial studies suggested that CE and DBE have a comparable diagnostic yield in patients with suspected SB disease, including OGIB, when the whole SB is visualized[2]. Now evidence is growing that CE misses a significant number of lesions detected on enteroscopy[3,4]. In a recent meta-analysis, the yield of DBE after previously-negative CE was 27.5%[5]. Nevertheless, CE remains the preferred initial diagnostic test because of its noninvasiveness, better tolerance, and ability to view the entire SB.

SB tumors are source of bleeding in some patients with OGIB, particularly younger patients. In a large series of patients undergoing CE, SB tumors were found in 2.4% (Rondonotti *et al*[6]), 8.9% (Cobrin *et al*[7]), 6.3% (Bailey *et al*[8]), and 4.3% (Cheung *et al*[9]) of cases. Malignant tumors were found in 4.2%, 4%, and 2.7% of patients, respectively. In a multicenter Belgian study, the percentage of malignant tumors was 2.5%[10]. The percentage of DBE procedures detecting SB tumors is higher than with CE, increasing up to 12% (27 of 225 patients in Choi *et al*[11]) and up to 13.9% in the largest series described, comprising 1035 Japanese patients of whom 42.4% were examined because a SB tumor was suspected[4].

A retrospective review of 183 cases in which DBE was performed at 7 North American centers found that DBE identified SB tumors in 15 patients who had prior CE, whereas lesions were found by CE in only 5 patients, and all 4 cases of primary adenocarcinoma were missed by CE[3].

We performed a retrospective study to characterize SB tumors detected in consecutive patients who underwent CE at our center. The second aim of this study was to identify any SB tumors missed by CE in these patients.

MATERIALS AND METHODS

The results of all consecutive CE examinations (PillCam SB1, Given Imaging, Israel), which were assessed by two readers between March 2003 and July 2009 at a single center, were reviewed and categorized. In a standard evaluation, CE findings were further classified as negative or positive. Positive findings were also classified as clinically significant or insignificant lesions. Clinically significant lesions included angioectasias, tumors or polyps $\geq$ 10 mm, active bleeding, blood clots, diverticula, mucosal breaks, and features consistent with celiac disease. Clinically insignificant lesions included red spots, white spots, erythema, focal atresia of villi, or small polyps.

As CE allows for only an approximate estimation of polyp size, a cut-off polyp diameter of 10 mm was used; this size is an accepted indication for polyp resection in patients with polyposis syndromes.

The preparation for CE included fasting from lunchtime and ingesting 3 L of glycol polyethylene the day before the examination. The patients ingested the capsule in the morning with 50 mL of water and 0.5 mL of simethicone (Espumisan, Berlin Chemie, Poland). The patients were allowed to drink more water no earlier than 2 h after capsule ingestion and eat no earlier than 4 h after capsule ingestion.

Follow-up data were obtained by reviewing hospital case notes. Results of push, double-balloon, single-balloon, and intraoperative enteroscopy performed following CE, and the results of any surgery performed were retrieved. Following the analysis of records, an attempt to contact the patients by phone or mail was made using a standardized interview. The questions referred to any serious diseases diagnosed following CE, including cancer, and operations performed.

Finally, the National Cancer Registry and the polish clinical gastrointestinal stromal tumor (GIST) Registry were searched to identify any neoplasms possibly missed in the study population.

The study was approved by the institutional review board in accordance with the guidelines of the Declaration of Helsinki revised in 1989.

Table 1 Characteristics of the study population *n* (%)	
Gender, males	**71/145 (49)**
Age	
(min, max)	(8, 85)
mean (SD)	50.1 (19.2)
Main indication for CE[1]	*n* = 150[2]
Overt obscure bleeding	58 (38.7)
Occult obscure bleeding	23 (15.3)
Anemia	19 (12.7)
Malabsorption	18 (12.0)
Abnormal SB follow through	9 (6.0)
Abdominal pain	7 (4.7)
Celiac disease	5 (3.3)
Neuroendocrine tumor	3 (2.0)
Peutz-Jeghers syndrome	2 (1.3)
Diarrhea	2 (1.3)
Crohn's disease	2 (1.3)
Polyposis syndrome	2 (1.3)

[1]Primary indication was given; [2]Capsule endoscopy (CE) was performed twice in 5 patients for the following reasons: incomplete examination (2 patients); recurring overt obscure gastrointestinal bleeding (OGIB) in a patient with normal first examination (1 patient) and recurring overt gastrointestinal bleeding in patients with abnormal first CE result and treatment instituted (mucosal breaks, angioectasias, 2 patients).

RESULTS

Over the study period, 145 patients underwent 150 CEs. The characteristics of the patients, including the indications for CE, are presented in Table 1. The most frequent indication for performing the procedure was OGIB (81 patients; 53.7%), which was occult in 23 patients (15.3%) and overt in 58 (38.4%).

The capsule reached the colon in 115 (76.6%) examinations. CE revealed no abnormalities in 29 (19.3%) procedures, was abnormal and clinically significant in 82 (54.6%), and abnormal but insignificant in 37 (24.7%) procedures. No conclusions were drawn in 2 cases (1.3%). In the initial studies, the cleansing conditions were not routinely assessed by the reader, so this parameter could not be reported for the whole study population. The results of the 150 procedures are shown in Table 2.

Tumors ≥ 10 mm were identified in 15 patients (10%). Fourteen tumors were surgically or endoscopically resected. The characteristics of these patients are presented in Table 3. Of the 14 resected tumors, 6 were malignant (4%), 4 were benign (2.6%), and 3 were non-neoplastic (2%) and the precise histology of one non-malignant tumor was not retrieved. The most frequent indication for CE that resulted in tumor detection was overt OGIB (6 patients).

Longer follow-up was available for 139 patients (95.8%). Sixteen patients died (11%). In 6 patients (4.1%), the medical records were unavailable or the patient could not be contacted by phone or mail. However, they were included in the registries search. The median observation time of the living patients in whom the follow-up was performed was 39 mo (*n* = 124, range 4.2-102.5 mo).

It was established that CE missed 2 SB GISTs and

Table 2 Results of 150 capsule endoscopy examinations *n* (%)	
Findings	*n* = 150
Significant findings	
Angioectasias	25 (16.7)
Mucosal breaks	20 (13.3)
Tumor or polyp(s) ≥ 10 mm	15 (10.0)
Diverticula	14 (9.3)
Celiac disease	5 (3.3)
Active bleeding with no visible origin	3 (2.0)
Insignificant findings	
Erythema or red spots	15 (10.0)
White spots	13 (8.7)
Other	7 (4.7)
Modeling of the bowel wall	2 (1.3)
Normal	29 (19.3)
Non-diagnostic	2 (1.3)

one SB mesenteric tumor. All three patients underwent CE due to overt OGIB.

In one patient, PE up to the ligament of Treitz was performed before CE and duodenal lymphangiectasis were seen. CE examination was complete and normal, but cleansing of the distal SB was poor. Following CE, CT angiography was performed and active SB bleeding was observed in the right mid-abdomen and a lesion within the ileocecal artery was suggested. Immediate surgery revealed bleeding in Meckel's diverticulum, and a non-bleeding jejunal 4-cm GIST that was 15 cm behind the ligament of Treitz. The mucosa covering the tumor was normal.

In the second patient CE was complete, but the SB cleansing was poor. On CE a diverticulum in the left mid-abdomen was seen. Subsequent laparoscopy revealed a 4-cm SB tumor that appeared to be a GIST. Unfortunately, the exact tumor location was not assessed. This patient did not undergo enteroscopy.

In the third patient, upper DBE was performed before CE and 150-170 cm of SB inspected. Upon withdrawal, a small clot firmly attached to the mucosa in the proximal jejunum was observed. A possible iatrogenic lesion was suspected and argon plasma coagulation performed. No other abnormalities were detected. Subsequent CE was complete and normal, however, contrast abdominal CT performed 11 mo later revealed a mass located between the pancreatic head and duodenum.

On laparotomy, a diagnosis of non-resectable mesenteric tumor was made, but intraoperative cytology and later histology did not confirm neoplastic disease. After 6 mo of observation without progression of the disease, the patient was lost to follow-up.

In addition, the National Cancer Registry and the Polish Clinical GIST Registry were searched for 144 patients (99.3%) whose national identity number was available, and this search did not identify any other (missed) SB neoplasms during the post-examination period. A plasmocytoma was diagnosed 14 mo after a normal CE in a female who underwent the procedure due to occult OGIB; this patient died 4 mo after the cancer diagnosis.

Table 3 Characteristics of patients with tumor or polyp(s) ≥ 10 mm detected on capsule endoscopy and the results of follow-up

No.	Age (yr)	Sex	Indication for CE	Bleeding on CE	CE reached the colon	DBE result	Surgery result	Neoplasm
1	21	F	PJS	-	√	NA	Dysplastic Peutz-Jeghers polyp	yes
2	25	M	Polyposis	-	-	NA	Dysplastic Peutz-Jeghers polyp	yes
3	30	M	Overt OGIB	-	-	NA	Meckel's diverticulum	no
4	36	F	Occult OGIB	√	-	NA	SB adenocarcinoma	yes
5	37	F	Occult OGIB	-	-	Dysplastic Peutz-Jeghers polyp in jejunum	Not operated on	yes
6	47	M	Malabsorption	√	√	NA	Unknown but benign	unknown
7	50	M	Abnormal SBFT	-	√	NA	Not operated on	unknown
8	53	M	Overt OGIB	√	-	NA	GIST	yes
9	53	M	PJS	-	√	NA	Dysplastic Peutz-Jeghers polyp	yes
10	56	M	Anemia, disseminated melanoma malignum	-	-	NA	Melanoma malignum metastasis	yes
11	59	F	Overt OGIB	√	√	Submucosal tumor	Venous malformation	no
12	60	F	Overt OGIB	-	√	NA	GIST	yes
13	64	F	Overt OGIB	-	√	NA	SB adenocarcinoma	yes
14	68	F	Occult OGIB	-	-	NA	Inflammatory tumor	no
15	68	F	Overt OGIB	-	√	NA	GIST	yes

CE: Capsule endoscopy; NA: Not applicable; DBE: Double-balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding; SBFT: Small bowel follow through; PJS: Peutz-Jeghers syndrome; GIST: Gastro-intestinal stromal tumor; M: Male; F: Female.

Based on these data, in a per patient analysis the sensitivity of CE for tumor detection was 83.3% and the negative predictive value was 97.6%. The specificity and positive predictive value were both 100%.

DISCUSSION

We performed a retrospective study of consecutive patients who underwent CE at our center for various reasons. We then followed these patients and found three tumors missed by CE. To the best of our knowledge, this is the first study with such a specific, tumor-oriented follow-up. The percentage of tumors found in our study (10%) was higher than in other CE series, which may be explained by the strict selection of patients who undergo CE at our center[6-10]. This may be the result of a lack of reimbursement for CE by the national health care system. OGIB, for which CE had the highest diagnostic yield, was the indication for CE in 81 (53.7%) examinations in our series and CE resulted in tumor detection in 9 OGIB patients (11.1%). The diagnostic algorithm included an upper and lower endoscopy and push enteroscopy. The latter was performed in 35 (24.1%) patients before CE and was negative, which eliminated proximal intestinal vascular abnormalities, making a tumor diagnosis more likely. In the studies which analyzed only OGIB patients, a SB mass was found in 10%[12] and 7.18%[13] of cases. In the CE results in the study by Cobrin *et al*[7], SB tumors were detected in 9% of patients with OGIB and the number of OGIB patients in the other CE series cited was not given.

In our study, the median follow-up was slightly over 3.2 years (39 mo). This seems sufficient for a serious symptomatic diagnosis, which might have been missed by

CE, to be made during complementary investigations.

During follow-up, we found two cases of GIST in the SB not detected by CE. Both lesions were diagnosed intra-operatively. The first lesion was located in the proximity of Treitz's ligament; the mucosa covering the tumor was normal and the source of active bleeding was Meckel's diverticulum. Thus, one might suppose that this tumor would not be recognizable on CE. The exact location of the second GIST could not be given precisely. Notably, the bowel cleansing for CE in these two patients was poor.

The third missed lesion was first found on contrast CT, and was located in the proximal SB. This is in concordance with observations made by others. Postgate *et al*[14] described 5 significant lesions missed by CE that were found using other imaging modalities [DBE in 3 patients, CT enterography (CTE), and magnetic resonance enterography (MRE) in the 2 remaining patients]; 4 of which were located in the proximal jejunum. Chong *et al*[15] described 4 tumors in the proximal ileum that were missed by CE but found with DBE. This particular location, where many lesions were missed, may be partly explained by a rapid transit of the capsule through the duodenum and the proximal jejunum that enhances the risk of missing a lesion in the proximal SB.

The complementary role of DBE in CE-positive and CE-negative patients is widely accepted. Among our patients, the first with GIST underwent PE that did not reach the segment with the tumor. The second patient with GIST did not undergo enteroscopy. In the third patient, DBE included the involved segment but failed to provide a diagnosis.

Radiological imaging is more readily available than BAE and remains the next diagnostic step at many cen-

ters. With respect to conventional radiological SB imaging, CE is superior in diagnosing mass lesions. A small study comparing CE to barium enterography in children with Peutz-Jeghers syndrome (PJS) showed that polyps with a diameter of 10 mm and more were detected with similar frequency with both modalities, but CE identified significantly more polyps < 10 mm[16]. The performance of CE compared with newer radiological SB imaging is still a subject of debate. The first study comparing CE and magnetic resonance imaging (MRI) in patients with PJS (4 patients) or familial adenomatous polyposis syndrome (FAP, 16 patients) showed that smaller polyps were seen much more often with CE, whereas polyps larger than 15 mm were detected at similar rates with both CE and MRI[17]. However, a subsequent study performed in 19 PJS patients showed that CE missed large polyps (> 15 mm) detected on MRE in three patients, suggesting that MRE may be less prone to miss large polyps and more reliable in their size assessment[18]. With regard to CTE, both CE and CTE were performed in 32 patients with OGIB described in a retrospective study by Khalife *et al*[19]. When CTE followed CE, it helped to identify tumors not detected by CE (*n* = 2) and excluded suspected tumors (*n* = 3). In another retrospective study of 17 patients with SB tumors who had both CE and CTE, CE detected SB tumors in 6 patients and CTE in 16, with a significant difference in the sensitivity of the two methods[20]. In a prospective comparison of CTE and CE in 58 patients with OGIB, the sensitivity of CTE for detecting SB bleeding sources and SB masses was significantly greater than that of CE[21]. In our study, (angio) CT followed CE and helped to establish the source of bleeding in two patients.

The risk of rebleeding in 42 patients with OGIB and negative CE was first evaluated by Macdonald *et al*[22] who observed bleeding episodes in only 2 overt OGIB patients during 17.3 mo of follow-up. Subsequently, Park *et al*[23] observed 57 OGIB patients, of whom 46 had overt OGIB, for a median time of 31.7 mo. They found a substantial cumulative rebleeding rate of 35.7% in CE-negative patients, recommending further investigation or close observation of such patients[23]. The results of these studies suggest that following a negative CE, overt OGIB patients were the most likely to benefit from further investigation.

In summary, in patients with overt OGIB and normal or insignificant CE, the risk of missing a lesion in the SB cannot be underestimated. In our opinion, BAE should be the next diagnostic tool used when symptoms strongly suggest that the source of the bleeding is located in the SB. In the remaining cases, or when BAE is not easily available, CT or MRI seem to be a rational choice in further evaluations. According to the most recent studies, CT or MRI enterography may be the best choice. Laparotomy remains a diagnostic option when these tests are normal or not available, with the advantage of therapeutic possibilities.

COMMENTS

Background
Capsule endoscopy (CE) has become a first-line diagnostic tool for obscure gastrointestinal bleeding (OGIB) and small bowel (SB) polyp or tumor detection, but the reliability of negative CE for excluding gross SB pathology is unclear.

Research frontiers
SB tumors detected by CE were characterized in a retrospective cohort. At follow-up, three additional SB tumors missed by CE were identified. In patients with overt OGIB, negative CE does not exclude significant disease.

Innovations and breakthroughs
This is the first study of CE with a specific, tumor-oriented, long-term follow-up.

Applications
In patients with significant clinical symptoms, the risk of missing a lesion in the SB cannot be underestimated. In overt OGIB, supplementary diagnostic methods should be used to visualize SB, including balloon enteroscopy, computed tomography, and magnetic resonance enterography.

Terminology
CE is a method of visualizing the walls of the gut and used mainly to investigate the SB, which is difficult to access by conventional endoscopy, but is also used to visualize the esophagus and colon. A small, pill-like camera is ingested by the patient and moves naturally throughout the gastrointestinal tract, taking thousands of pictures. These pictures are sent to a detector connected to the patient's skin during the examination and assessed later by a reader on the computer. The camera pill is excreted with the stool.

Peer review
This study has the strengths of large enrollment and SB tumor-oriented follow-up. It is well written.

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BRIEF ARTICLE

Serum concentrations of insulin-like growth factor-binding protein 5 in Crohn's disease

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Abstract

AIM: To investigate serum insulin-like growth factor-binding protein 5 (IGFBP-5) levels and intestinal IGFBP-5 expression in patients with Crohn's disease (CD).

METHODS: We analyzed the serum concentrations and intestinal expression of IGFBP-5 in 42 patients with CD, of whom 26 had endoscopically or radiologically proven stricture formation. Nine of the 42 patients had active disease, with a Crohn's disease activity index > 150. Serum IGFBP-5 levels were analyzed in 20 healthy controls matched by sex and age to the CD patients. Serum IGFBP-5 was measured using an enzyme-linked immunosorbent assay. Intestinal tissue was obtained from patients through endoscopic biopsies. IGFBP-5 expression was detected using immunohistochemistry and was scored semiquantitatively.

RESULTS: The median serum IGFBP-5 concentrations of CD patients were significantly lower compared with healthy controls [median 7.2 (IQR: 5.5-11.3) ng/mL *vs* 11.3 (8.0-44.6) ng/mL, $P < 0.001$]. There was no significant difference between median serum IGFBP-5 levels in CD patients with or without stricture formation [6.9 (5.5-11.3) ng/mL *vs* 7.8 (5.3-10.1) ng/mL, $P = 0.815$]. The serum IGFBP-5 levels were not significantly different between patients with active disease and inactive disease [7.2 (6.5-7.6) ng/mL *vs* 7.2 (5.5-11.3) ng/mL, $P = 0.890$]. However, a significant correlation was observed between serum IGFBP-5 levels and platelet count (PLT) ($r = 0.319$, $P = 0.0395$). No significant correlation was found between tissue IGFBP-5 immunohistochemical staining intensity scores and serum IGFBP-5 levels. No significant difference was found when comparing the serum IGFBP-5 levels among the patients with different tissue IGFBP-5 staining scores (absent/very weak, weak, moderate or strong). There was a significant correlation between tissue IGFBP-5 staining scores and white blood cell count ($r = 0.391$, $P = 0.01$) and PLT ($r = 0.356$, $P = 0.021$).

CONCLUSION: Our results indicate that serum IGFBP-5 concentrations were lower in CD patients compared to healthy controls regardless of disease activity or the presence of stricture formation.

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Key words: Crohn's disease; Insulin-like growth factor-binding protein 5; Stricture; Immunohistochemistry;

Enzyme-linked immunosorbent assay

Core tip: Previous studies have suggested that insulin-like growth factors are important for the growth and development of visceral smooth muscle. In particular, increased insulin-like growth factor-binding protein 5 expression has been described in inflamed and fibrotic intestinal tissue. In this study, we aim to investigate the possible role of insulin-like growth factor-binding protein 5 in Crohn's disease with stricture involvement. Crohn's disease patients had lower serum levels of IGFBP-5 compared to healthy controls. The results of the study suggest that additional research is necessary to explain the low circulating levels of IGFBP-5 in Crohn's disease patients.

INTRODUCTION

Crohn's disease (CD), a condition characterized by chronic inflammation of the alimentary tract, arises from a complex interaction between genetic, immunological, and microbial factors[1]. More than one-third of CD patients develop a distinct fibrostenosing phenotype with progressive intestinal strictures and potential intestinal obstruction[2]. Intestinal obstruction and fistulae are the main indications for surgery in patients with CD[3]. Stricture formation is caused by a combination of smooth muscle cell hyperplasia, smooth muscle cell hypertrophy, and excessive net extracellular matrix production by intestinal smooth muscle cells[4]. The factors underlying stricture development in CD are not completely understood. It is critical to develop markers that can be used to predict intestinal stricture formation in the early stages of CD.

The insulin-like growth factor (IGF) system has a critical role in regulating the growth and development of visceral and vascular smooth muscle[5]. Insulin-like growth factors (IGF-Ⅰ and IGF-Ⅱ) are transported in the serum by insulin-like growth factor-binding proteins (IGFBPs; IGFBP-1 to 6), which are produced in the liver. IGFBPs are also produced by non-liver tissues, in which they act in autocrine and paracrine manners to modulate responses to IGFs[6]. Despite their structural similarity, each IGFBP has unique characteristics and functions. Insulin-like growth factor-binding protein 5 (IGFBP-5) is the most conserved of the IGFBPs, has several regulatory functions, and is among the IGFBP subtypes that display IGF-independent effects. The most important in vivo regulator of IGFBP-5 expression is IGF-Ⅰ. In normal adult human serum, IGFBP-5 levels are positively correlated with IGF-Ⅰ concentrations[7]. In patients with CD,

IGF-Ⅰ expression is specifically upregulated in smooth muscle cells in regions of stricture compared to normal margins; this upregulation is accompanied by upregulated IGFBP-5 expression, which acts synergistically with IGF-Ⅰ in these cells[8]. Increased IGFBP-5 expression has also been described in two human fibrotic disorders: systemic sclerosis and idiopathic pulmonary fibrosis[9,10].

Several studies have investigated the serum concentrations of IGF-1 and IGFBP-3 in active inflammatory bowel disease (IBD) patients and found significantly decreased serum levels[11-16]. There are currently no data available regarding serum IGFBP-5 levels in patients with CD. It is unknown whether circulating IGFBP-5 proteins influence local IGFBP-5 tissue expression or whether the protein levels are reflective of stricture formation in patients with CD. Therefore, we aimed to investigate the serum concentration of IGFBP-5 and intestinal IGFBP-5 expression in tissue taken from CD patients with and without stricture formation and to determine the correlation between serum IGFBP-5 levels and intestinal IGFBP-5 expression.

MATERIALS AND METHODS

Patients and samples

Forty-two patients with CD [20 female patients and 22 male patients; mean age ($\pm$ SD) 38.79 $\pm$ 13.91 years; range 19-70 years, mean disease duration 4.74 $\pm$ 7.46 years] were enrolled from the inflammatory bowel disease outpatient clinic in Istanbul Medeniyet University, Goztepe Training and Research Hospital, Istanbul, Turkey, between March 2011 and September 2012. The study was conducted in accordance with the Declaration of Helsinki and according to the principles of Good Clinical Practice. The Goztepe Training and Research Hospital ethics committee approved the study (19/S-2012). All subjects gave informed consent. Twenty healthy controls who were matched to study patients by sex and age [10 female and 10 male, mean age ($\pm$ SD) 38.4 $\pm$ 8.73 years; range 26-56 years] were also enrolled, and they provided written consent for the collection of blood samples.

CD was diagnosed based on the established criteria of clinical, endoscopic, and histological findings. All patients (n = 42) were evaluated endoscopically or radiologically for the presence of stricture formation. Twenty-six of the 42 patients (61.9%) had endoscopically or radiologically proven stricture formation. Eighteen of these 26 patients (69.2%) had a history of intestinal resection. The Crohn's disease activity index (CDAI) was used in all patients to assess disease activity[17]. Nine of the 42 patients (21.4%) had active disease corresponding to a CDAI > 150. Only four of the 26 patients (15.4%) with stricture formation had active disease corresponding to a CDAI > 150.

All patients were subdivided into disease phenotypes according to the Montreal Classification[18]: ileal disease only (L1) (n = 9, 21.4%), colonic disease only (L2) (n = 2, 4.8%), and ileocolonic disease (L3) (n = 31, 73.8%). Clini-

cal data regarding each patient's duration and localization of the disease, history of bowel resection, and current medications were obtained and recorded. Exclusion criteria included the presence of liver fibrosis or cirrhosis, systemic sclerosis, idiopathic pulmonary fibrosis, or a history of cancer.

The recruited patients were scheduled to undergo an ileocolonoscopy. The reasons for the scheduled endoscopy were as follows: to assess the disease extent and activity (n = 24, 57.1%), to monitor response to therapy (n = 9, 21.4%), and to perform stricture dilation (n = 9, 21.4%). Endoscopy was performed by experienced gastroenterologists, who collected biopsies with standard-sized flexible endoscopic forceps from areas that were endoscopically strictured and/or ulcerated (ileal and/or colonic) in patients with stricture formation. The biopsies were taken from inflamed or ulcerated areas (ileal and/or colonic) in patients without stricture formation. At least 2 biopsy specimens were collected from each area. One specimen was stained with immunohistochemistry to determine IGFBP-5 expression, and the other was stained with hematoxylin and eosin (HE). Routine histological examination of the biopsy specimens was performed by an experienced pathologist. Blood samples were collected on the same day, and sera were frozen at -80 ℃ until testing was performed.

Determination of IGFBP-5 in serum
Human serum IGFBP-5 levels were determined using an ELISA kit (RayBiotech, Norcross, GA) according to the manufacturer's instructions. The sensitivity of the assay was less than 2 ng/mL. The intra- and inter-assay coefficients of variation (CVs) were < 10% and < 12%, respectively.

Immunohistochemical staining and evaluation
Immunohistochemical staining was performed using polyclonal antibodies against IGFBP-5 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Tissue samples from the diagnostic ileocolonoscopy (ileal and/or colonic) were stained with immunohistochemistry to determine IGFBP-5 expression. HE staining was performed on parallel sections of the tissue samples. Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (3 μm) were deparaffinized with xylene for 10 min. After deparaffinization, the sections were incubated in a 3% hydrogen peroxide block for 10 min to reduce nonspecific background staining due to endogenous peroxidase. After a wash in phosphate-buffered saline plus Tween 20 (20 ×) (PBS; ScyTek Laboratories, Logan, Utah, United States), the sections were incubated in an ultra V block (ScyTek Laboratories, Logan, Utah, United States) for 5 min at room temperature (RT) to block non-specific binding. A primary antibody against IGFBP-5 (dilution 1:50) was added to the tissue sections, and the sections were incubated at RT for 90 min, followed by incubation with a secondary antibody (dilution 1:200, Ultra Tek antipolyvalent biotinylated antibody, ScyTek Labo-

ratories Logan, Utah, United States) at RT for 15 min. After rehydration with PBS, Ultra Tek HRP (ScyTek Laboratories, Logan, Utah, United States) was added to the specimens. The DAB chromogen system (DAB substrate kit, ScyTek Laboratories, Logan, Utah, United States) was added to the specimens after rehydration with PBS. Mayer's hematoxylin stain was used as a counterstain.

IGFBP-5 immunohistochemical staining was scored semiquantitatively by an independent pathologist who was blinded to clinical information. Positive staining for IGFBP-5 was observed as diffuse brown staining. The intensity of staining was scored as follows: 0 = absent or very weak staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining.

Statistical analysis
The SPSS statistical software package (SPSS version 19.0, SPSS, Chicago, IL, United States) was used for data management and analyses. CD patients were matched by age and sex with healthy controls to minimize confounding factors; matched controls were included because the numbers of patients in the study was not large enough to carry out the modeling necessary to adjust for possible effects of age and gender. For continuous normally distributed variables, the mean and standard deviation were reported. Median and interquartile range were reported for non-normally distributed continuous variables. Frequencies and percentages were given for categorical variables. The Mann-Whitney test was used to evaluate the median difference between groups, and a t test was used to compare differences in mean scores. Fisher's exact test was used instead of the typical χ^2 test to compare the frequencies or categorical variables because there were few subjects in each category (n < 10 subjects). Spearman's correlation coefficient was used to assess the association between continuous variables in the CD group. The Kruskal-Wallis test was used to assess differences in serum concentrations of IGFBP-5 and expression in tissue specimens (*i.e.*, scores of 0, 1, 2, and 3). Statistical significance was set at a 95%CI level using a 2-sided P value.

RESULTS
The baseline characteristics of the CD patients and healthy controls are summarized in Table 1. The median duration of CD diagnosis was 1.2 years (IQR: 0.16-7). Of the 42 patients with CD, 31(73%) had ileocolonic disease, and 33 (78.6%) were treated with azathioprine.

The main clinical and biochemical characteristics of CD patients and healthy controls are presented in Table 2. The median values for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cell (WBC) and platelet count (PLT) were significantly higher in CD patients compared to controls (for ESR and CRP, P < 0.001; for PLT, P = 0.0001; and for WBC count, P = 0.036). However, the median levels of albumin (Alb) and hemoglobin (Hb) were significantly lower in the CD

Table 1 Baseline characteristics of the study population *n* (%)

	Crohn's disease (*n* = 42)	Healthy controls (*n* = 20)
Age, mean ± SD (yr)	38.7 ± 13.9	38.4 ± 8.7
Gender		
Female	20 (47.6)	10 (50)
Male	22 (52.4)	10 (50)
Disease localization		-
Ileum	9 (21.4)	
Colon	2 (4.8)	
Ileum + colon	31 (73.8)	
Medical treatment		-
None	9 (21.4)	
Corticosteroids	1 (2.4)	
5-aminosalicylate	8 (19)	
Azathioprine	33 (78.6)	
Sulfasalazine	1 (2.4)	
More than one drug	23 (54.8)	
Median disease duration (yr) (IQR)	1.2 (0.17)	-
Prior intestinal resection	18 (42.9)	-

IQR: Interquartile range.
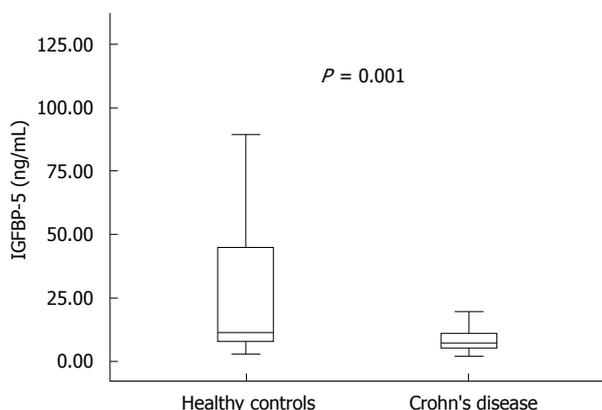
Figure 1 Serum Insulin-like growth factor-binding protein 5 concentrations in Crohn's disease patients and healthy controls. Serum insulin-like growth factor-binding protein 5 (IGFBP-5) (ELISA) levels were significantly decreased in patients with Crohn's disease compared to healthy controls (*P* < 0.001). *P* value from Mann-Whitney test.

group compared to healthy controls, with *P* values of < 0.001 and 0.0063, respectively. Serum IGFBP-5 levels were significantly reduced in patients with CD [7.2 (5.5 -11.3) ng/mL] compared to healthy controls [11.3 (8.0-44.6) ng/mL, *P* = 0.001] (Figure 1 and Table 2).

Table 3 shows the demographic and biochemical characteristics of CD patients with and without stricture formation. There were no significant differences between the CD patients with stricture formation and those without stricture formation with regards to age, gender, disease activity, disease localization or disease duration. Additionally, there was no significant difference in median values for biochemical parameters in CD patients with or without stricture formation. There was also no significant difference between serum IGFBP-5 levels in CD patients with and without stricture formation [6.9 (5.5-11.3) ng/mL and 7.8 (5.3-10.1) ng/mL (*P* = 0.815), respectively].

The serum median IGFBP-5 levels were not signifi-

Table 2 Clinical and biochemical parameters of Crohn's disease patients and healthy controls

	Crohn's disease (*n* = 42)	Healthy controls (*n* = 20)	*P* value[1]
CDAI	87.0 (52-138)	-	
ESR (mm/h)	35.5 (17.0-48)	12.0 (10.5-14.5)	< 0.001
CRP (mg/dL)	0.6 (0.3-1.5)	0.2 (0.1-0.3)	< 0.001
Hb (g/dL)	13.0 (11.8-13.9)	14.0 (12.9-15)	0.0063
WBC (× 10^3/mm^3)	7.7 (5.9-9.2)	6.2 (5.3-7.9)	0.0356
PLT (× 10^3/mm^3)	309.5 (245-359)	240.0 (229.0-243.5)	0.0001
Alb (g/L)	3.9 (3.7-4.4)	4.7 (4-5)	< 0.001
IGFBP-5 (ng/mL)	7.2 (5.5-11.3)	11.3 (8.0-44.6)	0.0019

[1]*P* value from Mann-Whitney test. Results are given as median. IQR: Interquartile range; CDAI: Crohn's Disease Activity Index; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; Hb: Hemoglobin; WBC: White blood cell count; PLT: Platelet count; Alb: Albumin; IGFBP-5, Insulin-like growth factor-binding protein 5.

Table 3 Characteristics of Crohn's disease patients with and without stricture formation *n* (%)

	CD patients with stricture formation (*n* = 26)	CD patients without stricture formation (*n* = 16)	*P* value[1]
Age mean ± SD (yr)	41.4 (15.0)	34.6 (11.0)	0.124
Gender (F/M)	11 (42.3)/15 (57.7)	9 (56.3)/7 (43.8)	0.527
Disease localization			0.083
Ileum	3 (11.5)	6 (37.5)	
Colon	1 (3.9)	1 (6.3)	
Ileum + colon	22 (84.6)	9 (56.3)	
Disease duration (yr)	1.25 (0.25-12)	1.2 (0-3.1)	0.254
CDAI	78.5 (50.0-122.0)	112.0 (74.5-165.5)	0.090
ESR (mm/h)	36.5 (17.0-54.0)	35.0 (21.0-39.5)	0.660
CRP (mg/dL)	0.4 (0.3-1.2)	1.0 (0.4-3.0)	0.239
Hb (g/dL)	13.0 (12.4-14.1)	12.4 (11.5-13.7)	0.468
WBC (× 10^3/mm^3)	7.25 (5.5-9.6)	8.8 (6.15-9.15)	0.509
PLT (× 10^3/mm^3)	309.5 (262-383)	288.5 (234.0-334.0)	0.399
Alb (g/L)	3.9 (3.6-4.4)	4.1 (3.8-4.5)	0.233
IGFBP-5 (ng/mL)	6.9 (5.5-11.3)	7.8 (5.3-10.1)	0.815

[1]*P* values are from *t* test, Fisher's exact and Mann-Whitney test statistics were appropriate. Results are given as median. IQR: Interquartile range; CDAI: Crohn's Disease Activity Index; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; Hb: Hemoglobin; WBC: White blood cell count; PLT: Platelet count; Alb: Albumin; IGFBP-5: Insulin-like growth factor-binding protein 5.

cantly different in patients with active CD [7.2 (6.3-7.6) ng/mL] compared to patients with inactive CD [7.2 (5.5-11.3) ng/mL; *P* = 0.8901]. However, patients with active disease had lower 75 percentile values compared to patients with inactive disease (7.6 *vs* 11.3) (data not shown).

The serum IGFBP-5 levels were not correlated with clinical baseline parameters reflecting disease activity (CDAI, Alb, and Hb) or the presence of stricture formation. However, there was a significant correlation between serum IGFBP-5 levels and PLT (*r* = 0.319, *P* = 0.0395).

We evaluated IGFBP-5 expression in ileal biopsies using immunohistochemistry in all CD patients. Figure 2 presents representative examples of different immunohistochemical staining intensity scores (scores 1, 2 and
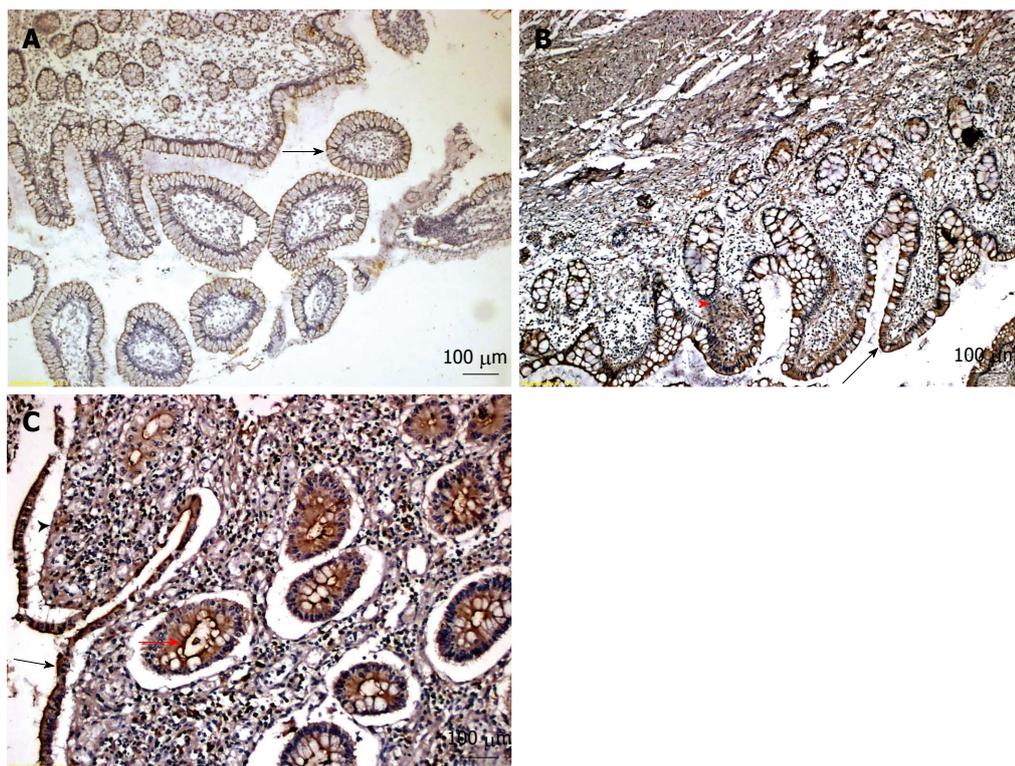
Figure 2 Representative examples of different immunohistochemical staining intensity scores for Insulin-like growth factor-binding protein 5 expression. Insulin-like growth factor-binding protein 5 (IGFBP-5) was diffusely expressed in the intestinal tissue. A: Weak staining (score = 1) (arrow) in epithelial cells in a Crohn's disease (CD) patient without stricture formation (ileal sample); B: Moderate staining (score = 2) both in epithelial (arrow) and stromal cells (arrowhead) in a CD patient with stricture formation (ileal sample); C: Strong staining (score = 3) in epithelial cells (arrow), stromal cells (arrowhead) and crypt lumen (red arrow) in a CD patient with active disease (colonic sample). Bar = 100 μm. Images were obtained using a light-field microscope, and edited using Adobe Photoshop CS5 (Adobe Systems Incorporated).

Table 4 Frequency of Insulin-like growth factor-binding protein 5 positive samples and the median intensity score of IGFBP-5 immunostaining (scores 0 to 3) in Crohn's disease patients with and without stricture formation *n* (%)

	CD patients with stricture formation (*n* = 26)	CD patients without stricture formation (*n* = 16)	*P* value[1]
Number of IGFBP-5 positive samples	16 (61.5)	8 (50)	0.463
Median intensity score of IGFBP-5 immunostaining	1	0.5	0.405

[1]*P* values are from Fisher's exact and Mann-Whitney test statistics were appropriate. IGFBP-5: Insulin-like growth factor -binding protein 5; CD: Crohn's disease.

3) for IGFBP-5 expression using tissue samples from 3 different CD patients. Among all 42 CD patients, 24 (57.1%) had tissue samples stain positive for IGFBP-5 expression. The median level of IGFBP-5 intensity score was 1.0. The frequency of IGFBP-5 positive samples and the median intensity score did not differ significantly between CD patients with stricture formation and those without stricture formation (Table 4) or between CD patients with active disease and those with inactive disease (data not shown). The IGFBP-5 intensity scores were positively correlated with WBC count ($r = 0.391$; $P = 0.01$) and PLT ($r = 0.356$; $P = 0.021$).

No significant correlation was found between ileal IGFBP-5 immunohistochemical expression and serum IGFBP-5 levels. The serum IGFBP-5 concentrations were not significantly different for individuals with biopsies with absent/very weak (0), weak (1), moderate (2), or strong (3) IGFBP-5 staining scores (Figure 3).

DISCUSSION

This study revealed that circulating levels of IGFBP-5 were significantly reduced in patients with CD compared to healthy controls [7.2 (5.5-11.3) ng/mL *vs* 11.3 (8.0-44.6) ng/mL ($P = 0.002$), respectively]. To our knowledge, this is the first study that demonstrates low serum IGFBP-5 levels in patients with CD compared to healthy controls. Despite the low serum IGFBP-5 levels described in CD patients, the authors did not observe any significant differences between the median serum IGFBP-5 levels for patients with and without strictures [6.9 (5.5-11.3) ng/mL *vs* 7.8 (5.3-10.1) ng/mL ($P = 0.815$), respectively]. Previous studies have mainly evaluated serum total and free IGF- I , IGFBP-1, IGFBP-2 and IGFBP-3 levels in the context of disease activity. Our results were similar to these studies, demonstrating low levels of IGF system proteins in IBD patients[11-16]. Katsanos *et al*[11] reported
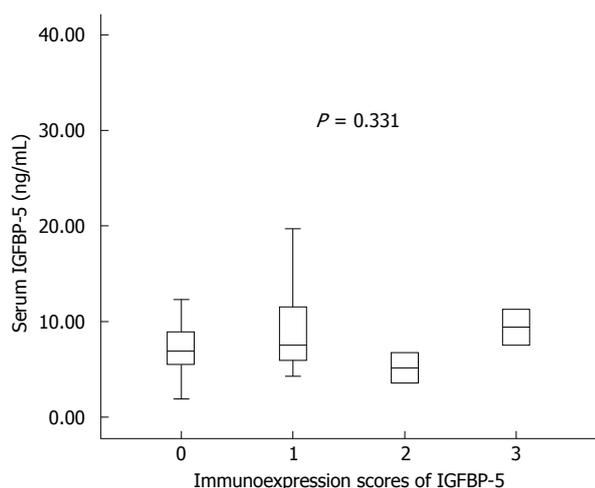
**Figure 3 Serum Insulin-like growth factor-binding protein 5 concentrations in patients with different immunohistochemical staining intensity scores for Insulin-like growth factor-binding protein 5 expression.** Serum insulin-like growth factor-binding protein 5 (IGFBP-5) concentrations were not significantly different for individuals with absent/very weak, weak, moderate, or strong IGFBP-5 staining intensity scores ($P = 0.331$). P value from Kruskal-Wallis test.

that circulating levels of IGF-I and IGFBP-3 were reduced in patients with active IBD[9]. Grønbek *et al*[12] demonstrated reduced serum total and free IGF-I and IGFBP-3 levels in patients with active IBD without complete normalization during high-dose prednisolone treatment and tapering[10]. Vespasiani Gentilucci *et al*[14] observed low IGF-1 and IGFBP-3 levels in active IBD patients before infliximab therapy, and a repeated drop in levels after normalization and clinical remission. However, we did not observe any significant difference between serum IGFBP-5 levels in patients with active and inactive disease [7.2 (5.5-11.3) ng/mL *vs* 7.2 (6.3-7.6) ng/mL ($P = 0.8901$), respectively]. Moreover, serum IGFBP-5 levels were moderately correlated with PLT, but not with hemoglobin, albumin levels, or CDAI. This finding may be explained by the low number of active CD patients ($n = 9$, 21.4%) in our study or by the presence of low-grade subclinical transmural inflammation, which is not detectable with clinical or biochemical markers. The most important in vivo regulator of IGFBP-5 expression is IGF-I [7]. In normal adult human serum, IGFBP-5 levels are positively correlated with IGF-I concentrations[19]. Although we did not evaluate serum IGF-I levels, the mechanism underlying low IGFBP-5 levels in CD patients may be similar to the mechanism for low IGF-I levels, as IGFBP-5 expression is mainly regulated by IGF-I. Previous studies have suggested that low IGF-1 levels in active IBD patients may be due to the direct adverse effects of circulating inflammatory cytokines[19,20]. Katsanos *et al*[11] also showed that serum IL-6 levels were increased in IBD patients with active disease compared to healthy controls. IGFBP-5 expression *in vitro* can be regulated by hormones and cytokines[7]. However, as inflammatory cytokines were not evaluated in our study, further investigations are needed to examine the effects

of cytokines on circulating IGFBP-5 levels. Previous studies demonstrated partially normalized or unchanged low IGF levels during corticosteroid and infliximab treatments[12,14-16], and the low serum IGFBP-5 levels in our study may be explained by the previously described poor correlation between clinical activity, endoscopic severity and biological parameters in CD patients[21].

To our knowledge, we are the first researchers to investigate the relationship between circulating IGFBP-5 concentrations and intestinal IGFBP-5 expression in CD patients. No correlation was found between circulating IGFBP-5 concentrations and intestinal IGFBP-5 expression. The serum IGFBP-5 concentrations were not significantly different between individuals with biopsies with absent/very weak, weak, moderate, or strong IGFBP-5 staining. Previous studies demonstrated that serum levels of hepatic-derived IGF-I and IGFBP-3 were lower in patients with active CD than in normal subjects, whereas the expression of IGF-I, IGFBP-5, and IGFBP-3 in smooth muscle cells in strictured intestines was increased compared to adjacent nonstrictured intestinal muscle from the margin of resected tissue[22-24]; however, the reasons for the discrepancy in the levels of these mediators in the serum compared to the expression in the muscle layer of the intestine remain unclear. It has been suggested that serum levels and intestinal expression of the IGF system are differentially regulated.

Three pathophysiologic events occurring within smooth muscle cells of the muscularis propria contribute to stricture formation in CD: increased smooth muscle cell hyperplasia, increased smooth muscle cell hypertrophy, and excess net extracellular matrix proteins, including collagen. IGF-I up-regulation is accompanied by IGFBP-5 up-regulation and collagen I, III, and V up-regulation[4,5,25]. Zimmermann *et al*[5] showed that IGF-I and IGFBP-5 mRNA was increased in inflamed/fibrotic intestines compared with normal-appearing intestines. However, we could not demonstrate increased intestinal IGFBP-5 expression in CD patients with stricture formation compared to those without stricture formation. Unfortunately, we were not able to analyze and compare intestinal IGFBP-5 expression in both fibrotic and normal-appearing tissue in CD patients with stricture formation. Moreover, the intestinal tissue was obtained with standard endoscopic biopsies, and the majority of our patients had inactive disease ($n = 33$, 78.6%), whereas previous studies analyzed active inflamed intestinal tissues from resection samples. Although we did not observe any significant difference between active and inactive patient groups regarding intestinal IGFBP-5 expression, intestinal IGFBP-5 expression was positively correlated with WBC count and PLT. This finding may be due to a poor correlation between clinical activity indices and actual endoscopic disease activity. One limitation of our study is the small sample size. Additionally, we were unable to obtain biopsies from normal-appearing intestinal mucosa of CD patients to compare with intestinal IGFBP-5 expression in inflamed/strictured mucosa. Moreover, intestinal IGFBP-5 expression could be affected by the area of the

biopsy samples.

In conclusion, our results indicate that serum IGFBP-5 concentrations are lower in CD patients compared to healthy controls regardless of disease activity or the presence of stricture formation. Serum IGFBP-5 concentrations were not associated with intestinal IGFBP-5 tissue expression. Therefore, our results do not answer the question of whether IGFBP-5 is involved in the stricture formation of CD, and thus, more research is necessary. Directions for future research include examination of other serum IGF system components, use of a larger patient population with active and inactive disease, endoscopic determination of disease activity and collection of biopsy tissue from both normal and inflamed/strictured areas.

COMMENTS

Background
Crohn's disease (CD) is a multifactorial disorder and its behavior may change throughout the course of the disease. Approximately 30 % of the CD patients will develop strictures and experience complications related to the stricture formation. It is crucial to understand the pathophysiology of bowel-wall stricturing in CD. Members of the insulin-like growth factor (IGF) system have been implicated as central players in stricture formation.

Research frontiers
It has been shown that both insulin-like growth factor 1 (IGF- I) and insulin-like growth factor-binding protein 5 (IGFBP-5) expressions are increased in inflamed/fibrotic intestine. Many studies showed that circulating levels of IGF- I and its binding protein proteins (IGFBPs) are decreased in inflammatory bowel disease. In the present study serum IGFBP-5 levels and intestinal IGFBP-5 expression was investigated in CD patients with and without stricture formation.

Innovations and breakthroughs
The serum levels of IGFBP-5 and intestinal expression of IGFBP-5 with immunohistochemistry in CD patients has not been studied previously. This study, for first time, reports that serum IGFBP-5 levels are decreased in CD patients regardless the presence of stricture formation or disease activity.

Applications
By understanding the circulating IGFBP-5 profile in CD patients, this study may represent a future strategy for prospective studies to understand the interaction between IGF system and CD pathophysiology, which may in turn aid in finding specific biomolecular targets for treatment of CD.

Terminology
IGFBP-5 is a member of six IGFBPs. It is binding to IGFs with high affinity and has several regulatory functions. IGFBP-5 stimulates muscle hyperplasia and collagen secretion in human intestinal smooth muscle. It has been suggested that IGFBP-5, may be important in the pathogenesis of intestinal fibrosis in inflammatory bowel disease.

Peer review
The authors examined the serum levels of IGFBP-5 and its intestinal expression in CD. It revealed that circulating IGFBP-5 levels are lower in CD patients compared to healthy controls. The results are interesting and may address the potential role of circulating IGFBP-5 in the pathophysiology of CD.

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BRIEF ARTICLE

Alvarado, Eskelinen, Ohhmann and Raja Isteri Pengiran Anak Saleha Appendicitis scores for diagnosis of acute appendicitis

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Abstract

AIM: To assess the reliability and practical applicability of the widely used Alvarado, Eskelinen, Ohhmann and Raja Isteri Pengiran Anak Saleha Appendicitis (RIPASA) scoring systems in patients with suspected acute appendicitis.

METHODS: Patients admitted to our tertiary center due to suspected acute appendicitis constituted the study group. Patients were divided into two groups. appendicitis group (Group A) consisted of patients who underwent appendectomy and were histopathologically diagnosed with acute appendicitis, and non-appendicitis group (Group N-A) consisted of patients who underwent negative appendectomy and were diagnosed with pathologies other than appendicitis and patients that were followed non-operatively. The operative findings for the patients, the additional analyses from follow up of the patients and the results of those analyses were recorded using the follow-up forms.

RESULTS: One hundred and thirteen patients with suspected acute appendicitis were included in the study. Of the 113 patients (62 males, 51 females), the mean age was 30.2 ± 10.1 (range 18-67) years. Of the 113 patients, 94 patients underwent surgery, while the rest were followed non-operatively. Of the 94 patients, 77 patients were histopathologically diagnosed with acute appendicitis. Our study showed a sensitivity level of 81% for the Alvarado system when a cut-off value of 6.5 was used, a sensitivity level of 83.1% for the Ohmann system when a cut-off value of 13.75 was used, a sensitivity level of 80.5% for the Eskelinen system when a cut-off value of 63.72 was used, and a sensitivity level of 83.1% for the RIPASA system when a cut-off value of 10.25 was used.

CONCLUSION: The Ohmann and RIPASA scoring systems had the highest specificity for the diagnosis of acute appendicitis.

Key words: Acute appendicitis; Alvarado; Eskelinen; Ohhmann; Raja Isteri Pengiran Anak Saleha Appendicitis

Core tip: Several scoring systems have been devised to aid decision making in doubtful acute appendicitis cases, including the Ohmann, Alvarado, Eskelinen, Raja Isteri Pengiran Anak Saleha Appendicitis and several others. These scores utilize routine clinical and laboratory assessments and are simple to use in a variety of clinical settings. However, differences in sensitivities and specificities were observed if the scores were applied to various populations and clinical settings, usually with

worse performance when applied outside the population in which they were originally created.

Erdem H, Çetinkünar S, Daş K, Reyhan E, Değer C, Aziret M, Bozkurt H, Uzun S, Sözen S, İrkörücü O. Alvarado, Eskelinen, Ohhmann and Raja Isteri Pengiran Anak Saleha Appendicitis scores for diagnosis of acute appendicitis. *World J Gastroenterol* 2013; 19(47): 9057-9062 Available from: URL: http://www.wjg-net.com/1007-9327/full/v19/i47/9057.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i47.9057

INTRODUCTION

Acute appendicitis is a common surgical condition that requires prompt diagnosis to minimize morbidity and avoid serious complications. Accurate identification of patients who require immediate surgery as opposed to those who will benefit from active observation is not always easy[1].

Several scoring systems have been devised to aid decision making in doubtful cases, including the Ohmann, Alvarado, Eskelinen, Raja Isteri Pengiran Anak Saleha Appendicitis (RIPASA) and several others[2-5]. These scoring systems utilize routine clinical and laboratory assessments and are simple to use in a variety of clinical settings. However, differences in sensitivities and specificities were observed if the scores were applied to various populations and clinical settings, usually with worse performance when applied outside the population in which they were originally created[2,3,6]. Additionally, geographic variation of the incidence and clinical pattern of the differential diagnosis of acute abdominal pain may impair their applicability[7]. Accurate diagnosis of acute appendicitis is especially difficult in women, where the inaccuracy of available diagnostic methods leads to an unacceptably high negative appendectomy rate due to gynecological disorders that frequently mimic appendicitis[8].

This study aimed to assess the reliability and practical applicability of the widely used Alvarado, Eskelinen, Ohhmann and RIPASA scoring systems in patients with suspected acute appendicitis.

MATERIALS AND METHODS

Study design

This prospective study was approved by the local Institutional Review Board (ANEAH 2011/2). Written informed consent was obtained from all subjects. Patients admitted to our tertiary center due to suspected acute appendicitis between October 2011 and March 2012 constituted the study group.

Patients were divided into two groups: appendicitis group (Group A) consisted of patients who underwent appendectomy and were histopathologically diagnosed with acute appendicitis, and non-appendicitis group (Group N-A) consisted of patients who underwent negative appendectomy, patients diagnosed to have patholo-

gies other than appendicitis, and patients that were followed non-operatively.

Outcome parameters

Patient data including age, gender, height, weight, the duration of hospital stay, accompanying disease history, operation or follow-up findings, and laboratory and imaging findings were recorded. Parameters from the Alvarado, Eskelinen, Ohmann and RIPASA scoring systems were combined in this form[2-5]. Decisions regarding operation and follow up were given according to the preferences of the surgeon, not the scoring results.

The scores were calculated using an automated Microsoft Excel sheet after the patients were discharged. Calculated values were recorded as having a low, medium or high probability for acute appendicitis (Table 1). Operative findings, additional analyses of follow-up patients and the results of those analyses were recorded using the follow-up forms. A diagnosis of appendicitis was given macroscopically during the operation (purulent formations, and edematous- necrotic changes on the appendix wall). The results were confirmed with histopathological findings.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences 19.0 for Windows (SPSS Inc., Chicago, IL, United States) and Medcalc (Mariakerke, Belgium) for Windows. The results for all of the items were expressed as the mean ± SD, assessed within a 95% reliance and at a level of $P < 0.05$ significance. The sample size calculation was based on a significance level of 0.05. We needed a sample of 103 patients to achieve 80% power. A normal distribution of the quantitative data was checked using the Kolmogorov-Smirnov test. Parametric tests were applied to the normally distributed data and non-parametric tests were applied to data with a questionably normal distribution. An independent sample t test and Mann-Whitney U test were used to compare the independent groups. Receiver operating characteristic curves were used to identify the optimal cut-off points. Cross tables were prepared for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the diagnostic accuracy values of the scoring systems. We used a χ^2 test to compare categorical measures regarding the diagnosis of acute appendicitis.

RESULTS

One hundred and thirteen patients with suspected acute appendicitis were included in the study. Of the 113 patients (62 males, 51 females), the mean age was 30.2 ± 10.1 (range 18 to 67) years. Of the 113 patients, 94 patients (83.19%) underwent surgery, while the rest (16.81%) were followed non-operatively. Of the 94 patients, 77 patients (81.91%) were histopathologically diagnosed with acute appendicitis, 6 (6.38%) were diagnosed with pathologies other than appendicitis (ovarian cyst rupture in three patients, inflammatory bowel disease in two patients, and a carcinoid tumor in one patient), and 11 patients (11.71%)

Table 1 Clinical approaches advised by the authors regarding the scoring systems

	High probability	Probable/should be followed	Appendicitis with high probability
Alvarado	< 4	5-6	> 7
Eskelinen	< 48	48-57	> 57
Ohhmann	< 6	6-11.5	> 12
RIPASA	< 5	5-7	> 7.5

Table 2 Frequency of symptoms, examination findings and laboratory results *n* (%)

		Group A	Group N-A	*P* value
Symptoms				
Loss of appetite	Yes	25 (69)	66 (86)	0.072
	No	11 (31)	11 (14)	
Nausea-Vomiting	Yes	20 (56)	52 (68)	0.294
	No	16 (44)	25 (32)	
Time pain started	< 48	21 (58)	59 (77)	0.074
	> 48	15 (42)	18 (23)	
Starting point of pain	Around stomach	8 (22)	37 (48)	0.021
	Lower right quadrant	25 (69)	38 (49)	
	Anywhere	3 (8)	2 (3)	
Relocalization of the pain to the lower right quadrant	Yes	7 (19)	33 (43)	0.020
	No	29 (81)	44 (57)	
Urinary system complaint	Yes	9 (25)	8 (10)	0.052
	No	27 (75)	69 (90)	
Similar pain history	Yes	19 (53)	13 (17)	< 0.001
	No	17 (47)	64 (83)	
Findings				
Sensitivity on lower right quadrant	Yes	36 (100)	76 (99)	0.999
	No	0 (0)	1 (1)	
Defense-rigidity	Yes	23 (64)	77 (100)	< 0.001
	No	13 (36)	0 (0)	
Rebound	Yes	16 (44)	75 (97)	< 0.001
	No	20 (56)	2 (3)	
Rowsing finding	Yes	7 (19)	31 (40)	0.034
	No	29 (81)	46 (60)	
Fever	> 37.3	5 (14)	28 (36)	0.015
	< 37.3	31 (86)	49 (64)	
Laboratory results				
Neutrophil	> %75	10 (28)	49 (64)	0.001
	< %75	26 (72)	28 (36)	
Leukocyte	< 10000	15 (42)	13 (17)	0.009
	≥ 10000	21 (58)	64 (83)	
Urine analysis	Normal	24 (67)	75 (97)	< 0.001
	Abnormal	12 (33)	2 (3)	

Group A: Appendicitis group; Group N-A: Non-appendicitis group.

underwent a negative appendectomy. Among the 19 patients who were followed non-operatively, urinary system disease was diagnosed in eight patients, gastroenteritis was diagnosed in four patients, mesenteric lymphadenitis was diagnosed in one patient, inflammatory bowel disease was diagnosed in one patient and gynecologic problems were diagnosed in one patient. A diagnosis was not established, and clinical improvement was observed in four patients.

Group A included 77 patients (46 males, 31 females) with a mean age of 29.5 ± 9 years, and Group N-A included 36 patients (16 males, 20 females) with a mean age of 31.8 ± 12.1 years. Both groups did not differ significantly in age and gender ($P = 0.560$ and $P = 0.157$, respectively). With respect to the mean height (168.9 ± 8.1 cm *vs* 168.1 ± 8.8 cm), mean weight (71.3 ± 12.8 kg *vs* 71.6 ± 16.4 kg), and duration of hospital stay (45.3 ± 20.1 d *vs* 57.9 ± 37.6 d), the two groups were not significantly different ($P = 0.634$, $P = 0.894$, and $P = 0.065$, respectively).

Regarding patient symptoms, there was no similar pain history among the 64 patients that were diagnosed with acute appendicitis, while 13 patients had a similar pain history. It was found that not having a similar pain history was statistically significant for acute appendicitis ($P < 0.001$). The studied groups differed significantly from each other with regard to the starting point of pain ($P = 0.021$) and relocalization of the pain to the lower right quadrant ($P = 0.020$). As for the examination findings, the defense-rigidity, rebound, and Rowsing findings differed significantly between the groups ($P < 0.001$, $P < 0.001$, and $P = 0.034$, respectively). Fever was also significantly different between the groups ($P = 0.015$). As for the laboratory results, the neutrophil rate, leukocyte count, and urine analysis results differed significantly between the groups ($P = 0.001$, $P = 0.009$, and $P < 0.001$, respectively) (Table 2). The operative and follow-up results for the patients were as follows: phlegmonous in 45 patients, catarrhal in 15 patients, gangrenous in 11 patients, vermiformis (negative appendectomy) in 11 patients, and perforated in six patients.

When the sensitivity and specificity levels of the scoring systems were assessed, they were 82% and 75% for the Alvarado, 100% and 28% for the RIPASA, 96% and 42% for the Ohmann, and 100% and 44% for the Eskelinen scores. When the negative appendectomy rates of the Alvarado, RIPASA Ohmann and Eskelinen scoring systems were assessed, they were found to be 12%, 25%, 22% and 21%, respectively (Table 3). When a cut-off value for the Alvarado system was set at 6.5, its sensitivity was calculated as 81%. When a cut-off value for the Ohmann system was set at 13.75, its sensitivity was calculated as 83.1%. When a cut-off value for the Eskelinen system was set at 63.72, its sensitivity was calculated as 80.5%. When a cut-off value for the RIPASA system was set at 10.25, its sensitivity was calculated as 83.1% (Figure 1 and Table 4).

DISCUSSION

The diagnosis of acute appendicitis still represents one of the most difficult problems in surgery[7]. It is generally accepted that the removal of a normal appendix is safer in questionable cases and that delaying surgery leads to an increased rate of perforation[8]. There have been many attempts to increase the accuracy of the diagnosis of acute appendicitis. In addition to clinical evaluation, with the

Table 3 Sensitivity, specificity, positive predictive value, negative predictive value, diagnostic accuracy and negative appendectomy values of the scoring systems

	Alvarado (cut-off = 7)	Ohhmann (cut-off = 12)	Eskelinen (cut-off = 57)	RIPASA (cut-off = 7.5)
Sensitivity	82%	96%	100%	100%
Specificity	75%	42%	44%	28%
PPV	88%	78%	79%	75%
NPV	66%	83%	100%	100%
Diagnostic accuracy	80%	79%	82%	77%
Neg. app. rate	12%	22%	21%	25%

PPV: Positive predictive value; NPV: Negative predictive value; Neg. app. rate: Negative appendectomy rate.
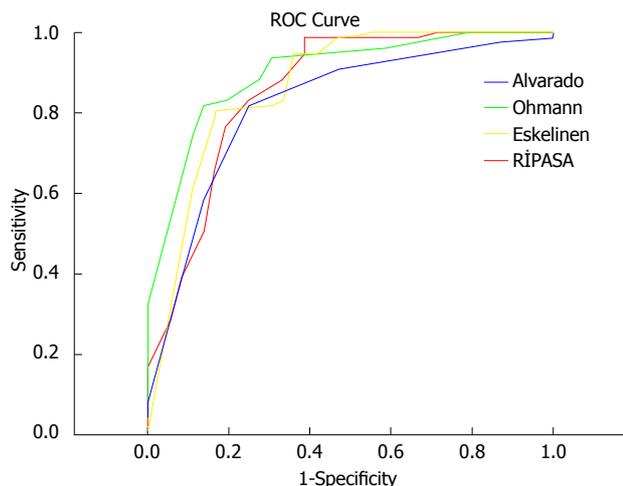
Figure 1 If we use high cut-off values for diagnostic methods and accept where the majority (at least three methods) are positive as positive and the others as negative, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy values of this new diagnostic method would be 98.7%, 55.6%, 82.6%, 95.2% and 84.9%, respectively.

Table 4 Cut-off values for the maximum sensitivity and specificity values of the scoring systems

Measurements	AUC	Cut-off	Sensitivity	Specificity
Alvarado	0.818	6.50	81.8	75.0
Ohmann	0.899	13.75	83.1	80.6
Eskelinen	0.867	63.72	80.5	83.3
RİPASA	0.857	10.25	83.1	75.0

AUC: Area under the curve.

ity, such as perforation and peritonitis. In the present study, 83.2% of the patients underwent surgery, while 16.8% were followed non-operatively. Of the patients who underwent surgery, 81.9% were histopathologically diagnosed with acute appendicitis, 6.38% were diagnosed to have pathologies other than appendicitis, and 11.71% underwent a negative appendectomy.

Acute appendicitis typically presents itself with pain that starts in the epigastrium or around the stomach and localizes to the lower right quadrant. A study by Ortega-Deballon *et al*[14] reported that the acute appendicitis diagnosis rate found in patients presenting with pain in the lower right quadrant was 65%. Similarly, Lane *et al*[15] reported this rate as 55%. In the present study, 68% of the patients who presented with pain in the lower right quadrant were histopathologically proven to have acute appendicitis.

Non-surgical pathologies can be found on physical examination and laboratory analyses in 20%-25% of the cases presenting with acute pain in the lower right quadrant, and these cases can be followed using conservative methods[16,17]. Furthermore, 5%-15% of cases with suspected acute appendicitis cannot be diagnosed despite an aggressive work-up[18]. In the present study, the rate of patients who could be followed non-operatively was 21%. The rate of patients with symptoms that receded clinically was 5%.

The idea of improving the diagnostic accuracy simply by assigning numeric values to defined signs and symptoms has been the goal of some of the scores that were previously described[1-5]. The parameters comprising the score usually include general signs of abdominal illness (*e.g.*, type, location and migration of pain, body temperature, signs of peritoneal irritation, nausea, vomiting, *etc*.) and routine laboratory findings (leukocytosis)[19]. Ohmann

variety of clinical signs and symptoms, many of the modern diagnostic tools have proved to be effective for the diagnosis of acute appendicitis[1,7,8]. Although sonography and CT increase the accuracy of the diagnosis of acute appendicitis, they are unfortunately still often unavailable in some emergency departments[9,10]. Several scoring systems that have been devised for the purpose of increasing both the sensitivity and specificity of the diagnosis of acute appendicitis have been repeatedly tested[2-5]. Scoring systems represent an inexpensive, non-invasive and easy to use diagnostic aid.

According to previous publications, the criteria for diagnostic quality have been postulated as a 15% rate of negative appendectomies, a 10% rate of negative laparotomies, a 35% rate of potential perforations, a 15% rate of overlooked perforations and a 5% rate of overlooked acute appendicitis[9,10]. Although the negative appendectomy rate reported by surgeons advocating early surgical intervention in suspected cases to prevent perforation varies between 20% and 40%, the generally accepted negative appendectomy rate is approximately 15%-20%[11-13]. Furthermore, misdiagnosis and late surgical intervention leads to complications with high morbidity and mortal-

et al[3] performed a multivariate analysis, and of the initial 15 parameters, eight were included in a regression model, resulting in different values being attributed to each parameter. Originally, it was proposed that patients with scores less than six should not be considered to have appendicitis. However, patients with scores of six or more should undergo observation, and those with scores of 12 or more should proceed to immediate appendectomy[3]. The Eskelinen score delivered acceptable clinical results after calibration to a cut-off value of 57[5]. The Alvarado score is widely used for the diagnosis of acute appendicitis. The score is calculated over 10 points, and a score higher than six is indicative of acute appendicitis. On the other hand, a score of less than four indicates that it is unlikely that the patient has appendicitis. For scores of 4-6, follow-up or imaging with computerized tomography is recommended[4]. Chaudhuri *et al*[20], in their series of 175 patients with a mean age of 30 years, reported a negative cut-off point of five. The RIPASA score is a relatively new diagnostic scoring system and has been shown to have a significantly higher sensitivity, specificity and diagnostic accuracy[2,21]. The RIPASA score is easy to apply and includes several parameters that are absent in the Alvarado score, such as age, gender and the duration of symptoms prior to presentation[22,23]. Our study calculated the sensitivity and specificity of the Alvarado scoring system as 82% and 75%, respectively, and calculated the sensitivity and specificity of the RIPASA scoring system as 100% and 28%, respectively. Although the diagnostic accuracy levels of these two scoring systems were comparable, the RIPASA scoring system is considered less accurate because of the higher negative appendectomy rates. The negative appendectomy rate calculated in our study was 12%. When the accuracy measures of all of the scoring systems included in our study were analyzed, they performed better, especially if the cut-off values were increased. A higher cut-off value leads to 100% sensitivity and a negative predictive value for the RIPASA and Eskelinen methods and leads to 96% sensitivity with an 83% negative predictive value for the Ohmann method. When these values are assessed, it is found that the Ohmann and Eskelinen methods are one step ahead in terms of detecting appendicitis, although they fail to meet expectations in terms of specificity. The disadvantage of the Eskelinen scoring system is the practicality of calculations because values in this system are decimals, and in other systems they are integers.

For the scoring systems, sensitivity and specificity values higher than 80% are acceptable[24,25]. This is why these scoring systems may prove more advantageous when the cut-off values are customized to clinical populations. Our study showed a sensitivity level of 81% for the Alvarado system when the cut-off value was set at 6.5, a sensitivity level of 83.1% for the Ohmann system when the cut-off value was set at 13.75, a sensitivity level of 80.5% for the Eskelinen system when the cut-off value was set at 63.72, and a sensitivity level of 83.1% for the RIPASA system when the cut-off value was set at 10.25.

The main limitation of our study is the relatively small number in our series. In addition, some details regarding the history and factors that may influence the outcome may not have been completely documented. Due to these restrictions, associations should be interpreted with caution.

In conclusion, the Ohmann and RIPASA scoring systems have the highest specificity for the diagnosis of acute appendicitis.

COMMENTS

Background
Several scoring systems have been devised to aid decision making in doubtful acute appendicitis cases, including the Ohmann, Alvarado, Eskelinen, Raja Isteri Pengiran Anak Saleha Appendicitis (RIPASA) and several others.

Research frontiers
To assess the reliability and practical applicability of the widely used Alvarado, Eskelinen, Ohhmann and RIPASA scoring systems in patients with suspected acute appendicitis.

Innovations and breakthroughs
The Ohmann and RIPASA scoring systems have the highest specificity for the diagnosis of acute appendicitis.

Applications
Accurate identification of patients who require immediate surgery as opposed to those who will benefit from active observation is always useful.

Terminology
The Alvarado, Eskelinen, Ohhmann and RIPASA are common scoring systems that are used in patients with suspected acute appendicitis.

Peer review
This study is a prospective one and was conducted over 5-mo period and managed to recruit 113 patients (62 males and 51 females); 94 patients underwent surgery. This could be the first study comparing the four scoring systems in term reliability in diagnosing appendicitis.

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BRIEF ARTICLE
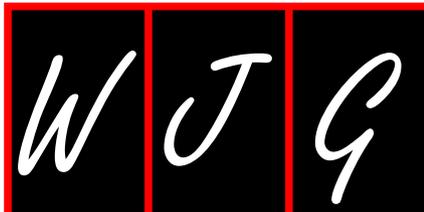
Seasonal variations in the onset of ulcerative colitis in Japan

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Abstract

AIM: To investigate seasonal variations in the onset and relapse of ulcerative colitis (UC) in Japanese patients.

METHODS: Between 1994 and 2006, 198 Japanese patients diagnosed with UC according to conventional criteria in an academic hospital were enrolled for onset evaluation. Among 265 Japanese patients with UC who were observed for more than 12 mo, 165 patients relapsed (239 times) and were enrolled for relapse evaluation. The patients' symptoms were recorded each month for 12 consecutive years.

RESULTS: There was monthly seasonality in symptom onset during October and March for UC. The onset of symptoms in UC patients frequently occurred during the winter. Variation in UC onset was observed according to both month ($P = 0.015$) and season ($P = 0.048$). Relapse commonly occurred in October, and variations in relapse were not significant either in month ($P = 0.52$) or season ($P = 0.12$). Upper respiratory inflammation was the main factor responsible for relapse.

CONCLUSION: Our results suggest that environmental factors associated with winter and spring seasonality may be responsible for triggering the clinical onset of UC in Japan.

Core tip: Monthly seasonality in the symptomatic onset of ulcerative colitis (UC) during October and March was observed in Japan. The onset of symptoms frequently occurred during the winter, whereas relapse of UC particularly occurred in October. Upper respiratory inflammation was one of the main factors responsible for relapse. Therefore, environmental factors associated with winter and spring seasonality may be responsible for triggering the clinical onset of UC in Japan.

Koido S, Ohkusa T, Saito H, Yokoyama T, Shibuya T, Sakamoto N, Uchiyama K, Arakawa H, Osada T, Nagahara A, Watanabe S, Tajiri H. Seasonal variations in the onset of ulcerative colitis in Japan. *World J Gastroenterol* 2013; 19(47): 9063-9068 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/

INTRODUCTION

Ulcerative colitis (UC) is a chronic relapsing disease characterized by alternating periods of remission and active disease. UC is the result of a complex interaction between genetic susceptibility[1], stimulation by bacterial antigens[2] in the lumen and occasional environmental triggers[3] that damage the mucosal barrier. Although the incidence and prevalence of UC is lower in Asia than in the West, recent population-based and referral center cohorts have shown a rising incidence and prevalence of UC in Asia[3]. Therefore, it is critical to gain a better understanding of the environmental factors that contribute to the onset and relapse of UC in Asia.

Furthermore, the environmental factors associated with UC are poorly defined. For example, previous studies examining seasonal variations in the onset and relapse of UC in Western populations[4-10] have demonstrated conflicting results. These studies used retrospectively collected or hospital admission data, which may have led to a bias in the results. Moreover, due to different cultural backgrounds, racial genetic predisposition and dietary habits, Japanese patients may show different clinical patterns from those of Western populations. Therefore, the present study was designed to investigate whether there were seasonal variations in the onset and relapse of symptoms in Japanese patients with UC.

MATERIALS AND METHODS

Patients

We performed an epidemiological cohort study of Japanese patients with UC who were diagnosed according to conventional criteria[11] between 1994 and 2006 at an academic hospital in Tokyo. The diagnosis of UC was confirmed by a typical history combined with the appropriate endoscopic, histopathological, and radiologic findings[11]. A total of 198 patients were enrolled in the onset evaluation (Figure 1). Data concerning the onset of symptoms were prospectively assessed using a standard interview focusing on symptoms accepted for UC (diarrhea, blood in stool, mucus or pus in stool, abdominal pain, fever, weight loss) and the period of time in which such symptoms occurred for the first time. The date of diagnosis was established according to the first investigation (endoscopy and histology, radiology or surgery) in which a diagnosis of UC could be defined. The onset of symptoms was recorded each month for 12 consecutive years. Relapse was defined as 3 or more increases in the symptom score[12], excluding patients who relapsed due to decreasing doses of steroids, 5-aminosalicylic acid (5-ASA), or salazosulfapyridine (SASP).

Among 265 Japanese patients with UC observed for more than 12 mo between 1994 and 2006, 165 patients relapsed (239 times). The symptom scores of these pa-
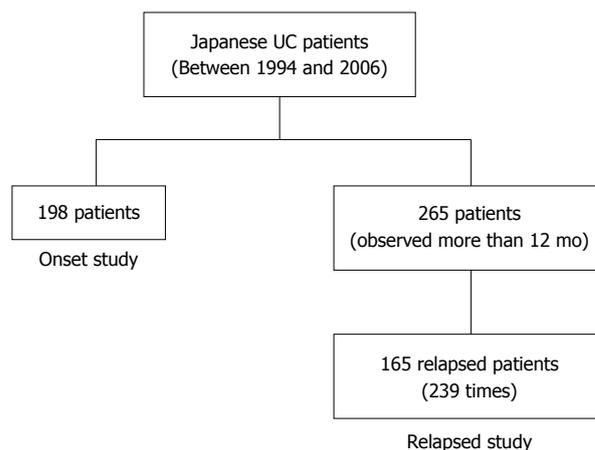
Figure 1 A flow chart of Japanese ulcerative colitis patients. Among 265 Japanese patients with ulcerative colitis observed for more than 12 mo between 1994 and 2006, a total of 198 patients were enrolled in the onset evaluation. Of these, 165 patients relapsed (239 times), and their symptom scores were recorded each month for at least one year in the relapse evaluation.

tients were recorded each month for at least one year (Figure 1). The frequencies of onset and relapse were compared for each month and the following four seasons: winter (December to February), spring (March to May), summer (June to August), and autumn (September to November).

Statistical analysis

The frequencies of onset and relapse were compared using the χ^2 test. In addition, the 12-mo seasonality was tested using Rogers' method. $P < 0.05$ was considered significant.

RESULTS

Variations in the onset of UC

A total of 198 Japanese patients with UC (132 males and 66 females) were investigated in the onset evaluation. The median age at diagnosis was 35 ± 14 years. The distribution of symptom onset according to month is shown in Figure 2. The timing of symptom onset was characterized by a clear monthly variation ($\chi^2_{(2\,df)} = 8.43$, $P = 0.015$), with a peak during October and March and a trough during June and September. Moreover, a seasonal pattern was observed ($\chi^2_{(3\,df)} = 7.88$, $P = 0.048$), as the onset rate was highest from autumn to spring [observed/expected (O/E): 53/49.36, 57/48.82, 55/49.91, respectively] (Figure 3).

Variations in the relapse of UC

Among 265 Japanese patients with UC observed for more than 12 mo, 165 patients relapsed (239 times), which was defined as 3 or more increases in the symptom score[12]. Patients who relapsed due to decreasing doses of steroids, 5-ASA, or SASP were excluded. The median age at relapse was 34 ± 12 years. Figure 4 shows the 165 observed UC patients according to month, taking into
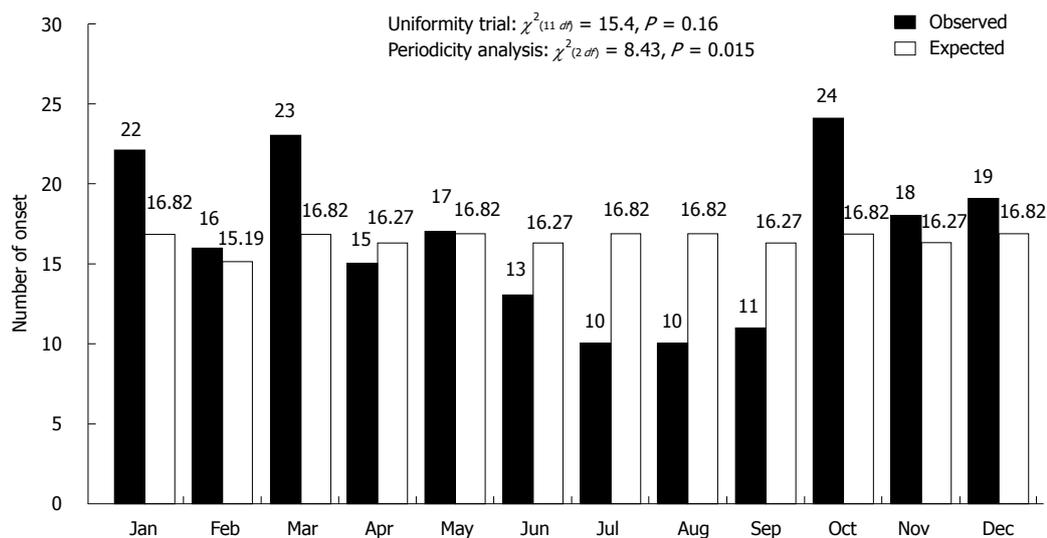
Figure 2 Monthly variations in the onset of ulcerative colitis. The highest onset rate was observed in October (24/198, 12.1%), followed by March (23/198, 11.6%). The lowest onset rate was observed in July and August (10/198, 5.1%). The timing of ulcerative colitis (UC) onset was characterized by a monthly variation ($\chi^2_{(2 df)}$ = 8.43, P = 0.015), with a peak during October and March and a trough during June and September.
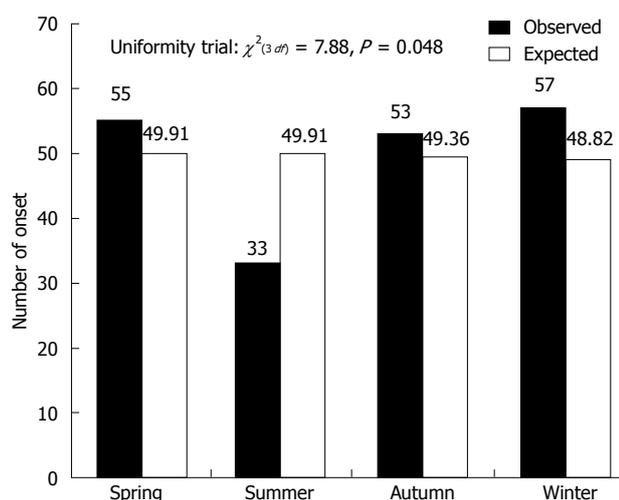
Figure 3 Seasonal variations in the onset of ulcerative colitis. The highest seasonal onset rate was observed in the winter (57/198, 28.8%), followed by the spring (55/198, 27.8%), autumn (53/198, 26.8%), and summer (33/198, 16.7%). A seasonal pattern was also observed ($\chi^2_{(3 df)}$ = 7.88, P = 0.048), as the onset rate was highest during autumn to spring (observed/expected: 53/49.36, 57/48.82, 55/49.91, respectively).

consideration the difference in the number of UC relapses each month. Relapse of symptoms in UC patients frequently occurred in October (O/E: 27/20.30). The lowest relapse rate was observed in January (O/E: 11/20.30). Variations in relapse were not found on a monthly basis ($\chi^2_{(2 df)}$ = 1.31, P = 0.52, Figure 4). Moreover, there was no variation in relapse on a seasonal basis ($\chi^2_{(3 df)}$ = 5.75, P = 0.12) (Figure 5).

Causes of UC relapse
In most cases, the causes of relapse were not identified. However, in cases with an identifiable cause, upper respiratory inflammation was the main factor responsible for relapse (Figure 6).

DISCUSSION

It is well known that environmental factors contribute to the induction of UC, although little is known about the relationship between seasonality and symptom flares in Asian UC patients, especially in the Japanese population. Therefore, we performed an epidemiological cohort study of patients with UC diagnosed between 1994 and 2006 at an academic hospital in Tokyo. The diagnosis of UC was confirmed by a typical history combined with the appropriate endoscopic, histopathological, and radiologic findings[11]. A total of 198 patients (132 males and 66 females) were enrolled in the onset evaluation.

A growing number of studies in Asia have reported an equal gender distribution in UC[12], although several studies have also demonstrated a male predominance[13]. In our academic hospital-based cross sectional study, there was a preponderance of male UC cases. These conflicting findings may, at least in part, reflect the small population numbers in the present study. Our results revealed seasonal variations in the onset of UC symptoms in Japanese patients, although there was no difference in the timing of relapse. We observed monthly seasonality in the symptomatic onset of UC in October and March, mainly in winter and spring. Previous studies have reported seasonal variations in the symptomatic onset of UC in December in the United Kingdom[5,14], December to January in Norway[15], and June to August in Spain[16]. Moreover, increased relapse rates for UC were reported in winter[4] and autumn[5] in the United Kingdom, spring to autumn in Greece[8], and winter in Sweden[7]; however, other studies reported no seasonality in the United Kingdom[17], Spain[16], or the United States[10,18]. These conflicting data may, at least in part, reflect the distinct genetic
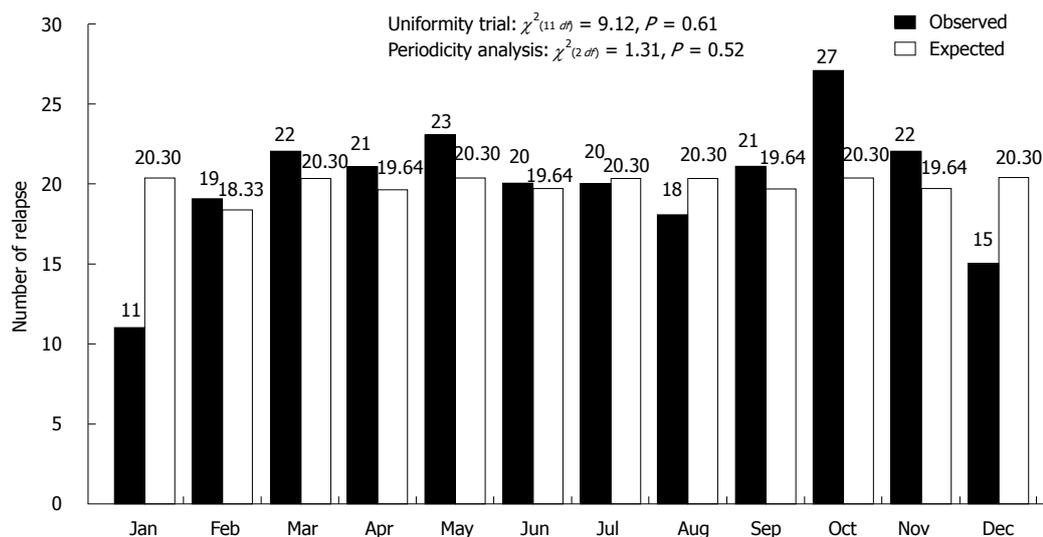
Figure 4 Monthly variations in the relapse of ulcerative colitis. The highest monthly relapse rate was observed in October (27/239, 11.3%), followed by May (23/239, 9.6%). The lowest relapse rate was observed in January (11/239, 4.6%). Variations in relapse were not found on a monthly basis ($\chi^2_{(2\ df)}$ = 1.31, P = 0.52).
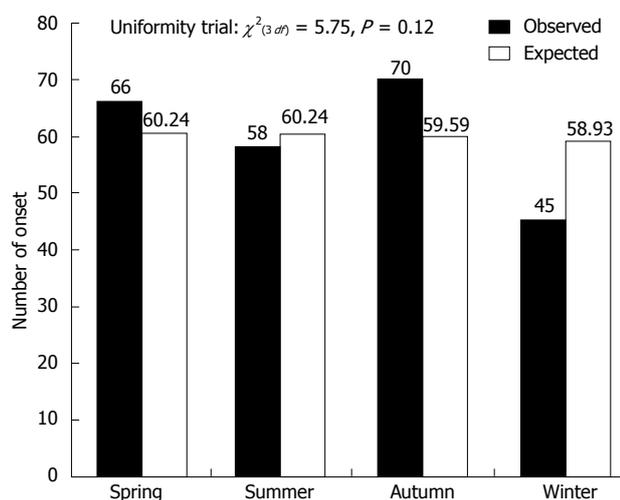
Figure 5 Seasonal variations in the relapse of ulcerative colitis. The highest seasonal relapse rate was observed in the autumn (70/239, 29.3%), followed by the spring (66/239, 27.6%), summer (58/239, 24.3%), and winter (45/239, 18.8%). There was no variation in relapse on a seasonal basis ($\chi^2_{(3\ df)}$ = 5.75, P = 0.12).
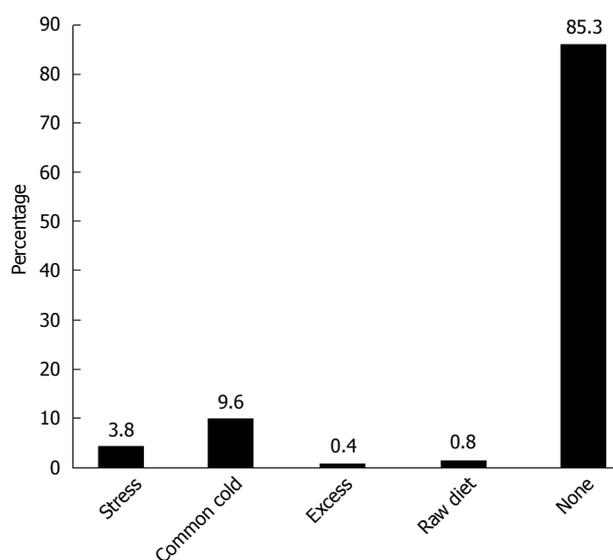
Figure 6 Causes of ulcerative colitis relapse. In most cases (85.3%), the causes of ulcerative colitis (UC) relapse were not identified (none). However, in cases with an apparent cause, upper respiratory inflammation was the main factor responsible for UC relapse in our Japanese sample.

backgrounds of the study populations. In addition, the different seasonal patterns observed in different countries could also reflect variations in the climate and related environmental triggering factors associated with the onset of UC.

The environmental factors that may induce the onset or relapse of UC are not well understood. Patients with UC often experience exacerbations following bacterial and viral infections, and it is interesting that enteric pathogens such as *Salmonella*, *Campylobacter*, *E. coli*, and *Clostridium difficile* may cause relapses in UC. We have also reported that bacteria such as *Fusobacterium varium* (*F. varium*) can modulate the gut immune response and contribute to UC[19]; however, *F. varium* infections do not show a seasonal pattern of occurrence. Smoking is a well-known

environmental factor for UC[20], and the use of non-steroidal anti-inflammatory drugs (NSAIDs)[21] and antibiotics[22] is also a risk factor for UC. In particular, during the winter, due to respiratory tract infections with organisms such as influenza and *Mycoplasma pneumoniae*, these drugs may be associated with the onset of UC. It has also been reported that respiratory and systemic viral infections are associated with the exacerbation of inflammatory bowel disease[23,24]. Also during the winter, intake of NSAIDs due to arthritic pain is common in Japan. In this study, cigarette smoking was not found to enhance the onset of UC, which is in contrast to previous reports concerning Crohn's disease[25]. Normally, patients with UC experience exacerbation when they attempt to quit smoking[25].

Cigarette smoking may especially enhance the onset of UC in combination with other seasonal environmental factors, such as the intake of NSAIDs and antibiotics in the winter[20], and these variables may explain the high incidence of disease onset observed during the winter. Our data showing that upper respiratory inflammation was the main factor responsible for relapse, with the exception of cases with no apparent cause for relapse, also support the high incidence of UC onset during the winter. Moreover, UC leads to inappropriate immune activation and increased levels of inflammatory cells and mediators, and UC can also be triggered by inappropriate immune activation in genetically predisposed individuals[26]. In addition, seasonal variations in immune responses have been reported[27]; unlike the summer and spring, immune functions and the levels of pro-inflammatory cytokines are decreased during the winter[28]. These differences in immune function across seasons may also explain the seasonal variations in the onset of UC.

In retrospective studies, UC relapse was shown to occur more frequently in spring and autumn in Western populations[5,8,10]. In our study of Japanese individuals, there were no significant differences in relapse on a monthly or seasonal basis. For most Japanese patients with UC, there may be no clear seasonal trigger for disease relapse, although long-term follow up studies with detailed microbiological surveillance of UC relapse should be performed, as antibiotic therapy can increase susceptibility to bacteria.

In conclusion, our results support the seasonality of UC onset in Japan. We found that the onset of UC in Japan typically occurred between October and March, although relapse rates did not show consistent seasonal variation. However, the present study was an epidemiological cohort study conducted at an academic hospital in Tokyo, and the study size was too small to draw valid statistical conclusions. Therefore, larger studies are needed in a Japanese population to assess the seasonal variations in UC onset and relapse.

COMMENTS

Background

The incidence and prevalence of ulcerative colitis (UC) has increased rapidly in Japan; however, the environmental factors that contribute to the course of UC have not been well defined. Therefore, it is critically important to gain a better understanding of the environmental factors that contribute to the onset and relapse of UC in a Japanese population.

Research frontiers

Seasonal variations in the onset or relapse of UC have previously been studied in Western populations with conflicting results. Due to different cultural backgrounds, racial genetic predispositions and dietary habits, Japanese patients may show different clinical patterns from those of Western populations. To date, the environmental factors in Japan have not been well defined, although the results of studies in Western populations suggest that there may be seasonal variation in the natural history of UC.

Innovations and breakthroughs

Environmental factors related to winter and spring seasonality are responsible for triggering the clinical onset of UC in Japan.

Applications

The symptomatic onset of UC occurred during October and March in Japan,

whereas relapse generally occurred in October. Upper respiratory inflammation was one of the main factors responsible for relapse. Thus, the results of this study shed light on the environmental factors that contribute to the onset and relapse of UC in Japanese patients.

Peer review

This manuscript reports statistically significant differences in the seasonal variation of UC incidence in a Japanese population. There was monthly seasonality in symptomatic onset during October and March; the onset of symptoms in UC patients generally occurred during the winter. The variation in UC onset was observed for both month (P = 0.015) and season (P = 0.048). In contrast, the variation in relapse was not significant either in month (P = 0.52) or season (P = 0.12). These conclusions support similar previous observations in Western populations.

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BRIEF ARTICLE

Anxiety and depression propensities in patients with acute toxic liver injury

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Abstract

AIM: To investigate anxiety and depression propensities in patients with toxic liver injury.

METHODS: The subjects were divided into three groups: a healthy control group (Group 1, *n* = 125), an acute non-toxic liver injury group (Group 2, *n* = 124), and a group with acute toxic liver injury group caused by non-commercial herbal preparations (Group 3, *n* = 126). These three groups were compared and evaluated through questionnaire surveys and using the Hospital Anxiety-Depression Scale (HADS), Beck Anxiety Inventory (BAI), Beck Depression Inventory (BDI), and the hypochondriasis scale.

RESULTS: The HADS anxiety subscale was 4.9 ± 2.7, 5.0 ± 3.0 and 5.6 ± 3.4, in Groups 1, 2, and 3, respectively. The HADS depression subscale in Group 3 showed the most significant score (5.2 ± 3.2, 6.4 ± 3.4 and 7.2 ± 3.4 in Groups 1, 2, and 3, respectively) ($P < 0.01$ *vs* Group

1, $P < 0.05$ *vs* Group 2). The BAI and BDI in Group 3 showed the most significant score (7.0 ± 6.3 and 6.9 ± 6.9, 9.5 ± 8.6 and 8.8 ± 7.3, 10.7 ± 7.2 and 11.6 ± 8.5 in Groups 1, 2, and 3, respectively) (BAI: $P < 0.01$ *vs* Group 1, $P < 0.05$ *vs* Group 2) (BDI: $P < 0.01$ *vs* Group 1 and 2). Group 3 showed a significantly higher hypochondriasis score (8.2 ± 6.0, 11.6 ± 7.5 and 13.1 ± 6.5 in Groups 1, 2, and 3, respectively) ($P < 0.01$ *vs* Group 1, $P < 0.05$ *vs* Group 2).

CONCLUSION: Psychological factors that present vulnerability to the temptation to use alternative medicines, such as herbs and plant preparations, are important for understanding toxic liver injury.

Key words: Liver injury; Herb; Toxic; Anxiety; Depression

Core tip: In South Korea, the number of toxic liver injuries caused by herbal and folk remedies is increasing. Although positive views on folk remedies are widespread and patients who have been hospitalized with toxic liver injury are often re-hospitalized, no studies have been conducted on the correlation between toxic liver injury and anxiety or depression. This multi-center nation-wide prospective study showed the anxiety and depression propensities in patients with toxic liver injury. Psychological factors that lead to vulnerability to the temptation to use alternative medicines, such as herbs and plant preparations, are important to better understand toxic liver injury.

INTRODUCTION

As interest in health is increasing due to the rising average life expectancy, the aging population, and increased income, the use of unconventional medicines processed from various natural substances is increasing[1]. The frequency of the use of unconventional medicines is currently far higher than previously reported. The extents of use and costs of such medicines are also more wide and expensive in the United States[2].

In South Korea, liver injuries caused by the abuse of oriental medicines and folk remedies with no clinical study results are increasing because many people still depend on easily accessible oriental medicines and folk remedies[3]. In particular, the groundless belief that natural

extracts and plant preparations have less adverse effects is widespread among the general public, and liver injuries caused by the misuse and abuse of such substances are gradually increasing[4]. A retrospective multi-center preliminary study, which was performed in 7 university hospitals in South Korea across the country in 2003, reported that the estimated annual incidence of hospitalization of patients with toxic hepatitis at a university hospital in South Korea was 2629.8[5]. Four years investigation of patients with acute liver injury in the Gyeongju area, located in southeast South Korea, found that 52% of patients had drug-induced liver injuries, and about 50% of such liver injuries were caused by plant preparations[6]. In a recent prospective nationwide study of drug-induced liver injury in South Korea, the most common cause of drug-induced liver injury was found to be "herbal medications"[7]. Toxic hepatitis is often seen in clinical settings, but the general public has a lower interest in and less knowledge of toxic hepatitis than viral hepatitis. Even though most people in South Korea are exposed to oriental medicines and supplementary health foods made of plant preparations that may induce hepatotoxicity, basic data about the frequency, the clinical catamnesis and the medical and social costs of hepatotoxicity caused by such substances are insufficient. Therefore, active reports on such clinical experiences are needed; however, it is difficult to definitively diagnose most cases of liver injuries that are presumed to be caused by plant preparations; thus, they are kept idle[8-10]. Furthermore, because repetitive exposures to preparations that cause liver injuries have been clinically observed, studies are needed on the psychoneurotic propensities of patients who are exposed to supplementary health foods, oriental medicines, and folk remedies that cause liver injuries.

The symptoms of anxiety and depression are often observed in psychiatry, and they are also often discovered in patients with non-psychiatric physical disease. Considerable research has been conducted regarding anxiety or depression in non-psychiatric general or medical practice[11-14].

As shown by the above-mentioned studies, it is a well-known fact that such medical diseases are accompanied by anxiety and depression symptoms. Even though positive views on folk remedies are widespread and patients who have been hospitalized with toxic liver injury are often re-hospitalized, no studies have been conducted on the correlation between toxic liver injury which is frequently observed in South Korea in patients with anxiety and depression. Thus, this multi-center nationwide study was intended to investigate the propensities associated with anxiety and depression in patients with toxic liver injury.

MATERIALS AND METHODS

Study design and population

This is a prospective, multi-center study using questionnaire surveys to determine the anxiety and depression

of patients with toxic liver injury who were selected by their Roussel Uclaf Causality Assessment Method (RU-CAM) scores, which were determined from their medical records. The RUCAM system is a means of assigning points for clinical, biochemical, serologic and radiologic features of liver injury, which gives an overall assessment score that reflects the likelihood that the hepatic injury is due to a specific medication[15].

The study groups were enrolled between May 1, 2010 and April 30, 2012. Ten university hospitals in South Korea (Konkuk University, Korea University, Catholic University of Daegu, Dongguk University, Soon Chun Hyang University, Yeongnam University, Chonbuk National University, Chungnam National University, Hallym University, and Hanyang University) participated in this study.

The subjects were divided into three groups: a control group, a non-toxic acute liver injury group, and a toxic acute liver injury group involving toxic hepatitis. The subjects were divided as follows; Control group (Group 1): some patients who visited the medical examination center of each hospital for the purpose of medical examination were selected randomly; Non-toxic acute liver injury group (Group 2): patients with acute liver injury due to non-toxic causes such as virus and metabolic causes; and Toxic acute liver injury group (Group 3): patients with acute liver injury caused by toxic causes.

To identify the cause of acute liver injury, careful history taking, physical examination, liver function test, viral hepatitis serological testing (anti-HAV IgM, HBsAg, anti-HBC IgM, anti-HCV antibody, CMV, EBV, HSV), anti-nuclear antibody, anti-mitochondrial antibody, or imaging studies (abdominal sonography or CT) were performed.

These three groups were compared and evaluated through the questionnaire survey using scales of anxiety and depression, and the causative factors were analyzed. Liver injury was defined as cases in which the serum alanine aminotransferase (ALT) or conjugated bilirubin values were increased more than twice the upper limit of normal, or that aspartate aminotransferase (AST), alkaline phosphatase and total bilirubin were increased together with at least one of them being more than twice the upper limit of normal[16]. The acute nature of liver injury was defined as cases in which the liver injury had been recovered within 3 mo.

Toxic liver injury was defined as an acute liver injury, caused by medicinal herbs, plant preparations, health foods and folk remedies, with the exception of commercial drugs, having a score of 4 or higher on the RUCAM scale. After the purpose of this study was explained to patients with liver injury, only the patients, who agreed to participate in the survey, were selected as subjects. The following patients were excluded from this study: patients whose cases have been diagnosed as or who are receiving treatment by a psychiatrist for depression disorder, dysthymic disorder, schizophrenia, schizoaffective disorder, or organic mental disorder, and patients who did not respond to the survey during outpatient visits.

Questionnaires including the following questions were answered by the selected subjects and were collected, and the characteristics of the clinical study groups were compared and analyzed. The questionnaire included general questions on age, sex, *etc*.; questions from the Hospital Anxiety-Depression Scale (HADS), the Beck Anxiety Inventory (BAI), the Beck Depression Inventory (BDI), and the hypochondriasis scale. The questionnaire surveys were conducted during the hospital visit for Group 1 and at the time of admission for Groups 2 and 3.

After collecting the 448 case questionnaires answered by the subjects, 73 case questionnaires were excluded because the patient failed to answer all of the questions, acute liver injury caused by commercial drugs, chronic liver disease, a RUCAM score of 4 or less, or an AST/ALT value that did not correspond with aute liver injury. The other 375 case questionnaires were analyzed. Accordingly, 125, 124, and 126 subjects were selected for Groups 1, 2, and 3, respectively.

HADS was developed by Zigmond and Snaith in 1983 to identify the caseness (possible and probable) of anxiety disorders and depression among patients in non-psychiatric hospital clinics[17]. The tool includes 14 items, seven related to anxiety and seven related to depression, each scored between 0 and 3. The authors recommended that a score above 8 on an individual scale should be regarded as a possible case and a score above 10 a probable case[18]. To rule out somatic disorders on the scores, all symptoms of anxiety or depression that were related to a physical disorder, such as dizziness, headaches, insomnia, anergia and fatigue, were excluded.

BAI is a 21 item self-report questionnaire measuring common symptoms of clinical anxiety, such as nervousness and fear of losing control. Respondents indicated the degree to which they are affected by each symptom. Each symptom is scored on a range from 0 to 3, with higher scores corresponding to higher levels of anxiety. Thirteen items assess physiological symptoms, five describe cognitive aspects, and three represent both somatic and cognitive symptoms[19]. A score above 21 is considered a breaking point and was distributed among each of the groups.

BDI is a 21 item self-reported questionnaire that measures the status of clinical depression without psychological diagnosis[20]. It consists of 8 items of physical depression and 13 items of non-physical depression. The BDI covers cognitive, emotional, and somatic symptoms, and its reliability, sensitivity and specificity have a high affinity for diagnosing depression[21,22]. Each item is scored ranging from 0 to 3, with higher scores corresponding to higher levels of depression. A score above 21 is considered a breaking point and was divided evenly among each of the groups.

To measure hypochondriasis, 33 questions corresponding to the hypochondriasis in the Minnesota Multiphasic Personality Inventory, which is widely used in psychia-
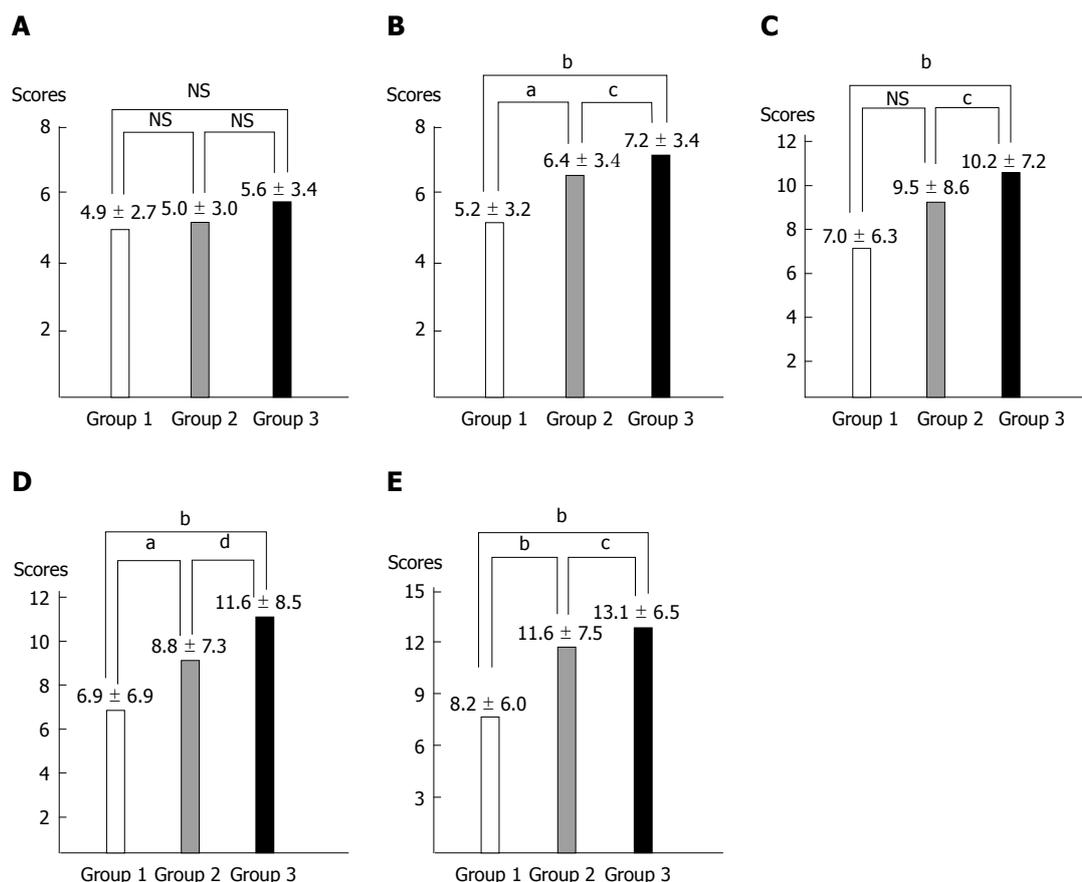
Figure 1 The anxiety and depression subscale of the Hospital Anxiety-Depression Scale, Beck anxiety inventory, Beck depression inventory and the hypochondriasis scores among the study groups. A: Group 3 showed the highest score, but there was no significant difference in the HADS anxiety subscale between groups; B: The HADS depression subscale in Group 3 showed the most significant score; C: The BAI in Group 3 showed the most significant score; D: The BDI in Group 3 showed the most significant score; E: Group 3 showed a significantly higher hypochondriasis score. Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. NS: Non significant; HADS: Hospital Anxiety-Depression Scale; BAI: Beck anxiety inventory; BDI: Beck depression inventory. [a]$P < 0.05$, [b]$P < 0.01$ *vs* Group 1; [c]$P < 0.05$, [d]$P < 0.01$ *vs* Group 2. *P* value by the Mann-Whitney *U* test.

try, were extracted, and the total score was used for data analysis.

Statistical analysis

The SPSS for Windows 19.0 (IBM, New York, NY, United States) statistics program was used for statistical processing of the data. Main results were presented as mean ± SD and the statistical significance was determined by *P* values smaller than 0.05. For comparison of continuous variables among the patient groups and the control groups or comparison of various variables depending upon different severity levels in the patient groups, non-parametric analyses (Kruskal-Wallis test and subsequent Mann-Whitney *U* test) were performed. The categorical variables were analyzed using the χ^2 test.

RESULTS

Clinical characteristics

Of the 375 total subjects, Groups 1, 2, and 3 consisted of 125, 124, and 126 subjects, respectively. The average age was 45.2 ± 13.7 in Group 1, 37.7 ± 14.3 in Group 2, and 48.4 ± 13.1 in Group 3. The male-female ratio was 1:1.5

in Group 1, 1:1.5 in Group 2, and 1:1.5 in Group 3. In Group 2, the primary cause of acute liver injury was acute viral hepatitis A (Table 1). In Group 3, with toxic liver injury, the mean RUCAM score was 7.14 ± 1.6 (4-11). As a cause of liver cell injuries by types, hepatocellular liver injuries were found in 96 cases (76.1%), cholestatic in 11 cases (8.7%), and a mixed type in 19 cases (15.0%).

HADS

The anxiety subscale of the HADS was 4.9 ± 2.7 (0-14) in Group 1, 5.0 ± 3.0 (0-15) in Group 2, and 5.6 ± 3.4 (0-16) in Group 3. Group 3 showed the highest score, but there was no significant difference in the HADS between groups (Table 2) (Figure 1). When the breaking point was set at 8, subjects, who had a score of 8 or higher and were thus deemed to have a high anxiety propensity, presented 20 cases (16.0%), 23 cases (18.5%) and 32 cases (25.3%) in Groups 1, 2, and 3, respectively. Group 3 had the largest number (Table 3).

The depression subscale of the HADS was 5.2 ± 3.2 (0-13) in Group 1, 6.4 ± 3.4 (0-16) in Group 2, and 7.2 ± 3.4 (0-15) in Group 3. Group 3 showed the highest score, which was significantly higher than Groups 1 and 2 (*P* <

Table 1 Clinical characteristics of the patients

Demographic data	Group 1 (*n* = 125)	Group 2 (*n* = 124)	Group 3 (*n* = 126)
Mean age (yr) (range)	45.2 ± 13.7 (20-78)	37.7 ± 14.3 (22-87)	48.4 ± 13.1 (18-72)
M:F (*n*)	1:1.5 (50:75)	1:1.5 (50:74)	1:1.5 (50:76)
Cause (*n*)	Healthy control (125)	Acute HAV (75)	Toxic hepatitis (126)
		Unknown (34)	
		Acute HBV (5)	
		Alcoholic hepatitis (3)	
		Gall stone (3)	
		NAFLD (1)	
		Cholecystitis (1)	
		Cholangitis (1)	
		Autoimmune hepatitis (1)	

Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. NAFLD: Non-alcoholic fatty liver disease.

Table 2 Mean scores of the Hospital Anxiety-Depression Scale, Beck anxiety inventory, Beck depression inventory and Hypochondriasis scores in each groups

Scale	Group 1 (*n* = 125)	Group 2 (*n* = 124)	Group 3 (*n* = 126)	*P* value
Anxiety mean scores of HADS	4.9 ± 2.7	5.0 ± 3.2	5.5 ± 3.4	0.110
(range)	(0-14)	(0-15)	(0-16)	
Depression mean scores of HADS	5.2 ± 3.1	6.4 ± 3.4	7.0 ± 3.5	0.000
(range)	(0-13)	(0-16)	(0-15)	
BAI mean scores	7.0 ± 6.5	9.5 ± 8.5	10.2 ± 7.4	0.000
(range)	(0-34)	(0-41)	(0-40)	
BDI mean scores	6.8 ± 7.0	8.8 ± 7.3	11.2 ± 8.6	0.000
(range)	(0-38)	(0-33)	(0-37)	
Hypochondriasis scores	8.2 ± 6.4	11.6 ± 7.5	12.7 ± 6.7	0.000
(range)	(0-27)	(0-31)	(0-25)	

Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. *P* value by the Kruskal-Wallis *U* test. HADS: Hospital Anxiety-Depression Scale; BAI: Beck anxiety inventory; BDI: Beck depression inventory.

Table 3 The number of cases with scores of 8 or higher on the Hospital Anxiety-Depression Scale and 21 or higher on the Beck anxiety inventory and Beck depression inventory in each groups *n* (%)

Scale	Group 1 (*n* = 125)	Group 2 (*n* = 124)	Group 3 (*n* = 126)	*P* value
Anxiety subscale of HADS				
No. of ⩾ 8 scores	20 (16.0)	23 (18.5)	32 (25.3)	0.157
Depression subscale of HADS				
No. of ⩾ 8 scores	33 (26.4)	44 (35.4)	57 (45.2)	0.008
BAI				
No. of ⩾ 21 scores	7 (5.6)	17 (13.7)	13 (10.3)	0.098
BDI				
No. of ⩾ 21 scores	6 (4.8)	10 (8.0)	19 (15.0)	0.017

Group 1: Control group; Group 2: Non-toxic acute liver injury group; Group 3: Toxic acute liver injury group involving toxic hepatitis. *P* value by the χ^2 test. HADS: Hospital Anxiety-Depression Scale; BAI: Beck anxiety inventory; BDI: Beck depression inventory.

0.01 *vs* Group 1, $P < 0.05$ *vs* Group 2) (Table 2) (Figure 1). When the breaking point was set at 8, the number of subjects, who had a score of 8 or higher and were thus deemed to have a high depression propensity, included were 33 cases (26.4%), 44 cases (35.4%) and 57 cases (45.2%) in Groups 1, 2, and 3, respectively. Group 3 had the most significant number ($P < 0.01$) (Table 3).

BAI
The mean of the BAI score, which was designed to assess

anxiety symptoms, was 7.0 ± 6.3 (0-34) in Group 1, 9.5 ± 8.6 (0-41) in Group 2, and 10.7 ± 7.2 (0-40) in Group 3. Group 3 showed the most statistically significant score ($P < 0.01$ *vs* Group 1, $P < 0.05$ *vs* Group 2) (Table 2) (Figure 1). When the breaking point was set at 21, subjects, who had a score of 21 or higher and were thus deemed to have a high anxiety propensity, were 7 cases (5.6%), 17 cases (13.7%) and 13 cases (10.3%) in Groups 1, 2, and 3, respectively. Group 2 had the largest number, but there was no statistical significance (Table 3).

BDI

The mean of the BDI score, which was designed to assess depression symptoms, was 6.9 ± 6.9 (0-38) in Group 1, 8.8 ± 7.3 (0-33) in Group 2, and 11.6 ± 8.5 (0-37) in Group 3. Group 3 showed the most statistically significant score (P < 0.01 *vs* Groups 1 and 2) (Figure 1 and Table 2). When the breaking point was set at 21, subjects, who had a score of 21 or higher and were thus deemed to have a high depression propensity, were 6 cases (4.8%), 10 cases (8.0%) and 19 cases (15.0%) in Groups 1, 2, and 3, respectively. Group 3 had the most statistically significant number (P < 0.05) (Table 3).

Hypochondriasis score

The mean of the hypochondriasis score was 8.2 ± 6.4 (0-27) in Group 1, 11.6 ± 7.5 (0-31) in Group 2, and 12.7 ± 6.7 (0-25) in Group 3. Group 3 showed the most statiscally significant score (P < 0.01 *vs* Group 1, P < 0.05 *vs* Group 2) (Figure 1 and Table 2).

DISCUSSION

The present study demonstrates that patients with toxic liver injury had high anxiety and depression propensities. Many studies have recently been conducted on anxiety and depression symptoms accompanying various medical diseases such as diabetes[23,24], cardiovascular diseases[25,26], and chronic obstructive pulmonary disease[27,28]. This study has a high value because this was the first study that evaluated patients with toxic liver injury from the psychiatric aspects of anxiety and depression. Furthermore, as a multi-center study, we are confident that the results of this study represent the nationwide pattern in South Korea.

From the above results, although the HADS levels were similar anxiety and depression tend to be observed slightly more in Group 3 compared to that of Group 2 and more in Group 2 compared to that of Group 1. In HADS, the anxiety subscale is not influenced by the 8 cut off point but the depression scale in Group 3 was significantly higher. In BAI and BDI, Group 3 showed a significantly higher rate of anxiety and depression. When subscale 21 was used as a cutoff point in BAI and BDI, the depression scale was particularly very high in the Group 3 patients. These observations show that both anxiety and depression are related to toxic hepatitis but that depression has a particularly high correlation. When the hypochondriasis scale is considered, Group 3 also displays a higher score. This general trend may be the result of the patients' tendency, due to anxiety and depression, to seek herbal supplements or alternative medicine when faced with toxic hepatitis.

Patients with toxic liver injury can be largely divided into two types: those whose liver injury was unavoidably caused by hospital prescriptions and those whose liver injury was caused by voluntary administration of sought out medicines. We can presume that patients of the latter type would have psychiatrically higher anxiety and depression than the general public, which is the main subject matter in this study.

When this study was designed, we took into consideration that acute liver injury alone could cause anxiety and depression. Therefore, we divided the acute liver injury group into toxic/non-toxic groups. Generally, toxic or drug-induced liver injury displays similar increases in AST and ALT levels. However, causes of the damage are of a different nature: drug-induced liver injury is usually due to the passive intake of prescribed medication as instructed by physicians, whereas toxic liver injury is due to active self medication. Therefore, patients with drug-induced acute liver injury were excluded in this study, and we have a separate study on drug-induced liver injury and toxic liver injury planned.

Generally, there are risk factors such as genetic, non-genetic host susceptibility and environmental factors for idiosyncratic drug-induced liver injury[29]. The risk factors for herbal toxic liver injury are not well known. This study is meaningful in that we found that among the risk factors for the herbal or dietary supplement induced toxic liver injury including sex, age, cumulative dosage and herb-drug interaction[30-33], the psycho-behavioral attitude of patients could also be an important risk factor. The reason that toxic liver injury broke out in a large number of females and people aged 50 or older is not known, but the results of this study suggests interpretationally that psychological factors should contribute greatly to such phenomenon.

It is thought that an effective method for preventing toxic liver injury is to treat patients with special care in collaboration with psychiatric treatment rather than simply telling them not to ingest plant preparations or health foods that could cause toxic liver injury. Although it may not be economic to treat all patients by administering drugs in collaboration with psychiatric treatment, it is thought that it could be a good guide to apply these study results, at least generally, to patients who visit the hospital repetitively due to toxic liver injury. This is because the patients, who had visited the hospitals at least 2 or 3 times repetitively due to the toxic liver injury, had higher anxiety and depression scores (data not shown).

This study has one limitation: most subjects in Group 2 were patients with acute hepatitis A. This was unavoidable because acute hepatitis A has recently broken out in a large number in South Korea[34]. However, no statistical difference was shown between the group with the acute liver injury caused by acute hepatitis A and the group with the acute liver injury caused by a small number of other causes. The reason for including Group 2 in the comparison was to determine whether anxiety and depression propensities were secondarily accompanied due to the acute liver injury, or whether the acute liver injury broke out because people with previously high anxiety and depression propensities had often searched for invigorants to rejuvenate their body.

This study is a cross-sectional study that was designed to try to understand the psychological states, such as

anxiety and depression, in patients with toxic liver injury who are taking herbal or folk remedies. The primary purpose of our study was achieved by determining the proportion of psychological conditions, such as anxiety and depression, suffered by patients with toxic liver injury taking herbal or folk remedies. However, there are issues that may be pointed out as a limitation of this cross-section study, such as "Does psychological state induce toxic liver injury?" and "Are changes in psychological state caused by toxic liver injury?" The relation can be demonstrated, but this research design may be limit our understanding of causalities. To overcome some of these limitations, we tried to compare the non-toxic acute liver injury group with the normal group. Through this process, we attempted to distinguish between anxiety and depression induced by hospitalization alone. This study demonstrated that the rate of anxiety and depression in patients with toxic liver injury is significantly higher than that of cases without toxic liver injury, even when taking into account the change in the psychological states due to hospitalization. We believe that this finding is a key result of our research. We plan to promote research to clarify the psychological risk factors such as anxiety and depression by comparing healthy individuals who are taking herbal preparations with toxic hepatitis patients taking herbal preparations through a case-control study.

It is thought that it will be necessary to develop scales specifically applicable to anxiety and depression in patients with toxic liver injury. Even though various conventional scales for anxiety and depression were used in this study, we feel that it is necessary to develop specific scales for anxiety and depression in patients with toxic liver injury.

We believe that meaningful results were derived from this study because psychiatric patients who were previously diagnosed with psychiatric disorders were excluded from this study. It is assumed that the results of this study would have a higher significance if a specific scale for patients with toxic liver injury had been used. .

In conclusion, patients with toxic liver injury showed high scores on the HADS, BAI, BDI, and hypochondriasis scales. It can be postulated from this result that patients with toxic liver injury have high anxiety and depression propensities. This fact suggests that high anxiety and depression propensities could be the psychological factors that lead patients with toxic liver injury to access factors that may cause toxic liver injury, such as folk remedies and oriental medicines. It is therefore thought that although it is important to treat toxic liver injury itself, it is necessary to take active measures to understand and improve anxiety and depression symptoms of patients in an effort to prevent toxic liver injury from occurring and recurring.

COMMENTS

Background
Even though positive views on herbal preparations or folk remedies are widespread and patients who have been hospitalized with toxic liver injury are often re-hospitalized, no studies have been conducted on the correlation between toxic liver injury which is frequently observed in South Korea and anxiety and depression in patients with toxic liver injury.

Research frontiers
Patients with toxic liver injury can be largely divided into two types: those whose liver injury was unavoidably caused by hospital prescriptions and those whose liver injury was caused by voluntary administration of sought out medicines. It can be presumed that patients of the latter type would have psychiatrically higher anxiety and depression than the general public, which is the main subject matter in this study.

Innovations and breakthroughs
This study has a high value because this was the first study that evaluated patients with toxic liver injury from the psychiatric aspects of anxiety and depression. This study indicates that patients with toxic liver injury showed high scores on the Hospital Anxiety-Depression Scale, the Beck Anxiety Inventory, the Beck Depression Inventory, and the hypochondriasis scales. It can be postulated from this result that patients with toxic liver injury have high anxiety and depression propensities. This fact suggests that high anxiety and depression propensities could be the psychological factors that lead patients with toxic liver injury to access factors that may cause toxic liver injury, such as folk remedies and oriental medicines.

Applications
This study indicates that although it is important to treat toxic liver injury itself, it is necessary to take active measures to understand and improve anxiety and depression symptoms of patients in an effort to prevent toxic liver injury from occurring and recurring.

Peer review
The authors present patients with herbal preparations-induced acute toxic liver injury had high anxiety and depression propensities. The results are interesting and indicate that psychological factors vulnerable to the temptation to use alternative medicines, such as herbs and plant preparations, are most important for understanding the toxic liver injury.

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BRIEF ARTICLE

Appropriateness, endoscopic findings and contributive yield of pediatric gastrointestinal endoscopy

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Abstract

AIM: To determine the predictability of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and American Society for Gastrointestinal Endoscopy (ASGE) guideline with regard to appropriate endoscopic practice in children, positive endoscopic findings and contributive yield in clinical practice.

METHODS: This was a descriptive, retrospective analysis, conducted at the Department of Paediatrics, University Malaya Medical Centre, Malaysia. All children who had esophagogastroduodenoscopy (EGD) and colonoscopy from January 2008 to June 2011 were included. An endoscopy was considered appropriate when its indication complied with the NASPGHAN and ASGE guideline. All endoscopic findings were classified as either positive (presence of any endoscopic or histologic abnormality) or negative (no or minor abnormality, normal histology); effecting a positive contributive (a change in therapeutic decisions or prognostic consequences) or non-contributive yield (no therapeutic or prognostic consequences).

RESULTS: Overall, 76% of the 345 procedures (231 EGD alone, 26 colonoscopy alone, 44 combined EGD and colonoscopy) performed in 301 children (median age 7.0 years, range 3 months to 18 years) had a positive endoscopic finding. Based on the NASPGHAN and ASGE guideline, 99.7% of the procedures performed were considered as appropriate. The only inappropriate procedure (0.3%) was in a child who had EGD for assessment of the healing of gastric ulcer following therapy in the absence of any symptoms. The overall positive contributive yield for a change in diagnosis and/or management was 44%. The presence of a positive endoscopic finding was more likely to effect a change in the therapeutic plan than an alteration of the initial diagnosis. A total of 20 (5.8%) adverse events were noted, most were minor and none was fatal.

CONCLUSION: The NASPGHAN and ASGE guideline is more likely to predict a positive endoscopic finding but is less sensitive to effect a change in the initial clinical diagnosis or the subsequent therapeutic plan.

Core tip: Since the publication of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and American Society for Gastrointestinal Endoscopy (ASGE) modification of the

guideline on the appropriate use of endoscopy in children, no study has been conducted to ascertain the applicability of this guideline in the pediatric population. The present study addressed the deficiency in the literature by conducting a retrospective review of the gastrointestinal endoscopies conducted in a university setting in an Asian country. The present study showed that the modified NASPGHAN and ASGE guideline is applicable universally, be it in a Western country or an Asian country.

INTRODUCTION

Endoscopy is a useful diagnostic tool in both adult and pediatric populations[1,2]. Endoscopy in the pediatric population is usually performed by a pediatric gastroenterologist, and occasionally by a pediatric surgeon. In settings where the expertise of a fully trained pediatric gastroenterologist is not available, an adult gastroenterologist, supported by a pediatrician, can perform simple, diagnostic endoscopy in children safely[3].

Esophagogastroduodenoscopy (EGD) and colonoscopy in children can be either diagnostic or therapeutic[2]. Common indications for diagnostic EGD and colonoscopy in children include the presence of symptoms indicative of an underlying organic pathology of the gastrointestinal (GI) tract[1,2,4,5].

Generally, diagnostic pediatric EGD and colonoscopy are safe[6]. The risks of therapeutic endoscopy depend on the nature of interventions, but if performed by a pediatric endoscopist with appropriate training, the complication rate is less than 1%[6,7]. Potential complications may be encountered in sedation and anesthesia provided during the procedure[8].

In 2000, the American Society for Gastrointestinal Endoscopy (ASGE) published a guideline on the appropriate use of GI endoscopy in the adult population[9]. Since then, many studies have found the ASGE guideline to be applicable in the adult population[10-12]. ASGE and the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) published a modification of the guideline for the pediatric population, where clear indications for both EGD and colonoscopy in children were recommended[2].

There are several studies on the appropriateness of endoscopy in the adult population[10-12]. However, similar studies in the pediatric population are limited[5,13,14]. We conducted a retrospective review to assess the appropriateness of GI endoscopy performed in children in our unit, based on the NASPGHAN and ASGE guideline[9]. In addition, the rates of positive and negative endoscopic findings as well as contributive and non-contributive yields to the diagnosis and management of the patients were also studied.

MATERIALS AND METHODS

This was a retrospective, descriptive study conducted at the Gastroenterology and Nutrition Unit, Department of Paediatrics, University of Malaya Medical Centre (UMMC), Malaysia; from 1st January 2008 to 30th June, 2011. The present study was approved by the institutional ethical committee of UMMC.

During the study period, all children who required GI endoscopy in the unit, including those who were referred from outside the unit, were screened initially by one of the three practicing pediatric gastroenterologists (Lee WS, Chai PF, Boey CCM). All endoscopic procedures were performed by one of these three pediatric gastroenterologists.

Case ascertainment

All consecutive patients younger than 18 years of age who had undergone EGD and colonoscopy during the study period were included. Patients were identified from the electronic database of the unit, and were cross-checked with the patient database from the endoscopic unit of the hospital. The case notes were reviewed. Patients who had inadequate data or incomplete procedures were not included.

Data collection

The following data were collected: basic demographic data, preliminary diagnosis, indication for endoscopy, sedation or anesthesia, endoscopic finding, adverse events encountered during and after the procedure, clinical course and final diagnosis.

Definitions

"Appropriate" and "inappropriate" indications for EGD and colonoscopy were defined according to the "Modifications in Endoscopic Practice for Pediatric Patients" by ASGE and NASPGHAN, published in 2008[9]. The indication for endoscopic procedures performed during the study period, if found to be compliant with the indications listed under "pediatric upper endoscopy" and "pediatric colonoscopy" in the ASGE and NASPGHAN guideline, was considered as "appropriate". An indication was classified as "inappropriate" if the indication of the procedure was not listed in the guideline.

Anesthetic techniques and drugs used: In the present study, the induction of anesthesia used in children was the inhalational technique with sevoflurane and oxygen. After endotracheal intubation, patient paralysis, if necessary, was achieved by intravenous atracurium. Maintenance anesthesia was achieved by inhalational sevoflurane. Reversal of anesthesia was achieved by neostigmine and atropine.

Table 1 Characteristics of 310 children undergoing 345 endoscopic procedures *n* (%)	
Age	
< 6 mo	3 (1)
6 mo-2 yr	32 (11)
2-10 yr	185 (61)
> 10 yr	81 (27)
Gender	
Male	158 (53)
Female	143 (47)
Weight-for-age	
< 3rd centile	123 (41)
3rd-50th centile	154 (51)
50th-95th centile	22 (6)
> 95th centile	4 (1.4)
Type of procedure	
Esophagogastroduodenoscopy	231 (77)
Colonoscopy	26 (9)
Both	44 (15)

Three hundreds and forty-five endoscopic procedures took place in University Malaya Medical Center, Kuala Lumpur; January 2008 to June 2011.

Table 2 Indications for esophagogastroduodenoscopy and colonoscopy in 310 children *n* (%)	
Indications	Value
Esophagogastroduodenoscopy	
Diagnostic	
Variceal surveillance/eradication	137 (48.9)
Hematemesis	41 (14.9)
Significant recurrent abdominal pain	37 (13.4)
Malenic stool	20 (7.3)
Chronic diarrhea/malabsorption	16 (5.8)
Recurrent vomiting	5 (1.8)
Malignancy surveillance	5 (1.8)
Dysphagia/odynophagia	4 (1.4)
Complicated gastroesophageal reflux disease	3 (1.0)
Unexplained anemia	1 (0.3)
Failure to thrive	1 (0.3)
Therapeutic	
Gastrostomy insertion	2 (0.7)
Foreign body removal	1 (0.3)
Colonoscopy	
Rectal bleeding	19 (27.0)
Monitoring of inflammatory bowel disease	19 (27.0)
Chronic diarrhea/malabsorption	18 (25.7)
Surveillance for polyp syndrome	6 (8.5)
Recurrent abdominal pain	5 (7.1)
Malignancy surveillance	3 (4.2)

Positive and negative findings

By screening the procedures report, the endoscopic findings were divided into positive (presence of any abnormality in the endoscopic findings, or presence of relevant histologic findings), or negative (no abnormality or minor abnormality, normal histology)[10].

Contributive and non-contributive yields

The endoscopic procedures were divided into two categories: a positive contributive yield (the procedure had a positive effect on therapeutic decisions or prognostic consequences; this included interventional procedures) and non-contributive yield (a procedure which has no therapeutic or prognostic consequences)[10]. The patient may have a negative endoscopic finding and yet the procedure may be considered as having a positive contributive yield (example: a negative EGD finding in a child with upper GI bleeding).

Adverse events

Adverse events which occurred during and after the procedures were noted. These were divided into sedation- or anesthesia-related, or procedure-related.

Statistical analysis

Data were collected and managed by using statistical software programs (SPSS version 20.0, SPSS Inc., Chicago, IL, United States). Data were analyzed using a two-tailed χ^2 test; OR and related 95%CI were calculated. A *P* value < 0.05 was considered significant. Multivariate analysis was performed on selected symptoms and signs predicting a positive contributive yield (change) on the initial diagnosis or subsequent therapeutic plan.

RESULTS

During the study period, a total of 362 procedures were

performed in 318 children. Of these, 17 procedures involving 17 patients were excluded from analysis: 8 had incomplete data (three for EGD, two each for colonoscopy and percutaneous endoscopic gastrostomy feeding tube, and one for foreign body removal), and 9 had an incomplete procedure (seven had colonoscopy abandoned because of poor bowel preparation, and two patients had EGD abandoned because of esophageal stricture). Thus, a total of 345 procedures involving 301 patients were analyzed. Of these, 231 patients had EGD alone, 26 had colonoscopy alone, while 44 had combined EGD and colonoscopy.

Patients' characteristics

The median age of these 301 children was 7.0 years old (range 3 mo to 18 years; Table 1). There were 158 (53%) males and 143 (48%) females. Almost half of the patients had a weight-for-age below the 3rd centile (*n* = 141, 41%).

Indications for endoscopy

The two most common indications for EGD were surveillance for esophageal varices (*n* = 137, 50%) and upper GI bleed (*n* = 73, 26%; Table 2), while the two most common indications for colonoscopy were per rectal bleeding (*n* = 19, 27%), and surveillance/diagnosis of inflammatory bowel disease (IBD; *n* = 19, 27%). Of the total 86 therapeutic procedures performed, three-quarters (74%) were rubber banding for esophageal varices (Table 3).

Appropriateness of endoscopy

Based on the NASPGHAN and ASGE guideline, 99.7% (*n* = 344) of the 345 procedures performed during the

Table 3 Therapeutic procedures *n* (%)

Procedures	Value
Esophageal varices eradication	
Rubber banding for esophageal varices	64 (74)
Sclerotherapy	18 (21)
Polypectomy	2 (2.3)
Foreign body removal	1 (1)
Insertion of percutaneous gastrostomy feeding tube	2 (2)
Total	86 (100)

Table 4 Probability of positive (abnormal) *vs* negative (normal) endoscopic findings *n* (%)

Procedures	Endoscopic findings		Total
	Positive	Negative	
Esophagogastroduodenoscopy	216 (79)	59 (21)	275 (100)
Colonoscopy	45 (64)	25 (36)	70 (100)
All	261 (76)	84 (24)	

study period were considered as appropriate. The only procedure (0.3%) which was considered as inappropriate was in a child who had an EGD for assessment of the healing of a gastric ulcer following medical therapy in the absence of any signs and symptoms.

Positive and negative findings

Three-quarters ($n = 261$, 76%) of the 345 procedures performed showed a positive (abnormal) endoscopic finding [EGD: 216 (79% of all EGD performed), colonoscopy: 45 (64% of all colonoscopy performed); Table 4] while the remaining 84 (24%) had a negative endoscopic finding. A rapid urease test from a mucosal biopsy taken from the stomach and duodenum for *Helicobacter pylori* (*H. pylori*) infection was performed in 62 patients and was positive in 10 patients (16%).

Factors predicting a positive endoscopic finding

Six clinical symptoms and four signs were analyzed to predict a positive contributive yield (effecting a change) in the initial diagnosis or subsequent therapeutic plan (Tables 5 and 6). On multivariate analysis, the presence of an enlarged liver or an enlarged spleen were least likely to effect a change in the diagnosis, while vomiting and abdominal pain were most likely to be associated with a change in the initial diagnosis. The presence of hematemesis was most likely to be associated with a change in therapeutic plan.

Contributive and non-contributive yields

The overall contributive yield was 44.3% (Table 7). All the 79 patients who had a change in the initial diagnosis (positive contributive yield in diagnosis) also had a change in the subsequent therapeutic plan (positive contributive yield in therapeutic plan).

The presence of a positive (abnormal) endoscopic finding confirmed the clinical diagnosis in 57% ($n = 197$, negative contributive yield) of patients, while it altered the diagnosis in 19% ($n = 64$, positive contributive yield) of patients (Table 7). Conversely, a negative (normal) endoscopic finding confirmed the clinical diagnosis in 20% ($n = 69$) of patients, while it altered the diagnosis in 4.3% ($n = 15$) of patients (Table 7). This was not statistically significant ($P = 0.234$). Of the 15 patients (4.3%) who had an alteration in the final diagnosis despite a negative endoscopic finding, most had an abnormal histology in the presence of normal endoscopic findings.

The presence of a positive endoscopic finding was more likely to effect a change in the management plan of a patient as compared to having a negative endoscopic finding (positive finding: $n = 145$, 42% *vs* negative finding: $n = 8$, 2.3%, $P < 0.001$; Table 7). Most of those ($n = 8$) who had a negative endoscopic finding but had a change in management plan were found to have a positive urease test for *H. pylori*. All had eradication therapy initiated.

Adverse events

A total of 20 (5.8%) adverse events were noted; most were minor (Table 8). Secondary bleeding following rubber banding or sclerotherapy for esophageal varices was noted in 12 patients, while the bleeding rate following EGD was 4.3%. All the bleeding episodes were seen in patients with ($n = 3$, aged 11 mo to 2 years) biliary atresia and liver cirrhosis who had rubber banding for esophageal varices. None had liver transplantation. All patients needed blood transfusion but none became hemodynamically unstable.

Two patients who had esophageal varices and large ascites complicating liver cirrhosis needed assistance in respiration for a few hours following general anesthesia. Three children developed fever after endoscopy. All recovered uneventfully following a course of oral antibiotics. Another patient developed transient bronchospasm following extubation.

Two iatrogenic perforations following colonoscopy were noted in two children who had Crohn's disease. Both had gross delay in referral, severe malnutrition and extensive colonic disease. Both had fecal diversion surgery and recovered following surgical repair. The perforation rate following colonoscopy was 2.9%. No death occurred as a result of endoscopy in the present study.

DISCUSSION

Generally, for a procedure to be considered as appropriate, its expected benefit should be greater than its expected negative consequences by a sufficiently wide margin to make the procedure worthwhile[15]. Benefit and negative consequences of a procedure are both defined in the broadest terms[10,15].

Guidelines on the appropriateness of endoscopic procedures have been devised to aid clinicians in selecting more appropriate patients for referral, especially to units with limited expertise and financial resources[2,9]. Recently, a guideline pertaining to the appropriate use of endoscopy in children was published by NASPGHAN and

Table 5 Univariate analysis for clinical parameters predicting a positive (abnormal) endoscopic finding

Clinical parameters	Positive contributive yield (a change in diagnosis)			Positive contributive yield (a change in treatment)		
	P value	OR	95%CI	*P* value	OR	95%CI
Symptoms						
Vomiting	< 0.001	4.5	2.0-10.3	0.456	1.4	0.5-3.5
Diarrhea	0.010	2.6	1.5-4.2	0.940	0.4	0.4-1.8
Abdominal pain	0.020	2.1	1.1-4.0	0.750	0.9	0.5-1.7
Hematemesis	0.321	1.7	0.5-5.5	0.001	4.3	1.7-10.3
Melena	0.048	0.7	0.05-0.5	0.027	0.6	0.27-1.4
Hematochezia	0.065	2.0	0.9-4.3	0.040	2.8	1.3-5.7
Signs						
Pallor	0.525	1.2	0.5-2.8	0.520	1.2	0.6-2.3
Hepatomegaly	0.551	1.4	0.43-4.7	0.825	0.9	0.5-1.7
Splenomegaly	< 0.001	0.082	0.029-0.2	0.227	0.6	0.3-1.2
Abdominal tenderness	0.396	0.5	0.14-2.1	0.242	0.37	0.07-1.9

Table 6 Multivariate analysis for clinical parameters predicting a positive (abnormal) endoscopic finding

Clinical parameters	Positive contributive yield (a change in diagnosis)			Positive contributive yield (a change in treatment)		
	P value	OR	95%CI	*P* value	OR	95%CI
Symptoms						
Vomiting	< 0.001	4.5	2.0-10.3	0.827	0.9	0.4-2.6
Diarrhea	0.014	2.6	1.5-4.7	0.448	0.8	0.4-1.4
Abdominal pain	< 0.001	3.7	2.1-6.4	0.664	0.6	0.4-1.6
Hematemesis	0.251	1.5	0.7-3.3	< 0.001	4.3	1.8-9.5
Melena	0.080	1.7	0.9-4.3	0.022	2.9	1.0-5.2
Hematochezia	0.010	3.1	1.6-6.3	0.020	1.9	1.1-4.5
Signs						
Pallor	0.768	1.4	0.5-2.1	0.113	2.0	0.8-3.0
Hepatomegaly	0.004	0.2	0.1-0.5	0.629	1.3	0.6-1.9
Splenomegaly	< 0.001	0.08	0.04-0.17	0.198	1.3	0.8-2.0
Abdominal tenderness	0.267	1.9	0.5-2.1	0.097	0.2	0.05-3.0

Table 7 Endoscopic findings and a subsequent contributive yield *n* (%)

Endoscopic findings	Positive contributive yield (a change in diagnosis)		Positive contributive yield (a change in management)		Total
	Yes	No	Yes	No	
Positive	64 (18.6)[1]	197 (57.1)	145 (42.0)[2]	116 (33.6)	261 (75.7)
Negative	15 (4.3)	69 (20.0)	8 (2.3)	76 (22.0)	84 (24.3)
Total	79 (22.9)	266 (77.1)	153 (44.3)	192 (55.7)	345 (100)

[1]P = 0.234 (χ^2 test); [2]P < 0.001 (χ^2 test).

ASGE[2]. We believe that, although there are unavoidable socio-cultural and geographical differences as well as pattern of diseases, the NASPGHAN and ASGE guideline can be applied universally. Thus, for the present study the NASPGHAN and ASGE guideline was chosen.

In addition, little is known about pediatric endoscopic practice and its appropriateness in Asian countries, where human and financial resources, funding model, pattern of GI and liver diseases are different from the more advanced Western countries.

There have been several studies on the appropriateness of EGD in various clinical situations in children[13,14]. However, none are based on the NASPGHAN and ASGE guideline. For example, Jantchou *et al*[13], based on the recommendations by the French-language Pediatric Hepatology, Gastroenterology and Nutrition Group (GF-HGNP), noted that 18% of the 251 EGD procedures

performed were considered as inappropriate, a figure which was higher among outpatient referrals. Guariso *et al*[5], using a model of expert consensus from theoretical scenarios, noted that except in cases with a positive family history of peptic ulcer and/or *H. pylori* infection, children aged 10 years of older, or with persistent symptoms, not all EGD in children with dyspeptic symptoms could be considered as appropriate. Miele *et al*[14] found that the publication of Rome II criteria for functional GI disorders has a positive impact on the appropriateness of GI endoscopy, with inappropriate procedures reduced significantly after its publication. Nevertheless, 26% of all procedures were still considered as inappropriate[14].

In contrast, although using different standards, the overall inappropriateness for pediatric endoscopy in the present study was 0.3%, with an overwhelming 99.7% of the cases being considered as appropriate. The only case

Table 8 Adverse events encountered in 345 endoscopic procedures

Complications	*n*
Procedure-related	
Secondary bleeding following rubber banding or sclerotherapy	12
Bowel perforation during colonoscopy	2
Anesthesia/sedation-related	
Delayed extubation due to ascites	2
Post-extubation bronchospasm	1
Secondary fever	3
Total	20

in the present study which was deemed to be inappropriate was an EGD reassessment of a healing gastric ulcer, in the absence of any symptoms and signs. This figure compares favorably with 18% of inappropriateness noted by Jantchou *et al*[13] and 26% found by Miele *et al*[14].

There is, at present, limited availability of human resources in pediatric gastroenterology practice in Malaysia. The pediatric gastroenterology and nutrition unit of UMMC is only one of two pediatric gastroenterology units in Malaysia providing regular pediatric endoscopic services. The model of practice is not an open-access system. Thus, in the present study, all referrals for GI endoscopy from office-based pediatricians were screened initially by one of the practicing gastroenterologists before being subjected to endoscopy, hence reducing potentially inappropriate cases.

Nevertheless, some authors argued that the probability of detecting a clinically relevant lesion is considered as important as the appropriateness of the procedure[16,17]. Gonvers *et al*[18] found that when applying the ASGE criteria to 450 outpatients who underwent EGD, there were no significant differences in clinically relevant findings in those patients who had an appropriate *vs* an inappropriate EGD.

Thus, we also studied the probability of finding a positive endoscopic finding in addition to studying the appropriateness of endoscopy. In the present study, the overall probability of detecting a positive endoscopic finding was 76%, higher in EGD (79%) than in colonoscopy (64%).

In the present study, the positive contributive yield for a change in the initial diagnosis was only 23% (Table 5). This is mainly because in over half of the cases (57%), a positive endoscopic finding confirmed the initial diagnosis, thus the contributive yield was considered as negative. However, what is equally important was a negative finding which has a positive contributive yield. Examples included the reassuring negative EGD finding in a child with upper GI bleeding. In the present study, a positive endoscopic finding was more likely to effect a change in the management plan than to effect a change in the initial diagnosis.

Although endoscopic procedures in the pediatric population are generally safe, adverse events and complications related to anesthesia and the endoscopic procedure itself are well documented[7,8,19]. Most of the adverse events encountered in the present study were minor and transient in nature. The perforation rate of colonoscopy

in the present study was 2.9%, higher than similar figures in the literature[7,19]. Both cases were children with Crohn's disease who had severe delay in referral, advanced malnutrition and total colonic involvement. Nevertheless, efforts should be initiated to reduce the complications rate further by improving the training of endoscopy in the unit[20].

The main shortcoming in the present study was its retrospective nature. Thus, it may not be entirely accurate in ascertaining whether an endoscopic finding effected any alteration in the initial diagnosis and subsequent therapeutic plan. In addition, the age range of the patients in the present study was wide, and the indications for endoscopy in young children may not be similar to adolescents. Thirdly, the present study was conducted in a university hospital setting and the procedures were performed by experienced pediatric gastroenterologists. Thus, the findings of the present study may not be entirely applicable in other settings.

In conclusion, the present study showed that the modified NASPGHAN and ASGE guideline is applicable universally, be it in a Western country or an Asian country. Although the NASPGHAN and ASGE guideline on the appropriateness of pediatric endoscopy is useful in helping clinicians selecting the most appropriate patient for GI endoscopic procedures, nevertheless its predictability of a positive endoscopic finding is moderate, and it is not very sensitive in predicting whether a procedure has any positive contributive yield in the diagnosis and management of the patients.

COMMENTS

Background
Esophagogastroduodenoscopy (EGD) and colonoscopy in children can either be diagnostic or therapeutic. Generally, diagnostic pediatric EGD and colonoscopy are safe, but the risks of therapeutic endoscopy depend on the nature of interventions. If performed by an experienced pediatric endoscopist with appropriate training, the complication rate is less than 1%. Generally, for a procedure such as gastrointestinal endoscopy to be considered as appropriate, its expected benefit should be greater than its expected negative consequences by a sufficiently wide margin to make the procedure worthwhile. There have been several publications on the appropriateness of gastrointestinal endoscopy in the adult population. But studies of a similar nature in the pediatric population are limited.

Research frontiers
The present study planned to address the deficiency in the literature on the appropriateness of pediatric gastrointestinal endoscopy by conducting a retrospective review on the gastrointestinal endoscopies conducted in a university setting in an Asian country.

Innovations and breakthroughs
The present study was the first major study to ascertain the applicability of the Modified Guidelines on the Appropriate use of Gastrointestinal Endoscopy in children by North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) and American Society for Gastrointestinal Endoscopy (ASGE). It is also the first study from an Asian country to determine the indications of pediatric gastrointestinal endoscopy in children. There have been several publications on the indications of childhood gastrointestinal endoscopy from the Western countries, but none from an Asian country.

Applications
The results of the present study showed that the vast majority of the pediatric gastrointestinal endoscopies performed in a university hospital setting were appropriate according to the modified guidelines. Thus, other pediatric endoscopists performing pediatric gastrointestinal endoscopy should consider referring

to the "Modified Guidelines" for the purpose of benchmarking.

Terminology

"Appropriate" and "inappropriate" indications for pediatric gastrointestinal endoscopies were defined according to the "Modifications in Endoscopic Practice for Pediatric Patients" by the American Society for Gastrointestinal Endoscopy and North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. A contributive yield was defined as a procedure that had a positive effect on therapeutic decisions or prognostic consequences in a patient. A non-contributive yield was defined as a procedure that had no therapeutic or prognostic consequences.

Peer review

This article from an Asian country aimed to determine the predictability of the NASPGHAN and ASGE guideline in endoscopic practice for children on positive endoscopic finding and contributive yield in clinical practice in children. Although there are some unavoidable socio-cultural and geographical differences as well as pattern of diseases, the NASPGHAN and ASGE guidelines, as the present study shows, can be applied universally. The overall study is interesting, and no similar study was detected in the literature.

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BRIEF ARTICLE

Routine lymph node dissection may be not suitable for all intrahepatic cholangiocarcinoma patients: Results of a monocentric series

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Abstract

AIM: To investigate the indications for lymph node dissection (LND) in intrahepatic cholangiocarcinoma patients.

METHODS: A retrospective analysis was conducted on 124 intrahepatic cholangiocarcinoma (ICC) patients who had undergone surgical resection of ICC from January 2006 to December 2007. Curative resection was attempted for all patients unless there were metastases to lymph nodes (LNs) beyond the hepatoduodenal ligament. Prophylactic LND was performed in patients in whom any enlarged LNs had been suspicious for metastases. The patients were classified according to the LND and LN metastases. Clinicopathologic, operative, and long-term survival data were collected retrospectively. The impact on survival of LND during primary resection was analyzed.

RESULTS: Of 53 patients who had undergone hepatic resection with curative intent combined with regional LND, 11 had lymph nodes metastases. Whether or not patients without lymph node involvement had undergone LND made no significant difference to their survival (*P* = 0.822). Five patients with multiple tumors and involvement of lymph nodes underwent hepatic resection with LND; their survival curve did not differ significantly from that of the palliative resection group (*P* = 0.744). However, there were significant differences in survival between patients with lymph node involvement and a solitary tumor who underwent hepatic resection with LND and the palliative resection group (median survival time 12 mo *vs* 6.0 mo, *P* = 0.013).

CONCLUSION: ICC patients without lymph node involvement and patients with multiple tumors and lymph node metastases may not benefit from aggressive lymphadenectomy. Routine LND should be considered with discretion.

Core tip: The indications for lymph node dissection (LND) in patients with intrahepatic cholangiocarcinoma (ICC) are still controversial. Our findings may provide a reference to the criterion for LND in ICC patients. Routine LND should be considered with discretion for ICC patients without lymph node involvement and patients with multiple tumors and lymph node metastases.

Li DY, Zhang HB, Yang N, Quan Y, Yang GS. Routine lymph node dissection may be not suitable for all intrahepatic cholangiocarcinoma patients: Results of a monocentric series. *World J Gastroenterol* 2013; 19(47): 9084-9091 Available from: URL:

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC), arising from second order or more peripheral branches of the intrahepatic bile duct, is the second most common primary liver cancer after hepatocellular carcinoma (HCC), accounting for 5%-10% of primary malignancies of the liver[1,2]. It is considered a highly malignant neoplasm because it is frequently associated with lymph node (LN) involvement, intrahepatic metastasis, and peritoneal dissemination[3,4].

Hepatic resection remains the most effective therapy for patients with ICC. LN status, a definite prognostic factor in oncologic surgery, significantly affects long-term survival, as reported by the tumor staging system of the International Union Against Cancer[5]. Regional lymph node dissection (LND) is already a standard procedure, in combination with hepatic resection, for carcinoma arising from the extrahepatic bile duct [6,7]. Although LN metastasis is considered to be the most important prognostic factor for survival of ICC patients[8,9], the indications for, and roles of, LND in patients with ICC are still subject to discussion. It is important to define the role of LND because it is a modifiable factor by a surgeon during hepatic resection, but no clear guidelines yet exist. Although some consider the standard surgical procedure for ICC is hepatectomy combined with extensive nodal dissection, not all centers support routine LND[10]. Some institutions have reported selective LND and limited application of this procedure[11]. Concerns remain about routine performance of LND in patients with liver tumors because it is reportedly associated with an increased operative risk compared with hepatic resection alone[3,12].

We performed a retrospective analysis of consecutive patients at our hospital to examine the outcomes of ICC patients undergoing hepatic resection. We assessed the influence of LND on patient survival to clarify the indications for this procedure in surgical treatment of ICC, especially when LN metastases are absent.

MATERIALS AND METHODS

Patients

Altogether, 152 patients were diagnosed with ICC and underwent surgical dissection at Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China) from January 2006 to December 2007. Twelve patients only underwent laparotomy and biopsy because they had peritoneal dissemination. The remaining 140 patients were included in the present study. Among them, only 124 (88.6%) were followed sufficiently to allow subsequent data analysis, and the remaining 16 patients were lost to follow-up. The reasons for their loss to follow-up are unknown but include inability to contact them and possibly death. ICC was defined as adenocarci-

noma arising from second order or more distal branches of the intrahepatic bile ducts[10,11]. Patients with combined HCC and cholangiocarcinoma or bile duct cystadenocarcinoma were excluded from this study. The study protocol was approved by the Clinical Research Ethics Committee of our hospital. Written informed consent was obtained from all patients in the study according to the requirements of this committee.

Preoperative investigations

Resectability of the ICCs was assessed by ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) before a decision to perform surgery was made. Liver function was evaluated according to the Child-Pugh classification. Patients aged over 60 years were routinely subjected to formal cardiopulmonary evaluation and evaluation of their general condition preoperatively. Resection criteria were constant over the study period and included the number of resectable tumors, presence or absence of tumor thrombi and gross metastatic foci, and adequate hepatic functional reserve, as described in our previous study[13]. Patients were deemed to have resectable disease only if the tumor could be completely removed while preserving a sufficient functional liver remnant with adequate vascular inflow and hepatic venous outflow. If the estimated liver resection volume exceeded 60% of the whole liver as calculated by CT, preoperative percutaneous transhepatic portal embolization was performed on the liver segment to be resected, in order to induce compensatory hypertrophy of the future remnant liver.

Surgical procedures and definitions of parameters

Patients with peripheric ICC underwent hepatectomy while patients with hilar ICC underwent hemihepatectomy or trisectionectomy. Bisectionectomy or more was defined as a major hepatectomy. Sectionectomy or less was defined as a minor hepatectomy. Extended hepatectomy was defined as removal of 5 or more segments. Liver resection was performed using finger fracture and clamp crushing with intermittent Pringle's maneuver at room temperature. Initial intraoperative assessment consisted of careful examination and palpation of the hepatic hilum and hepatoduodenal ligament by the chief surgeons to detect any enlarged LNs. Any enlarged LN was considered suspicious for metastases. Because of the patient's old age, poor general condition, peripheral tumor location in the liver and the known increased risk of adding this procedure to hepatic resection, prophylactic LND was not performed in patients in whom LN involvement had not been identified by preoperative imaging (CT and MRI) and intraoperative assessment. These patients were clinically defined as not having LN metastases. If LN metastases were clinically recognized, regional LND was performed. However, curative resection was not attempted when there were metastases to LNs beyond the hepatoduodenal ligament. Regional LND included complete excision of soft tissue and LNs at the hepatic hilum, hepatoduodenal liga-

Table 1 Operative procedures for intrahepatic cholangiocarcinoma

Operative modality	*n*
Hepatic resection (*n* = 124)	
Major resection (*n* = 65)	
Partial hepatectomy	38
Right trisectionectomy	1
Left trisectionectomy	2
Extended left hemihepatectomy	2
Right hemihepatectomy	4
Left hemihepatectomy	15
Central bisectionectomy	3
Minor resection (*n* = 59)	
Partial hepatectomy	37
Right anterior sectionectomy	4
Right posterior sectionectomy	3
Left lateral sectionectomy	8
Bisegmentectomy	7
Additional procedures (*n* = 67)	
Spleen resection	2
Gallbladder resection	12
Lymph node dissection	53

ment, posterior to the upper portion of the pancreatic head, and common hepatic artery stations. The extent of LND was similar for right- and left-sided tumors, except that dissection of LNs along the lesser curvature of the stomach was added for tumors located in the left lobe of the liver.

Intrahepatic cholangiocarcinoma was classified by gross appearance, as proposed by the Liver Cancer Study Group of Japan[14]. These types include mass-forming (MF), periductal infiltrating (PI), and intraductal growth (IG), with mixed types being expressed as MF + PI or MF + IG. Tumor-node-metastasis (TNM) staging of tumors followed the guidelines of the seventh edition of the American Joint Committee on Cancer/International Union against Cancer. In this study, multiple tumors were defined as more than one involved node (including micrometastases that were discovered only on pathological examination); tumor size referred to the maximum tumor diameter; resection of three or more hepatic segments was classified as major liver resection; and resection of one or two hepatic segments as minor liver resection. Curative resection was defined as negative surgical margins on microscopic examination of the resected specimen, surgical findings of macroscopic absence of intrahepatic metastases in the residual liver, and absence of visible abdominal dissemination.

Follow-up
Clinical data for all patients were collected retrospectively. After resection, follow-up included routine blood tests, physical examination, and abdominal ultrasonography every 3 mo postoperatively for the first 2 years and twice a year thereafter at our hospital. Suspected recurrences were confirmed by CT or MRI. If the patients were unable to attend for these assessments, they were followed by telephone or letter yearly.

Statistical analysis
Overall survival (OS) was measured from the date of surgery. Recurrence-free survival (RFS) was calculated from the date of surgery to the date of the first clinically documented disease recurrence. Comparison between groups was examined by the χ^2 test or Fisher's exact test. The OS and RFS were calculated using the Kaplan-Meier method. The log-rank test was used to assess differences. All statistical analyses were performed with software package SPSS 18.0 (SPSS, Chicago, IL, United States). Statistical significance was defined as $P < 0.05$.

RESULTS
Clinicopathological characteristics
We continued follow-up of patients until death or the final date of the study, June 30, 2012. Data for analysis were available for 124 patients, including 96 men and 28 women with a median age of 56 years (range, 28-79). According to the Union for International Cancer Control TNM classification, 65 patients had stage I disease, 8 had stage II, 51 had stage III, and none had stage IV. As for liver function as defined by the Child-Pugh classification, 113 patients had class A, 11 had class B, and none had class C.

Of the 124 patients, 65 underwent major liver resection and 59 underwent minor resection. We performed additional procedures in 67 patients (Table 1). Surgical complications occurred in eight patients, including biliary leakage in three, subphrenic infection in two, liver abscess in one, bowel obstruction in one, and bleeding in one. LND did not increase the rates of postoperative complications or death.

Of the 124 patients, 10 had microscopically positive resection margins (palliative resection group). Of the 114 patients who underwent resection with curative intent, 61 did not undergo LND [LND (-) group]. Of the 53 patients who underwent LND, 42 did not have LN metastases [LND (+) LN (-) group] and 11 did [LND (+) LN (+) group]. In all, 318 LNs were analyzed histologically. The median number of retrieved LNs was 6 (1-16). We found LN metastases in the hepatoduodenal ligament in 10 patients and along the common hepatic artery in three patients. We found a single LN metastasis in nine patients.

OS and RFS of patients who did not undergo LND and those who did and had no LN metastases detected
The clinical and pathological characteristics of the patients in the LND (-) and LND (+) LN (-) groups are summarized in Table 2. There were no significant differences between these groups. Figure 1 presents the Kaplan-Meier survival analysis comparing patients in the LND (-) group with those in the LND (+) LN (-) group. There were no differences in their survival curves ($P = 0.822$). The 1-, 3-, and 5-year OS rates were 69%, 26% and 15%, respectively, in the LND (-) group and 64%, 31%, and 17%, respectively, in the LND (+) LN (-) group. Recurrence occurred in 69 of these patients (67.0%).
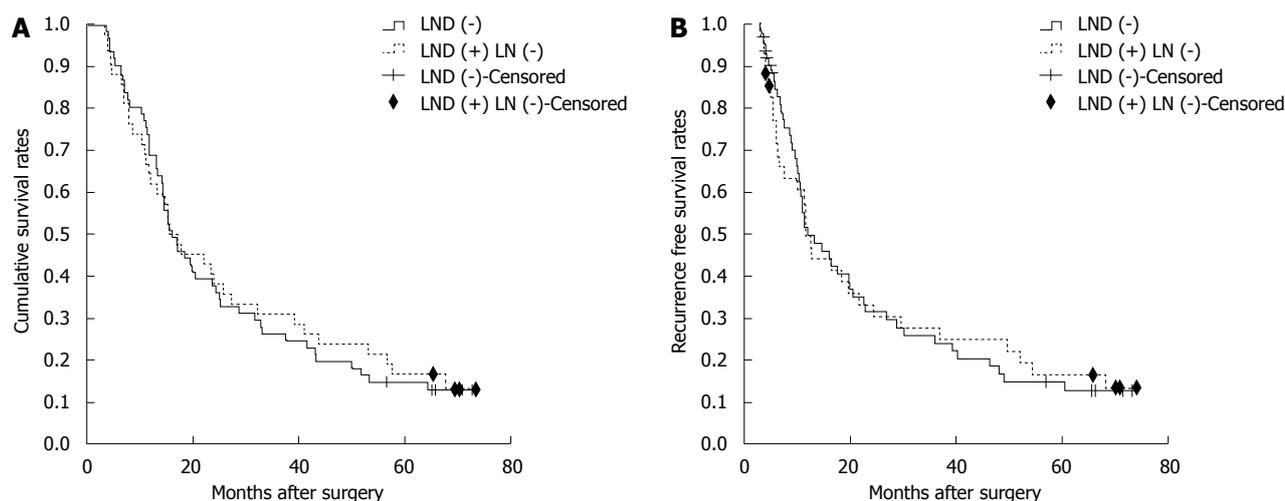
Figure 1 Overall survival and recurrence-free survival curves of intrahepatic cholangiocarcinoma patients without lymph node involvement. A: Survival curves of patients in the lymph node dissection (LND) (-) and LND (+) LN (-) groups. There is no significant survival difference between the two groups (P = 0.822). The censored represented the cases who were still alive at the endpoint; B: Recurrence-free survival curve of patients in LND (-) and LND (+) LN (-) groups. There is no significant survival difference between the two groups (P = 0.970). The censored represented the cases who were still alive at the endpoint or died for other reasons instead of tumor recurrence.
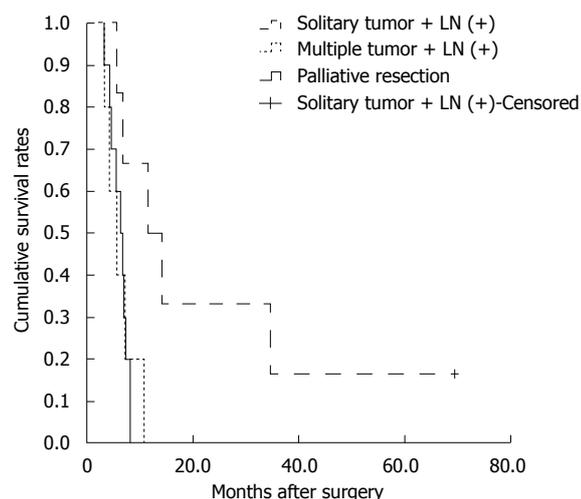
Figure 2 Survival curves of patients in the palliative resection and lymph node dissection (+) lymph node (+) groups. There are significant differences between the palliative resection group and patients with lymph node (LN) involvement and a solitary tumor (P = 0.013). There are no significant differences between the palliative resection group and patients with LN involvement and multiple tumors (P = 0.744).

RFS rates at 1-, 3-, and 5-year were 53%, 25%, and 15%, respectively, in the LND (-) group and 52%, 29%, and 17%, respectively, in the LND (+) LN (-) group. There was no significant difference in RFS between the LND (-) and LND (+) LN (-) groups (P = 0.970) (Figure 1). The sites of recurrence are shown in Table 3. The most common recurrence site was the remnant liver. Among the 61 patients who did not undergo LND, the initial recurrence site was LNs in nine. Recurrence in LNs occurred in four patients who had undergone LN dissection.

OS of patients in the palliative resection group and patients who underwent LND and had positive LNs

Five patients with LN involvement and multiple tumors underwent hepatic resection with LND. As for the subgroup analysis, the median survival times of the palliative resection group and patients with LN involvement and multiple tumors were 6.0 mo and 5.5 mo, respectively (Figure 2). There were no significant survival differences between the two groups (P = 0.744). However, there was a significant difference between patients with a solitary tumor and LN involvement who underwent hepatic resection with LND and the palliative resection group (P = 0.013), and their median survival times were 12 mo and 6.0 mo, respectively (Figure 2).

DISCUSSION

Although curative resection provides the only chance of long-term survival for patients with ICC, the prognosis after surgical resection remains poor because this tumor exhibits aggressive invasion locally and frequently metastasizes, tending especially to spread via the lymphatic system[3,15-25]. The rate of perihepatic LN positivity detected at surgery reportedly ranges from 36% to 62%[3,15-25]. In our current study of 53 patients who underwent regional lymphadenectomy, the incidence of LN metastasis was 20.8% (11/53), which is slightly lower than those reported in previous studies. The most common site of LN metastases was the hepatoduodenal ligament (10/11).

Many investigators have used multivariate analysis to determine useful prognostic factors for ICC after surgical resection in recent 5 years (Table 4)[8,9,26-37]. According to these reports, potentially significant prognostic factors

Factor	LND (-) $n = 61$	LND (+) LN (-) $n = 42$	P value
Gender			1.000
Female	13	9	
Male	48	33	
Age			0.516
⩽ 60	44	27	
> 60	17	15	
Viral hepatitis			1.000
Yes	36	24	
No	25	18	
Cirrhosis			0.694
Yes	28	21	
No	33	21	
Child-Pugh class			0.735
A	55	39	
B	6	3	
CA19-9 (U/mL)			0.553
⩽ 37	30	18	
> 37	31	24	
Histologic differentiation			0.498
Well or Moderate	44	33	
Poor	17	9	
Gross type			0.433
MF	43	28	
PI	4	7	
IG	5	2	
MF + PI	5	4	
MF + IG	4	1	
Tumor number			0.510
Single	45	28	
Multiple	16	14	
Tumor size (cm)			0.318
< 5	33	18	
⩾ 5	28	24	
TNM classification			0.080
Early (stage Ⅰ, Ⅱ)	47	25	
Advanced (stage Ⅲ, Ⅳ)	14	17	
Width of resection margin (cm)			0.229
< 1	33	17	
⩾ 1	28	25	
Surgical procedure			0.153
Major hepatectomy	41	22	
Minor hepatectomy	20	20	

CA19-9: Carbohydrate antigen 19-9; IG: Intraductal growth; LN: Lymph node; LND: Lymph node dissection; MF: Mass-forming; PI: Periductal infiltrating; TNM: Tumor-node-metastasis.

Site of initial recurrence	LND (-) $n = 61$	LND(+) LN (-) $n = 42$	LND(+) LN (+) $n = 11$
Liver, lymph nodes	6	3	2
Liver, lung	2	0	1
Liver	25	18	6
Lymph nodes	3	1	0
Peritoneum	5	2	2
Wound site	0	1	0
Bone	0	1	0
Lung	1	1	0
Total No. of recurrence	42	27	11

LN: Lymph node; LND: Lymph node dissection.

theoretical potential to improve long-term survival. LND for nodal metastases has reportedly resulted in a few long-term survivors[38,39]. But some authors have reported that extended LN dissection in patients with ICC does not seem to offer any advantage without control of liver metastases, because most recurrences are in the liver[3,40].

In the current study, we showed that patients who did not undergo LND and those who did, but had negative LNs, had similar survival (1-year: 69% *vs* 64%; 3-year: 26% *vs* 31%; 5-year: 15% *vs* 17%, P = 0.822). These findings suggest that LND does not improve the survival significantly in LN negative patients. The commonest recurrence pattern was intrahepatic, which is similar to other reported findings[5,25,41,42]. We also found no statistically significant difference in RFS between patients who did and did not undergo LND (P = 0.970). It seems that LND does not improve the prognosis because it has no effect on liver metastases.

Prophylactic LND has been advocated to prevent LN recurrence, not only because there can be microscopic LN metastases around the perihepatic LNs, but also because it allows removing a frequent site of recurrence. Among the 61 patients who did not undergo LND, three developed LN recurrence as the primary recurrence site. The chance of benefiting from LND seems to be only about 3/61 (4.9%) of all patients with ICC. Choi *et al*[37] found that the patients who underwent LND but had negative LDs appear to show slightly worse survival than LND (-) group in the earlier time of the follow-up period, although it was not statistically significant because of the small sample size. Thus, prophylactic LND may be not beneficial to the clinically LD negative patients.

One might question the reliability of our intra-operative LN examination and indications for lymphadenectomy. For patients who had not undergone LN dissection, the N status cannot be ascertained. Clearly, some patients have microscopic nodal involvement that is beyond detection by conventional radiographic imaging or even direct palpation. Some authors have advocated routine lymphadenectomy for all patients undergoing hepatic resection as a staging procedure[8,9,40,43]. However, clinical assessment of LN negativity without histopathologic

include multiple tumors, LN metastasis, serum CA 19-9 level, vascular invasion, tumor size, histological grade, intrahepatic metastases, histological grade, and resection margin. LN metastasis was confirmed to be one of the most significant independent prognostic factors for patients with ICC. Although LN metastasis was considered a significant prognostic factor, whether routine LND should be adopted is still controversial.

It is unclear whether prophylactic clearance of the route of LN metastasis improves survival. Ribero *et al*[8] reported that LN metastases and multiple tumors are associated with decreased survival rates. Lymphadenectomy should be considered for all patients according to its

Table 4 Selected published series of intrahepatic cholangiocarcinoma patients after resection

Author	Year	Cases	Prognostic factors	Median survival time	5-yr survival rate	Routine LND
Ribero *et al*[8]	2012	434	LN metastases Multiple tumors CA19.9 level	33	32.90%	No
de Jong *et al*[9]	2011	449	Tumor number Vascular invasion LN metastasis	27.3	30.7	No
Saxena *et al*[26]	2010	88	CA 19.9 level Clinical stage Histological grade LN metastases	33	28	No
Ercolani *et al*[27]	2010	72	LN metastasis Blood transfusion	57.1	48	Yes
Cho *et al*[28]	2010	63	Old age CA19-9 level LN metastasis Narrow resection margin	Not available	31.8	Yes
Shirabe *et al*[29]	2010	60	Lymphatic invasion index Histological grade	Not available	30.6	No
Guglielmi *et al*[30]	2009	81	LN metastasis Vascular invasion	40	20	Not available
Tamandl *et al*[31]	2009	93	Lymph node ratio	25.5	Not available	No
Choi *et al*[37]	2009	64	LN metastasis	39	39.5	No
Shimada *et al*[32]	2009	104	Intrahepatic metastases LN metastasis	25	37	No
Yedibela *et al*[33]	2009	67	Resection margin LN metastasis Blood transfusion	26	27	No
Nakagohri *et al*[34]	2008	56	Intrahepatic metastasis	22	32	Not available
Uenishi *et al*[35]	2008	133	Intrahepatic metastasis LN metastases Tumor at the margin	18.4	29	Yes
Shimada *et al*[36]	2007	57	LN metastasis	62	56.8	No

LN: Lymph node; LND: Lymph node dissection; ICC: Intrahepatic cholangiocarcinoma.

confirmation appears to be associated with a small risk of subsequent LN metastases. Grobmyer *et al*[44] stated that the incidence of truly occult metastatic disease to perihepatic LNs is low in patients with primary and metastatic liver cancer. Of patients with negative preoperative imaging and intraoperative assessment, none had involved perihepatic nodes. This conclusion is consistent with another report of a low incidence of missed diagnosis of LN metastases[45]. In addition to the increased operative time associated with lymphadenectomy, surgeons should factor potential complications into decisions about performing this procedure in these patients without LN involvement.

In addition, hepatectomy with LND might not contribute to long-term survival in patients with multiple tumors and LN metastases. These patients had similar survival to patients who underwent palliative resection (*P* = 0.744), possibly because both LN involvement and multiple tumors are poor prognostic factors[8]. However, patients with a solitary tumor and LN involvement might benefit from LND. Suzuki *et al*[21] reported that hepatic resection with LND may be curative for patients with a solitary tumor and a single LN metastasis. Nakagawa *et al*[24] also reported that curative resection with LND could improve the prognosis of patients with a solitary tumor

and no more than two LN metastases. In our series of patients with ICC, those with a solitary tumor and LN involvement had better survival than did palliative resection patients. The median survival time was 12 mo *vs* 6.0 mo (*P* = 0.013). Although LN metastasis is an independent prognostic factor, it seems that LND can prolong the survival time of patients with a solitary tumor and LN metastases. However, more studies are needed.

Because our study was based on retrospectively available medical records, and more than five surgeons were involved in treating the study patients, it is difficult to draw definite conclusions about the indications for LND.

In conclusion, ICC patients without LN involvement and patients with multiple tumors and LD metastases may not benefit from aggressive lymphadenectomy. Without sufficient evidence, routine LND for all the ICC patients would be dogmatic. Routine LND should be considered with discretion.

COMMENTS

Background

Surgical resection is considered to improve the survival of patients with intrahepatic cholangiocarcinoma (ICC). Lymph node (LN) involvement significantly affects survival adversely. However, the benefit of lymph node dissection (LND) is still controversial.

Research frontiers

Although LN metastasis was considered one of the most significant prognostic factors for patients with ICC, whether routine LND should be adopted is still controversial. Some consider that LN metastasis should not be considered a selection criterion that prevents patients from undergoing a potentially curative resection. Lymphadenectomy should be considered for all patients. On the other hand, some consider that routine use of LND in patients with ICC is not recommended, because no difference in survival was observed in patients with negative LN metastases, irrespective of the use of LND.

Innovations and breakthroughs

The indications for, and roles of, LND in patients with ICC are still subject to discussion. It is important to define the role of LND because it is a modifiable factor by a surgeon during hepatic resection, but no clear guidelines yet exist. In this study, they found that ICC patients without LN involvement and patients with multiple tumors and LN metastases may not benefit from aggressive lymphadenectomy.

Applications

It is unclear whether prophylactic clearance of the route of LN metastasis improves survival. Thire findings may provide a reference to the criterion for LND in ICC patients. Routine LND should be considered with discretion for ICC patients without LN involvement and patients with multiple tumors and LN metastases. Routine LND can be performed for the patients with a solitary tumor and LN metastases for a better survival.

Peer review

The authors investigated the benefit of LND in the patients with ICC. They concluded that ICC patients without LN involvement and patients with multiple tumors and LN metastases may not benefit from aggressive lymphadenectomy, so routine LND should be considered with discretion. It is a well written manuscript that addresses an interesting topic. It also provides useful data on recurrences. The design is appropriate and the conclusion is reasonable.

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BRIEF ARTICLE

Separate calculation of DW-MRI in assessing therapeutic effect in liver tumors in rats

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Abstract

AIM: To explore whether the antitumor effect of a vascular disrupting agent (VDA) would be enhanced by combining with an antiangiogenic agent, and whether such synergistic effects can be effectively evaluated with separate calculation of diffusion weighted magnetic resonance imaging (DW-MRI).

METHODS: Thirty-seven rats with implanted liver tumors were randomized into the following three groups: (1) ZD6126, a kind of VDA; (2) ZDTHA, ZD6126 in combination with an antiangiogenic, thalidomide; and (3) control. Morphological DW-MRI were performed and quantified before, 4 h and 2 d after treatment. The apparent diffusion coefficient (ADC) values were calculated separately for low b values (ADC_{low}), high b values (ADC_{high}) and all b values (ADC_{all}). The tissue perfusion contribution, ADC_{perf}, was calculated as ADC_{low}-ADC_{high}. Imaging findings were finally verified by histopathology.

RESULTS: The combination therapy with ZDTHA significantly delayed tumor growth due to synergistic effects by inducing cumulative tumor necrosis. In addition to delaying tumor growth, ZDTHA caused tumor necrosis in an additive manner, which was verified by HE staining. Although both ADC_{high} and ADC_{all} in the ZD6126 and ZDTHA groups were significantly higher compared to those in the control group on day 2, the entire tumor ADC_{high} of ZDTHA was even higher than that of ZD6126, but the significant difference was not observed for ADC_{all} between ZDTHA and ZD6126. This indicated that the perfusion insensitive ADC_{high} values calculated from high b value images performed significantly better than ADC_{all} for the monitoring of tumor necrosis on day 2. The perfusion sensitive ADC_{perf} derived from ADC_{low} by excluding high b value effects could better reflect the reduction of blood flow due to the vessel shutdown induced by ZD6126, compared to the ADC_{low} at 4 h. The ADC_{perf} could provide valuable perfusion information from DW-MRI data.

CONCLUSION: The separate calculation of ADC is more useful than conventional averaged ADC in evaluating the efficacy of combination therapy with ZD6126 and thalidomide for solid tumors.

Key words: Diffusion weighted imaging; Magnetic resonance imaging; Therapeutic assessment; Liver tumor; Rats; Vascular disrupting agent; Antiangiogenic agent;

Animal model; Rodents

Core tip: The combination therapy with ZD6126 and thalidomide significantly delayed liver tumor growth due to synergistic effects by inducing cumulative tumor necrosis in rodents. The apparent diffusion coefficient (ADC)$_{high}$ performed significantly better than ADC$_{all}$ for the monitoring of tumor necrosis on day 2. The ADC$_{perf}$ could better reflect the reduction of blood flow due to the vessel shutdown induced by ZD6126, compared to the ADC$_{low}$. The ADC$_{perf}$ could provide valuable perfusion information from diffusion weighted magnetic resonance imaging data.

INTRODUCTION

Tumor vasculature has become an attractive target for therapy. One of such therapies is to use vascular disrupting agents (VDAs), which can selectively destroy existing tumor blood vessels by disrupting the microtubules of the cytoskeleton in endothelial cells; this leads to ischemic central necrosis of the tumor[1]. However, tumors can rapidly rebound from the residual viable rim when VDAs are used alone; this compromises the therapeutic utility of these agents[2]. Another therapy is to prevent new tumor blood vessel formation with antiangiogenic agents. Therefore, current efforts have gradually shifted from the single use of VDA to the combination of a VDA with an antiangiogenic agent[3,4]. As the latter may inhibit the growth of new tumor vessels, the combination of two approaches thus is likely to have synergistic therapeutic efficacy.

As an established non-invasive technique, *in vivo* magnetic resonance imaging (MRI) has played an important role in the evaluation of tumor response to treatment. Diffusion-weighted MRI (DW-MRI), due to its ability to detect molecular water motion at the cellular level, *i.e.*, the measurement of tissue apparent diffusion coefficient (ADC), has become a favorite choice of measures in a variety of oncological studies and tissue viability assessments[5]. Technological innovations in recent years have enabled the increasing use of high-quality and quantitative DW-MRI in monitoring tumor treatment. However, it has been realized that the information acquired from conventional calculation of DW-MRI data actually represents the combined effects of tissue microcirculation perfusion and pure tissue diffusivity in each imaging voxel at DW-MRI. This may hinder the appropriate interpretation of the DW-MRI data[6]. Therefore, there is growing interest in applying more sophisticated approaches, such as separate ADC (calculating different

ADC values based on various combinations of *b* values with a monoexponential fitting algorithm)[7-9] and intra-voxel incoherent motion (IVIM)[10,11], to differentiate the fraction of microcirculation perfusion from pure diffusivity within the DW-MRI data.

The purpose of the present study was to test our hypotheses that the antitumor effect of a VDA, ZD6126, would be enhanced by combining with an antiangiogenic agent, thalidomide, and that the effect can be monitored and better elucidated with separate calculation of ADC values compared to conventional ADC value in a rat liver tumor model. To our knowledge, the application of separate calculation of ADC maps has not been reported in such a combined antitumor therapy.

MATERIALS AND METHODS

Experimental design

A total of 37 rats were randomly assigned into the following 3 groups: (1) ZD6126 group (*n* = 14): ZD6126 (AstraZeneca, Cheshire, United Kingdom) was dissolved with 4 portions of 8.4% sodium carbonate and 1 portion of phosphate-buffered saline (PBS), pH 7.4. On day 0, one dose of 50 mg/kg ZD6126 was injected *iv* into each animal; (2) ZDTHA group (*n* = 13): Stock solutions of thalidomide (Pharmaceutical Factory, Changzhou, China) were prepared in DMSO (Sigma-Aldrich NV/SA, Bornem, Belgium) and injected *ip* at a dose of 200 mg/kg three times at a interval of one day during the entire experiment[12]; the first dose of thalidomide was injected 24 h prior to ZD6126 administration, and the second and third doses of thalidomide were given immediately and 2 d after ZD6126 administration, respectively; and (3) Control group (*n* = 10): Animals were *iv* and *ip* injected with the vehicles (solvents) of both agents at the same time points that the other groups were injected. For all groups, MRI was performed before, and 4 h and 2 d after the initial ZD6126 treatment. At the end of the experiment, all animals were sacrificed for histopathological examinations.

Animal model

This study was approved by the institutional ethical committee for the use and care of laboratory animals. Adult WAG/Rij rats (Iffa Credo, Brussels, Belgium) with existing subcutaneous rhabdomyosarcomas were used as donors. The tumor tissues were excised and implanted into 37 normal adult WAG/Rij rats that weighed 225-275 g, as described previously[13].

MRI

All rats were initially anesthetized by inhalation of 2% isoflurane and maintained with 0.8% isoflurane for MRI. A clinical 1.5T MRI system (Sonata, Siemens, Erlangen, Germany) was used with a maximum gradient capability of 40 mT/m. The following sequences were acquired in the transverse plane for all rats, with a slice thickness of 2 mm and an inter-slice gap of 0.2 mm: (1) Fat saturated

T2-weighted fast spin echo MRI (T2W-MRI) with a repetition/echo time (TR/TE) of 3860/106 ms, a turbo factor of 19, a field of view (FOV) of 140 mm × 70 mm, and an acquisition matrix of 256 × 256 (in-plane resolution: 0.5 mm × 0.3 mm). Three signals were acquired, in a scan time of 1 min 25 s; (2) Contrast-enhanced fat saturated T1-weighted fast spin echo MRI (CE-T1W-MRI) immediately after an *iv* bolus of 0.3 mmol/kg gadoterate meglumine (Dotarem®, Guerbet, France), with the following parameters: a TR/TE of 535/9.2 ms, a turbo factor of seven, a FOV of 140 mm × 70 mm, and an acquisition matrix of 256 × 256 (in-plane resolution: 0.5 mm × 0.3 mm). Four signals were acquired, in a scan time of 1 min 24 s; (3) DW-MRI with a 2-dimensional (2D), spin echo, echo-planar imaging sequence. We used a TR/TE of 1700/83 ms, a FOV of 140 mm × 82 mm, and an acquisition matrix of 192 × 91 (in-plane resolution: 0.7 mm × 0.9 mm). For the DW-MRI, six signals were acquired, including repeated measurements for 10 different *b* values (0, 50, 100, 150, 200, 250, 300, 500, 750, and 1000 s/mm^2) in 3 directions (x, y, and z) and averaged for the calculation of the isotropic ADC value. A parallel imaging technique was applied to reduce susceptibility artifacts and examination times. The total examination time was 4 min 51 s.

Tissue processing and histology

All rats were euthanized for tissue processing and histology at the end of the experiment. First, animals were over-anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) (Nembutal, Sanofi Sante Animale, Brussels, Belgium). Then, the livers were collected, fixed with formalin, embedded in paraffin, and sliced into transverse sections. The sections were 2 mm thick, and were positioned on the same planes used for the MRI scans, based on a grid (Agar Scientific, England). The tumor slices (5 μm thick) were stained with hematoxylin and eosin (HE).

MRI analysis

An off-line LINUX workstation with dedicated software (Biomap, Novartis, Basel, Switzerland) was used for image analyses. Two experienced radiologists delineated the entire tumor with operator-defined regions of interest (ROI) in consensus to obtain robust measurements and to facilitate comparisons between different treatment approaches. All ROIs were larger than 10 pixels in size. For each imaging parameter, tumor and normal liver were measured with ROIs on all tumor-containing image slices, and mean values were obtained for each tumor and the liver, respectively. After that, the mean value and standard deviation for each parameter were calculated for each group at each time point for statistical analysis.

T2 weighted and contrast enhanced-T1 weighted MRI: The residual viable tumor or rim after treatment was visualized as contrast-enhanced, high signal region on the CE-T1W-MRI. The tumor necrotic areas were contoured on CE-T1W-MRI based on the unenhanced, low-signal areas within the tumors that were observed after injection of a contrast agent. Relative volumes (%) of tumor necrosis were calculated by normalizing them to the entire tumor volume. For each lesion, the tumor areas were delineated at T2W-MRI on all slices and automatically combined into the total tumor volume. The tumor volume change (%) was calculated using the following formula: [(volume$_{post}$ - volume$_{pre}$)/volume$_{pre}$] × 100.

Separate calculation of tumor ADC: For calculating different ADC values, the first step was to measure the entire tumor signal intensity (SI) from original DW-MRI images of 10 *b* values, respectively. Briefly, for each tumor, freehand delineations were performed on all slices of the original DW-MRI at a *b* value of 1000 s/mm^2. These delineations were merged to form one 3D volume of interest per lesion. The volume of interest was then automatically copied to all images with different *b* values and the average SI of each lesion per *b* value was determined. The second step was to obtain separate ADC values according to a monoexponential model using all 10 *b* values[14]. To differentiate the individual contributions of tissue microcapillary perfusion and pure tissue diffusivity, the ADC values of each tumor were obtained separately for low *b* values (b = 0, 50, and 100 s/mm^2; ADC$_{low}$) and high *b* values (b = 500, 750, and 1000 s/mm^2; ADC$_{high}$) from the average SI per tumor and per *b* value. Each ADC value was calculated by using a least squares solution of the following system of equations: ADC$_{all}$: $S_k = S_0 \times exp$ (-$b_k \times$ ADC$_{low}$), for k = 0, 50, 100; 150, 200, 250, 300, 500, 750, 1000; ADC$_{low}$: $S_i = S_0 \times exp$ (-$b_i \times$ ADC$_{low}$), for i = 0, 50, 100; ADC$_{high}$: $S_j = S_0 \times exp$ (-$b_j \times$ ADC$_{high}$), for j = 500, 750, 1000; where S_k, S_i, and S_j are the SI measured on the DW-MRI acquired with the corresponding *b* values b_k, b_i and b_j, S_0 represents the exact SI (without the influence of noise induced by the MR measurement) with *b* value equal to 0 s/mm^2. ADC$_{low}$ is perfusion sensitive, while ADC$_{high}$ is perfusion insensitive. Although ADC$_{low}$ is perfusion sensitive, it is also affected by diffusion effects in tissue[15]. Therefore, an approximate indicator, ADC$_{perf}$, for the tissue perfusion contribution can be calculated as ADC$_{low}$-ADC$_{high}$[7]. Imaging software (MeVisLab 2.2.1, MeVis Medical Solutions AG, Bremen, Germany) was used to generate the maps of ADC$_{all}$, ADC$_{high}$, ADC$_{low}$ and ADC$_{perf}$.

Microscopic analysis

Microscopic image analyses were performed by a pathologist blinded to the experimental detail with magnifications ranging from × 50 to × 400. On HE stained macroscopic sections, image analysis software (ImageJ 1.34s, NIH, United States) was used to quantify the percentages of amorphous eosinophilic necrosis in the total tumor area.

Statistical analysis

Statistical analysis was carried out with the SPSS for win-

dows software package (release 18.0, SPSS Inc., Chicago, United States). A general linear model, with repeated-measures, was used to compare changes in various parameters over time among groups. The nonparametric Kruskal-Wallis analysis of variance was performed for comparing parameters between groups at certain time points, followed by post-hoc group-wise comparisons using a Bonferroni correction for multiple tests. A P value < 0.05 was considered statistically significant.

RESULTS

A total of 37 rats (72 tumors) were included in the study. Four rats in the ZDTHA group were found to have minor hemorrhage around the eye socket and perianal area on day 1 after the first thalidomide treatment. This was probably due to a venous thromboembolism induced by thalidomide[16].

Tumor volume growth

As shown on T2W-MRI images, ZD6126 and ZDTHA both induced a significant tumor volume growth delay from pretreatment to 2 d after administration, compared to the control group (P < 0.0001 for both). Furthermore, ZDTHA performed significantly better than ZD6126 in delaying tumor growth on day 2 after treatment (P < 0.0001) (Figure 1).

Perfusion insensitive ADC$_{high}$: Before treatment: there were no significant differences in ADC$_{high}$ among the three groups (P > 0.05 for all). At 4 h, there was no significant change in the ADC$_{high}$ (P > 0.05 for both) in both the ZDTHA and ZD6126 groups, compared to the control group. On day 2, the therapy-induced tumor necrosis caused a significant rise in the ADC$_{high}$ in both ZDTHA and ZD6126 groups compared to the control group (P < 0.0001 and = 0.0004, respectively). Furthermore, the ADC$_{high}$ was much higher in the ZDTHA group than in the ZD6126 group (P = 0.03) (Table 1 and Figure 2A-C).

ADC$_{all}$: At 4 h, the ADC$_{all}$ in both the ZDTHA and ZD6126 groups was significantly reduced compared to the control group (P = 0.01 and 0.02, respectively). In contrast, the ADC$_{all}$ in both the ZDTHA and ZD6126 groups showed a sharp increase on day 2 compared to the control group (P < 0.0001 for both). However, no difference in ADC$_{all}$ was found between the ZDTHA and ZD6126 groups (P = 0.08) (Table 1 and Figure 2A-C).

Comparison of ADC$_{high}$ with ADC$_{all}$: The performance of ADC$_{all}$ was different with that of ADC$_{high}$ at the following time points. At 4 h, the ADC$_{all}$ in both the ZDTHA and ZD6126 groups showed a significant decrease compared to the control group; however, this was not observed for ADC$_{high}$ in the same two groups. On day 2, the ADC$_{high}$ of ZDTHA was significantly greater than that of ZD6126 (P = 0.03), but the significant difference was not observed for ADC$_{all}$ between the ZDTHA and

ZD6126 groups (P = 0.08) (Table 1 and Figure 2A-C).

ADC$_{low}$ and perfusion sensitive ADC$_{perf}$

ADC$_{low}$: The ADC$_{low}$ of ZDTHA was significantly lower than that of ZD6126 before treatment (P < 0.05), but it was not for the control group (P = 0.12). No significant differences in ADC$_{low}$ were observed among the three groups at 4 h (P > 0.05 for all). On day 2, the ADC$_{low}$ of ZDTHA was much higher compared to the control group (P = 0.02) (Table 1 and Figure 2D-F).

ADC$_{perf}$: Compared to the control group, tumor ADC$_{perf}$ in both the ZD6126 and ZDTHA groups decreased dramatically at 4 h, most likely due to a rapid vascular shutdown induced by ZD6126 (P = 0.016 and 0.047, respectively). This was followed by a rapid rebound on day 2 in both the ZD6126 and ZDTHA groups (no longer significantly different compared to the control group, P = 0.979 and 0.525, respectively) (Figure 2D-F). A significant reduction in the tumor ADC$_{perf}$ of ZDTHA was noted at 4 h compared to the ZD6126 group (P = 0.025). The ADC$_{perf}$ of ZDTHA still showed a lower level compared to the ZD6126 at 2 d, although there was no significant difference (P = 0.44) (Table 1 and Figure 2D-F).

Histology

Two days after treatment, the percentages of necrosis compared to the total tumor areas on HE stained tumor sections were significantly higher in both the ZDTHA and ZD6126 groups compared to the control group (P = 0.000 for both). No significant difference was found in the necrotic areas of the ZDTHA and ZD6126 groups (P = 0.09) (Figure 1).

DISCUSSION

We have demonstrated three main findings in the present study. First, tumor growth was significantly delayed by both ZD6126 and ZDTHA treatments compared to the control group, and a significant delay could be observed only two days after application of a single dose of ZD6126. In addition, ZDTHA significantly delayed tumor growth than ZD6126, indicating a synergistic anticancer effect of ZD6126 and thalidomide. It has been known that tumors can rapidly regrow due to the residual viable rim when ZD6126 was used alone[2]. It has also been reported that thalidomide, which was reintroduced into clinical practice with its antiangiogenic properties, had little or no effect on full-grown tumors like those in our patients, when used alone[4]. Therefore, the synergistic effects of ZDTHA may have contributed to the enhanced antitumor effect in this study. In the combination therapy, ZD6126-induced tumor necrosis, which may then promote tumor angiogenesis, could provide the appropriate conditions for THA to indirectly inhibit angiogenesis[17], because thalidomide is effective only in the early stages of tumor formation[4]. Thalidomide indirectly inhibits angiogenesis *via* tumor necrosis factor and
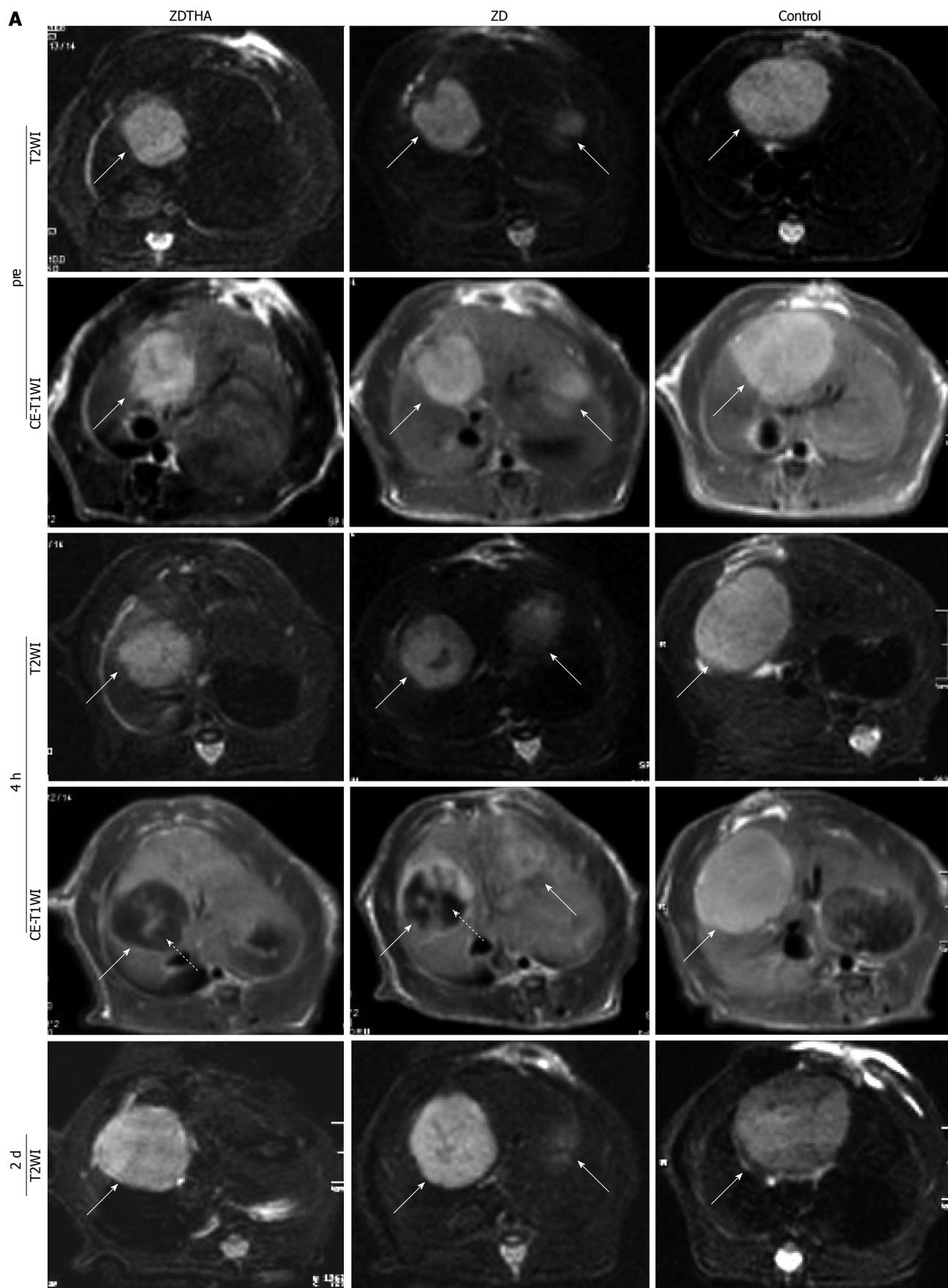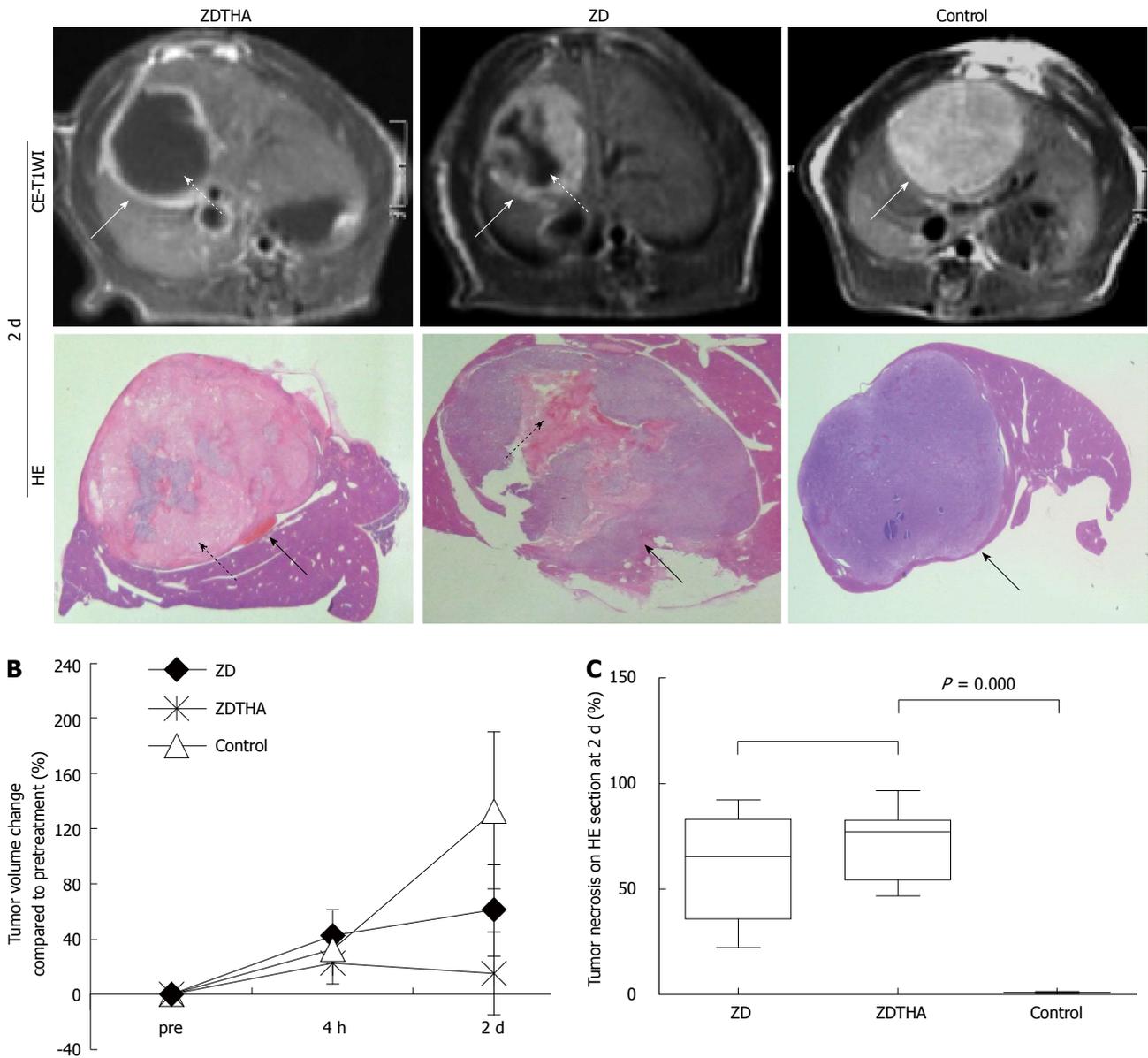
Figure 1 Tumor growth delay. A: Representative axial magnetic resonance images of liver tumors acquired with T2-weighted images (T2WI) [repetition/echo time (TR/TE) = 3860/106 ms], contrast enhanced T1-weighted images (CE-T1WI) (TR/TE = 535/9.2 ms) and HE stained sections. Top row magnetic resonance images: After the combination therapy with ZDTHA, the tumor (solid arrow) in the right liver lobe showed delayed growth with massive central necrotic area (dotted arrow) compared to the control tumor on day 2; Middle row magnetic resonance images: After ZD6126 treatment, the right tumor also showed delayed growth compared to the control tumor on day 2 , however, the tumor necrotic area was reduced (dotted arrow) because the tumor regrew from viable rim; Bottom row magnetic resonance images: In a control animal, the tumor grew remarkably at 2 d; HE sections: The tumor (solid arrow) and central necrotic areas (dotted arrow) in different groups were verified by HE staining; B: The graph indicated that ZDTHA induced a significant tumor volume growth delay during the experiment, compared to both the ZD6126 and control groups (^bP < 0.01 for both; C: The box plots showed significantly higher percentages of necrotic area (necrosis/tumor) on HE stained sections in both the ZDTHA and ZD6126 groups compared to the control group (P = 0.000 for both). No significant difference in necrosis was found between the ZDTHA and ZD6126 groups (P = 0.09).

the prostaglandin E pathway[17]. Therefore, the proposed combination therapy with ZD6126 and thalidomide may have some potential applications for solid tumor treatment in clinic.

Second, ADC$_{high}$, a separate ADC value calculated from high b value images, performed significantly better than ADC$_{all}$ for the monitoring of tumor necrosis. In addition to delaying tumor growth, ZDTHA caused tumor necrosis in an additive manner, which was verified by HE staining. Our results showed that although both the ADC$_{high}$ and ADC$_{all}$ of ZDTHA and ZD612 were significant-

ly higher compared to those of the control group on day 2, the entire tumor ADC$_{high}$ of ZDTHA was even higher than that of ZD6126, but the significant difference was not observed for ADC$_{all}$ between ZDTHA and ZD6126. This was due to that ADC$_{high}$ was more sensitive to the diffusion change resulting from the therapeutic necrosis of the tumor on day 2. It has been reported that VDA can cause massive central necrosis 2 d after treatment[18]. Thalidomide can directly induce apoptosis or G1 phase arrest[19]. Consequently, tumor cells treated with ZD6126 and thalidomide underwent increased necrosis compared

A
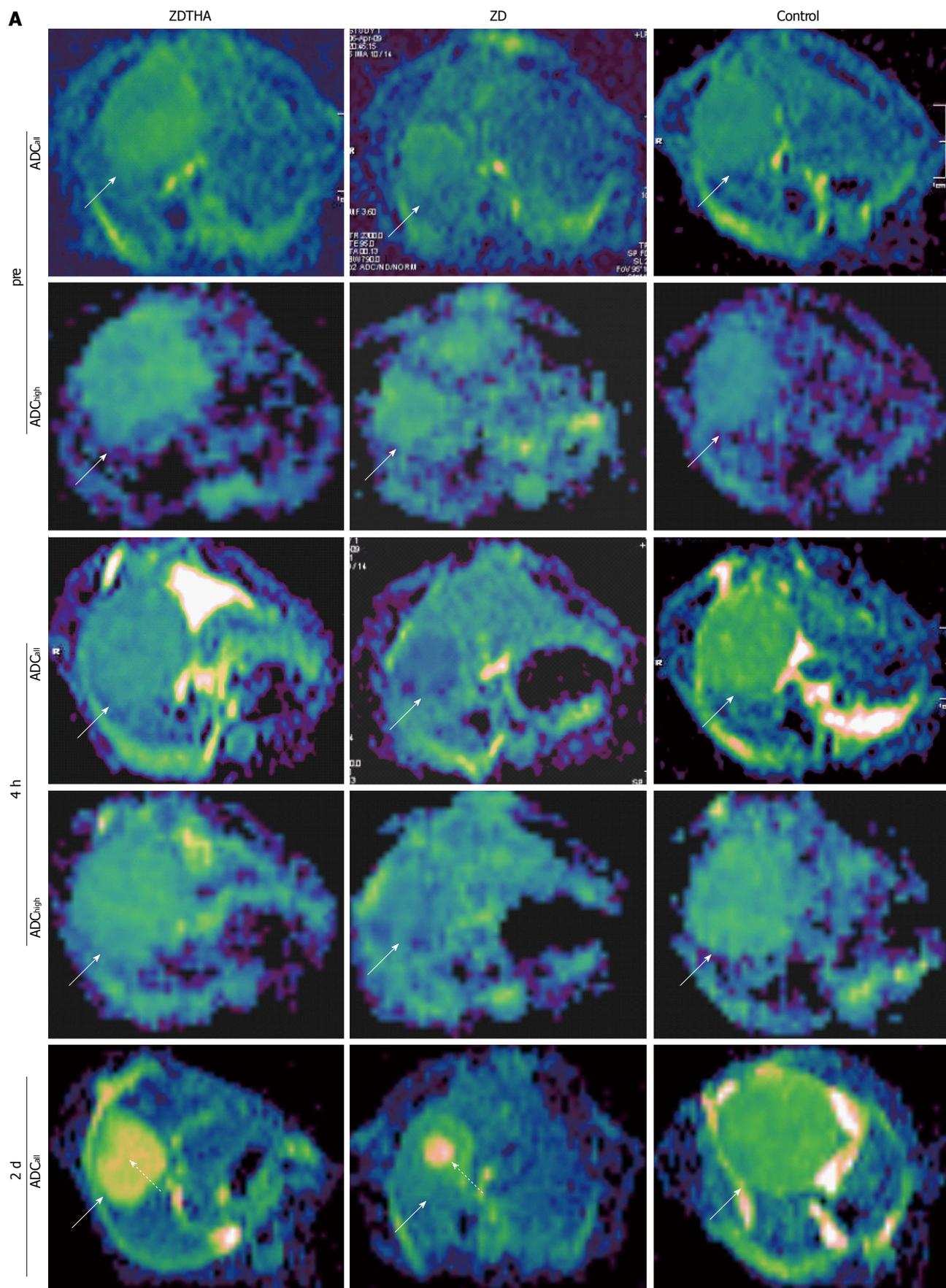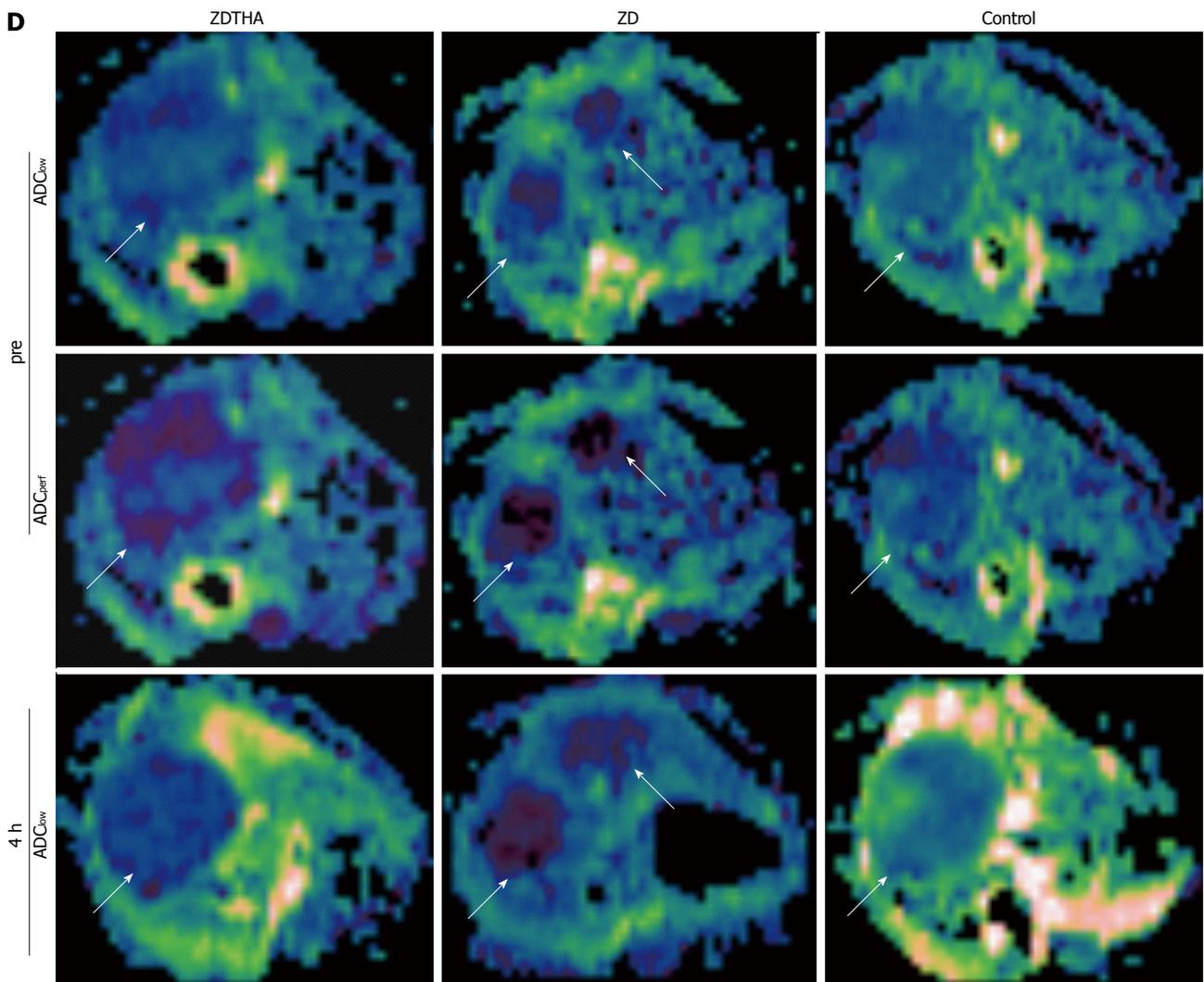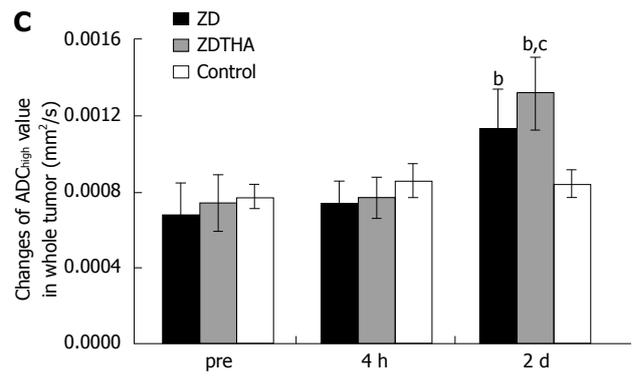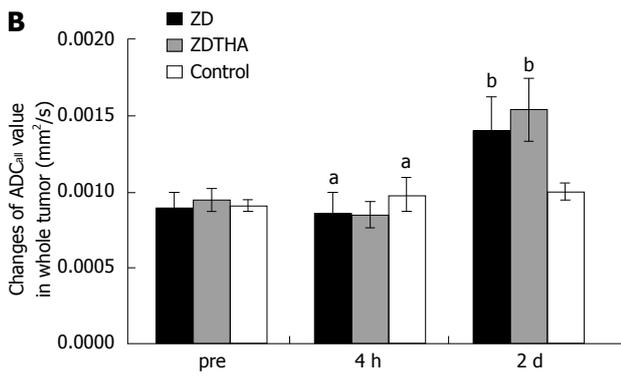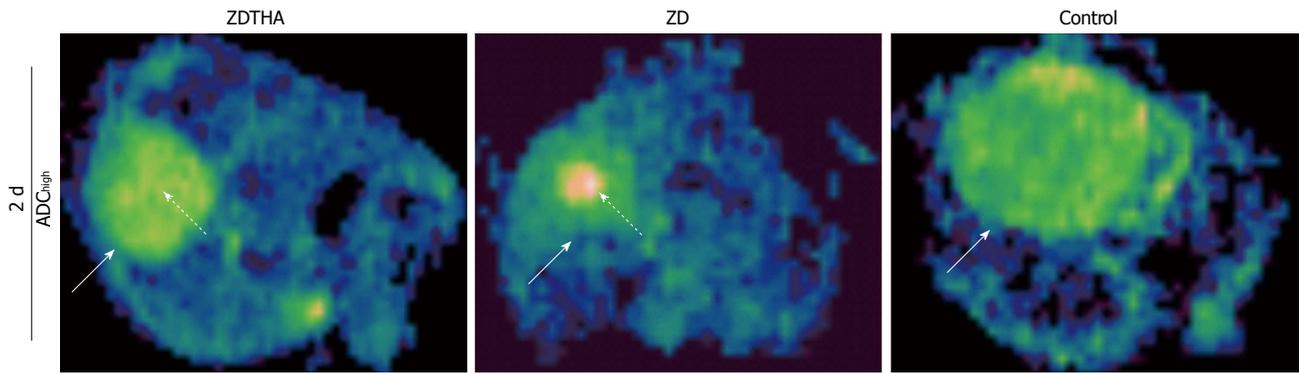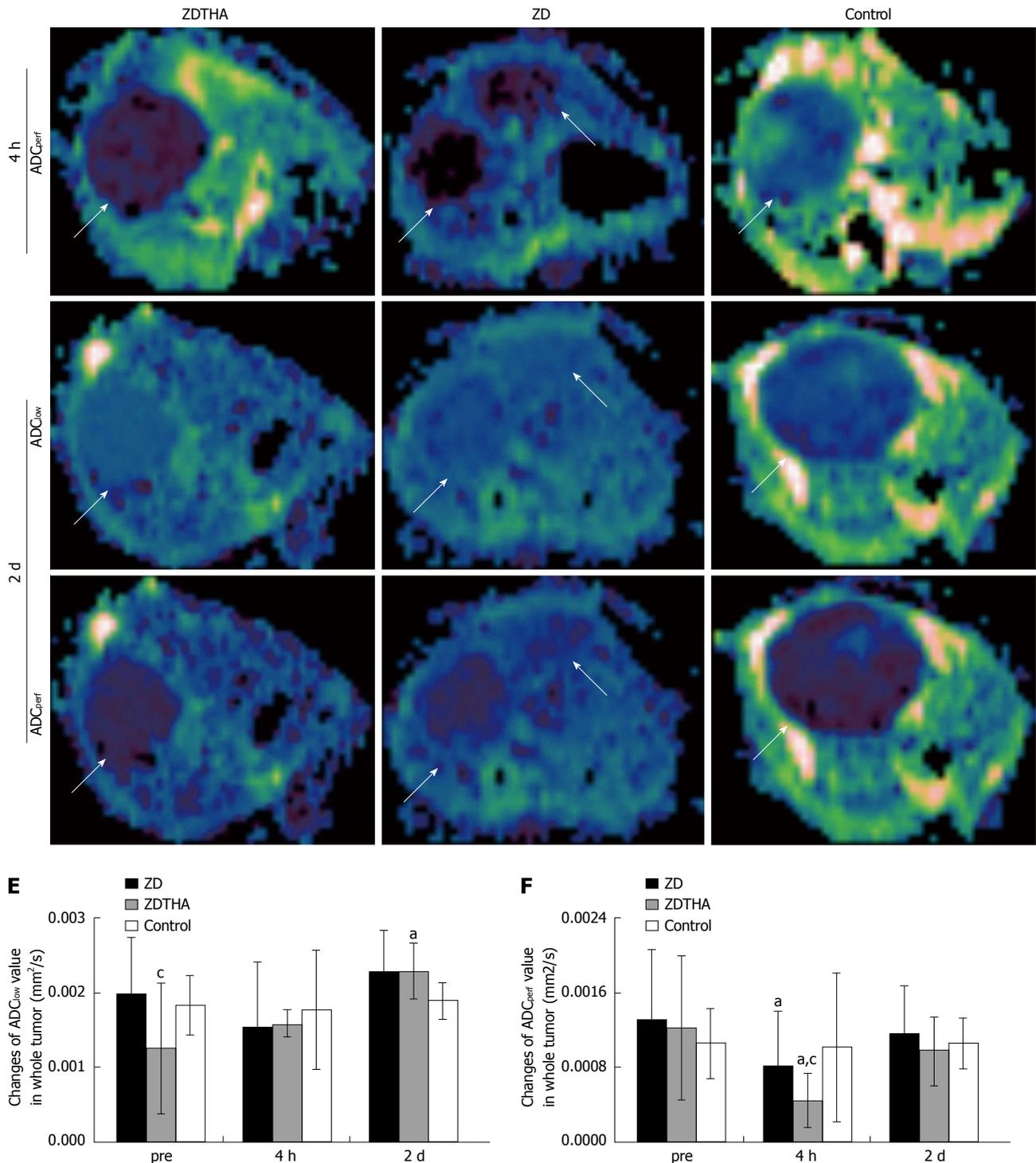
Figure 2 Apparent diffusion coefficient maps for tumors. A: Representative maps of apparent diffusion coefficient (ADC)all and ADChigh for liver tumors (solid arrow) in three groups. The area of therapy-induced necrosis (dotted arrow) was significantly larger in the ZDTHA group than those observed in both the ZD6126 and control groups; B: The dynamic change of ADCall during the experiment ([a]$P < 0.05$, [b]$P < 0.01$ *vs* control; respectively); C: The dynamic change of ADChigh during the experiment. Compared to the ADCall, the increased diffusion due to therapeutic necrosis was better reflected with ADChigh in both the ZDTHA and ZD6126 groups on day 2 ([b]$P < 0.01$ *vs* control; [c]$P < 0.05$ *vs* ZD6126); D: Representative maps of ADClow and ADCperf for liver tumors (arrow) in the three groups. The signal intensities observed on ADClow maps were always higher than those observed on ADCperf maps at each time point in each group, because ADClow combines both the perfusion and diffusion effects; E: The dynamic change of ADClow during the experiment ([a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* ZD6126); F: The dynamic change of ADCperf during the experiment. Compared to the ADClow, the perfusion reduction due to the shutdown of tumor vessels was better reflected with ADCperf in both the ZDTHA and ZD6126 groups at 4 h ([a]$P < 0.05$ *vs* control). Furthermore, the ADCperf in the ZDTHA group was even lower compared to the ZD6126 group at 4 h ([c]$P < 0.05$ *vs* ZD6126).

to the single use of ZD6126 at the end of the study; this synergistic effect on necrosis could be better reflected with ADChigh as shown in this study.

Third, ADCperf, a separate ADC value calculated as ADClow minus ADChigh, can provide valuable perfusion information from DWI data. Although the ADClow was

Table 1 Average apparent diffusion coefficient of entire tumor before and after treatment (mean ± SD)

Group and time	ADC$_{all}$ (× 10^{-3} mm^2/s)	ADC$_{high}$ (× 10^{-3} mm^2/s)	ADC$_{low}$ (× 10^{-3} mm^2/s)	ADC$_{perf}$ (× 10^{-3} mm^2/s)
ZD6126				
Pre	0.90 ± 0.10	0.68 ± 0.16	1.98 ± 0.75	1.30 ± 0.74
4 h	0.86 ± 0.13	0.73 ± 0.12	1.54 ± 0.87	0.81 ± 0.70
2 d	1.40 ± 0.22	1.13 ± 0.21	2.28 ± 0.54	1.15 ± 0.52
ZDTHA				
Pre	0.95 ± 0.08	0.70 ± 0.15	1.25 ± 0.87	1.22 ± 0.77
4 h	0.85 ± 0.08	0.77 ± 0.11	1.21 ± 0.59	0.44 ± 0.50
2 d	1.54 ± 0.21	1.31 ± 0.19	2.28 ± 0.37	0.97 ± 0.36
Control				
Pre	0.91 ± 0.04	0.77 ± 0.07	1.82 ± 0.39	1.06 ± 0.37
4 h	0.98 ± 0.11	0.85 ± 0.09	1.76 ± 0.79	1.01 ± 0.80
2 d	1.00 ± 0.06	0.95 ± 0.08	1.90 ± 0.24	1.06 ± 0.27

Pre: Pretreatment; ZD: ZD6126; ZDTHA: ZD6126 + thalidomide; ADC: Apparent diffusion coefficient; ADC$_{all}$: Calculated from the entire b value setting (b = 0, 50, 100, 150, 200, 250, 300, 500, 750, 1000 s/mm^2); ADC$_{high}$: calculated from the high b values (b = 500, 750, 1000 s/mm^2); ADC$_{low}$: Calculated from the low b values (b = 0, 50, 100 s/mm^2); ADC$_{perf}$: Calculated from ADC$_{low}$-ADC$_{high}$.

calculated from low b value images and perfusion sensitive, it was still contaminated with diffusion effects in tissues[15]. Therefore, ADC$_{low}$ was not satisfactory in evaluating the tumor response to treatment as indicated in this study. However, ADC$_{perf}$ was calculated from ADC$_{low}$ by excluding high b value effects; it would be more perfusion sensitive. In this study, for instance, strikingly reduced perfusion in response to treatment was detected with ADC$_{perf}$ at 4 h, but not with ADC$_{low}$ for both the ZDTHA and ZD6126 groups compared to the control group. Furthermore, the reduction of ADC$_{perf}$ in ZDTHA was even lower; this indicated a more pronounced decrease in blood perfusion induced by ZDTHA. The ADC$_{perf}$ of ZDTHA still showed a lower level compared to ZD6126 on day 2, although there was no significant difference. This could be explained by the fact that besides the vascular shutdown effect of ZD6126, thalidomide may also induce a transient normalization of tumor vasculature *via* aggressive vascular pruning and improve pericyte coverage on vessels. As a result, tumor perfusion was reduced[20,21]. Our results indicated that ADC$_{perf}$ allowed the early monitoring of therapeutic effects, because it was more sensitive to the microcapillary perfusion and could detect perfusion in response to therapy before the appearance of tumor necrosis without the administration of contrast media.

Similarly, such a significant perfusion reduction at 4 h was also detected with ADC$_{all}$ in both the ZDTHA and ZD6126 groups, however, this was not observed for ADC$_{high}$, compared to the control group. The reason is that ADC$_{all}$ was derived from 10 b values including low and high b values; consequently it was affected by both diffusion and perfusion in the tumor. Even though, the perfusion change measured with ADC$_{all}$ was not as striking as that noted with ADC$_{perf}$ due to the influence of diffusion contribution. Despite the delayed growth and the massive central necrotic areas in both the ZDTHA and ZD6126 groups, tumors began to relapse evidenced by the recovery of tumor ADC$_{perf}$ and ADC$_{low}$, as well as the enhanced rim visualized on CE-T1WI, due to residue

viable tumor cells on day 2 after therapy. These results are consistent with previous findings[18,22]. However, ZDTHA demonstrated significantly less tumor relapse than ZD6126, suggesting the benefit of applying the combination therapy.

It remains controversial regarding the option of mono- or biexponential model in extracting diffusion and perfusion information from DWI data. Because each model has its own advantages and drawbacks[15,23]. As a pioneering work in the mid-1986s, Le Bihan *et al*[24,25] proposed the concept of IVIM to address the microscopic movements in image voxel in MRI. In biologic tissue, the motions include the molecular diffusion of water and the microcirculation of blood or capillary perfusion. With the biexponential model of IVIM, the fraction of capillary perfusion can be separated from diffusion. Therefore, there is growing studies using IVIM from DWI data[26-29]. However, the clinical benefit of the biexponential model as compared to the monoexponential model has not been comprehensively established[10,15]. Our study supports that the ADC$_{high}$ values are similar to the diffusion coefficient derived from IVIM model. The separate calculations of ADC$_{all}$, ADC$_{high}$, ADC$_{low}$ and ADC$_{perf}$ using a monoexponential fitting algorithm are relatively simple to estimate and are readily available for most users of clinical MR scanners. However, a lack of direct comparison of diffusion parameters derived from mono- and biexponential model may be a limitation of the present study.

In conclusion, we have demonstrated that ZDTHA combination treatment significantly delayed tumor growth due to synergistic effects by inducing cumulative tumor necrosis. The perfusion insensitive ADC$_{high}$ values calculated from high b value images performed significantly better than ADC$_{all}$ values for the monitoring of tumor necrosis. The perfusion sensitive ADC$_{perf}$ values derived from ADC$_{low}$ by excluding high b value effects could provide valuable perfusion information from DWI data. Therefore, the *in vivo* separate calculations of ADC values derived from monoexponential model are more useful than conventional averaged ADC values in the

successful evaluation of tumor therapeutic effects.

COMMENTS

Background
Diffusion weighted magnetic resonance imaging (DW-MRI), due to its ability to detect molecular water motion at the cellular level, *i.e.*, the measurement of apparent diffusion coefficient (ADC), has become a favorite choice of measures in a variety of oncological studies and tissue viability assessments.

Research frontiers
Technological innovations in recent years have enabled the increasing use of high-quality and quantitative DW-MRI in monitoring tumor treatment. However, it has been realized that the information acquired from conventional calculation of DW-MRI data actually represents the combined effects of tissue microcirculation perfusion and pure tissue diffusivity in each imaging voxel at DW-MRI. This may hinder the appropriate interpretation of the DW-MRI data. Therefore, there is growing interest in applying more sophisticated approaches, such as separate ADC (calculating different ADC values based on various combinations of *b* values with a monoexponential fitting algorithm) and intravoxel incoherent motion, to differentiate the fraction of microcirculation perfusion from pure diffusivity within the DW-MRI data.

Innovations and breakthroughs
The combination therapy with ZD6126 and thalidomide significantly delayed liver tumor growth due to synergistic effects by inducing cumulative tumor necrosis in rodents. The ADC_{high} performed significantly better than ADC_{all} for the monitoring of tumor necrosis on day 2. The ADC_{perf} could better reflect the reduction of blood flow due to the vessel shutdown induced by ZD6126, compared to the ADC_{low}. The ADC_{perf} could provide valuable perfusion information from diffusion weighted MRI data.

Applications
The separate calculation of ADC is more useful than conventional averaged ADC in evaluating the efficacy of combination therapy with ZD6126 and thalidomide for solid tumors.

Terminology
DW-MRI is an *in vivo* imaging technique to detect molecular water motion at the cellular level by using the ADC parameter. Separate ADC measurement is to calculate the different ADC values based on various combinations of *b* values with a monoexponential fitting algorithm.

Peer review
The authors present ADC is more useful than conventional averaged ADC in evaluating the efficacy of combination therapy with ZD6126 and thalidomide for solid tumors. It would be of interest to perform the same study in a vascular tumor such as infantile hemangioma or a malignant vascular tumor such as angiosarcoma or hemangiopericytoma.

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[131]I-labeled metuximab combined with chemoembolization for unresectable hepatocellular carcinoma

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Abstract

AIM: To investigate the safety and effectiveness of combined ^{131}I-metuximab and transcatheter arterial chemoembolization (TACE) for hepatocellular carcinoma (HCC).

METHODS: One hundred and eighty-five patients (159 men and 26 women) with advanced HCC were enrolled in this study from February 2009 to July 2011. There were 95 patients in the combined metuximab and TACE group, and 90 patients in the TACE only group. The patients were followed for 12 mo. Clinical symptoms, blood cell counts, Karnofsky Performance Score (KPS) evaluation and therapeutic effects according to the Response Evaluation Criteria in Solid Tumors were recorded and evaluated.

RESULTS: The 1-mo effective rates (complete response + partial response + stable disease) of the test group and control group were 71.23% and 38.89%, respectively ($P < 0.001$). The 6-, 9- and 12-mo survival rates were 86.42%, 74.07% and 60.49% for the test group and 60.0%, 42.22% and 34.44% for the control group ($P < 0.001$). The incidence of adverse events (gastrointestinal symptoms, fever and pain) and blood cell toxicity were significantly higher for the test group than for the control group ($P < 0.001$). No severe ^{131}I-metuximab-related complications were identified. With respect to efficacy, patients in the test group had greater improvement in tumor-related pain ($P = 0.014$) and increase in KPS ($P < 0.001$) than those in the control group.

CONCLUSION: Combination of ^{131}I-metuximab and TACE prolonged the survival time in patients with HCC compared with TACE alone. The combination treatment was safe and effective.

Core tip: ^{131}I-metuximab has high affinity with a target antigen highly expressed on hepatocellular carcinoma (HCC) cells and a limited area of action. The combination of metuximab and transcatheter arterial chemoembolization had a synergistic effect in the treatment of HCC. It may represent a promising treatment modality for patients with advanced HCC, especially for those patients with multiple nodules who have a heavy tumor burden.

He Q, Lu WS, Liu Y, Guan YS, Kuang AR. ^{131}I-labeled metuximab combined with chemoembolization for unresectable hepatocellular carcinoma. *World J Gastroenterol* 2013;

INTRODUCTION

Hepatocellular carcinoma (HCC) has traditionally been regarded as a radioresistant tumor because external beam radiation does great harm to the surrounding normal tissue. On the contrary, since the 1980s, radioimmunotherapy has become a promising treatment modality for HCC, due to the specificity of the antibodies and the killing power of the radionuclides, resulting in improvement of clinical efficacy with fewer side effects.

A therapeutic anti-HCC radioimmunological agent, ^{131}I-metuximab, generated by ^{131}I labeling of the murine monoclonal antibody (mAb) fragment HAb18 F(ab')2 derived from HAb18G/CD147, has been approved for the treatment of primary HCC by the China State Food and Drug Administration (Registration No. S20050039).

Transcatheter arterial chemoembolization (TACE) is currently one of the widely used treatment modalities for unresectable advanced HCC. However, the long-term survival rate of such patients remains low, with a reported 5-year survival rate of 17%[1]. Although ^{131}I-metuximab monotherapy has been shown to be effective, both in the treatment of HCC and in the prevention of HCC recurrence after orthotopic liver transplantation[2], its efficacy in combination with other established treatment modalities such as TACE has seldom been tested. Theoretically, the TACE can enhance the antitumor effects of ^{131}I-metuximab, because of substantially reduced blood flow to the tumor that prolongs retention of ^{131}I-metuximab in the tumor tissues. Radioimmunotherapy combined with TACE may provide a new concept in radiotherapy for patients with HCC.

In this study, the safety and efficacy of ^{131}I-metuximab in combination with TACE were evaluated in patients with advanced HCC to demonstrate that the combination of ^{131}I-metuximab with TACE could produce better results than TACE alone.

MATERIALS AND METHODS

[131]I-metuximab injection

[131]I-metuximab injection (Licartin; Chengdu Hoist Hi-tech Co. Ltd., Chengdu, China) is an ^{131}I-labeled HAb18 F(ab')2 fragment of murine mAb against the HCC-associated antigen HAb18G/CD147. Before metuximab therapy, 0.5 mL of iodine solution should be taken orally tid for 3 d and continued for 7 d after treatment for thyroid protection. A vial of ^{131}I-metuximab injection solution that had been prepared at the standard dose of 0.75 mCi/kg was removed from a lead box containing ice at a temperature of 0 ℃. The thawed solution was diluted with 1 mL of saline and sucked into a 5-mL syringe for arterial injection.

Patient cohort

One hundred and eighty-five patients (159 men and 26 women, aged 12-87 years) with advanced unresectable HCC were enrolled in this study from February 2009 to July 2011. Patients with a Karnofsky Performance Score (KPS) < 60 or severe heart, kidney of hematological disease were excluded to ensure at least a 3-mo lifespan in the enrolled patients, so as to have enough time for follow-up. Patients with a history of allergy to biological products, pregnant or breast-feeding women, or patients receiving other therapies within 4 wk of the clinical trial were also excluded. All patients in our study gave written informed consent. Patients in the test group underwent ^{131}I-metuximab therapy and TACE while those in the control group received TACE only. The patients received local ethanol injection, microwave coagulation, resection or liver transplantation before and after TACE or ^{131}I-metuximab therapy if needed. All tumors were diagnosed according to pathological examination or distinctive findings on computed tomography (CT), conventional angiography, magnetic resonance imaging (MRI), or serum tumor markers [α-fetoprotein (AFP)].

Procedure of TACE and ^{131}I-metuximab intra-arterial injection

TACE and ^{131}I-metuximab injection were performed through the femoral artery using the Seldinger technique with local anesthesia. Arteriography of the celiac trunk and superior mesenteric artery was performed to visualize the arterial vascularization of the liver and evaluate portal vein patency, and anticancer drugs were injected. The angiographic catheter was superselected into the hepatic artery where the target tumor was located. An embolic agent (mainly Lipiodol) was continuously injecting through the artery until the rate of blood flow to the tumor mass fell below 25%, or minimal hepatic vein appeared to protect the liver tissues around the tumor so that Licartin is more liable to stay in tumor tissues by minimizing the effect of quick or slow blood flow. Patients in the test group underwent ^{131}I-metuximab therapy immediately after TACE. At each injection, ^{131}I-metuximab was administered at a dose of 0.75 mCi/kg according to the patient's weight and the intra-arterial injection usually lasted 1-2 min. Patients in the control group received TACE only. In both groups, the dose of Lipiodol, ranging from 3-20 mL, was determined according to the size and number of tumors and functional hepatic reserve. Anticancer drugs for each patient enrolled in this trial were 5-fluorouracil (800-1000 mg) and epirubicin-adriamycin (30-40 mg) according to the body surface area. Therapy for patients in both groups was repeated according to the patient's clinical condition and the iconography exams at a 1-6-mo interval.

Follow-up protocol and efficiency evaluation

Clinical symptoms, blood cell counts and KPS evaluation were recorded before and after treatment. After treatment, ultrasound, CT scan or MRI was performed every

Table 1 Baseline characteristics

Characteristics	Test group (n = 95)	Control group (n = 90)	Statistical analysis
Age (yr)	50.2 (22-80)	51.4 (12-87)	NS
Sex (M/F)	83/12	76/14	NS
Child–Pugh classification			NS
Child class A	91	88	
Child class B	4	2	
BCLC stage			NS
C	95	90	
Size of main tumors			
≥ 5 cm	80	81	NS
< 5 cm	15	9	NS
Tumor/liver volume ratio			
0%–50%	10	13	NS
≥ 50%	85	77	NS
Hepatitis B/C	70	76	NS
KPS	75.16 ± 7.42	73.89 ± 11.39	NS

BCLC: Barcelona clinic liver cancer; KPS: Karnofsky performance status; NS: Not significant.

Table 2 Clinical symptoms immediately after treatment n (%)

Group	Fever[1]	Gastrointestinal[1] symptoms	Pain[1]	Sudden death
Test group	90 (94.74)	74 (77.89)	93 (97.89)	0
Control group	28 (31.11)	16 (17.78)	19 (21.11)	1 (1.11)

[1]$P < 0.001$.

1-3 mo, with or without contrast enhancement, to evaluate the features of Lipiodol deposit and the therapeutic effect according to the Response Evaluation Criteria in Solid Tumors (RECIST). If elevated tumor marker (AFP), diminished Lipiodol, enlarged lesions or new nodules were observed, the patients were readmitted for angiography and treatment. The starting point of survival analysis was regulated as the day of initial treatment. The Kaplan-Meier method was used to analyze the survival rates in the two groups.

Statistical analysis

The primary endpoint of this study was overall survival and the secondary endpoint was short-term (1 mo) treatment response. Survival analysis was estimated by the Kaplan-Meier method. Survival probabilities were estimated using the life-table method, and between-group differences in survival rates were compared using the log-rank test. All statistical analyses were carried out with SPSS version 17.0 (SPSS, Chicago, IL, United States). All reported P values were two-sided, with $P < 0.05$ considered statistically significant.

RESULTS

Patient population

The patients were divided into the test group ($n = 95$) with a mean age of 50.2 years (range: 22-80 years) and the control group ($n = 90$) with a mean age of 51.4 years (range: 12-87 years). All the patients in this trial were classified as Barcelona Clinic Liver Cancer Stage C. Both the test group and control group had a high percentage of patients (89.47% and 85.56%, respectively) with a tumor/liver volume ratio > 50%. Thus, the patients enrolled in this clinical trial had advanced HCC. Although this was a nonrandomized prospective cohort study, no significant difference was observed in baseline characteristics between the two groups (Table 1).

The patients were followed for 12 mo. At the time of analysis, 46 and 80 patients had died while 14 and zero were lost to follow-up in the test and control groups, respectively. Causes of death in the test and control groups included tumor progression in 46 and 72 patients, digestive tract hemorrhage in zero and four, tumor rupture in zero and one, acute renal failure in zero and one, and other causes in zero and two, respectively; none was possibly related to treatment.

Two hundred and forty-one (mean: 2.56 procedures) and 261 (mean: 2.90 procedures) procedures of interventional therapy were performed in the test and control groups, respectively. Arterial portal vein shunt (AVS), arterial hepatic vein shunt (APS) and/or portal vein involvement, which indicate high invasion and poor prognosis were found in 35.79% (34/95) of patients in the test group and 33.3% (30/90) of patients in the control group. No difference was observed in the time of therapy and the incidence of malignancy signs such as AVS, APS or portal vein involvement between the two groups.

Safety

The clinical symptoms were carefully recorded after treatment (Table 2). Overall, although ^{131}I-metuximab in combination with TACE was well tolerated, the patients in the test group obviously suffered more frequent adverse events than those in the control group. The most frequent adverse event in the test group was abdominal pain. Of the 95 patients in the test group, 93 (97.89%) suffered from abdominal pain, 90 (94.74%) had fever of 37.2 ℃-40 ℃, which usually occurred 0.5-10 h after ^{131}I-metuximab injection and lasted for 1-14 d, and 74 (77.89%) had anorexia and/or vomiting, which often faded away in several days. The corresponding numbers of patients in the control group were 19 (21.11%), 28 (31.11%) and 16 (17.78%) ($P < 0.001$). The changes in blood cell count and liver function before and 1 mo after treatment were evaluated. Statistical analysis showed that the changes in leukocytes and platelets were significant. Changes in total bilirubin, albumin, aspartate aminotransferase, alanine transaminase and hemoglobin were not significant (Table 3).

One patient in the test group had hypothyroidism and was prescribed oral thyroxin, and one sudden death occurred in the control group, possibly because of liver rupture. No human anti-murine antibody immune responses, anaphylactic reaction and changes in myocardial zymograms were observed.

Table 3 Changes in blood cells and liver function before and 1 mo after treatment

Group	Test group	Control group	P value
Leukocytes	–1.25 ± 1.79	2.04 ± 11.51	0.0270
Platelets	–36.69 ± 49.62	12.74 ± 52.59	< 0.001
TB	1.7	0.95	0.860
Alb	–0.86 ± 6.89	–1.30 ± 5.36	0.6708
AST	5	7.5	0.631
ALT	–1	0	0.5137
HGB	–5.86 ± 16.42	–7.98 ± 20.26	0.515
KPS			< 0.001
Mass-associated pain			0.014

Total bilirubin (TB), Aspertate aminotransferase (AST), Glutamic-pyruvic transaminase (ALT), Karnofsky and pain (Wilcoxon rank sum test); others (*t* test). Alb: Albumin; HGB: Hemoglobin; KPS: Karnofsky performance score.

Table 4 Therapeutic effect evaluated according to Response Evaluation Criteria in Solid Tumors at 1 mo after treatment *n* (%)

Group	CR	PR	SD	PD	Effective rate (CR + PR + SD)
Test group	4 (4.11)	38 (39.73)	26 (27.40)	27 (28.77)	68 (71.23)
Control group	1 (1.11)	14 (15.56)	20 (22.22)	55 (61.11)	35 (38.89)

P < 0.001. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Efficacy

The palliative rate of mass-associated pain 1 mo after treatment was 71.1% (27/38) for patients in the test group, which was higher than that in the control group (31.6%, 24/76) (*P* = 0.014). For changes in KPS, the patients in the test group had a greater increase than those in the control group (*P* < 0.001, Table 3). The therapeutic effect was evaluated following the RECIST after treatment. Rates of complete response (CR), partial response (PR), stable disease (SD) and progressive disease in the two groups are listed in Table 4. The total effective rates (CR + PR + SD) were 71.23% and 38.89% for the test group and control group, respectively. Wilcoxon rank sum test showed that the therapeutic effects in the two groups were significantly different (*P* < 0.001). The survival rates at 6, 9 and 12 mo after treatment were 86.42%, 74.07% and 60.49% in the test group, and 60.0%, 42.22% and 34.44% in the control group, suggesting that the survival rates for the test group were significantly higher than for the control group (*P* < 0.001, Figure 1).

DISCUSSION

HCC is a highly malignant tumor with high morbidity and mortality rates. Although TACE, as a palliative treatment for unresectable HCC, has become one of the most common interventional therapies[3-6], its effect is limited due to the lack of appropriate and reliable embolic agents, and the infiltrative or hypovascular nature, too large or
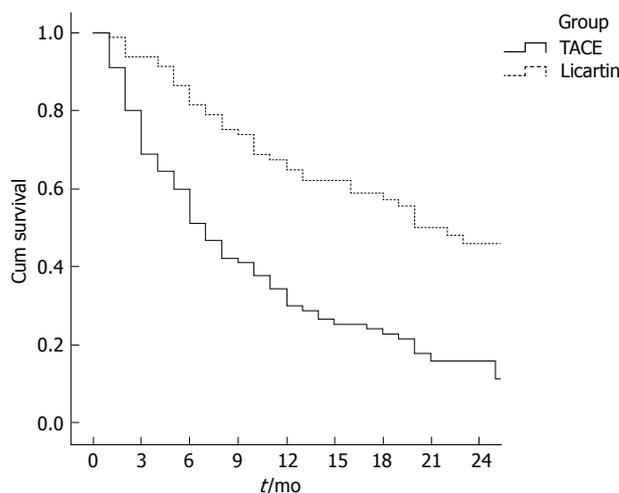
Figure 1 Kaplan-Meier curves of survival of patients in the test group receiving combination therapy (Licartin) and those in the control group receiving transcatheter arterial chemoembolization only (*P* < 0.001). TACE: Transcatheter arterial chemoembolization.

small in size[7-9]. Another limitation of TACE is the need for repeated treatment, thus resulting in deterioration of liver function[10]. Therefore, many efforts have been made to explore other new therapies in order to achieve better efficacy. Percutaneous ethanol injection, radiofrequency ablation, targeted molecular therapies, and gene therapy in combination with TACE have been found to improve survival in patients with advanced HCC[11-13] and decrease the risk of liver failure[14-16]. However, in spite of all the above, advanced HCC with wide metastasis, especially in patients with multiple lesions who always have a high tumor/liver volume ratio, still has a poor prognosis and lack of efficient therapeutic modalities.

The target antigen for ^{131}I-metuximab, HAb18G/CD147, a member of the CD147 family, is highly expressed on HCC cells. The binding rate of HAb18 to human 7721 hepatoma cells, determined by flow cytometry, is up to 99.55%[17,18]. Immunohistochemistry performed with HAb18 showed that the positive rate of HCC staining was 75% and had no cross-reaction to normal tissues[17,19]. Moreover, the results of drug safety studies showed that ^{131}I-metuximab injection caused no impairment to cardiovascular, respiratory, or nervous systems[2].

The mechanism by which ^{131}I-metuximab may benefit patients with HCC has been investigated both *in vitro* and *in vivo*, as well as in clinical trials[2,17,20]. ^{131}I-metuximab is specific to and has high affinity for a target antigen highly expressed on HCC cells. This allows for concentration of conjugated ^{131}I in HCC tissues, both in the liver and metastatic nodules, which kills tumor cells directly. In addition, the target antigen, HAb18G/CD147, is a cell adhesion molecule with multiple functions and is closely related to tumor metastasis. This antigen is involved in the adhesion and motion of tumor cells, angiogenesis, and signal transduction and can induce fibroblasts to produce matrix metalloproteinases (MMPs), including MMP-1, MMP-2, and MMP-9. These MMPs can degrade the ex-

tracellular matrix and promote the metastasis of HCC cells. Injection of ^{131}I-metuximab into HCC cells inhibits oncogenesis and metastasis within and outside the liver, blocking and destroying cells carrying HAb18G/CD147 and inhibiting HCC metastasis[1].

The combination of ^{131}I-metuximab and TACE ought to have a synergistic effect in the treatment of HCC. First, TACE may enhance the efficacy of ^{131}I-metuximab due to its arterial embolization effect, substantially reducing blood flow to the HCC and resulting in prolonged retention of ^{131}I-metuximab in the tumor. Second, retention of the anticancer drug in the tumor may have a radiosensitizing effect on ^{131}I-metuximab. Third, ^{131}I-metuximab can eliminate residual cancer cells after TACE for its continuous radiation. Taken together, these mechanisms may explain, at least in part, the ability of combination therapy to enhance survival, compared with conventional TACE alone, in patients with advanced HCC. Of course, they are the reasons why the test group suffered more blood cell toxicity and adverse events.

In the present study, the results for the control group were similar to those of earlier trials of TACE in patients with HCC. In those studies, the tumor response rate according to WHO criteria ranged from 12%-57.9%[1,21-23], median survival ranged from 7-19 mo (mean: 13.63 ± 6.10 mo), and 1- and 2-year survival rates ranged from 42%-72% (mean: 58.30 ± 10.14%) and from 0%-55% (mean: 28.74% ± 15.88%), respectively[24-28]. Moreover, treatment of patients with HCC with ^{131}I-metuximab alone resulted in 6-mo and 12-mo survival rates of 82.63% and 58.68%, respectively, with a median survival time of 19 mo and an objective response rate (CR + PR) of 15.53% according to World Health Organization (WHO) criteria[1]. In comparison, patients receiving combination therapy in our study showed 6- and 12-mo survival rates of 86.42% and 60.49%, respectively; a median survival time of 20.0 mo; and an objective response rates of 43.84% according to WHO criteria. All of these studies were performed in patients staged as Child-Pugh class A/B, and their baseline characteristics were similar to those of our patients, therefore, it can be suggested that the combination of ^{131}I-metuximab and TACE tested here exhibited better clinical efficacy than either treatment alone.

Given the high level of expression of the high-affinity target antigen on HCC cells and the limited area of action of ^{131}I-metuximab, we thought we would observe more advantages in our clinical trial because most of the enrolled patients had multiple nodules and a high tumor/liver volume ratio. And we did find that the median survival of patients in the test group was significantly longer than that in the control group (20.0 mo *vs* 7.0 mo). This improvement was more marked than that in two similar earlier trials (21.15 mo *vs* 17.73 mo and 26.7 mo *vs* 20.6 mo). The most notable difference between the present and previous studies is that our patients had more advanced HCC and a greater tumor burden (tumor/liver volume ratio ≥ 50%; 87.56% *vs* < 29%)[29,30].

It is commonly accepted that HCC patients with countable nodules often have more treatment choices, better treatment efficacy, and longer lifespan than those with countless nodules. Our clinical trial proved that ^{131}I-metuximab combined with TACE had an extensive range of therapeutic function, especially for advanced liver cancer with wide metastasis and multiple lesions. The combination of ^{131}I-metuximab and TACE may greatly improve the treatment efficacy in these patients and extend their poor life expectancy.

The present study had several limitations. First, the relatively short follow-up period may have resulted in underestimation of survival. Second, most patients were treated with combination therapy only once, a few twice and followed by TACE again. Repeated combination therapy may have a more significant effect on survival. Third, the effects of Chinese traditional medicine, which the patients used when discharged from the hospital, were uncertain. These were hard to control and might have affected the final results.

In conclusion, our findings indicate that combination of ^{131}I-metuximab and TACE was safe and more effective than TACE alone. It may represent a promising treatment modality for patients with advanced HCC. Nevertheless, caution should be exercised, and a few questions remain. What is the best method for delivering ^{131}I-metuximab with the TACE procedure? Does the 25% be the best point to administer Licartin? Whether and how much could the Licartin injected into the hepatic artery concentrate in the metastasis outside the liver? All these questions require well-designed, prospective randomized controlled trials.

COMMENTS

Background
Transcatheter arterial chemoembolization (TACE) is currently one of the widely used treatment modalities for unresectable advanced hepatocellular carcinoma (HCC). However, the long-term survival rate of such patients remains poor. At the same time, ^{131}I-metuximab monotherapy has been shown to be effective, both in the treatment of HCC and in the prevention of HCC recurrence after orthotopic liver transplantation. However, its efficacy in combination with TACE has seldom been tested. Theoretically, TACE can enhance the antitumor effects of ^{131}I-metuximab, because of substantially reducing blood flow to the tumor, which prolongs retention of ^{131}I-metuximab in the tumor tissues.

Research frontiers
[131]I-metuximab (Licartin) has high affinity with a target antigen highly expressed on HCC cells and has a limited area of action. The combination of ^{131}I-metuximab and chemoembolization has a synergistic effect in the treatment of HCC. It may especially benefit patients with multiple nodules who always have a high tumor/liver volume ratio.

Innovations and breakthroughs
It is commonly accepted that HCC patients with countable nodules often have better outcome than patients with countless lesions. At present, patients with multiple lesions still have a poor prognosis and lack of efficient therapeutic modalities. However, in our trial, they managed to treat the same types of patients with ^{131}I-metuximab combined with TACE and prolonged their lifespan significantly. This improvement was more notable than in earlier similar trials. The combination of ^{131}I-metuximab and TACE had an extensive range of therapeutic function, especially for advanced liver cancer with wide metastasis and multiple lesions. Combination therapy may greatly improve the treatment efficacy in these patients and extend their poor life expectancy.

Applications
Radioimmunotherapy combined with TACE may provide a new concept in radiotherapy for patients with HCC.

Peer review
The authors performed a study on 185 patients (159 men and 26 women) with advanced HCC. The data indicate that combination of ^{131}I-metuximab with TACE was safe and more effective than TACE alone. The title of the paper accurately reflects the content of the article. The abstract contains a short description of the study. The abstract and article are written in accordance with the journal requirements. The introduction gives sufficient information about the research objectives. It is important that authors state their motivation to conduct this investigation. The design of the study is simple and understandable.

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META-ANALYSIS

Efficacy of mosapride plus proton pump inhibitors for treatment of gastroesophageal reflux disease: A systematic review

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Abstract

AIM: To assess the potential benefits of mosapride plus proton pump inhibitors (PPIs) in the treatment of gastroesophageal reflux disease.

METHODS: A literature search was performed through MEDLINE, EMBASE, and the ISI Web of Knowledge. The clinical trials that compared the benefit of mosapride plus PPI treatment with that of PPI monotherapy were analyzed. The rate of responders was evaluated by the pooled relative risk (PRR) and improvement in symptom scores was assessed by single effect size of a standardized mean, while Hedges'g was used as the effect size. Pooled effect sizes with 95%CIs were calculated using a fixed-effects model. Between-study heterogeneity was assessed using Q test and I^2 analyses. In addition, studies that assessed the additional efficacy of mosapride in PPI-resistant patients were also reviewed.

RESULTS: This systematic review included information on a total of 587 patients based on 7 trials. Four trials compared the efficacy of combination therapy of mosapride plus a PPI with that of PPI monotherapy. The statistical analysis for the effect of additional mosapride showed equivocal results (PRR = 1.132; 95%CI: 0.934-1.372; P = 0.205; Hedges'g = 0.24; 95%CI: 0.03-0.46; P = 0.023). No heterogeneity and publication bias were found among the studies. Three open-labeled trials assessed the additional efficacy of mosapride in PPI-resistant patients. However, since these trials did not set the control group, the results may be considerably biased.

CONCLUSION: Mosapride combined therapy is not more effective than PPI alone as first-line therapy. Whether it is effective in PPI-resistant patients needs to be determined.

Key words: Mosapride; Proton pump inhibitor; Gastroesophageal reflux disease; Systematic review; Combined therapy

Core tip: Prokinetic agents have been widely used to relieve the gastroesophageal reflux disease (GERD) symptoms, and mosapride is a selective 5-HT$_4$ receptor agonist that can be safely used. We conducted a systematic review and meta-analysis to assess the potential benefits of the addition of mosapride to proton pump inhibitors (PPIs) in the treatment of GERD. Based on this research, mosapride combined therapy seems to be not more effective than PPI alone as first-line therapy. Whether it is effective in PPI-resistant patients needs to be determined.

INTRODUCTION

Gastroesophageal reflux disease (GERD) encompasses a spectrum of clinical presentations in which gastric content refluxes into the esophagus leading to symptoms with or without visible damage to the esophageal mucosa. It is the most common gastrointestinal diagnosis recorded during visits to outpatient clinics[1]. Population-based studies suggest that GERD is a common condition with a prevalence of 10%-20% in Western Europe, while in Asia it is lower, less than 5%[2,3]. Traditionally, the treatment for GERD should be focused on symptom control, and abundant data from randomized trials show benefits of inhibiting gastric acid secretion in patients with GERD. Treatment with proton pump inhibitors (PPIs) heals reflux esophagitis in 83% of patients with comparable symptom relief, an outcome that is superior to treatment with histamine 2-receptor antagonists[4].

However, GERD patients present with a wide range of symptom severity and frequency, sometimes do not respond to PPI therapy. Several mechanisms have been proposed for the pathogenesis of refractory GERD, including weakly acidic reflux, visceral hypersensitivity and delayed gastric emptying[5].

An Asia-Pacific consensus on the management of GERD showed that the use of prokinetic agents either as monotherapy or adjunctive therapy to PPIs may have a role in the treatment of GERD in Asia[6]. Prokinetic agents like cisapride, which act on the 5-hydroxytryptamine (5-HT)$_1$-receptor, have been found to be associated with potentially fatal heart rhythm abnormalities. However, mosapride, a selective 5-HT$_4$ receptor agonist, is an alternative prokinetic agent that can be safely used in patients with upper gastrointestinal disorders[7-9], while stimulating gastrointestinal motility and gastric emptying[10-12]. Many studies have shown that mosapride can reduce acid reflux episodes and esophageal clearance of refluxate, theoretically, suggesting potential efficacy in the treatment of GERD[13,14]. In a randomized trial, mosapride combined with PPIs achieved a better therapeutic effect than use of a PPI alone[15]. However, another clinical trial showed the additional effect of mosapride was limited[16].

In this study, our aim was to clarify the data on the treatment of GERD by systematically reviewing the literatures on the efficacy of mosapride plus PPIs with regard to initial symptom relief. The additional treatment effect of mosapride in PPI-resistant GERD patients was also assessed.

MATERIALS AND METHODS

Study retrieval and selection

The present meta-analysis follows the guidelines for Preferred Reporting Items for Systematic Reviews and Meta-Analyses PRISMA[17]. We performed a literature search using the following databases: MEDLINE, EMBASE and the ISI Web of Knowledge. The search pool was enlarged by references found in these initial articles. Three authors (Liu Q, Feng CC and Wang EM) independently searched from the beginning of indexing for each database to May 10th, 2013, using the key terms ("gastroesophageal reflux disease" or "reflux esophagitis" or "non-erosive reflux disease") and ("mosapride" or "mosapride citrate" or "prokinetic" or "prokinetics"). Only the articles written in English were included.

Three authors (Liu Q, Feng CC and Wang EM) independently evaluated all of the retrieved studies according to pre-specified selection criteria. Discrepancies between the three investigators were resolved by discussion. Studies were included based on the following criteria: (1) published as original articles; (2) investigations of adults; (3) clinical trials that evaluated the efficacy of mosapride. Studies were excluded if they had the following features: (1) without specific description for the diagnosis of GERD; (2) reported duplicated results that have been published in other articles as repeated data; (3) other primarily identifiable causes of GERD symptoms such as esophageal neoplasm and esophageal stricture; (4) use of mosapride was not designed as an additional drug in combination with a PPI; and (5) included participants who were taking medications that could have complicated interpretation of results.

Data extraction and analysis

The following data were abstracted from each article: the author(s), publication year, country, study design, numbers of enrolled patients, age, gender distribution and body mass index of the subjects, definition of GERD, treatment dose and duration, effects of treatment. Data extraction was performed independently by two reviewers (Liu Q and Feng CC). We validated a priority of data from intention-to-treat (ITT) analysis other than per-protocol (PP) analysis when the data obtained from two approaches were both available in certain studies.

Subsequently, we arranged the clinical trials using thematic analysis. The overarching categories included the controlled trials showing parallel comparisons between efficacy of mosapride and PPI group with that of PPI alone group, and open trials to assess the additional efficacy of mosapride to PPI-resistant patients.

Using the data from the controlled trials in which treatment efficacy was evaluated by comparing the rate of responders and improvement in symptom scores in a group receiving mosapride plus a PPI with those in patients receiving PPI alone, we assessed the drug effect based on the pooled relative risk (PRR) and single effect

A

Potential articles identified through literature searches
MEDLINE (*n* = 392)
EMBASE (*n* = 522)
ISI Web of Knowledge (*n* = 454)

Duplication removed (*n* = 510)

Potential articles included in the review (*n* = 858)

Excluded after title/abstract review (*n* = 838)

Potential articles included for full-text assessment (*n* = 20)

Subjects not GERD patients (*n* = 7)
Mosapride not used in combination with PPI (*n* = 5)
Reported duplicated data (*n* = 1)

Articles included (*n* = 7)

B

Figure 1 Flow chart of study selection and risk of bias summary. A: Flow chart of study selection; B: Risk of bias summary.

size of a standardized mean. The PRR was calculated using the Mantel-Haenszel method[18], and continuous variables were transformed from the means and standard deviation to determine a standardized effect size. We used the Hedges'g effect size, which is a variation of Cohen's *d*, to correct for bias associated with small sample sizes[19]. Statistical heterogeneity across the various studies was then tested with the use of Q-statistic[20]. A P_Q-value < 0.10 indicated a significant statistical heterogeneity across studies, allowing for the use of a random effects model. Additionally, we calculated I^2 statistics, which quantifies the percentage of variation across studies caused by heterogeneity, rather than chance, and, therefore are less biased by the number of studies included in a meta-analysis[21]. Finally, publication bias was quantified using Egger's test[22]. A two-tailed $P < 0.05$ was considered to be statistically significant. The above analyses were performed using Stata 11.0 (Stata Corp, College Station, TX, United States). Risk of bias was assessed using Cochrane Review guidelines[23].

RESULTS

Search results and study characteristics
The search strategy generated 858 references, 20 of which were selected for further assessment by full-text reading (Figure 1A). In this step, 7 articles were excluded because the subjects in the study were not GERD patients[24-30], 5 articles were excluded because mosapride was not used in combination with PPI[13,14,31-33], and one trial reported duplicated data[34]. Ultimately, 7 studies were included in this systematic review which contained information on a total of 587 patients, with the characteristics shown in Table 1. The diagnostic criteria of

GERD in the 7 articles we included were basically based on typical reflux-associated symptoms (heartburn and/or regurgitation) which occurred at least twice a week, although the duration was obscure in three studies[16,37,39]. The subjects in 3 articles were non-erosive reflux disease (NERD) patients[16,38,39], but one study focused on reflux esophagitis (RE) patients[35]. With respect to the dose of mosapride, only one trial used this agent at a dose of 10 mg thrice daily[36]. All others employed 5 mg three times per day. Various PPIs were used in these studies including rabeprazole, omeprazole, pantoprazole, lansoprazole and esomeprazole.

Quality and methodology of trials
Risk of bias was assessed using criteria specified by the Cochrane group. Overall, the risk of bias was high in some studies[37,39] and low in others[15,16,36,38] (Figure 2). A summary of individual quality assessment can be found in Figure 1B.

There was significant heterogeneity between trials with regard to methodology. In 3 studies[35,38,39], symptom evaluation was based on a frequency scale for the symptoms of GERD (FSSG), a GERD-specific questionnaire developed in Japan has been used for screening GERD patients[40]. The gastrointestinal symptom rating scale (GSRS) questionnaire[41] was adopted from another trial[37]. Two articles presented an explicit symptom assessment approach[15,36], and one used a visual analogue scale to evaluate the symptom[16].

Trials comparing mosapride plus PPI combination therapy with PPI monotherapy
Four trials compared the efficacy of combination therapy of mosapride plus a PPI with that of PPI monothera-

Table 1 Characteristics of the included studies

Ref.	Year	*n*	Male (%)	Mean age	BMI	Study design	Treatment agent (daily), dose	Treatment duration	Outcome measures
Trials comparing mosapride plus PPI combined therapy with PPI monotherapy									
Madan *et al*[15]/	2004	Cases 33	57.6	34.7	Unclear	Double-blind	Pantoprazole 80 mg	8 wk	Symptom
India		Controls 28	75.0	36.5	Unclear	Randomized controlled trial	+ mosapride 15 mg Pantoprazole 80 mg + placebo		Questionnaire
Hsu *et al*[35]/Taiwan	2010	Cases 50	46.0	47.0	23.7 ± 3.6	Double-blind	Lansoprazole 30 mg	4 wk	FSSG
		Controls 46	54.3	47.0	23.9 ± 4.6	Randomized Crossover trial	+ mosapride 15 mg Lansoprazole 30 mg + placebo		Questionnaire
Miwa *et al*[16]/Japan	2011	Cases 97	38.1	52.1	22.3 ± 3.3	Double-blind	Omeprazole 10 mg	4 wk	VAS
		Controls 95	36.8	52.2	22.0 ± 3.6	Randomized Controlled trial	+ mosapride 15 mg Omeprazole 10 mg + placebo		
Cho *et al*[36]/	2012	Cases 24	62.5	49.0	21.3 ± 2.3	Double-blind	Esomeprazole 40 mg	4 wk	Reflux- symptoms
South Korea		Controls 19	47.4	43.0	21.5 ± 2.3	Randomized Controlled trial	+ mosapride 30 mg Esomeprazole 40 mg + placebo		Questionnaire
Trials on addition of mosapride to PPIs for the treatment of PPI-resistant GERD patients									
Miyamoto *et al*[37]/ Japan	2008	34	Unclear	53.1[1]	23.0 ± 0.3[1]	Open trial	Rabeprazole 10 mg + mosapride 15 mg	12 wk	FSSG Questionnaire
Futagami *et al*[38]/ Japan	2010	44	50%	42.8	23.0 ± 1.9	Open trial	Omeprazole 20 mg + mosapride 15 mg	12 wk	GSRS Questionnaire
Miyamoto *et al*[39]/ Japan	2010	117	Unclear	47.4[1]	23.0 ± 3.6[1]	Open trial	PPI therapy[2] + mosapride 15 mg	4 wk	FSSG Questionnaire

[1]Data calculated based on the included participants at the beginning of study; [2]Patients were randomly administered rabeprazole 10 mg or lansoprazole 30 mg or omeprazole 20 mg or lansoprazole 15 mg or omeprazole 10 mg. PPI: Proton pump inhibitor; GERD: Gastrooesophageal reflux disease; FSSG: Frequency scale for the symptoms of GERD; GSRS: Gastrointestinal symptom rating scale; VAS: Visual analogue scale; BMI: Body mass index.
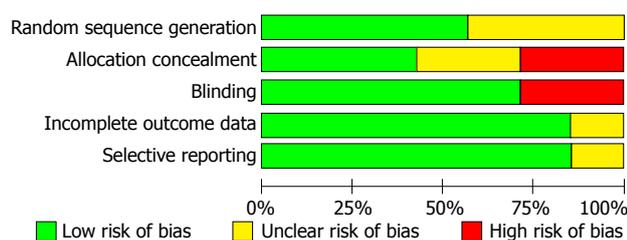
Figure 2 Risk of bias in trials.

py[15,16,35,36], all of which were designed as double-blind, randomized, placebo-controlled trials.

Madan *et al*[15] demonstrated that the combination therapy with pantoprazole and mosapride was more effective than pantoprazole alone in providing symptomatic relief to patients with erosive GERD. However, the number of patients who responded to therapy was not statistically different between combination therapy and monotherapy with pantoprazole (89.2% *vs* 69.7%). However, at the end of the treatment duration, the mean symptom score was significantly lower in patients receiving combination therapy (1.67 *vs* 3.78, *P* = 0.009).

Hsu *et al*[35] conducted a double-blind randomized trial studying the effects of adding mosapride to lansoprazole for the management of reflux esophagitis. The reduction in symptom score after 4 wk of treatment with lansoprazole and mosapride was not significantly higher compared with lansoprazole plus placebo (13.42 *vs* 10.85, *P* = 0.103), indicating little benefit from the addition of mosapride to a PPI in RE patients. However, in the subgroup of severely symptomatic patients, the difference was marginally significant (*P* = 0.039), indicating that mosapride as an adjunct to PPI may be beneficial in patients with severe symptoms.

Miwa *et al*[16] targeted on patients with NERD in a double-blind placebo-controlled study and found that there was no significant difference between the rates of responders from omeprazole plus mosapride, and omeprazole plus placebo groups in ITT (46% *vs* 44%) and PP (50% *vs* 43%) analyses. The change in symptom score in the treatment group was not significantly different from the placebo group in ITT analysis (-3.8 *vs* -3.4, *P* = 0.128). Therefore, the addition of mosapride to omeprazole was not found to be more effective than omeprazole alone in NERD patients.

Theoretically, prokinetic drugs can improve GERD by increasing lower esophageal sphincter basal pressure, improving esophageal peristalsis, accelerating esophageal acid clearance and facilitating gastric emptying. Cho *et al*[36] focused on the change of high-resolution manometry parameters to evaluate the efficacy of mosapride on esophageal motility and reflux symptoms in patients with GERD when used in combination with a PPI. The authors found that a combination of mosapride with esomeprazole affected esophageal peristalsis by improving esophageal contractibility and lowering intrabolus pressure that could lead to facilitation of esophageal bolus transit in patients with GERD. However, with regard to symptom assessment, treatment responsiveness in the combined therapy group was not different from that of the monotherapy group (79% *vs* 68%).

Of note, for the statistical analysis, one study was
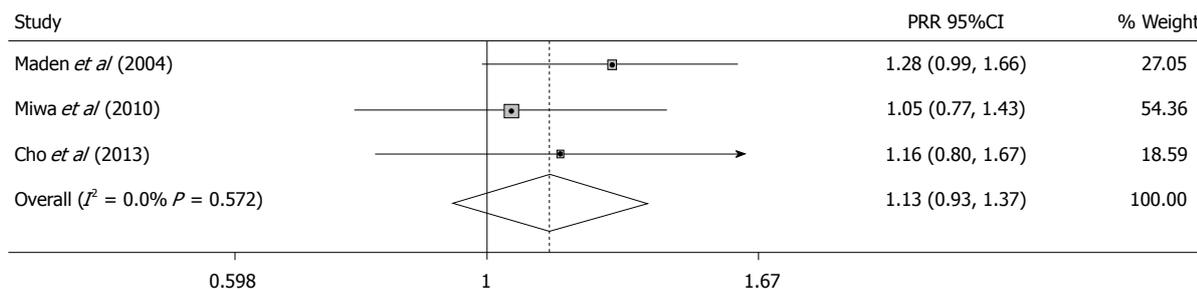
Figure 3 Meta-analysis of three trials that used mosapride as combined therapy with proton pump inhibitor compared with placebo in gastroesophageal reflux disease, a fixed-effects model was used and pooled relative rate was the measure of effect size. I^2, total variation across studies that is attributable to heterogeneity rather than to chance; PRR: Pooled relative rate.
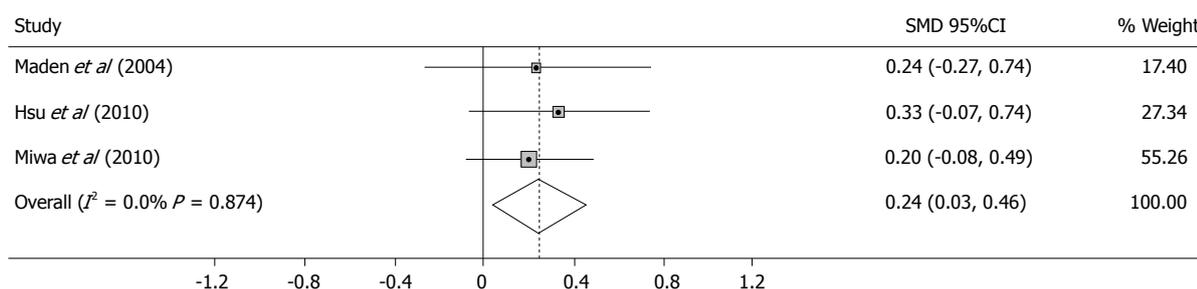

Figure 4 Meta-analysis of three trials that used mosapride as combined therapy with proton pump inhibitor compared with placebo in gastroesophageal reflux disease, a fixed-effects model was used and Hedges'g was the measure of effect size. I^2, total variation across studies that is attributable to heterogeneity rather than to chance; SMD: Standardized mean difference.

excluded from the above 4 trials for the calculation of responder rate[35] and change in symptom scores[36] respectively because of insufficient information. Concerning the comparison between mosapride combined therapy and PPI monotherapy, use of mosapride did not significantly elevate the rate of responders (PRR = 1.132; 95%CI: 0.934-1.372; P = 0.572; I^2 = 0.0%) (Figure 3). However, the treatment arm achieved a greater symptom relief than that in placebo arm (Hedges'g = 0.24; 95%CI: 0.03-0.46; P = 0.874; I^2 = 0.0%) (Figure 4). No heterogeneity was found among the studies, both Egger's tests (P = 0.587; P = 0.636) failed to show significance for these studies, indicating no statistically significant publication bias. Only one trial[16] reported a safety analysis, which showed a similar incidence of adverse effects in the two groups.

Trials on addition of mosapride to PPIs for the treatment of PPI-resistant GERD patients

Three open-labeled trials evaluated the additional efficacy of mosapride in PPI-resistant patients. Miyamoto *et al*[37] used an FSSG questionnaire which comprised 12 questions concerning not only acid-related symptoms, but also dysmotility symptoms. They treated 163 GERD patients with rabeprazole 10 mg daily for 3 mo. Thirty-four patients were dissatisfied with the PPI monotherapy and, therefore, were considered to be PPI-resistant. Three months of combined therapy with mosapride resulted in high efficacy. Futagami *et al*[38] explored the function of gastric emptying in PPI-resistant NERD patients, and found that PPI-resistant NERD patients showed

significant disturbances of gastric emptying compared to healthy volunteers. Moreover, administration of mosapride in addition to omeprazole alleviated reflux symptoms and improved gastric emptying in PPI-resistant NERD patients. Another study by Miyamoto *et al*[39] analyzed FSSG-reflux score (RS) and -dyspeptic score (DS) of PPI-resistant NERD patients. Significant improvement in FSSG-total score and FSSG-DS was observed after the addition of mosapride in PPI non-response NERD patients. These results indicate that patients with significant dysmotility and functional dyspepsia were more likely to be PPI-resistant and suggest the need for the addition of a prokinetic agent to PPI therapy.

DISCUSSION

With respect to the comparison between mosapride combined treatment with PPI and PPI monotherapy, similar efficacy was found between these two groups in most[16,35,36] of the four randomized controlled trials, the meta-analysis showed similar treatment responsiveness but a significant difference in symptom score improvement between the treatment arm and the placebo arm. However, all the four trials used different symptom scores, the one point improvement should not mean the same symptom relief in different scoring systems. They cannot be standardized, compared or combined easily. Therefore, the rate of responders is more appropriate as the measure of effect size, since the criteria of improvement in each paper was decided to be feasible at least by

the author of each paper. The results indicated that the addition of mosapride to PPI therapy might be useful for patients with GERD, but could not achieve satisfactory effects. Of note, type II error should also be considered as a reason for the failure to show a significant difference in the rate of responders. The number of patients may not have been enough. In addition, the analysis of open-label trials showed that mosapride plus PPIs might be of benefit to PPI-resistant GERD patients. However, since these trials did not set the control group, the results may be considerably biased.

Mosapride is a selective 5-HT$_4$ receptor agonist with no affinity for 5-HT$_1$, 5-HT$_2$ or dopamine D$_2$ receptors[42]. It is devoid of anti-dopaminergic and direct cholinomimetic effects. It is well tolerated. Diarrhea, dry mouth, malaise and headache are the most frequent side effects and they were reported in < 5% of patients[43]. Currently, mosapride is commercially available in many Asian countries, but not in United States and Europe. An interesting feature of mosapride is that its action seems to differ along the gastrointestinal tract. Mosapride decreases acid reflux to the esophagus by modulating esophageal motor function in patients with GERD[14], or improving gastric emptying for both solids and liquids in healthy volunteers and diabetic patients[44,45]. It is known that gastric motility is impaired in some NERD patients, and mosapride improves the symptoms in such patients[32]. Mosapride has a distinctly lower affinity to receptors located in the colon[13]. These findings indicate that mosapride selectively stimulates upper gastrointestinal motility, and interacts heterogeously with 5-HT$_4$ receptors. Mosapride has also been shown to modulate visceral sensation *via* raising the threshold of visceral pain caused by balloon expansion in rat stomach[46]. Moreover, it has been reported that mosapride increased the pharmacokinetics of PPIs such as rabeprazole[47], indicating that it is able to facilitate the acid inhibitory effect of PPIs. However, the current results showed that mosapride as an add-on therapy was not more effective than PPI alone in the treatment of GERD. This may be partially due to the effect of the PPI, which might be beneficial to the relief of dyspeptic symptoms[48,49] and obscure the effect of prokinetic treatment.

The strengths of our systematic review could be summarized as follows. We sought to find as many publications as possible using various search approaches. The explicit, detailed eligibility criteria were set up to minimize the selection bias. And we used Cochrane Review Guidelines to assess the quality of the evidence. We also placed emphasis on calculating the possibility of publication bias by Egger's test and evaluating bias across studies, while no heterogeneity was found in our statistical analysis.

There are several limitations of this review. First, the number of patients in the individual studies was relatively small. Second, with respect to PPI-resistant subjects, all of the three studies were open trials, without a placebo control group who did not receive the additional prokinetic agents, therefore, the results could be considerably biased by placebo effect of mosapride administration in

this setting. Further randomized, placebocontrolled trials should be performed. Moreover, in two[38,39] out of these three studies, the investigators focused mainly on dyspeptic rather than reflux symptoms. Third, the subtypes of GERD (RE and NERD) patients were not discussed in this review due to little available information. Moreover, there was significant heterogeneity between trials with regard to methodology. Standardized methodology for GERD symptom questionnaire are needed to facilitate the comparison of outcomes and minimize the operating bias.

In conclusion, this review shows that mosapride combined therapy is not more effective than PPI alone as the first-line therapy in GERD patients. Whether it is effective in the treatment of PPI-resistant reflux disease needs to be determined. However, the results of this review are still at the level of descriptive analysis. Further clinical studies with better design and larger number of participants are needed to verify the efficacy of this combined therapy.

COMMENTS

Background
Gastroesophageal reflux disease (GERD) is the most common gastrointestinal diagnosis recorded during visits to outpatient clinics, and the patients sometimes do not respond to proton pump inhibitor (PPI) therapy. Prokinetic agents have been widely used to relieve the GERD symptoms, and mosapride is a selective 5-HT$_4$ receptor agonist that can be safely used. However, the potential benefit of the addition of mosapride to PPIs in the treatment of GERD is unclear.

Research frontiers
Mosapride is a selective 5-HT$_4$ receptor agonist devoid of anti-dopaminergic and direct cholinomimetic effects. Many studies have shown that mosapride can reduce acid reflux episodes and esophageal clearance of refluxate, suggesting potential efficacy in the treatment of GERD.

Innovations and breakthroughs
Based on this systematic review and meta-analysis, mosapride combined therapy was not more effective than PPI alone as the first-line therapy. This has not been identified clearly in previous studies.

Applications
The addition of mosapride to PPI therapy might be useful for patients with GERD, but could not achieve satisfactory effects. Moreover, the fact that mosapride is not yet available in Europe and the United States makes this study more relevant giving physicians some basis for the use in these countries once it is licensed.

Peer review
This is a well-performed systematic review of currently available studies on the potential benefits of the addition of mosapride to PPIs in the treatment of GERD. The authors found that mosapride combined therapy was not more effective than PPI alone as first-line therapy. The conclusions are unbiased and give informative clues to the readers.

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META-ANALYSIS

Fast-track rehabilitation *vs* conventional care in laparoscopic colorectal resection for colorectal malignancy: A meta-analysis

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Abstract

AIM: To evaluate the fast-track rehabilitation protocol and laparoscopic surgery (LFT) *vs* conventional care strategies and laparoscopic surgery (LCC).

METHODS: Studies and relevant literature comparing the effects of LFT and LCC for colorectal malignancy were identified in MEDLINE, the Cochrane Central Register of Controlled Trials and EMBASE. The complications and re-admission after approximately 1 mo were assessed.

RESULTS: Six recent randomized controlled trials (RCTs) were included in this meta-analysis, which related to 655 enrolled patients. These studies demonstrated that compared with LCC, LFT has fewer complications and a similar incidence of re-admission after approximately 1 mo. LFT had a pooled RR of 0.60 (95%CI: 0.46-0.79, $P < 0.001$) compared with a pooled RR of 0.69 (95%CI: 0.34-1.40, $P > 0.5$) for LCC.

CONCLUSION: LFT for colorectal malignancy is safe and efficacious. Larger prospective RCTs should be conducted to further compare the efficacy and safety of this approach.

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Key words: Laparoscopic surgery; Fast-track rehabilitation; Enhanced recovery; Colorectal surgery; Complications; Readmission

Core tip: Fast-track rehabilitation in laparoscopic colorectal resection has become the most fashionable way to treat colorectal malignancy. Complications after fast-track rehabilitation protocol and laparoscopic surgery (LFT) and conventional care strategies and laparoscopic surgery (LCC) of colorectal resection have generally been discussed in China, as well as in other countries. This study clarified that compared with LCC, LFT has fewer complications and has a similar incidence of re-admission after approximately 1 mo.

Li P, Fang F, Cai JX, Tang D, Li QG, Wang DR. Fast-track rehabilitation *vs* conventional care in laparoscopic colorectal resection for colorectal malignancy: A meta-analysis. *World J Gastroenterol* 2013; 19(47): 9119-9126 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i47/9119.htm DOI: http://dx.doi.

INTRODUCTION

Fast-track rehabilitation in laparoscopic colorectal resection has become the most fashionable way of treating colorectal malignancy. During the mid-1990s, fast-track rehabilitation, involving dieticians, nurses, surgeons and anesthesiologists, was developed by Kehlet *et al*[1,2], Wilmore *et al*[3] and Basse *et al*[4]. Common to the other enhanced recovery rehabilitations, it is an attempt to reduce the stress response, decrease complications, speed up recovery, shorten the hospital stay and reduce health costs, all without compromising patient safety. The laparoscopic approach to colorectal surgery has been shown to accelerate dietary intake and return of bowel function[5], to facilitate postoperative mobilization[6], to reduce the length of stay in hospital[5,7] and to have a positive effect on postoperative mortality[5,7-9].

Recently, laparoscopic surgery has been generally applied in the treatment of gastrointestinal cancer, which can significantly attenuate trauma and accelerate the rehabilitation of patients after surgery. It was reported that the hospital stay time is shorter and the complication and readmission rate are lower after laparoscopic surgery[10,11].

Despite all the major benefits of laparoscopy, elective colorectal resection is still associated with a morbidity rate between 20% and 30% and a postoperative hospital stay of 7-10 d[12]. Both laparoscopic surgery and FT perioperative care have been reported to be safe and effective, and to result in a shorter hospital stay with earlier recovery of gastrointestinal function[13-16] and lower morbidity than open colorectal surgery and standard care[17-19]. Many recently published randomized controlled trials are available that have compared fast-track rehabilitation to conventional care in laparoscopic colorectal resection for colorectal malignancy. The safety after fast-track rehabilitation protocol and laparoscopic surgery (LFT) of colorectal resection has generally been discussed; therefore, this study analyzed and compared the complications and re-admission between LFT and conventional care strategies and laparoscopic surgery (LCC). The primary aim of this meta-analysis was to evaluate LFT *vs* LCC; the secondary aim was to assess LFT.

MATERIALS AND METHODS

Publication search

PubMed, the Cochrane Central Register of Controlled Trials and EMBASE were searched for all relevant literature, including articles referenced in the publications. The medical subject headings (MeSH) and keywords collected for individually and in combination were as follows: "laparoscopic surgery" "open surgery" and "fast track" or "enhanced recovery" and "colorectal". The last search was done on May 10th, 2013. References, lists of retrieved articles, reviews and meta-analyses were then scanned for additional articles. Internet search engines were also used to perform a manual search for abstracts from international meetings, which were then downloaded and studied.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) randomized controlled trials; (2) studies that provided information on at least one of the outcome measures; (3) studies published in English. When a study reporting the same patient cohort was included in several publications, only the most recent or complete study was selected; and (4) detailed patient information provided. The exclusion criteria were as follows: (1) case reports; (2) articles that were not full text, or non-comparative studies; and (3) open operations, not by laparoscopic surgery.

Study selection

The inclusion criteria were met in studies if they involved LFT for colorectal malignancy in adult patients (*i.e.*, those 18 years and older) and used LCC as a control. All studies that used chemotherapy, or a rehabilitation protocol had to include less than seven of the seventeen FT items among the interventions in the FT group (programs using epidural or local anesthesia, minimally invasive techniques, optimal pain control and aggressive postoperative care) to achieve early recovery after colorectal surgery; and more than two of the conventional care strategies were included, were excluded. Studies that could not provide actual frequencies of complications or re-admission after approximately 1 mo were also excluded. Both full-length publications and abstract publications were selected. Letters, reviews without original data, non-English papers and animal studies were excluded. If any doubt regarding the suitability remained after the abstract was examined, the full manuscript was obtained.

Data extraction

All included studies were assessed for the quality of their methodology and relevance to the objective of our meta-analysis. Conduct and reporting were in accordance with the QUOROM statement. Data on complications or re-admission approximately 1 mo from each trial were extracted and compared independently by the two investigators.

Statistical analysis

In statistical analysis, Review Manager (RevMan) software version 5.0.0 was used (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). A pooled RR and a pooled Mean Difference with 95%CI were used to assess the outcomes of the studies. Statistical heterogeneity was tested by the χ^2 test. According to the forest plot, heterogeneity was limited, so the Mantel-Haenszel fixed effect model was adopted. The significance of the pooled RR was determined by the *Z* test and statistical significance was considered at $P < 0.05$. Publication bias was estimated by the use of a funnel plot with an Egger's linear regression test, and funnel plot
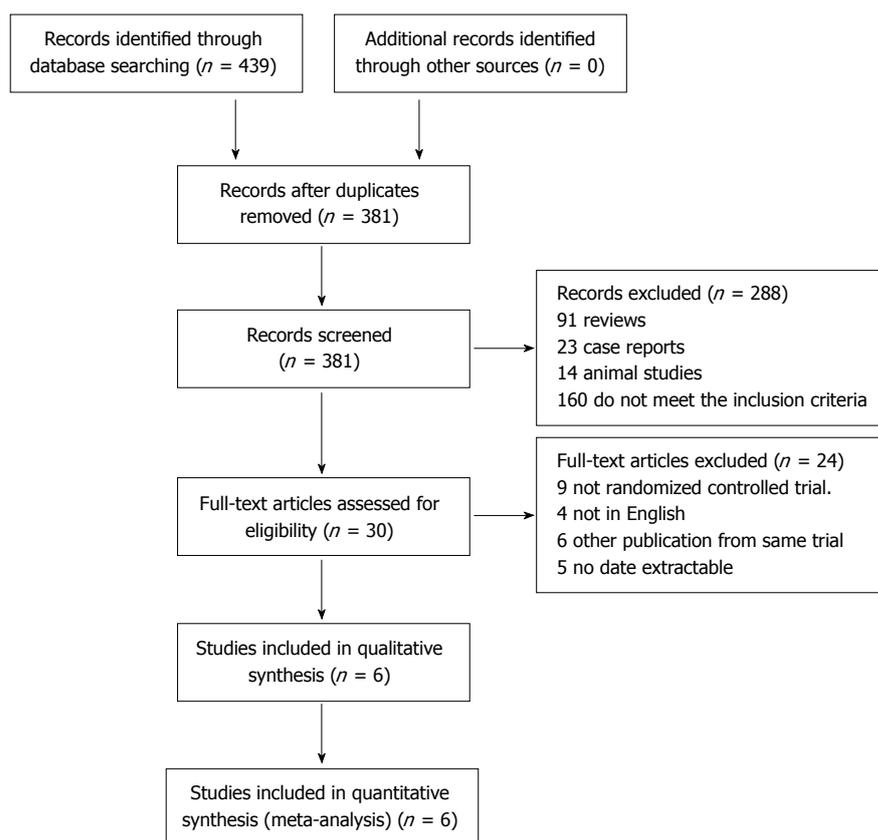
Figure 1 Selection of studies.

Table 1 Main characteristics of the six included studies

	n		Age (yr)		Sex (male/female)	
	LFT	**LCC**	**LFT**	**LCC**	**LFT**	**LCC**
Wang *et al*[20]	41	42	57.2 ± 18.1	55.4 ± 16.8	24/17	25/17
Vlug *et al*[21]	93	98	66 ± 10.3	66 ± 7.1	54/39	59/39
van Bree *et al*[22]	18	18	64 ± 10.1	66 ± 6.9	11/7	11/7
Veenhof *et al*[23]	17	20	65	68	9/8	14/6
Wang *et al*[24]	40	38	71	72	22/18	20/18
Wang *et al*[25]	106	104	57	55	65/41	60/44

LFT: Fast-track rehabilitation protocol and laparoscopic surgery; LCC: Conventional care strategies and laparoscopic surgery.

asymmetry on the natural logarithm scale of the RR was measured by a linear regression approach.

RESULTS

Search results

A total of 439 references were identified from medical journal databases. Upon examination of the abstracts, 409 articles were rejected based on the rejection criteria outlined in Figure 1. A study of the complete manuscripts for the 30 remaining articles led to elimination of 14 papers that contained no data pertaining to the outcome of LFT for colorectal resection, four papers not in English and six papers explaining the effect of analgesia. The remaining six non-duplicated randomized controlled trials (RCTs) that compared LFT with LCC were included

in the meta-analysis.

Characteristics of the selected RCTs

Characteristics of the six RCTs[20-25] included in the meta-analysis are summarized in Table 1. These studies were published between 1985 and 2013 and investigated a total of 665 patients: 323 received LFT and 332 received LCC.

Meta-analysis results

Complication: Data were collected from six studies (655 patients) on complications for LFT *vs* LCC. In the LFT group, 19.81% patients (64/323) had complications, while in the LCC group, 33.13% patients (110/332) had complications. Pooling the results indicated that LFT could significantly reduce complications compared with LCC. The weighted mean difference (WMD) was 0.60 (95%CI: 0.46-0.79, $P < 0.05$), $\chi^2 = 12.33$ ($P = 0.03$) and $I^2 = 59\%$, indicating heterogeneity among the studies.

Anastomotic leak: Data were collected from four studies (497 patients) on anastomotic leak for LFT *vs* LCC. 4.94% (12/243 patients) had an anastomotic leak in the LFT group and 4.72% (12/254) in the LCC group. Pooling the results indicated that LFT and LCC had similar risks of anastomotic leak. The WMD was 1.07 (95%CI: 0.50-2.32, $P > 0.05$, Figure 2), $\chi^2 = 2.13$ ($P = 0.55$) and $I^2 = 0\%$, which excludes heterogeneity among the studies.

Wound infection: Data were collected from four stud-

| | FT | | CC | | | Risk ratio | Risk ratio |
Study or subgroup	Events	Total	Events	Total	Weight	M-H, Fixed, 95%CI	M-H, Fixed, 95%CI
3.1.1 complications							
van Bree SH 2011	3	18	11	18	6.4%	0.27 [0.09, 0.82]	
veenhof AA 2012	2	19	9	23	4.7%	0.27 [0.07, 1.10]	
Vlug MS 2011	34	100	37	109	20.5%	1.00 [0.69, 1.46]	
Wang G 2011	20	106	39	104	22.8%	0.50 [0.32, 0.80]	
Wang G 2012	3	40	6	40	3.5%	0.50 [0.13, 1.86]	
Wang Q 2011	2	40	8	38	4.8%	0.24 [0.05, 1.05]	
Subtotal (95%CI)		323		332	62.6%	0.60 [0.46, 0.79]	
Total events	64		10				

Heterogeneity: $\chi^2 = 12.33$, $df = 5$ ($P = 0.03$); $I^2 = 59\%$
Test for overall effect: $Z = 3.74$ ($P = 0.0002$)

3.1.2 Anastomotic leak							
van Bree SH 2011	1	18	1	18	0.6%	1.00 [0.07, 14.79]	
veenhof AA 2012	0	19	3	23	1.8%	0.17 [0.01, 3.13]	
Vlug MS 2011	7	100	6	109	3.3%	1.27 [0.44, 3.66]	
Wang G 2011	4	106	2	104	1.2%	1.96 [0.37, 10.48]	
Subtotal (95%CI)		243		254	6.9%	1.07 [0.50, 2.32]	
Total events	12		12				

Heterogeneity: $\chi^2 = 2.13$, $df = 3$ ($P = 0.55$); $I^2 = 0\%$
Test for overall effect: $Z = 0.18$ ($P = 0.86$)

3.1.3 Wound infection							
veenhof AA 2012	1	19	1	23	0.5%	1.21 [0.08, 18.09]	
Vlug MS 2011	6	100	8	109	4.4%	0.82 [0.29, 2.27]	
Wang G 2011	4	106	7	104	4.1%	0.56 [0.17, 1.86]	
Wang Q 2011	1	40	3	38	1.8%	0.32 [0.03, 2.91]	
Subtotal (95%CI)		265		274	10.8%	0.66 [0.33, 1.32]	
Total events	12		19				

Heterogeneity: $\chi^2 = 0.85$, $df = 3$ ($P = 0.84$); $I^2 = 0\%$
Test for overall effect: $Z = 1.18$ ($P = 0.24$)

3.1.4 complications							
veenhof AA 2012	1	19	1	23	0.5%	1.21 [0.08, 18.09]	
Vlug MS 2011	7	100	8	109	4.4%	0.95 [0.36, 2.53]	
Wang G 2011	2	106	5	104	2.9%	0.39 [0.08, 1.98]	
Wang Q 2011	0	40	2	38	1.5%	0.19 [0.01, 3.84]	
Subtotal (95%CI)		265		274	9.4%	0.67 [0.32, 1.42]	
Total events	10		16				

Heterogeneity: $\chi^2 = 1.78$, $df = 3$ ($P = 0.62$); $I^2 = 0\%$
Test for overall effect: $Z = 1.04$ ($P = 0.30$)

3.1.5 complications							
van Bree SH 2011	1	18	2	18	1.2%	0.50 [0.05, 5.04]	
Vlug MS 2011	6	100	7	109	3.9%	0.93 [0.32, 2.69]	
Wang G 2011	4	106	6	104	3.5%	0.65 [0.19, 2.25]	
Wang G 2012	1	40	3	40	1.7%	0.33 [0.04, 3.07]	
Subtotal (95%CI)		264		271	10.3%	0.69 [0.34, 1.40]	
Total events	12		18				

Heterogeneity: $\chi^2 = 0.81$, $df = 3$ ($P = 0.85$); $I^2 = 0\%$
Test for overall effect: $Z = 1.03$ ($P = 0.30$)

Total (95%CI)		1360		1405	100.0%	0.66 [0.53, 0.81]	
Total events	110		175				

Heterogeneity: $\chi^2 = 19.56$, $df = 21$ ($P = 0.55$); $I^2 = 0\%$
Test for overall effect: $Z = 3.83$ ($P = 0.0001$)

0.1 0.2   0.5   1   2   5   10
Favours experimental   Favours control

Figure 2 Forest plot comparing fast-track rehabilitation protocol and laparoscopic surgery *vs* conventional care strategies and laparoscopic surgery in colorectal resection, outcome: complications. LFT: Fast-track rehabilitation protocol and laparoscopic surgery; LCC: Conventional care strategies and laparoscopic surgery.

Figure 3 Comparison between the fast-track rehabilitation protocol and laparoscopic surgery, and conventional care strategies and laparoscopic surgery in laparoscopic colorectal resection for colorectal malignancy, outcome: complications.

ies (539 patients) on wound infection for LFT *vs* LCC; 4.53% (12/265 patients) had wound infection in the LFT group and 6.93% (19/274) in the LCC group. Pooling the results indicated that LFT did not significantly reduce wound infections compared with LCC. The WMD was 0.66 (95%CI: 0.33-1.32, $P > 0.05$), $\chi^2 = 0.85$ ($P = 0.84$) and $I^2 = 0$%, which excludes statistical heterogeneity among the studies.

Obstruction: Data were collected from four studies (539 patients) on obstruction for LFT *vs* LCC. 3.77% (10/265 patients) had obstructions in the LFT group and 5.84% (16/274) in the LCC group. Pooling the results indicated no significant difference in the risk of obstruction. The WMD was 0.67 (95%CI: 0.32-1.42, $P > 0.05$), $\chi^2 = 1.78$ ($P = 0.62$) and $I^2 = 0$%, which excludes heterogeneity among the studies.

Re-admission: Data were collected from four studies (535 patients) on re-admission for LFT *vs* LCC. 4.55% (12/264 patients) were readmitted in the LFT group and 6.64% (18/271) in the LCC group. Pooling the results indicated no apparent difference in re-admission. The WMD was 0.69 (95%CI: 0.34-1.4, $P > 0.05$), $\chi^2 = 0.81$ ($P = 0.85$) and $I^2 = 0$%, which excludes heterogeneity among the studies.

Publication bias

Funnel plots were created to access the publication bias of the literature. The shapes of the funnel plots did not show any obvious asymmetry (Figure 3).

DISCUSSION

The straightforward conclusion from the six included studies is that LFT is a more reliable treatment for colorectal malignancy, compared with LCC. LFT reduced complications, but carried similar risks of anastomotic

leak, wound infection, obstruction and re-admission.

Complications after LFT and LCC of colorectal resection have generally been discussed in China, as well as in other countries. A recently published multivariate analysis identified male gender[26], preoperative education, anesthesia[27] and early postoperative oral nutrition[28] as potential risk factors for complications after colorectal surgery. In addition, some studies have found an increased risk of anastomotic leaks in males, which is consistent with the results of this study (10.1% of the men required re-operation for anastomotic leak *vs* 3.3% of the women)[29-32].

Preoperative education of patients has a crucial role in LFT. It is necessary to demonstrate the detailed treatment program, the different steps of fast-track rehabilitation program and relevant measures for the patients to make them better understand and accept the fast-track rehabilitation program.

Better cooperation of patients can bring better outcomes of LFT. Generally, since the gastric emptying time of solid meal and fluid are 6 and 2 h, respectively[33], the patients should be encouraged to have liquid meal 2 h before the operation instead of fasting. It has been shown that preoperative oral carbohydrate is safe and can efficiently reduce complications[34-36].

The role of epidural anesthesia or regional anesthesia in LFT should be stressed. Postoperative epidural analgesia can avoid stress-induced neurological, endocrinological and homeostatic changes or the blocking of sympathetic nerve-related surgical stress response, reduce complications such as nausea, vomiting and enteroparalysis after operation, promote early ambulation, improve the intestinal function and shorten the hospital stay time of patients after resection of colorectal cancer[26,37-42].

Early postoperative oral nutrition is regarded as an essential part of LFT. Food intake can stimulate gastrointestinal peristalsis, and early feeding during the first 24 h after surgery promotes the recovery of an obstruction. It has been illustrated that early postoperative oral nutrition attenuates catabolism and potentially decreases infectious complications[27,43].

Several studies have shown that American Society of Anesthesiologists grade III or higher is associated with increased postoperative morbidity[44-46].

LFT can improve the rehabilitation of patients after resection of colorectal cancer better than LCC, thus benefiting their surgery, anesthesia, pain management, physical therapy and social work. The primary work of LFT is the preoperative education of patients to make them understand the whole plan and the aim of each stage. Therefore, it is vital to obtain cooperation from the nursing staff.

However, we should still regard these outcomes with caution and evaluate them critically for the following reasons. Firstly, although there was no detectable publication bias, as tested by the funnel plots, the overall methodological quality and reporting of the included studies were poor. Secondly, the number of studies found was relatively low, and the aforementioned quality issues may have biased the results significantly. Therefore, more

large trials with better separation between LFT and LCC for colorectal malignancy seem necessary. Furthermore, in light of current evidence, LFT should not yet be considered the new standard for colorectal malignancy. Long-term data on outcome, as well as important other factors in making a decision for an intervention, are also lacking. Quality of life data and data on physiological performance after 5 years have never been described, nor have data on cost-effectiveness or economic evaluations of LFT. These parameters may play an important part in recommending LFT treatment in colorectal resection. However, we believe that, with greater awareness and the increasing popularity of LFT, more long-term follow-up reports will eventually be published.

There have been eight previously published systematic reviews, including meta-analyses on this topic[16,17,47-52]. These included three reviews of controlled clinical trials and randomized controlled trials[17,47,49], and five reviews of randomized controlled trials only[47,50-52]. The present study is the first meta-analysis to compare fast-track rehabilitation with conventional care in laparoscopic colorectal resection for colorectal malignancy. This also the first meta-analysis of patients undergoing elective colorectal surgery to demonstrate that LFT is associated with a significant reduction in postoperative complications, but no significant reduction in readmission rates. The increased number of included studies supported the quality of the evidence from the present study.

In conclusion, this meta-analysis demonstrated that LFT is safe and feasible for colorectal surgery. As LFT comes into even wider use, additional large, prospective RCTs should be conducted to further compare the efficacy and safety of this approach.

COMMENTS

Background
Fast-track rehabilitation in laparoscopic colorectal resection has become the most fashionable way of treating colorectal malignancy. Complications after fast-track rehabilitation protocol and laparoscopic surgery (LFT) and conventional care strategies and laparoscopic surgery (LCC) of colorectal resection have generally been discussed in China, as well as in other countries.

Research frontiers
Over the past three decades, many studies have assessed the performance of LFT. However, comparisons of LFT and LCC have not been published.

Innovations and breakthroughs
Based on this meta-analysis, LFT for colorectal malignancy is safe and efficacious. Similar associations were indicated in subgroup analyses of East Asian, Western, cohort, and high-quality studies. These findings were not presented clearly in previous systematic reviews.

Applications
LFT appears to be neither directly nor indirectly associated with risk. Further studies should seek to clarify this conclusion.

Peer review
LFT is rapidly becoming the focal point of attraction for specialists worldwide. This article shows the advantages of the procedure. This analysis has great practical value for clinicians.

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CASE REPORT

Localized type 1 autoimmune pancreatitis superimposed upon preexisting intraductal papillary mucinous neoplasms

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Abstract

A 70-year-old woman was found to have 2 cystic lesions in the head of the pancreas on abdominal ultrasonography during a routine medical examination. Endoscopic ultrasonography (EUS) and magnetic resonance cholangiopancreatography showed multilocular cysts in the head of the pancreas without dilation of the main pancreatic duct. The patient was followed-up semiannually with imaging studies for suspected branch duct-type intraductal papillary mucinous neoplasm (IPMN). At 3 years after initial presentation, hypoechoic lesions were observed around each pancreatic cyst by EUS. Diffusion-weighted imaging showed high-intensity regions corresponding to these lesions. Therefore, a diagnosis of invasive carcinoma derived from IPMN could not be excluded, and sub-total stomach-preserving pancreaticoduodenectomy was performed. The macroscopic examination of the surgical specimen showed whitish solid masses in the head of the pancreas, with multilocular cysts within each mass. Microscopically, each solid mass consisted of inflammatory cells such as lymphocytes and plasma cells. Furthermore, immunochemical staining revealed immunoglobulin G4-positive cells, and many obliterating phlebitides were observed. The cysts consisted of mucus-producing epithelial cells and showed a papillary growth pattern. Based on these findings, we diagnosed multiple localized type 1 autoimmune pancreatitis occurring only in the vicinity of the branch duct-type IPMN.

Core tip: We herein report a case of localized type 1 autoimmune pancreatitis (AIP) superimposed upon preexisting multifocal intraductal papillary mucinous neoplasms (IPMNs) of the branch duct. Although few reports have shown AIP associated with IPMN, in our case, AIP had developed only around the IPMN, which was under progressive observation. Therefore, the IPMN may have influenced the pathogenesis of AIP.

Urata T, Naito Y, Izumi Y, Takekuma Y, Yokomizo H, Nagamine M, Fukuda S, Notohara K, Hifumi M. Localized type 1 autoimmune pancreatitis superimposed upon preexisting intraductal papillary mucinous neoplasms. *World J Gastroenterol* 2013; 19(47): 9127-9132 Available from: URL: http://www.wjgnet.

INTRODUCTION

Autoimmune pancreatitis (AIP) is classified into 2 groups according to the International Consensus Diagnostic Criteria reported in 2011[1]. Type 1 AIP is characterized by the pathological condition termed lymphoplasmacytic sclerosing pancreatitis, whereas type 2 AIP is characterized by idiopathic duct centric pancreatitis. Although the pathogenesis of AIP remains unknown, it is considered to involve certain immune mechanisms. Typical parenchymal imaging features of AIP include diffuse enlargement with delayed enhancement and occasional segmental or focal enlargement. Given these imaging characteristics, distinguishing AIP with focal enlargement (f-AIP) from carcinoma of the pancreas is particularly difficult.

Although intraductal papillary mucinous neoplasm (IPMN) is recognized as a cystic mucus-producing tumor, its association with AIP has not been reported thus far. Herein, we report a rare case of AIP occurring only in the vicinity of IPMN, which was diagnosed during the follow-up examinations for IPMN.

CASE REPORT

A 70-year-old woman was found to have cystic lesions in the head of the pancreas on abdominal ultrasonography (US) during a routine medical checkup in December 2008. She had no symptoms, and the blood test results were essentially normal. Endoscopic ultrasonography (EUS) and magnetic resonance cholangiopancreatography (MRCP) showed multilocular cysts in the head of the pancreas without dilation of the main pancreatic duct (Figure 1). Diffusion-weighted imaging (DWI) showed no uptake in the pancreas (Figure 2A and B). The patient was followed-up by semiannual imaging studies for suspected branch duct-type IPMN.

In December 2011, solid lesions were observed in or around both the IPMNs on EUS (Figure 3A and B). DWI showed high-intensity signals corresponding to these lesions (Figure 2C and D), and MRCP showed a reduction in the diameter of the IPMNs (Figure 3C). Contrast-enhanced computed tomography (CT) revealed that these lesions had a lower density than the surrounding pancreatic parenchyma during the pancreatic parenchymal phase and showed iso-density in the equilibrium phases (Figure 4). ERP revealed a normal main pancreatic duct and no communication between the main pancreatic duct and the cystic lesions. The pancreatic juice cytology was negative for malignancy.

Based on these results, our principal differential diagnoses included inflammatory pseudo-tumors such as AIP; however, the possibility of invasive carcinoma derived from IPMN could not be excluded.

We performed a subtotal stomach-preserving pancre-
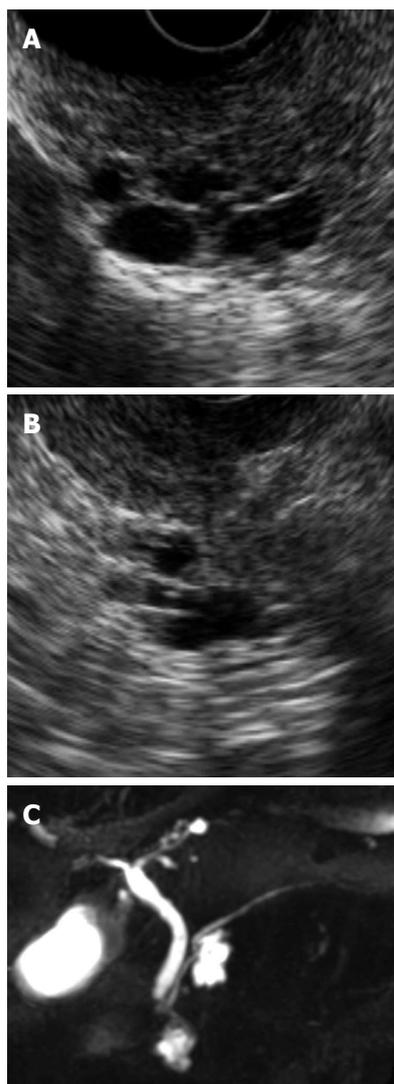
Figure 1 Imaging studies from the initial examination. A, B: Endoscopic ultrasonography showed 2 multilocular cysts in the pancreas head; C: Magnetic resonance cholangiopancreatography showed 2 cystic lesions without dilation of the main pancreatic duct in the pancreas head.

aticoduodenectomy after fully explaining the possibility of malignancy and the risks of the surgery to the patient.

Pathological findings

The macroscopic examination of the surgical specimen showed multilocular cysts with 2 whitish solid lesions (Figure 5). The 2 lesions were solitary and indicated no gross continuity with each other. Microscopically, each solid lesion presented a striform pattern consisting of fibroblasts/myofibroblasts mixed with lymphoid follicles and inflammatory cells (Figure 6A), particularly lymphocytes and plasma cells that tested positive for immunoglobulin IgG4 on immunochemical staining (Figure 6B). In addition, many obliterating phlebitides (Figure 6C) were observed. The multilocular cysts demonstrating periductal inflammation consisted of mucus-producing epithelial cells and showed a papillary growth pattern (Figure 6D). The epithelial cells were MUC2-negative
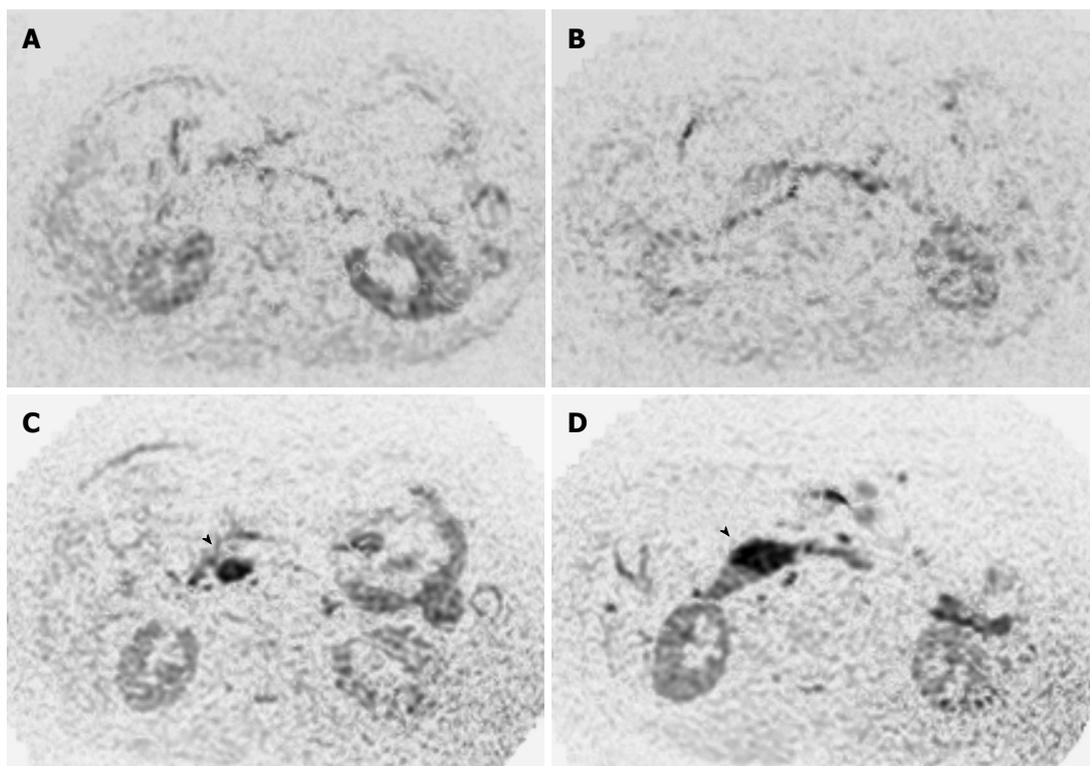
Figure 2 Diffusion-weighted magnetic resonance imaging. A, B: In 2008, diffusion-weighted imaging showed no signal in the pancreas; C, D: In 2011, diffusion-weighted imaging showed high-intensity signals (arrow-head) corresponding to both cystic lesions in the pancreas.
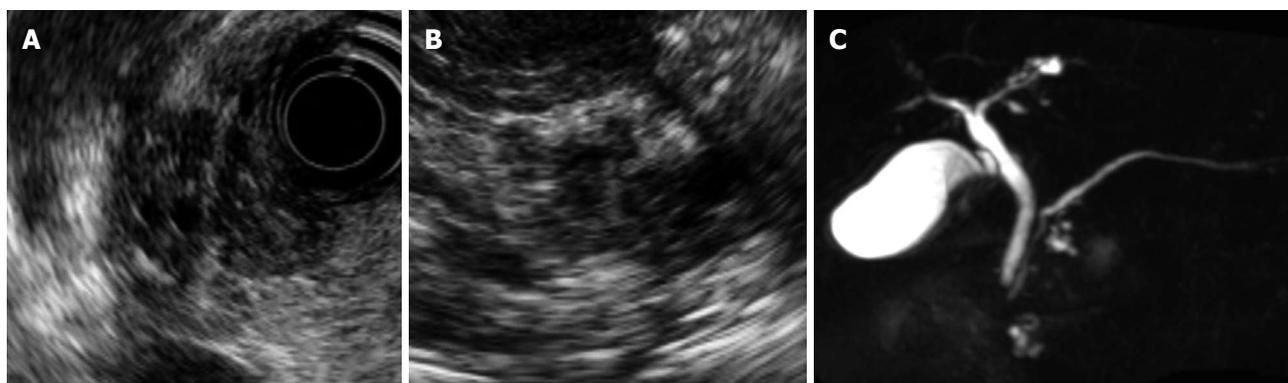

Figure 3 Imaging studies at follow-up examination. A, B: Endoscopic ultrasonography revealed solid lesions in or around both cystic lesions in the pancreas head. Hyperechoic foci and strands were observed in these lesions; C: Magnetic resonance cholangiopancreatography showed a reduction in the diameter of both cystic lesions.

and MUC5AC-positive on immunochemical staining. We therefore diagnosed multiple localized type 1 AIP involving only the vicinity of branch duct-type IPMN.

DISCUSSION

Type 1 AIP is histologically characterized by the following 4 characteristic features: (1) Dense infiltration of plasma cells and lymphocytes, particularly in the periductal regions; (2) Peculiar storiform fibrosis; (3) Venulitis with lymphocytes and plasma cells often leading to obliteration of the affected veins; and (4) Abundant [> 10 cells per high-power field (HPF)] IgG4-positive plasma cells[1]. Our case fulfilled all of these histological features,

indicating a diagnosis of AIP.

In diagnostic imaging, typical AIP is characterized by the diffuse enlargement of the pancreas with stenosis of the main pancreatic duct. However, at times, AIP can present as segmental or focal enlargement in the pancreas, and differentiating such cases of f-AIP from pancreatic carcinoma is rather difficult[2]. Although US and EUS show hypoechoic masses in both f-AIP and pancreatic carcinoma, the characteristic findings of f-AIP include hyperechoic foci and lobularity, reflecting an inflammatory tumor environment. In the Rosemont criteria[3], the EUS-based major criteria for the diagnosis of chronic pancreatitis (CP), including AIP, comprise hyperechoic foci with shadowing, calculi in the main
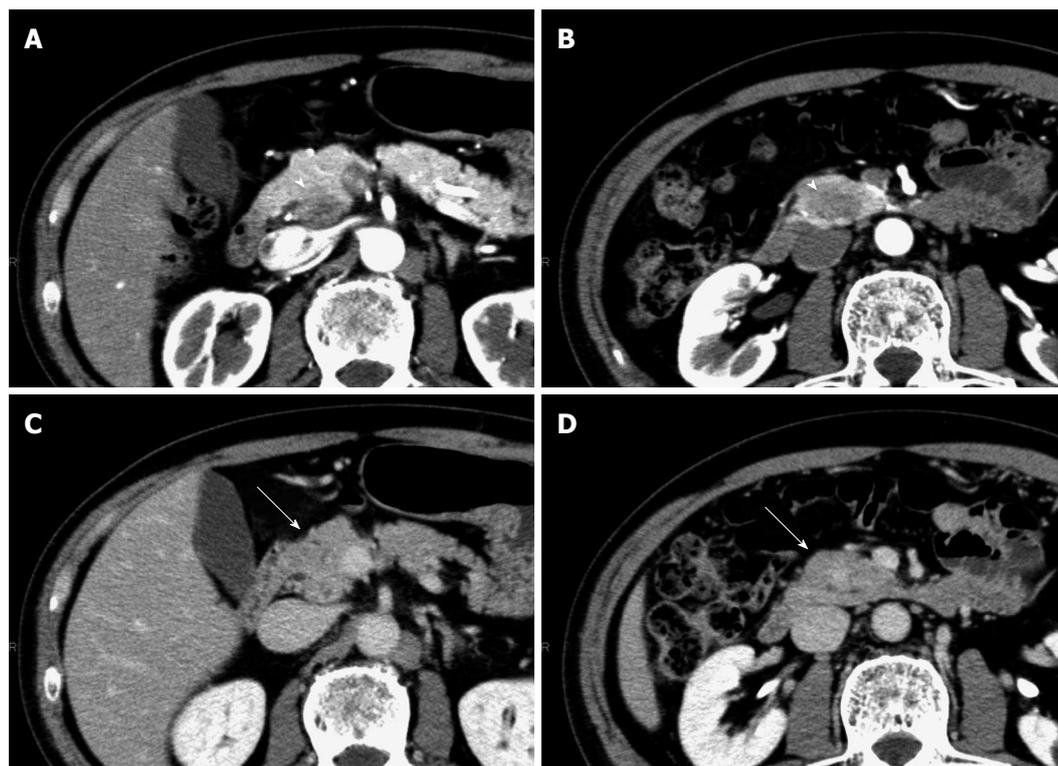
Figure 4 Computer tomography scan. A, B: Both cystic lesions (arrow-head) showed a lower density than the surrounding pancreatic parenchyma during the pancreatic parenchymal phase; C, D: Both cystic lesions (arrow) appeared as iso-dense in the equilibrium phases.
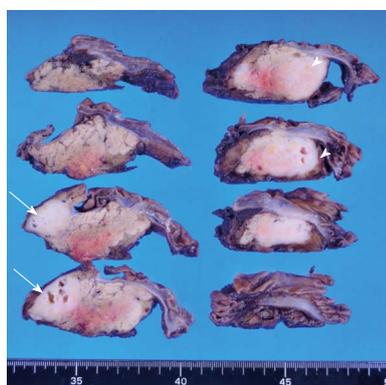

Figure 5 Macroscopic findings. The macroscopic examination revealed 2 whitish solid lesions with multilocular cysts (arrow, and arrow-head). Each lesion was solitary and indicated no gross continuity.

pancreatic duct, and lobularity with a honeycomb appearance. The minor criteria for CP include the presence of cysts, dilated ducts ≥ 3.5 mm, irregular pancreatic duct contours, dilated side branches ≥ 1 mm, a hyperechoic duct wall, the presence of strands, non-shadowing hyperechoic foci, and lobularity with noncontiguous lobules. Our case revealed features similar to CP, as the hypoechoic tumor observed within the vicinity of the cystic lesions demonstrated certain characteristic findings of CP, including hyperechoic foci and the presence of strands. However, distinguishing between f-AIP and PC on other imaging modalities may be difficult[2,4-7]. In the present case, the tumor showed high-intensity signals

on DWI and delayed enhancement on dynamic CT, although both f-AIP and PC may present similar findings in these types of imaging studies[7,8]. However, simultaneous carcinomatous changes in 2 IPMN lesions are very rare; in such a case, the possibility that such lesions are inflammatory rather than carcinomatous is rather high.

Among other diagnostic methods, Kanno *et al*[9] reported that EUS-guided fine-needle aspiration (EUS-FNA) with a 22-G needle may provide adequate tissue for histological examination. However, many reports have suggested that the material required to perform a confirmed diagnosis of AIP cannot be obtained with this approach, although EUS-FNA in cases of AIP can eliminate the possibility of carcinoma[10-12]. In our case, we could not perform EUS-FNA because the cyst was located near the puncture site.

The pathogenesis of AIP has not yet been clarified. The involvement of certain immunological mechanisms is suspected due to the presence of autoantibodies, the predominant infiltration of CD4 and CD8 T cells, and the expression of HLA-DR antigens in the pancreas in such cases[13]. In our patient, AIP was restricted to the vicinity of the IPMNs and did not develop in the remaining pancreatic parenchyma. Although this finding suggests that IPMN is related to the pathogenesis of AIP, proving a causal relationship is difficult because few studies have reported an association between these 2 clinical conditions[14]. According to previous reports, Naitoh *et al*[14] supported the hypothesis in which IPMN appears in the background of AIP. However, in our
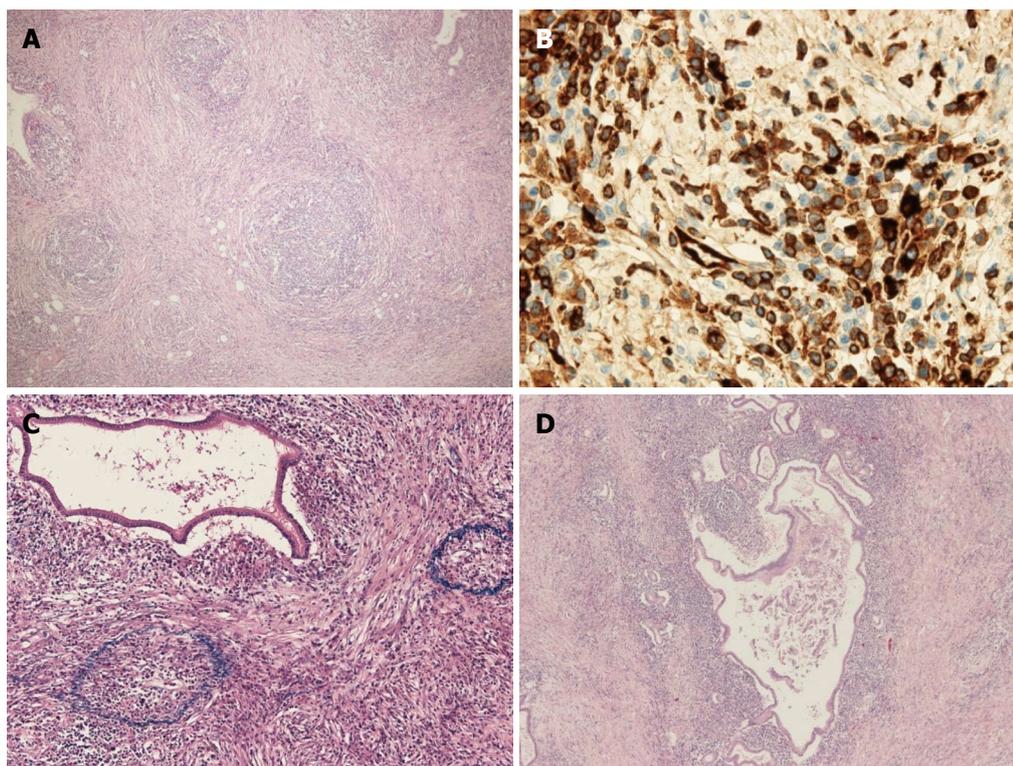
Figure 6 Microscopic findings. A: Each solid lesion presented a striform pattern with lymphoid follicles and inflammatory cells (HE, original magnification × 12.5); B: The plasma cells showed positivity for immunoglobulin G4 (HE, original magnification × 200); C: Many obliterating phlebitides were observed (HE, original magnification × 100); D: The multilocular cysts produced mucus and demonstrated a papillary pattern (HE, original magnification × 12.5).

case, because AIP developed during the progressive observation of IPMN, IPMN may have influenced the pathogenesis of AIP. However, the further examination of such cases of AIP and IPMN for an improved understanding of the relationship between these clinical entities is necessary.

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CASE REPORT

DOG1 is useful for diagnosis of KIT-negative gastrointestinal stromal tumor of stomach

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Abstract

Approximately 80%-95% of gastrointestinal stromal tumors (GISTs) show positive staining for KIT, while the other 5%-20% show negative staining. If the tumor is negative for KIT, but is positive for CD34, a histological diagnosis is possible. However, if the tumor is negative for KIT, CD34, S-100, and SMA, a definitive diagnosis is often challenging. Recently, Discovered on GIST-1 (DOG1) has received considerable attention as a useful molecule for the diagnosis of GIST. DOG1, a membrane channel protein, is known to be overexpressed in GIST. Because the sensitivity and specificity of DOG1 are higher than those of KIT, positive staining for DOG1 has been reported, even in KIT-negative GISTs. KIT-negative GISTs most commonly arise in the stomach and are mainly characterized by epithelioid features histologically. We describe our experience with a rare case of a KIT-negative GIST of the stomach that was diagnosed by positive immunohistochemical staining for DOG1 in a patient who presented with severe anemia. Our findings suggest that immunohistochemical staining for DOG1, in addition to gene analysis, is useful for the diagnosis of KIT-negative tumors that are suspected to be GISTs.

Core tip: We describe our experience with a rare case of a KIT-negative gastrointestinal stromal tumor (GIST) of the stomach that was diagnosed by positive immunohistochemical staining for Discovered on GIST-1 (DOG1) in a patient who presented with severe anemia. Our findings suggest that immunohistochemical staining for DOG1, in addition to gene analysis, is useful for the diagnosis of KIT-negative tumors that are suspected to be GISTs.

Wada T, Tanabe S, Ishido K, Higuchi K, Sasaki T, Katada C, Azuma M, Naruke A, Kim M, Koizumi W, Mikami T. DOG1 is useful for diagnosis of KIT-negative gastrointestinal stromal tumor of stomach. *World J Gastroenterol* 2013; 19(47): 9133-9136 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i47/9133.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i47.9133

INTRODUCTION

A gastrointestinal stromal tumor (GIST) is a mesenchymal tumor derived from the mesoderm that arises in the gastrointestinal tract. The estimated incidence is 2 cases per 100000 people per year. The most common age at
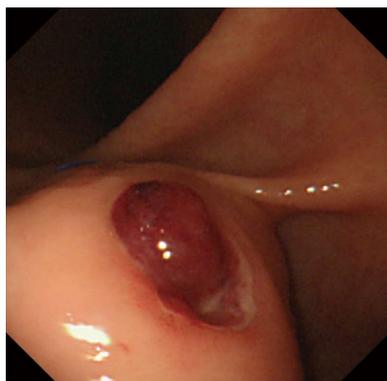
Figure 1 Findings on upper gastrointestinal endoscopy. A submucosal tumor accompanied by an ulcer with an adherent clot was found in the superior portion of the anterior wall of the gastric antrum.
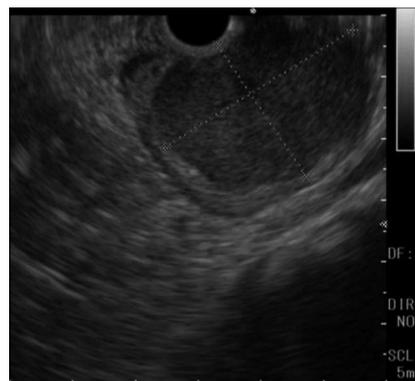

Figure 2 Findings on upper endoscopic ultrasonography. A homogeneous, hypoechoic, well-demarcated mass, approximately 4 cm in diameter with a flat border, arising from the fourth layer of the gastric wall.

diagnosis is 50-60 years. KIT protein is characteristically expressed by immunohistochemical staining. Gain-of-function mutations of the *c-kit* gene (approximately 90%) or the platelet-derived growth factor receptor alpha (*PDGFRA*) gene (approximately 5%) are the major causes of GISTs[1]. Immunohistochemical staining and gene analysis are considered useful for diagnosis, but if the tumor is negative for KIT, CD34, S-100, and smooth muscle actin (SMA), a definitive diagnosis is often challenging. We describe our experience with a patient in whom immunohistochemical staining for Discovered on GIST-1 (DOG1) enabled the diagnosis of a KIT-negative GIST[2].

CASE REPORT

A 60-year-old man was referred to the Department of Gastroenterology of our hospital because of wooziness, shortness of breath on effort, and tarry stools. A blood test showed that the hemoglobin level was 3.6 g/dL, indicating severe anemia. Upper gastrointestinal endoscopy disclosed a submucosal tumor accompanied by an ulcer with an adherent clot, arising in the superior portion of the anterior wall of the gastric antrum (Figure 1). Endoscopic ultrasonography (EUS) revealed a well-demarcated, homogeneous, hypoechoic mass with a flat border. The mass was approximately 4 cm in diameter and arose from the fourth layer of the gastric wall (Figure 2). Endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNAB)[3,4] was performed to obtain a definitive diagnosis and showed aggregations of cells with spindle-like or polygonal nuclei. However, immunohistochemical staining was negative for KIT, CD34, S-100, and SMA. A GIST was strongly suspected, but a definite diagnosis was not reached. Gene analysis could not be performed because the tissue sample was too small. However, the patient had a symptomatic, submucosal tumor with no distinct evidence of distant metastasis or direct invasion on enhanced computed tomography of the chest and abdomen. Surgery was, therefore, indicated according to the clinical practice guidelines for GIST in Japan[5], and a distal gas-

trectomy was performed. On macroscopic examination, the surgically resected specimen showed no evidence of bleeding or necrosis. The tumor measured 45 mm in diameter, and the resection margins were tumor negative. Histopathological examination showed that the tumor consisted of mixed components, including diffuse proliferations of spindle cells with eosinophilic cytoplasm, as well as epithelioid cells in some regions. One mitosis was found per 50 high-power fields, and the MIB-1 index was 3%. Immunohistochemical staining was negative for KIT, CD34, S-100, and SMA, but it was positive for vimentin and DOG1, a membrane channel protein (Figure 3). The tissue specimen obtained by EUS-FNA also stained positively for DOG1 (Figure 4). Genetic analysis showed a mutation in exon 18 (D842V) of the *PDGFRA* gene, with no mutation in the *c-kit* gene. On the basis of these results, a KIT-negative GIST with low risk according to Fletcher's classification[6], and very low risk according to Miettinen's classification, was diagnosed[7]. The patient recovered uneventfully after surgery. As of 3 years after surgery, the patient has been followed up on an outpatient basis and remains free of metastasis and recurrence.

DISCUSSION

GIST is a mesenchymal tumor of the mesoderm arising from the interstitial cells of Cajal in the gastrointestinal tract. The most common site is the stomach (60%), followed by the small intestine (30%), duodenum (5%), and large intestine (4%)[8]. GIST can be associated with diverse clinical symptoms, such as gastrointestinal bleeding, abdominal pain, and tumor obstruction. Histopathologically, GIST can be classified into 3 categories: spindle-cell type, epithelioid-cell type, and mixed type. Epithelioid-cell type accounts for approximately 70% of all GISTs, epithelioid-cell type accounts for approximately 20%, and mixed type, as was found in our patient, accounts for approximately 10%[8].

At present, specific tumor markers for the diagnosis of GIST are unavailable. A definite diagnosis is established by immunostaining tissue specimens obtained by
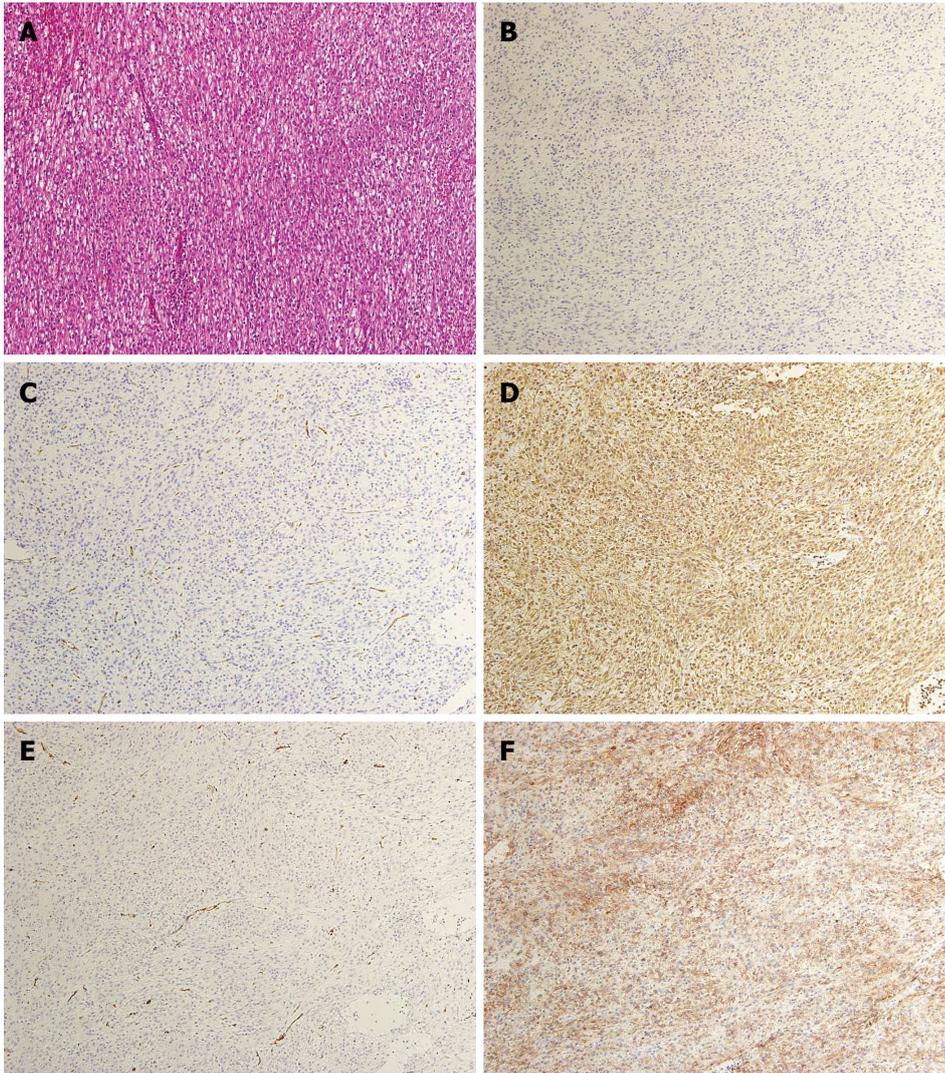
Figure 3 Histopathological findings of the surgically resected specimen. A: Hematoxylin and eosin staining (× 100). The tumor consisted of mixed components, including spindle cells with eosinophilic cytoplasm, as well as epithelioid cells in some regions; B: KIT staining (× 100), KIT staining was negative; C: CD34 staining (× 100), CD34 staining was negative; D: Vimentin staining (× 100); vimentin staining was positive. E: Smooth muscle actin (SMA) staining (× 100), SMA staining was negative; F: DOG1 staining (× 100); Immunostaining for DOG1 was positive mainly in the cell membrane and cytoplasm. DOG1: Discovered on gastrointestinal stromal tumor-1.
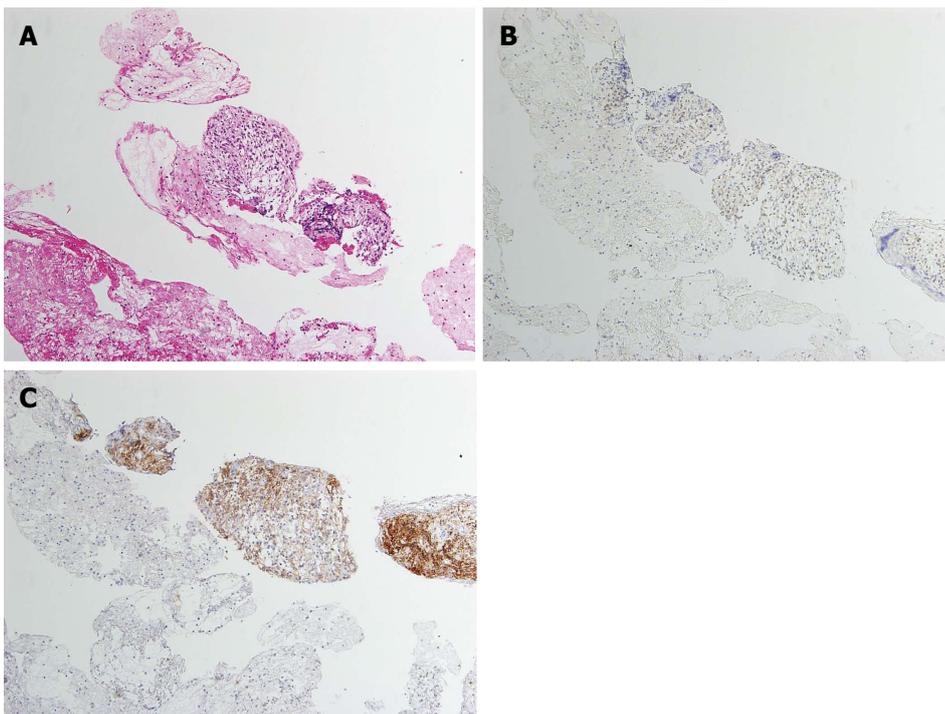
Figure 4 Histopathological findings (specimen obtained by endoscopic ultrasound-guided fine needle aspiration biopsy). A: Hematoxylin and eosin staining (× 100).The tumor consisted of mixed components, consisting of spindle cells with eosinophilic cytoplasm, as well as cells with epithelioid features in some regions; B: KIT staining (× 100), KIT staining was negative; C: DOG1 staining (× 100). Immunostaining for DOG1 was positive mainly in the cell membrane and cytoplasm. DOG1: Discovered on gastrointestinal stromal tumor-1.

EUS-FNAB or at surgery for KIT, CD34, SMA, desmin, S-100, and Ki-67[4,6]. Approximately 80%-95% of GISTs show positive staining for KIT, while the other 5%-20% show negative staining. If the tumor is negative for KIT but positive for CD34, a histological diagnosis is possible; however, if the tumor is negative for KIT, CD34, S-100, and SMA, similar to our patient, a definitive diagnosis is often challenging.

Recently, DOG1 has received considerable attention as a useful molecule for the diagnosis of GIST[2]. DOG1, a membrane channel protein, is known to be overexpressed in GIST. Because the sensitivity and specificity of DOG1 are higher than those of KIT, positive staining for DOG1 has been reported even in KIT-negative GIST[9-11]. KIT-negative GISTs most commonly arise in the stomach and are mainly characterized by epithelioid features histologically. KIT-negative GISTs are often associated with *PDGFRA* gene mutations[8]. Rizzardi *et al*[12] genetically analyzed a DOG1-positive, KIT-negative GIST of the stomach and reported the presence of a deletion in exon 14 of the *PDGFRA* gene, with no mutation in the *c-kit* gene.

In our patient, pathological examination of the surgically resected specimen showed a mixed-type GIST, including epithelioid cells. Immunostaining was negative for both KIT and CD34 but was positive for DOG1. Consistent with these findings, a mutation was found in exon 18 (D842V) of the *PDGFRA* gene, with no mutation in the *c-kit* gene. Because the D842V mutation of the *PDGFRA* gene is resistant to imatinib, sunitinib is prescribed when recurrence is found[1,13].

In histological specimens obtained by EUS-FNAB before surgery, immunostaining was negative for KIT. A definite diagnosis could not be made. Immunohistochemical staining for DOG1 was additionally performed and showed that the cytoplasm of the tumor cells was positively stained. Hwang *et al*[14] reported that DOG1 was a useful marker for the cytologic diagnosis of GIST in tissue specimens obtained by EUS-FNAB. However, one study reported that approximately 30% of KIT-negative GISTs are negative for DOG1, suggesting that tumors suspected to be GIST should be comprehensively evaluated, including analysis of other genes[10].

We have described our experience with a rare case of KIT-negative GIST of the stomach that was diagnosed by positive immunostaining for DOG1 in a patient who presented with severe anemia. Our findings suggest that immunostaining for DOG1, in addition to gene analysis, is useful for the diagnosis of KIT-negative tumors suspected to be GISTs.

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LETTERS TO THE EDITOR
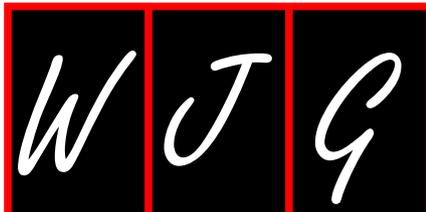
Assessment of proximal gastric accommodation in patients with functional dyspepsia

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Abstract

Impaired gastric accommodation is one of the most important etiologic factors in the pathophysiology of functional dyspepsia. Ultrasound is a potential alternative method to study changes in gastric volume as a reflection of gastric accommodation. Ultrasound is suitable for patients because it is a non-invasive, easily repeated and non-radioactive procedure, and a previous study has demonstrated the feasibility of 3-dimensional ultrasound in examining functional dyspepsia. The brief article by Fan *et al* demonstrated that both the proximal gastric area and volume, measured by 2- and 3-dimensional ultrasound respectively, were significantly smaller in patients with functional dyspepsia than in healthy controls. These results are very interesting, but we raise the relevant point that it should have been mandatory to study both changes in gastric volume and their relationship with upper gastrointestinal symptoms in functional dyspepsia. In fact, the relationship between cardinal symptoms and several pathophysiologic mechanisms in functional dyspepsia remains a matter of debate. Moreover, further evaluation of distal gastric volume that has been previously implicated in the origin of functional dyspeptic symptoms is advisable.

Therefore, impaired gastric accommodation does not serve as a clear marker of the cardinal symptoms experienced by patients with functional dyspepsia in daily life.

Core tip: Proximal gastric area and volume measured respectively by 2- and 3-dimensional ultrasound were significantly smaller in patients with functional dyspepsia compared to those of healthy controls. Hence, they could be used to assess accommodation impairment, but further prospective studies are needed to establish their clinical role in diagnosis of functional dyspepsia.

Iovino P, Santonicola A, Ciacci C. Assessment of proximal gastric accommodation in patients with functional dyspepsia. *World J Gastroenterol* 2013; 19(47): 9137-9138 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i47/9137.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i47.9137

TO THE EDITOR

We read with great interest the article by Fan *et al*[1] showing that post-prandial measurement of both proximal gastric area, measured by 2-dimensional ultrasound (US), and proximal gastric volume, measured by 3-dimensional US, could be useful for assessment of the proximal gastric accommodation in healthy controls and in patients with functional dyspepsia (FD). The authors, therefore, concluded that US measurement of gastric area and volume could help to predict FD.

This article is welcomed because US is a potential al-

ternative method for studying changes in gastric volume as a measure of gastric accommodation that is impaired in a subgroup of about 40% of patients with FD[2]. US is suitable for patients because it is a non-invasive, easily repeated and non-radioactive procedure, and a previous study has demonstrated the feasibility of 3-dimensional US in FD[3].

Nevertheless, the clinical significance of the conclusions of the study by Fan *et al*[1] should be regarded with a degree of caution, as the isolated determination of the lower gastric area and volume in FD compared with healthy controls is not sufficient to prove a clinical impact of this methodology in predicting FD as the authors suggest in the core tip of the study.

Dyspeptic symptoms are very common in the general population, with prevalence estimates ranging between 10% and 45%[4]. The results of prevalence studies are strongly influenced by the criteria used to define dyspepsia. FD, according to Rome III criteria, is a common disorder seen in daily clinical practice, and is characterized by the presence of pain or discomfort in the upper abdomen in the absence of organic, systemic, or metabolic disease[5]. FD patients complain about a variety of symptoms, which are frequently intermittent, and mostly related to food intake[5,6]. Therefore, it should have been mandatory to study both changes in gastric volume and their relationship with upper gastrointestinal sensations. Previous studies on this topic demonstrated that the relationship between specific upper abdominal sensations and several pathophysiologic mechanisms such as delayed gastric emptying, impaired proximal gastric accommodation, and visceral hypersensitivity, remain a matter of debate. Moreover, a further evaluation of the distal gastric volume is advisable on the basis of the previous results that showed that the distal gastric volume was larger in patients with functional dyspepsia[7] - an indirect finding in line with the observation by Caldarella *et al*[8] of antrofundic dysfunctions in FD.

Consequently, impaired gastric accommodation does not serve as a clear marker for the symptoms experienced by FD patients in daily life. These new findings warrant further research on this interesting topic that could also expand our knowledge in other subgroups of patients suffering dyspeptic symptoms.

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